WHO FOOD ADDITIVES SERIES: 54

Safety evaluation of certain food additives

Prepared by the
Sixty-third meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA)

IPCS

International Programme on Chemical Safety
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PREFACE

The monographs contained in this volume were prepared at the sixty-third meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), which met at WHO Headquarters in Geneva, Switzerland, 8–17 June 2004. These monographs summarize the safety data on selected food additives reviewed by the Committee.

The sixty-third report of JECFA has been published by the World Health Organization as WHO Technical Report No. 928. Reports and other documents resulting from previous meetings of JECFA are listed in Annex 1. The participants in the meeting are listed in Annex 3 of the present publication; a summary of the conclusions of the Committee is given in Annex 4. Some of the substances listed in Annex 4 were evaluated at the meeting only for specifications. Annex 5 contains a summary of the safety evaluation of secondary components for flavouring agents with minimum assay values of less than 95%.

Specifications that were developed at the sixty-third meeting of JECFA have been issued separately by FAO as Food and Nutrition Paper, No. 52, Addendum 12. The monographs in the present publication should be read in conjunction with the specifications and the report.

JECFA serves as a scientific advisory body to FAO, WHO, their Member States, and the Codex Alimentarius Commission, primarily through the Codex Committee on Food Additives and Contaminants and the Codex Committee on Residues of Veterinary Drugs in Foods, regarding the safety of food additives, residues of veterinary drugs, naturally occurring toxicants and contaminants in food. Committees accomplish this task by preparing reports of their meetings and publishing specifications or residue monographs and toxicological monographs, such as those contained in this volume, on substances that they have considered.

The toxicological monographs contained in the volume are based on working papers that were prepared by Temporary Advisers. A special acknowledgement is given at the beginning of each monograph to those who prepared these working papers.

Many proprietary unpublished reports are unreferenced. These were voluntarily submitted to the Committee by various producers of the food additives under review, and in many cases represent the only data available on those substances. The Temporary Advisers based the working papers they developed on all the data that were submitted, and all of these reports were available to the Committee when it made its evaluation. The monographs were edited by H. Mattock, Illkirch-Graffenstaden, France.

The preparation and editing of the monographs included in this volume were made possible through the technical and financial contributions of the Participating Organizations of the International Programme on Chemical Safety (IPCS), which supports the activities of JECFA.

The designations employed and the presentation of the material in this publication do not imply the expression of any opinion whatsoever on the part of the

organizations participating in the IPCS concerning the legal status of any country, territory, city, or area or its authorities, or concerning the delimitation of its frontiers or boundaries. The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the organizations in preference to others of a similar nature that are not mentioned.

Any comments or new information on the biological or toxicological properties of the compounds evaluated in this publication should be addressed to: Joint WHO Secretary of the Joint FAO/WHO Expert Committee on Food Additives, International Programme on Chemical Safety, World Health Organization, Avenue Appia, 1211 Geneva 27, Switzerland.

FOOD ADDITIVES

α-CYCLODEXTRIN (addendum)

First draft prepared by

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1. EXPLANATION

α-Cyclodextrin (synonyms: cyclohexaamylose, cyclomaltohexaose, α-Schardinger dextrin) is a non-reducing cyclic saccharide comprising six glucose units linked by α-1,4 bonds. α-Cyclodextrin was evaluated by the Committee at its fifty-seventh meeting (Annex 1, reference *154*), when the Committee concluded that, on the basis of the results of available studies with α-cyclodextrin and with the structurally related compounds β-cyclodextrin (seven glucose units) and γ-cyclodextrin (eight glucose units), for which acceptable daily intakes (ADIs) had

been allocated, there was sufficient information to allocate an ADI 'not specified' for α-cyclodextrin.

At its fifty-seventh meeting, the Committee evaluated α-cyclodextrin on the basis of known uses under good manufacturing practice as a carrier and stabilizer for flavours, colours, and sweeteners, as a water-solubilizer for fatty acids and certain vitamins, as a flavour modifier in soya milk, and as an absorbent in confectionery. The annular (doughnut-shaped) structure of α-cyclodextrin provides a hydrophobic cavity that allows the formation of inclusion complexes with a variety of non-polar organic molecules of appropriate size, while the hydrophilic nature of the outer surface of the cyclic structure causes such complexes to be soluble in water. α-Cyclodextrin is produced by the action of cyclodextrin glucosyltransferase and may contain residues of 1-decanol, which is used in the purification process.

At its present meeting, the Committee evaluated α-cyclodextrin for use as a food ingredient, suggested by the manufacturer to be a dietary fibre. It is stressed that the Committee only evaluated the safety of the estimated intake of α-cyclodextrin resulting from the proposed use levels. The Committee did not assess the efficacy of α-cyclodextrin used as a dietary fibre.

2. BIOLOGICAL DATA

2.1 Biochemical aspects: absorption, distribution, metabolism, and excretion

The biochemical aspects related to absorption, distribution, metabolism and excretion were described in the previous monograph (Annex 1, reference *154*), and no new relevant data in animals were available. α-Cyclodextrin, like β-cyclodextrin, is not digested in the gastrointestinal tract but is fermented by the intestinal microflora. In germ-free rats, α-cyclodextrin is almost completely excreted in the faeces, while γ-cyclodextrin is readily digested to glucose by the luminal and/or epithelial enzymes of the gastrointestinal tract. Overall, studies indicate that α-cyclodextrin can be absorbed intact at a level of approximately 1% from the small intestine. Absorbed intact α-cyclodextrin is excreted rapidly in the urine (Van Ommen & de Bie, 1995). All the nondigested and non-absorbed α-cyclodextrin reaches the microbially colonized segments of the gut, where the α-cyclodextrin ring is readily opened by microbial enzymes (certain amylases and cyclodextrinase). The resulting linear malto-oligosaccharides are then further hydrolysed and fermented via well established metabolic pathways to short-chain fatty acids (Antenucci & Palmer, 1984). Absorption of the metabolites of α-cyclodextrin leads to slow removal, mainly in exhaled carbon dioxide (CO_2) or in the urine (Van Ommen & de Bie, 1995).

2.2 Toxicological studies

No new data on toxicology in animals treated orally became available since the recent evaluation of α-cyclodextrin (Annex 1, reference *154*). The Committee concluded that the acute toxicity of α-cyclodextrin was low, but when given by the

intraperitoneal or intravenous route it can cause 'osmotic nephrosis' (also described in the literature as 'resorptive vacuolization') at high doses, which may lead to renal failure. The results of short-term (28- and 90-day) studies of toxicity indicated that α-cyclodextrin has little effect when given orally to rats or dogs. After administration of a very high concentration of α-cyclodextrin in the diet (20%, corresponding to a dose of 13.9 g/kg bw per day in rats and 10.4 g/kg bw per day in dogs), caecal enlargement and associated changes were seen in both species. This effect is likely to result from the presence of a high concentration of an osmotically active substance in the large intestine. No studies of intravenous administration were available to permit a comparison of the systemic toxicity of this compound with that of β- and γ-cyclodextrin.

Studies in mice, rats, and rabbits given α-cyclodextrin at concentrations of up to 20% in the diet (corresponding to doses of 49.3, 20 and 5.6–7.5 g/kg bw per day, respectively) did not indicate any teratogenic effects. Similarly, the results of assays for genotoxicity were negative. No long-term studies of toxicity, carcinogenicity, or reproductive toxicity have been conducted with α-cyclodextrin, but at its fifty seventh meeting (Annex 1, reference *154*) the Committee concluded that, given the known fate of this compound in the gastrointestinal tract, such studies were not required for an evaluation.

2.2.1 Special studies

(a) Skin irritation and/or sensitization

The potential of α-cyclodextrin to induce cutaneous delayed hypersensitivity was examined in guinea-pigs (a control group of five animals of each sex and a treated group of 10 animals of each sex), which were induced in two steps. First, a 3% solution of α-cyclodextrin with Freund complete adjuvant was injected intradermally. The controls received water with or without Freund complete adjuvant. One week later, a 30% dilution of α-cyclodextrin in vaseline was applied topically (controls received vaseline only). After two more weeks, a challenge treatment was made by topically applying vaseline with 0% (control), 10% or 30% α-cyclodextrin. The challenge treatment did not provoke signs of hypersensitivity (erythema, oedema) at 24 or 48 h after the challenge. It was concluded that α-cyclodextrin is not a sensitizer (Prinsen, 1992).

(b) Skin irritation and corrosion

The potential of α-cyclodextrin to induce dermal irritation and corrosion was examined in three albino rabbits. A mixture of α-cyclodextrin (0.5 g) with water (0.3 g) was applied to the shaven skin for 4 h. Skin irritation scores were recorded at 1, 24, 48 and 72 h after removal of the test material. No sign of skin irritation were observed at any time in any animal (Prinsen, 1991a).

(c) Ocular irritation

To examine potential ocular irritation, 0.062 g of α-cyclodextrin was instilled as a dry powder in the conjunctival cul-de-sac of the right eye of three albino rabbits.

The reaction was examined at 1, 24, 48 and 72 h and 7 and 14 days after administration. Different signs of acute ocular irritation were seen starting at 1 h after treatment. At 7 days after treatment, eye effects had cleared completely in one rabbit, whereas ischaemic necrosis of the nictitating membrane, slight redness and slight swelling of the conjunctivae were still observed in the other two rabbits. At 14 days after treatment, these eye effects had also cleared completely. It was concluded that dry α-cyclodextrin powder is irritating but not corrosive to the eye (Prinsen, 1990). Two groups of three rabbits received α-cyclodextrin in solution (7.25% and 14%, w/v), instilled in the conjunctival cul-de-sac of the right eye. The ocular reactions were examined after 1, 24, 48 and 72 h. The treatments caused slight redness and slight swelling of the conjunctivae in some animals. All eye effects had cleared completely at 24 h after treatment. It was concluded that solutions of α-cyclodextrin are not irritating and not corrosive to the eye (Prinsen, 1991b).

(d) Cell membrane and intestinal permeability

Effects on the cell membrane and on intestinal permeability were described in the previous evaluation of α-cyclodextrin (Annex 1, reference *154*). In vitro, α-cyclodextrin, like β-cyclodextrin, sequestered components of the membranes of erythrocytes, causing haemolysis. The threshold concentration for this effect was, however, higher than that observed for β-cyclodextrin. Similarly, of the three cyclodextrins α-cyclodextrin had the smallest effect on absorption in situ in rats.

(e) Digestibility in vitro

Early experiments on the digestibility of cyclodextrins by amylases in vitro demonstrated that pancreatic juice of dogs does not cleave α-cyclodextrin (Karrer, 1923) and that salivary amylase leaves α-cyclodextrin intact, hydrolyses β-cyclodextrin only very slowly, but hydrolyses γ-cyclodextrin at a rate of about 1% that for starch. At that time, α-cyclodextrin was called 'diamylose' and 'tetraamylose' (French, 1957). Recent studies in vitro showed that human salivary amylase, like human or porcine pancreatic amylases, are unable to hydrolyse α-cyclodextrin and β-cyclodextrin to any measurable extent, but readily hydrolyse γ-cyclodextrin (Marshall & Miwa, 1981; Kondo et al., 1990; McCleary, 2002).

(f) Interaction with the absorption of lipophilic nutrients

It has been demonstrated that the solubility of retinol acetate and vitamin K1 in water is higher in the presence of α-cyclodextrin (Pitha, 1981).

α-Cyclodextrin is known not to form complexes with vitamin D or vitamin E (Pitha, 1981).

(g) Interaction with the absorption of minerals

The possibility that the absorption of vitamins and minerals might be impaired by the consumption of increased amounts of dietary fibre has been addressed in

several reviews (e.g. Kelsay, 1990; Rossander et al., 1992; Gorman & Bowman, 1993; Gordon et al., 1995). Invariably it was concluded that dietary fibre, at recommended levels of intake, does not adversely affect the vitamin and mineral status of the average consumer. For resistant starch this was demonstrated recently in a study in which rats and pigs received diets with 6% native starch or retrograded high-amylose starch. The ingestion of the resistant starch did not significantly affect the absorption or retention of calcium, phosphorus, magnesium or zinc (De Schrijver et al., 1999). In addition, the low viscosity of α-cyclodextrin and its lack of anionic or cationic groups, make it unlikely that the absorption of minerals from the small intestines would be impaired.

(h) Impurities

The enzyme cyclodextrin-glycosyl transferase, which is used in the production of α-cyclodextrin, is derived from a non-genotoxic, non-toxigenic source and is completely removed during the purification of α-cyclodextrin (Annex 1, reference *154*).

1-Decanol is used as complexant for the precipitation of α-cyclodextrin. 1-Decanol has been used as a flavour for many years (estimated intake, 7–28 μg/person per day) and has been evaluated previously by the Committee (Annex 1, reference *132*). No data are available on the absorption, distribution, metabolism and excretion of 1-decanol; however, it is generally assumed that ingested aliphatic primary alcohols are absorbed and oxidized to the corresponding aldehyde, which is then rapidly oxidized to the acid. Acids with an even number of carbons are metabolized via β-oxidation to acetyl-coenzyme A, which then enters the citric acid cycle (Annex 1, reference *132*).

The safety of 1-decanol has been examined in studies of genotoxicity, acute oral toxicity and embryotoxicity and teratogenicity (inhalation and oral administration). An assay for gene mutation with *B. subtilis* H17 (rec^+) and M45 (rec^-) using 17 μg of 1-decanol per disk yielded a negative result (Oda et al., 1978, cited in Annex 1, reference *132*).

The acute oral toxicity of 1-decanol was examined in two studies in rats. Median lethal doses (LD_{50}) of >5 and 12.8 g/kg bw were reported (Henkel, K.G.A., unpublished data, Archive No 281; Bär & Griepentrog, 1967). In mice, a LD_{50} of 6.5 g/kg bw was observed. In a study of embryotoxicity and teratogenicity in Sprague-Dawley rats, the dams were exposed to 1-decanol by inhalation (100 mg/m^3; 6 h per day) on days 1–19 of gestation. No maternal toxicity was observed. The reproductive outcome (number of resorptions, litter size, fetal weights) was not adversely affected by the treatment, and there were no signs of fetotoxicity or teratogenicity (Nelson et al., 1990).

In a study of embryotoxicity and teratogenicity, unspecified random-bred albino rats were given a series of primary alcohols, including 1-decanol, by oral administration. A group of 10 female rats received daily doses of 1-decanol of 400 mg (equal to 2 g/kg bw per day) mixed with 600 mg of water by gavage on days 1–15 of gestation. A control group of 20 rats received 1 ml of water per day by gavage. No signs of maternal toxicity were reported. Pre- and postimplantation losses were

significantly increased with 1-decanol, but size and weight of the fetuses was not impaired. No teratogenic activity was observed. It was concluded that all the primary alcohols (C1, C2, C4, C9, C10) tested increased the number of pre- and postimplantation losses. 1-Decanol and nonanol were clearly less active than ethanol or methanol. Retardation of fetal development was observed with all the alcohols tested, except 1-decanol. None of the alcohols tested had teratogenic activity (Barilyak et al., 1991).

2.3 Observations in humans

2.3.1 Studies in human volunteers

In an early study of the metabolism of α-cyclodextrin, two patients with type-2 diabetes received 50 g of α-cyclodextrin of unknown purity per day. The substance was given with a low-carbohydrate diet. Nausea was noted in one subject on one out of two experimental days, about 10–12 min after ingestion. Other side-effects did not occur. The authors attributed this effect to an (unknown) impurity rather than to α-cyclodextrin itself (Von Hoesslin & Pringsheim, 1923).

In a subsequent series of experiments, a preparation of purified cyclodextrin (consisting mainly of α-cyclodextrin with some β- and γ-cyclodextrin) was given at a dose of 50 to 100 g/day. Some, but not all, volunteers (proportion not specified) reported nausea and, occasionally, diarrhoea. Urine analyses of four diabetic patients (two of whom were presumably type-1 diabetics) were presented and demonstrated that ingestion of the cyclodextrin preparation did not lead to an elevation of urinary glucose excretion, as was seen after the ingestion of bread (Von Hoesslin & Pringsheim, 1927).

The gastrointestinal tolerance of α-cyclodextrin was examined in 12 healthy male volunteers in the context of a study on its glycaemic effects. A single bolus dose of α-cyclodextrin of 25 g (dissolved in 250 ml of water) was administered to men who had fasted overnight. One man reported diarrhoea and three others reported abdominal discomfort. These effects were rated as 'mild' and did not prevent the volunteers from further participation in the study.

The ingestion of 10 g of α-cyclodextrin (dissolved in 250 ml of water) together with 100 g of fresh white bread was not associated with any intestinal side-effects in any of the men (Diamantis & Bär, 2002).

2.3.2 Digestibility in humans

In ileostomic subjects, more than 90% of an oral dose of β-cyclodextrin may be recovered from the ileal effluent (Flourie et al., 1993). β-Cyclodextrin and α-cyclodextrin are similarly resistant to the hydrolytic action of pancreatic amylase in vitro; it is therefore expected that the digestibility of α-cyclodextrin in vivo is as low as that of β-cyclodextrin. Direct proof for the low degree of digestibility of α-cyclodextrin stems from a study in which 12 healthy male volunteers received single doses of 25 g of α-cyclodextrin, 50 g starch (in the form of about 100 g of white bread), and a mixture of 50 g of starch and 10 g of α-cyclodextrin. Blood was collected at regular intervals over a period of 3 h for analysis of glucose and insulin.

Whereas the ingestion of 50 g of starch produced the expected rise in blood concentrations of glucose and insulin, no significant increase in blood concentrations of glucose and insulin was noted after the intake of 25 g of α-cyclodextrin (Diamantis & Bär, 2002).

Two diabetic subjects were given α-cyclodextrin (of unknown purity) at a dose of 50 g/day with a low-carbohydrate diet. No increase in urinary glucose excretion was observed, in contrast to that observed after consumption of 50 g of white bread (Von Hoesslin & Pringsheim, 1923). Similar results were noted in diabetic patients (including at least two type-1 diabetics) receiving mixed cyclodextrins (consisting mainly of α-cyclodextrin with some β- and γ-cyclodextrin) at a daily dose of 50 to100 g (Von Hoesslin & Pringsheim, 1927).

2.3.3 Attenuation by α-cyclodextrin of the glycaemic response to food containing starch

Diamantis & Bär (2002) examined the ability of α-cyclodextrin to reduce the glycaemic index. Twelve healthy male volunteers received, on separate days after overnight fasting, single doses of 25 g of α-cyclodextrin, 50 g of starch (in the form of about 100 g of fresh white bread) and a mixture of 50 g of starch (bread) and 10 g of α-cyclodextrin. Capillary blood was collected in regular intervals over a period of 3 h for analysis of glucose and insulin. The consumption of 50 g of starch produced the expected rise in blood concentrations of glucose and insulin. In contrast, no significant increase in blood concentrations of glucose and insulin was noted after the intake of 25 g of α-cyclodextrin. After intake of starch (bread) with α-cyclodextrin, the glycaemic and insulinaemic responses were delayed and reduced by 55% compared with those observed after intake of starch (bread) only. Similar observations were made with certain other types of soluble dietary fibre (Bär, 2004).

While only a few studies with α-cyclodextrin in humans are available, studies on other carbohydrates of low digestibility (such as inulin, fructooligosaccharides, polydextrose, resistant (malto)dextrins and other oligosaccharides) provide additional information. The largest number of studies is probably available for fructooligosaccharides and inulin. In a review of the safety data on fructans, including data on intestinal tolerance in children and adults (Carabin & Flamm, 1999), it was concluded that abdominal complaints would occur in adults after a single dose of ≥20 g. Children of school age tolerated supplementation of the diet with fructooligosaccharides at a level of 3–9 g (single dose). Ingestion of a single dose of 10 g of α-cyclodextrin (dissolved in 250 ml of water) together with 100 g of fresh white bread was not associated with any intestinal side-effects.

3. INTAKE

At its fifty-seventh meeting (Annex 1, reference *154*), the Committee estimated the potential intake of α-cyclodextrin from its known food uses. The predicted mean intake of α-cyclodextrin by consumers, based on individual dietary records for the USA and proposed maximum levels of use in a variety of foods, was 1.7 g/person

per day. For consumers at the 90th percentile of intake, the predicted daily intake of α-cyclodextrin was 3 g per person.

The intended use levels of α-cyclodextrin from its proposed new use as an ingredient in a number of food products range from a maximum of 10 g/kg in non-alcoholic beverages to a maximum of 100 g/kg in bakery products.

Assuming that α-cyclodextrin would be added to all possible food categories at the maximum proposed use levels and using the data on 'European diet' food consumption in the Global Environment Monitoring System — Food Contamination Monitoring and Assessment Programme (GEMS/Food) database, the Committee calculated a total intake of α-cyclodextrin of 65 g/person per day (see Table 1). This estimate is very conservative since it is unlikely that α-cyclodextrin would be consumed simultaneously from all sources on a regular basis.

An intake assessment was provided by Australia and New Zealand based on a national 1-day recall survey. It was assumed that α-cyclodextrin would be present at the highest proposed concentrations in all foods for which use was intended. The average dietary intake from intended uses was estimated to be 16 g/person per day and the 95th percentile of intake was estimated to reach 37 g.

In order to estimate the potential intake of α-cyclodextrin in a single eating occasion, the Committee used the GEMS/Food 'large portion' database, which contains the highest figures for 97.5th percentile consumption (eaters only) reported in national surveys. The highest estimated potential ingestion of α-cyclodextrin per eating occasion is between 19 and 38 g for bread only, depending on the proposed use level.

Table 1. Simulation of exposure to α-cyclodextrin using the European GEMS/Food[a] diet

	Maximum proposed use level (g/kg)	Mean food consumption (g/day)	Exposure (g/day)	% of total exposure
Cereals	50–100	222	11–22	16–32
Sugar	150	107	16	23
Margarine	200	17	3.4	5
Stimulant	0.088	14	0.0012	—
Milk	25	336	8.4	12
Nonalcoholic beverage	10	1500*	15	21
Total			65	

[a] Global Environment Monitoring System — Food Contamination Monitoring and Assessment Programme.

[b] Consumption for soft drinks is not available in the GEMS/Food database; therefore, this figure is an estimate.

4. COMMENTS

Only small quantities (1% or less of the administered dose) of intact α-cyclodextrin are absorbed from the small intestine. Absorbed α-cyclodextrin is rapidly excreted in the urine. α-Cyclodextrin, like β-cyclodextrin, is not digested in the gastrointestinal tract but is fermented to short-chain fatty acids by the intestinal microflora. These fatty acids are absorbed, oxidized, and eliminated largely as exhaled CO_2.

α-Cyclodextrin is not hydrolysed by human salivary and pancreatic amylases in vitro. Indirect proof that α-cyclodextrin is not digested in humans is drawn from experiments showing that the intake of 25 g of α-cyclodextrin does not lead to an increase in blood concentrations of glucose and insulin.

The results of short-term (28- and 90-day) studies of toxicity indicate that α-cyclodextrin has low oral toxicity in rats and dogs. After administration of α-cyclodextrin at a very high concentration in the diet (20%, corresponding to a dose of 13.9 g/kg bw per day in rats and 10.4 g/kg bw per day in dogs), caecal enlargement and associated changes were seen in both species. This effect is likely to result from the presence of a high concentration of an osmotically active substance in the large intestine.

Studies of embryotoxicity and teratogenicity in mice, rats, and rabbits fed diets containing α-cyclodextrin at a concentration of up to 20% (corresponding to a dose of 49.3 g/kg bw per day in mice, 20 g/kg bw per day in rats, and 5.9–7.5 g/kg bw per day in rabbits) did not indicate any adverse effects.

α-Cyclodextrin is neither an irritant nor a sensitizer after dermal application.

α-Cyclodextrin showed no effects in assays for genotoxicity in vitro and in vivo. No long-term studies of toxicity, carcinogenicity, or reproductive toxicity have been conducted with α-cyclodextrin, but the Committee reiterated its conclusion from the fifty-seventh meeting (Annex 1, reference *154*), stating that such studies were not required for the evaluation, in view of the known fate of this compound in the gastrointestinal tract.

It is possible that the potential interaction of α-cyclodextrin with lipophilic nutrients might impair their absorption. Although this has not been studied specifically for α-cyclodextrin, such an effect was considered to be unlikely by analogy to the results of studies with β-cyclodextrin. Complexes between fat-soluble vitamins and β-cyclodextrin have been shown to have a greater bioavailability than uncomplexed forms. In this context, α-cyclodextrin is known to enhance the solubility of retinol acetate and vitamin K1 in water, but does not form complexes with vitamin D and vitamin E.

It is also considered unlikely that the consumption of large amounts of α-cyclodextrin would impair the absorption of minerals, since it is known that the ingestion of resistant starch does not significantly affect the absorption or retention of calcium, phosphorus, magnesium or zinc. Moreover, α-cyclodextrin is of low viscosity, and its chemical structure lacks anionic or cationic groups.

A few studies in human volunteers indicate that flatulence, bloating, nausea and soft stools may occur in some individuals upon ingestion of α-cyclodextrin at a high dose. This is a well-known phenomenon for carbohydrates of low digestibility, particularly if ingested in liquid form on an empty stomach. It is partly caused by an influx of water in the small intestine (achieving isotonicity) and partly by the ensuing fermentation process in the more distal parts of the gut. Mild abdominal discomfort occurred in four out of twelve men, who had fasted overnight, given a single dose of 25 g of α-cyclodextrin in water, while no effects were reported after administration of 10 g of α-cyclodextrin in water together with white bread. In studies with other carbohydrates of low digestibility, such as inulin, fructooligosaccharides, polydextrose, resistant (malto)dextrins and other oligosaccharides, abdominal complaints were reported after a single dose of ≥20 g in adults, and children of school age tolerated supplementation of the diet with fructooligosaccharides at a single dose of 3–9 g.

Evaluation of potential impurities

The enzyme cyclodextrin-glycosyl transferase, which is used in the production of α-cyclodextrin, is derived from a nontoxigenic microorganism. The enzyme is completely removed from α-cyclodextrin during purification and is therefore of no safety concern. 1-Decanol, which is used as complexant for the precipitation of α-cyclodextrin, may be present in the final product at a concentration of <20 mg/kg. For example, an assumed intake of α-cyclodextrin of 65 g/person per day would correspond to an intake of 1-decanol of <1.3 mg/person per day. This is not a safety concern because 1-decanol is rapidly oxidized in the intestinal mucosa to the corresponding fatty acid, which then undergoes β-oxidation.

Intake

At its fifty-seventh meeting (Annex 1, reference *154*), the Committee estimated the potential intake of α-cyclodextrin from known food uses. The predicted mean intake of α-cyclodextrin by consumers, based on individual dietary records for the USA and maximum proposed levels of use in a variety of foods, was 1.7 g/person per day. For consumers at the 90th percentile of intake, the predicted daily intake of α-cyclodextrin was 3 g.

The intended use levels from the proposed new use of α-cyclodextrin as an ingredient in a number of food products range from a maximum of 10 g/kg in non-alcoholic beverages to a maximum of 100 g/kg in bakery products.

Assuming that α-cyclodextrin would be added to all possible food categories at the maximum proposed use levels, and using the GEMS/Food database, 'European diet' food consumption figures, the Committee calculated a total intake of α-cyclodextrin of 65 g/person per day. This estimate is very conservative since it is unlikely that α-cyclodextrin would be consumed simultaneously from all sources on a regular basis.

An intake assessment based on a national 1-day recall survey was provided by Australia and New Zealand. It was assumed that α-cyclodextrin would be

present at the highest proposed concentrations in all foods for which use was intended. The average dietary intake from intended uses was estimated to be 16 g/person per day and the 95th percentile of intake was estimated to reach 37 g.

In order to estimate the potential intake of α-cyclodextrin in a single eating occasion, the Committee used the GEMS/Food 'large portion' database, which contains the highest figures for 97.5th percentile consumption (eaters only) reported from national surveys. The highest estimated potential ingestion of α-cyclodextrin per eating occasion is between 19 and 38 g for bread only, depending on the proposed use level.

5. EVALUATION

At its present meeting, the Committee evaluated the safety of α-cyclodextrin based on its known use as food additive and on its proposed use as food ingredient.

A very conservative assessment of international exposure to α-cyclodextrin suggested that intakes could reach 65 g/person per day, while more realistic estimates at a national level suggested that intakes were likely to be 30–50% of this value. α-Cyclodextrin has been tested in various studies in animals, and no toxicity was observed at the highest doses tested, which were 10–100 times higher than the different estimates of potential intake by humans.

With respect to the previously evaluated use of α-cyclodextrin as a food additive and the present consideration of α-cyclodextrin as a food ingredient, the Committee concluded that there were no safety concerns at the proposed use levels and resulting predicted consumption.

The fact that the ingestion of ≥20 g of α-cyclodextrin on a single eating occasion may cause gastrointestinal effects in humans should be taken into account when considering appropriate levels of use.

The previously established ADI 'not specified' for the food additive uses of α-cyclodextrin as a carrier and stabilizer for flavours, colours, and sweeteners, as a water-solubilizer for fatty acids and certain vitamins, as a flavour modifier in soya milk, and as an absorbent in confectionery was retained.

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BENZOYL PEROXIDE

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1. EXPLANATION

Benzoyl peroxide is used as a bleaching agent in flour, in milk for production of cheeses and in whey from the manufacture of cheeses in which annatto and carotenoid pigments are present. At its present meeting, the Committee evaluated the safety of benzoyl peroxide at a maximum concentration of 100 mg per kg used as a bleaching agent in whey.

At its seventh meeting (Annex 1, reference *7*), the Committee evaluated benzoyl peroxide used as a bleaching agent in flour, and concluded that treatment of flour with benzoyl peroxide at concentrations of up to 40 mg per kg of flour was acceptable. At that meeting, the Committee noted that when benzoyl peroxide is used as a bleaching agent in flour, it reacts with oxidizable constituents of the flour and is almost totally converted to benzoic acid; any remaining traces of benzoyl peroxide are further reduced during the baking process and converted into benzoic acid. On this basis, the issues requiring consideration for use of benzoyl peroxide as a bleaching agent were determined to be the presence of small amounts of benzoic acid in bread and bakery products, the possible effects of oxidative treatment on the nutritional value of flour, and the possible formation of harmful substances or anti-metabolites.

At the fifty-fifth meeting of the Committee (Annex 1 reference *149*), the evaluation of the nutritional and toxicological implications of treatment of foods with benzoyl peroxide, with respect to potential effects on proteins, vitamins, antioxidants and physiologically important lipids, was postponed, owing to lack of information.

Benzoyl peroxide is manufactured by the reaction of benzoyl chloride, sodium hydroxide and hydrogen peroxide. During cheese-making or whey-drying, nearly all (>91%) benzoyl peroxide is converted to benzoic acid.

Concerning residues of benzoic acid, at the forty-first meeting of the Committee (Annex 1, reference *107*) a group acceptable dietary intake (ADI) of 0–5 mg/kg bw for benzoic acid and its calcium, potassium and sodium salts, benzyl acetate, benzyl alcohol, benzaldehyde and benzyl benzoate was established, and this was maintained by the Committee at its forty-sixth meeting (Annex 1, reference *122*). At its fifty-fifth meeting (Annex 1, reference *149*), the Committee noted that the intake of benzoic acid from foods treated with benzoyl peroxide should be considered together with intake from other dietary sources of benzoates in the group ADI of 0–5 mg/kg bw.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution, metabolism, and excretion of benzoyl peroxide

When benzoyl peroxide is used as a processing aid in food preparation, much of it is converted to benzoic acid during heat treatment or storage (Annex 1, reference *7*). Absorption of any residual benzoyl peroxide after oral ingestion may occur; however, most ingested benzoyl peroxide will be converted to benzoic acid (Nencki & Zaleski, 1899). Any remaining benzoyl peroxide that is absorbed is likely to be subject to the first-pass effect in the liver and be metabolized.

Chang et al. (1977) demonstrated that 91.7% of ^{14}C-labelled benzoyl peroxide was converted to [^{14}C]benzoic acid after reaction with whey; in addition, 7% of the radiolabel became tightly bound to nondialysable (6.84%) or dialysable (0.6%)

whey components, and this bound radiolabel was recovered as [^{14}C]benzoic acid after hydrolysis with hydrochloric acid (6 mol/l) and extraction. Minor amounts of hydroxybenzoic acids, phenol and phenyl benzoate were also formed upon reaction of [^{14}C]benzoyl peroxide with whey; only a very small amount of unreacted benzoyl peroxide remained. This investigation verified that residual benzoic acid in whey after the use of benzoyl peroxide to bleach this foodstuff is an issue to be considered.

Animals

Benzoyl peroxide may be metabolized by cleavage of the peroxide bond, resulting in benzoyloxyl radicals (Swauger et al., 1991). These radicals can either degrade to phenyl radicals and carbon dioxide, or remove hydrogen from molecules such as nicotinamide adenine dinucleotide, reduced (NADH) to form benzoic acid. Peroxidases catalyse the insertion of two hydrogen ions (donated by NADH) between the two oxygen atoms of hydrogen peroxide to form two molecules of water (Devlin, 1993). Benzoyl peroxide is likely to be converted to benzoic acid by the same mechanism; thus, benzoic acid is the major stable metabolite of benzoyl peroxide produced by keratinocytes (Nacht et al., 1981; Rothman & Pochi, 1988).

After metabolism of benzoyl peroxide, the major product, benzoic acid, is excreted in the urine, either as benzoate (Rothman & Pochi, 1988) or as a conjugate with glycine (benzoyl glycine; hippuric acid) (Life Science Research Office, 1980).

Humans

In a study using human keratinocytes in vitro, alkyl radical adducts were formed when benzoyl peroxide was incubated with 5,5-dimethyl-1-pyrroline-*N*-oxide (Kensler et al., 1988), consistent with formation of benzoyloxyl and phenyl radicals (Hazlewood & Davies, 1996). Production of free radicals occurs in freshly harvested and cultured keratinocytes at nontoxic concentrations of benzoyl peroxide (Iannone et al., 1993).

2.1.2 Effects on other biochemical parameters

Kaul & Khanduja (1999) found that benzoyl peroxide stimulates the formation of superoxide anion radicals in murine peritoneal macrophages in vitro. Benzoyl peroxide also increased the accumulation of diacylglycerol in these cells, with a concurrent release of choline metabolites. Eluants from dental resin disks containing benzoyl peroxide were found to cause a decrease in the free fatty acid pool and an increase in diacylglycerol formation in hamster oral epithelial cells in vitro (Lefebvre et al., 1996). The presence of benzoyl peroxide or its metabolic products could stimulate phospholipase C and/or D, which would result in higher levels of diacylglycerols that could alter cellular growth. Diacylglycerols have been shown to activate protein kinase C and stimulate the formation of superoxide anion radicals by inflammatory cells. Thus, with the accumulation of diacylglycerol, the pro-

duction of superoxide anion radicals by inflammatory cells is also likely to increase (Kaul & Khanduja, 1999).

Many studies have demonstrated the involvement of free radicals and reactive oxygen species, such as superoxide anion radicals, in the process of tumour promotion (Zhang & Mock, 1992; Kaul & Khanduja, 1999). Inflammatory cells, such as neutrophils and macrophages, are major sources of superoxide anion radicals during the promotional phase of skin carcinogenesis. In a study by Odukoya & Shklar (1984), chronic inflammation with infiltrates of lymphocytes and histiocytes, but no tumours, was present in the control group of hamsters administered benzoyl peroxide only, without a carcinogenic initiator. In a study by de Rey et al. (1994), treatment with benzoyl peroxide produced an increase in mast cells in the dermis.

Evidence that benzoyl peroxide generates free radicals involved in tumour promotion is supported by observations that exogenous antioxidants (e.g. butylated hydroxytoluene, disulfiram and ascorbic acid) have a general inhibitory effect on the incidence of skin tumours in studies of co-carcinogenesis. Free radical scavengers, such as glutathione and *n*-acyl dihydroxylamines also have an inhibitory effect (Slaga, 1995).

Benzoyl peroxide has been shown to bind covalently to protein, but not to DNA, and benzoyloxyl radicals likely covalently bind to macromolecules in a similar manner. Benzoic acid does not bind to either protein or DNA under similar conditions (Swauger et al., 1990). Potassium ion-activated phosphatase and sodium and potassium ion transporting ATPase (Na^+- and K^+-ATPase) are inhibited by benzoyl peroxide in rabbit dental pulp in vitro (Abiko et al., 1978; Kono et al., 1981). Haemolytic effects have also been reported (Fujisawa, 1978).

2.2 Toxicological studies

2.2.1 Acute toxicity

Mouse

Evidence concerning the acute toxicity of benzoyl peroxide in mice has been summarized by the Life Science Research Office (1980). The acute oral toxicity of benzoyl peroxide is low, with a median lethal dose (LD_{50}) of 2127 mg/kg bw (Antonyuk, 1969). Studies to determine toxicity after intraperitoneal injection were not considered to be relevant to the determination of the safety of benzoyl peroxide as a food additive (Life Science Research Office, 1980).

Rats

Benzoyl peroxide also has low acute oral toxicity in rats, with reported LD_{50} values in the range of 400–6400 mg/kg bw. When lethal doses were administered, animals displayed central nervous system depression within 30–40 min and death occurred within 24 h (Life Science Research Office, 1980).

Rabbits

In Draize tests for irritation conducted by Wazeter & Goldenthal (1973) in New Zealand white rabbits, benzoyl peroxide was not found to be a dermal irritant; however, it did cause transient ocular irritation. Conjunctivitis and swelling were noted, but no ulcerations or opacities. Benzoyl peroxide dust irritated the eyes of albino rabbits when not washed out within 5 min after administration, while dermal application of a 10% solution of benzoyl peroxide in propylene glycol caused slight to moderate erythema in guinea-pigs (IARC, 1985).

Humans

In humans, benzoyl peroxide is known to produce dermal irritation and sensitization reactions, particularly after multiple topical applications for acne treatment (Zesch, 1986; Kraus, 1995; National Toxicology Program, 2002).

2.2.2 Short-term studies of toxicity

Mice

In a 4-month study of toxicity (Antonyuk, 1969), mice were given benzoyl peroxide at a dose corresponding to 2, 5 or 10% of the calculated LD_{50} (148 mg/kg) by intraperitoneal administration. Decreased body-weight gains were noted in each group, but no deaths were observed.

Rats

Two short-term studies of toxicity with benzoyl peroxide administered by intraperitoneal injection were conducted.

In a 4-month study, rats given benzoyl peroxide at a dose of 7.5,18.7 or 37.3 mg/kg bw per day survived the observation period. A decrease in body-weight gain was measured (Antonyuk, 1969).

Mkhitaryan et al. (1974) demonstrated that concentrations of α-tocopherol in the brain decreased in rats given benzoyl peroxide at a dose of 48 mg/kg bw per day.

Dogs

Dogs fed flour treated with benzoyl peroxide for 12 weeks showed no evidence of toxicity (Newell et al., 1947). The flour was treated with 8 g of benzoyl peroxide per 45 kg (corresponding to a dose of approximately 5 mg/kg bw per day). After treatment, the flour was steamed before being given to the dogs. Concentrations of benzoyl peroxide were not determined after steaming. In a very similar study (Radomski et al., 1948), three dogs were fed flour treated with benzoyl peroxide (one ounce of benzoyl peroxide per pound of flour, i.e. 28 g per 454 g, equal to aproximately 15.6 mg/kg bw per day) for 6 weeks apparently did not show signs of toxicity. Again, concentrations of benzoyl peroxide were not determined after steaming.

Table 1. Results of studies of carcinogenicity in mice fed with benzoyl peroxide

Strain	No. per group	Route; exposure	Dose	Duration (weeks)	Result	Reference
Albino	25M, 25F	Oral, dietary; ad lib	28, 280, 2800 mg/kg of diet	80	No tumours observed	Sharrat et al. (1964)
Albino	25M, 25F	Subcutaneous; single dose	50 mg	80[a]	No tumours observed	Sharrat et al. (1964)
Unknown	30	Subcutaneous; implantation	0.5–3%	>12	No tumours observed	Oppenheimer et al. (1955)

F, female; M, male.
[a] Animals were apparently given a single dose and then observed for approximately 80 weeks.

2.2.3 Long-term studies of carcinogenicity

Mice

The results of studies of carcinogenicity in mice fed with benzoyl peroxide in mice, as cited by Kraus et al. (1995) and the International Agency for Research on Cancer (IARC, 1999), are summarized in Table 1. Many of these studies were previously reviewed by IARC (IARC, 1985, 1999).

Benzoyl peroxide was not carcinogenic in one dietary study in which groups of 25 male and 25 female mice received benzoyl peroxide at a dose of 28, 280 or 2800 mg/kg of diet (equal to a dose of 4.2, 42 and 420 mg/kg bw) for 80 weeks. In the same study, subcutaneous injection of a single dose of benzoyl peroxide of 50 mg per mouse (equal to a dose of approximately 2500 mg/kg bw) did not lead to tumour formation. In these studies, the final concentration of benzoyl peroxide in the diet given to the animals was not determined (Sharrat et al., 1964).

The carcinogenicity of benzoyl peroxide administered by dermal application has been thoroughly evaluated in mice. Sixteen studies (duration, 20–80 weeks) gave negative results. In almost all these studies, benzoyl peroxide was administered at doses varying from 20 to 40 mg, applied topically one to three times per week, except in one study in which benzoyl peroxide was applied six times per week (Sharrat et al., 1964).

Two studies gave positive results. In the first, benzoyl peroxide caused a statistically significant increase in skin tumours (8 out of 20 mice), of which 5 out of 20 were squamous cell carcinomas (Kurokawa et al., 1984).

In the second study, which used a transgenic line of mice with genetically initiated skin, the incidence of papillomas in heterozygous males was 0/5, 0/5, 3/5, 4/5 at 0, 1, 5 or 10 mg of benzoyl peroxide, respectively. In groups of three homo-

zygous mice, an increased incidence of papilloma over time was noticed in females receiving 5 or 10 mg of benzoyl peroxide topically, twice per week. Since these mice were prone to develop skin tumours, this study supports the promoting nature of benzoyl peroxide (Spalding et al., 1993).

In light of the negative results reported by most of the available studies, the positive results obtained in a single study are surprising, and probably show that the mice used were extremely sensitive to skin irritation and the development of skin tumours.

In a summary of all studies in mice treated with benzoyl peroxide by topical application to the skin (one to two times per week, at doses ranging from 10 to 40 mg) after initiation with carcinogens, benzoyl peroxide was shown to be a promoter of skin tumours in most cases, although different strains showed different sensitivities (Kraus et al., 1995).

Rats

The results of studies of carcinogenicity with benzoyl peroxide in rats have been summarized by Kraus et al. (1995).

In albino rats given benzoyl peroxide at a dose of 28, 280 or 2800 mg/kg of diet (equal to approximately 1.9, 19 or 190 mg/kg bw for males, and 2.3, 23 and 230 mg/kg bw for females) for 120 weeks, no carcinogenic effect was revealed; the incidence of malignant and/or benign tumours was not different between treated groups and controls. There was a significant increase in testis atrophy in the males at the highest dose, which according to the authors, was probably due to vitamin E deficiency. Body-weight gains in females at the highest dose and in the males at the intermediate dose were significantly reduced. The authors speculated that these weight depressions of about 10% were caused by marginal nutritional deficiencies, because an increased intake of food reversed the phenomenon. In these studies, the concentration of benzoyl peroxide in the final diet given to the animals was not determined (Sharrat et al., 1964).

Benzoyl peroxide was not carcinogenic in three studies in at least three different strains of rat treated by subcutaneous administration; however, the single subcutaneous injection apparently administered in the study by Sharrat et al. (1964) is not typical for a long-term study of toxicity or carcinogenicity.

Hamsters

Benzoyl peroxide was found not to be carcinogenic in hamsters when applied dermally at a dose of 160 mg, three times per week for 16 months. However, when 7,12-dimethylbenz[α]anthracene (DMBA) was administered as a single dose of 10 mg/kg bw by gavage, followed by 80 or 160 mg of benzoyl peroxide applied topically on the dermis, an increase in dermal melanotic foci, considered to be a precursor of melanotic tumours, and an increase in melanotic tumours were found (Schweizer, 1987).

Eight male and eight female hamsters were painted in the cheek pouch with an 0.1% solution of DMBA in mineral oil for 10 weeks, three times per week. After a subsequent non-treatment period of 6 weeks, the animals were painted, three times per week, with a 40% solution of benzoyl peroxide in acetone (approximately 20 mg each time). Six hamsters served as controls and were treated with benzoyl peroxide only. In both groups inflammatory changes and hyperkeratosis occurred. In the hamsters pretreated with DMBA, carcinoma in situ and epidermoid carcinomas were found in all animals at the application site, while no lesions were observed in organs examined histopathologically (heart, lungs, liver and kidneys) (Odukoya & Shklar, 1984).

Twenty-two male hamsters were painted with a 0.5% solution of DMBA in mineral oil, followed by 27 weeks of painting with a 40% solution of benzoyl peroxide in acetone, three times per week. A control group of six hamsters was treated only with benzoyl peroxide, using a similar dose and schedule. In the hamsters pretreated with DMBA, severe dysplastic changes, carcinoma in situ and early invasive squamous cell carcinomas were noticed in a nonspecified number of animals. The control animals treated only with benzoyl peroxide showed acanthosis, ulceration and inflammation of the painted areas, with severe dysplasia in one animal. Unexpectedly, benzoyl peroxide caused a reduction in DMBA-induced γ-glutamyltranspeptidase (GGT) foci in the liver (Zhang & Mock, 1992).

Thus benzoyl peroxide acts as a promoter for oral, topical and dermal carcinogenesis in hamsters, and this is consistent with benzoyl peroxide acting as a promoter in studies of carcinogenicity in mouse skin (see above).

In a 120-week study of carcinogenicity, mice and rats given diets containing benzoyl peroxide did not show an increase in the incidence of tumours, although a significant decrease in body weight (10%) was measured in females at the highest dose (230 mg/kg bw per day) and in males at the intermediate dose (19 mg/kg bw per day), but not in males at the highest dose (190 mg/kg bw per day). On the basis of these data and the concentrations of benzoyl peroxide added to the diet (up to 2000 mg/kg of diet), the Committee decided that the treatment of whey with benzoyl peroxide would not have an adverse effect on its nutritional value nor result in the formation of harmful substances or anti-metabolites in the whey.

During its deliberations, the Committee also considered the potential adverse effects of oxidation products of bixin and norbixin (carotenoids contained in annatto) formed from benzoyl peroxide, but found no evidence that this was a safety concern. In an evaluation by IARC, it was concluded that benzoyl peroxide was not classifiable as to its carcinogenicity to humans (IARC Group 3) (IARC, 1985).

2.2.4 Reproductive toxicity: developmental toxicity

Chickens

Benzoyl peroxide was dissolved in acetone at doses of 0, 0.05, 0.10, 0.21, 0.42, 0.83, and 1.7 μmol and injected into the air chamber of 3-day-old eggs from white Leghorn chickens, 30 eggs per dose group. There was a dose-related

increase in early embryonic deaths at all except the lowest dose. The dose–response curve was flat at the three higher doses, which indicates saturation of penetration. Only 1/80 controls were malformed; however, the rate of malformation was increased in all treatment groups and varied from 13 to 33%, without an apparent dose–response relationship (Korhonen et al., 1984; IARC, 1985).

2.2.5 Genotoxicity and other cellular effects

The results of studies of genotoxicity with benzoyl peroxide are summarized in Table 2.

As can be concluded from the data in this table, benzoyl peroxide is not mutagenic, it inhibits cellular communication, and it can cause single-strand breaks in DNA.

While benzoic acid did not produce DNA damage in a cell-free system utilizing ΦX-174 plasmid DNA in the presence of copper, benzoyl peroxide did produce DNA damage under these conditions. However, there was no apparent covalent binding of benzoyl peroxide to DNA (Swauger et al., 1991).

2.3 Observations in humans

2.3.1 Carcinogenicity in workers exposed in industry or in patients treated for acne

Epidemiological and clinical studies were carried out to determine whether exposures of workers to benzoyl peroxide during industrial use or of acne patients treated with benzoyl peroxide were associated with carcinogenicity. These studies have been reviewed by IARC (IARC, 1985, 1999) and by Kraus (1995). Topical preparations of benzoyl peroxide have been used in the treatment of acne for more than 30 years, with no reports of adverse effects that could be related to carcinogenicity. Adverse effects are usually limited to dermal irritation and sensitization reactions (Kraus et al., 1995).

A population-based case–control study of acne treatments as risk factors for skin cancer of the head and neck was performed in Canada. Women and men aged 10–51 years or 10–56 years, respectively, were asked to fill out questionnaires relating to a list of widely used medications for the treatment of acne. The response rate for participation for the 964 cases was 91%, and for the 3856 controls was 80%. Of the respondents, 92.3% had basal cell carcinoma, 4.8% had squamous cell carcinoma, and 2.9% had melanoma. Benzoyl peroxide had apparently been used in the treatment of acne for 9% of the cases and 10.1% of the controls. The odds ratio for use of benzoyl peroxide was 0.8 (95% confidence interval (CI), 0.5–1.3) for all cases of skin cancer of the head and neck combined; there was no association with use of benzoyl peroxide (Hogan et al., 1991).

2.3.2 Reproductive toxicity

Although no studies in pregnant women have been performed, years of clinical use of benzoyl peroxide in preparations used for the treatment of acne appear to

Table 2. Results of studies of genotoxicity with benzoyl peroxide

End-point	Test system	Concentration /dose	Results	Reference
In vitro				
Reverse mutation	*S. typhimurium*, TA1535, TA1537, TA1538	NS	Negative[b]	Litton Bionetics, Inc. (1975)
Reverse mutation	*Saccharomyces cerevisiae*, strain D4	NS	Negative[b]	Litton Bionetics, Inc. (1975)
Reverse mutation	*S. typhimurium*, TA100, TA1535, TA1537, TA98, TA92, TA94	2500 μg/ml	Negative[b]	Ishidate (1980)
Reverse mutation	*S. typhimurium*, TA100, TA102, TA104, TA 97a	100 μg/plate	Negative[b]	Dillon et al. (1998)
Chromosomal aberration	Chinese hamster lung cells	200 μg/ml	Negative[c]	Ishidate (1980)
Aneuploidy	Chinese hamster lung cells	200 μg/ml	Negative[c]	Ishidate (1980)
DNA single-strand breaks and DNA–protein cross-links	Human bronchial epithelial cells	242 μg/ml	Positive[c]	Saladino et al. (1985)
Increase in intercellular communication	Syrian hamster embryo cells	242 μg/ml	Positive[c]	Mikalsen & Sanner (1994)
Inhibition of gap-junctional intercellular communication	Primary mouse keratinocytes	40 μg/ml	Positive[c]	Jansen et al. (1996)
Inhibition of gap-junctional intercellular communication	Initiated primary mouse keratinocytes	10 μg/ml	Positive[c]	Jansen & Jongen (1996)
Inhibition of metabolic cooperation	Chinese hamster V79 cells	0.1–1.5 μg/ml	Positive, dose-dependent[d]	Slaga et al. (1981)
Sister chromatid exchange	Chinese hamster ovary cells	NS	Positive, dose-dependent response with metabolic activation; negative without metabolic activation	Jarventaus et al. (1984) (abstract)
Inhibition of metabolic cooperation	Human keratinocytes	0.5–3.6 μg/ml	Positive, dose-dependent[d]	Lawrence et al. (1984)

Table 2. (contd)

End-point	Test system	Concentration /dose	Results	Reference
DNA damage	Cell-free system using ΦX-174 plasmid DNA	1 mmol/l	Negative[d]	Swauger et al. (1991)
		0.1–1 mmol/l + copper (Cu)	Positive[d]	
In vivo				
Dominant lethal mutation	Mice	62 mg/kg bw[e]	Negative[c]	Epstein et al. (1972)

NS, not stated.
[a] Lowest effective dose or highest ineffective dose.
[b] With or without metabolic activation, source not stated.
[c] With metabolic activation; not tested without metabolic activation (not applicable in the case of the test for dominant lethal mutation in mice in vivo).
[d] Without metabolic activation; not tested with metabolic activation.
[e] Single dose, administered intraperitoneally.

indicate that benzoyl peroxide causes no detrimental reproductive effects in humans. Since benzoyl peroxide absorbed after topical administration is metabolized to benzoic acid in the skin and subsequently excreted as benzoic acid or as a conjugate of glycine, adverse systemic effects are unlikely to occur (Rothman & Pochi, 1988).

2.3.3 Immune response

Incidences of allergenic responses have been documented in workers exposed to benzoyl peroxide used as a bleaching agent in flour. A young male baker working with flour treated with benzoyl peroxide suffered for a year with asthmatic wheezing and severe dermatitis of the face, neck, shoulders, and arms. When the baker substituted 'unimproved' wheat flour for that treated with benzoyl peroxide, the allergic reactions disappeared. Two years later, when he was again exposed to flour treated with benzoyl peroxide, the baker promptly developed dermatitis (Baird, 1945).

Leyden & Kligman (1977) reported that benzoic acid was not sensitizing in a series of patients who were sensitive to benzoyl peroxide. Positive patch tests with benzoyl peroxide were reported in 38 out of 400 bakers tested (Grosfeld, 1951). In a study by Haustein el al. (1985), benzoyl peroxide was only a weak allergen but a strong irritant; only 11 out of 155 patients exhibited intolerance to the preparation and of those, 10 were able to continue use of the preparation.

Benzoyl peroxide is widely used as a topical agent, particularly in the treatment of acne, but also for other skin diseases, such as chronic skin ulcers, tinea pedis, and tinea versicolor (Hogan, 1991; IARC, 1999). Dermatologists have reported reactions among patients receiving various topical preparations of benzoyl peroxide for the treatment of acne; however, the reported incidences of contact sensitization to benzoyl peroxide varied widely among the various investigators.

The reported incidence of positive patch test reactions varied from 0 to 76% (Hogan, 1991). Leyden & Kligman (1977) reported a high incidence of contact sensitization with benzoyl peroxide. These investigators applied squares of cloth saturated with either 5% or 10% benzoyl peroxide gel to 25 patients for five periods of 48 h. The sensitization rate was 76% among these subjects, regardless of dose. The highest incidence (76%) of an allergenic response was reported in patients receiving benzoyl peroxide at high concentrations, applied under occlusive patches to treat chronic leg ulcers (Agathos & Bandmann, 1984). However, the incidence of positive patch tests does not appear to increase with the duration of use of benzoyl peroxide, and most patients exhibiting a reaction were able to continue using preparations containing benzoyl peroxide (Hogan, 1991).

In a double-blind study with 196 patients with acne, one group was treated with a placebo while three groups were treated with different lotions each containing 5.5% benzoyl peroxide. The lotions were applied one to four times daily for 4 weeks and left on the skin for at least 3–4 h each time. None of the patients exhibited dermal sensitization, nor were any significant systemic effects observed during the study (Ede, 1973).

In a study by Poole et al. (1970), 40% of adult volunteers became sensitized to an ointment containing 1% sulfur and 10% benzoyl peroxide. The investigators applied the preparation nine times for 24 h, within a period of 3 weeks. The preparation was reapplied after a 2-week interval. As a result of this challenge, 25 out of 69 subjects exhibited severe dermal sensitization reactions. Two months after the first applications, ten subjects who had exhibited only moderate reactivity were rechallenged with the ointment and all reacted severely (Poole et al., 1970).

In conclusion, most studies and clinical experience have demonstrated that benzoyl peroxide is a sensitizer when used in the treatment of acne, and that benzoyl peroxide can be a severe irritant.

3. TECHNOLOGICAL DATA

3.1 Secondary effects of treatment of food with benzoyl peroxide

In evaluating the health aspects of benzoyl peroxide, any secondary, possibly deleterious effects that might result from its use in foods should also be considered. Three possible effects of such action include: the formation of harmful degradation products; the destruction of essential nutrients; and the production of toxic substances from the food components (Life Science Research Office, 1980).

3.1.1 Degradation products of benzoyl peroxide

As indicated earlier, benzoyl peroxide in food is rapidly and almost completely converted to benzoic acid during processing. This results in an increase in the benzoic acid content of the treated food that is roughly equal to two molecules of benzoic acid per molecule of benzoyl peroxide employed. The direct addition of benzoic acid and sodium benzoate to food is approximately two to three times this

amount (Subcommittee on review of the GRAS List, 1972). Furthermore, benzoic acid is naturally found in several foods, including fruit, spices, milk products, meats, and beverages (Van Straten, 1977). A daily intake of 4–6 g of benzoic acid in humans causes no toxic symptoms, apart from slight gastric irritation (Goodman & Gilman, 1975). At its fifty-fifth meeting (Annex 1, reference *149*), the Committee reaffirmed that there was sufficient information on the toxicity of benzoic acid and related compounds to maintain the earlier established ADI of 0–5 mg/kg bw per day of benzoic acid equivalents.

3.1.2 Destruction of essential nutrients

Benzoyl peroxide reduces the vitamin A content of products containing fat. As whey is essentially fat-free, the treatment of whey is not affected by this problem.

No data are available on the fate of other essential nutrients in foods bleached with benzoyl peroxide, although results obtained in studies with hydrogen peroxide may be relevant in this connection. Treatment of milk for 24 h at 30 °C, or for 30 min at 51 °C with 0.3% hydrogen peroxide almost completely destroyed the small amounts of ascorbic acid and α-tocopherol present (Luck, 1958a; 1958b). These treatments had no effect on thiamin, riboflavin, or pyridoxine. No reduction in methionine content was noted when fish protein concentrates were treated with 1.25% hydrogen peroxide at 50 °C for 20 min, and only a slight reduction (8%) after treatment with 5% hydroperoxide (Rasekh, 1972). It should be noted that these latter concentrations of hydrogen peroxide are two orders of magnitude greater than those used in the proposed bleaching of whey with benxoyl peroxide.

3.1.3 Production of toxic compounds

It is possible that benzoyl peroxide might react with various constituents in whey. Chang et al. (1977) reported that the rate of decomposition of benzoyl peroxide in whey followed first-order kinetics, such that the rate depended on the size of the benzoyl peroxide particles and the agitation velocity. Moreover, they affirmed that the pH of whey had little effect on the decomposition rate of benzoyl peroxide and that benzoic acid was the major product. Minor amounts of hydroxybenzoic acids, phenyl benzoate, phenol, and benzoyl peroxide were also found.

4. INTAKE

As noted above, most of the benzoyl peroxide used in the bleaching treatment of whey is converted to benzoic acid. Subsequent processing will further reduce any traces of benzoyl peroxide that might remain in the whey that is used as a food ingredient. If any benzoyl peroxide were ingested, it would be subjected to further destruction in the gastrointestinal tract and by tissue peroxidases. Therefore, the major question requiring consideration is the acceptability of small amounts of benzoic acid being added to the diet by the consumption of food products to which bleached whey has been added.

In the Food and Agricultural Organization of the United Nations (FAO) food balance sheet for the year 2000, it was reported that 89 million tonnes of whey are produced annually worldwide. Estimates based on the production figures in the FAO STAT 2000 food balance sheet tables suggest that <15% of the world's whey production would be subject to this bleaching process. The worldwide consumption of whey, both bleached and unbleached, was 0.8 kg/person per year and the highest consumption was 15.4 kg/person per year in the USA. The latter would result in a total daily exposure to benzoic acid of 0.15×1540 mg of benzoic acid per year, or a daily exposure of 0.01 mg/kg bw (for a person with a body weight of 60 kg), assuming complete conversion of benzoyl peroxide.

5. COMMENTS

Almost all the benzoyl peroxide used in food processing is converted to benzoic acid during heat treatment or storage. While traces of benzoyl peroxide may be present in the processed food, most, if not all, of the benzoyl peroxide ingested will be degraded to benzoic acid in the intestine. It is likely that any benzoyl peroxide absorbed will be metabolized to benzoic acid in the liver. Finally, benzoic acid will be excreted in the urine, either as benzoate or as a conjugate with glycine. On this basis, the major issues to be considered when benzoyl peroxide is used as a bleaching agent in whey are the presence of small amounts of benzoic acid residues and the potential nutritional effects on whey.

During the metabolism of benzoyl peroxide, superoxide anion radicals may be produced. The low concentration of radicals formed will not, however, saturate superoxide dismutase and do not pose a safety concern.

Clinical studies have shown that benzoyl peroxide can be a severe dermal irritant, and is a dermal sensitizing agent in humans. The short-term studies of toxicity that are available are of limited quality. Benzoyl peroxide did not cause significant toxicity in rats or mice after repeated intraperitoneal injection. Benzoyl peroxide has been shown to cause single-strand breaks in DNA and to disrupt intercellular communication in vitro. However, it was not mutagenic and did not bind covalently to DNA. Benzoyl peroxide was not carcinogenic after subcutaneous or after dermal application. Benzoyl peroxide was shown to be a promoter in assays for initiation–promotion in mice treated dermally.

In a long-term study of carcinogenicity, the incidence of tumours did not increase in rats and mice receiving diets containing benzoyl peroxide. These and additional, although limited, data indicate that it is unlikely that treatment of food with benzoyl peroxide will have an effect on the nutritional value of whey, or result in the formation of harmful substances.

Epidemiological and clinical studies did not find an association between the incidence of skin cancer in industrial workers or acne patients and exposure to benzoyl peroxide. Adverse effects were usually limited to dermal irritation and sensitization reactions.

Intake

In the FAO food balance sheet for the year 2000, it was reported that 89 million tonnes of whey are annually produced in the world. Estimates based on the production figures in the FAO STAT 2000 food balance sheet tables suggest that <15% of the world's whey production would be subject to this bleaching process. The worldwide consumption per capita of whey (both bleached and unbleached) was 0.8 kg per year, and the highest consumption per capita was 15.4 kg per year in the USA. This results in a total daily exposure to benzoic acid of 0.01 mg/kg bw (for a 60 kg person), assuming complete conversion of benzoyl peroxide.

6. EVALUATION

The Committee considered the acceptability of small amounts of benzoic acid residues added to the diet by the consumption of food products containing bleached whey.

Assuming that 15% of cheese whey were bleached, the per capita intake of benzoic acid was estimated to be 0.01 mg/kg bw per day. The Committee concluded that this was a minor contribution to the total dietary intake of benzoic acid, for which a group ADI was established at the forty-first meeting, and that treatment of whey with benzoyl peroxide at a maximum concentration of 100 mg/kg did not pose a safety concern.

The Committee restated its conclusion from the fifty-first meeting (Annex 1, reference *122*) that it was possible that the intake of benzoic acid from all dietary sources by some consumers could exceed the ADI, and concluded that more precise intake data were required to estimate the number of such consumers and the magnitude and duration of intakes that are greater than the ADI.

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HEXOSE OXIDASE FROM CHONDRUS CRISPUS *EXPRESSED IN* HANSENULA POLYMORPHA

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1. EXPLANATION

The enzyme preparation under evaluation contains the active enzyme hexose oxidase (D-hexose: oxygen 1-oxidoreductase), which has not been evaluated previously by the Committee. Hexose oxidase is an enzyme that catalyses the oxidation of C6 sugars to their corresponding lactones, with the concomitant formation of hydrogen peroxide. Hexose oxidase has the highest affinity for D-glucose and D-galactose.

The hexose oxidase is produced from a nonpathogenic and nontoxigenic genetically modified strain of the yeast *Hansenula polymorpha* containing the hexose oxidase gene derived from the red alga *Chondrus crispus*. The enzyme activity is expressed in hexose oxidase units (HOXU).

Hexose oxidase can be used as a processing aid in the production of a range of foods at doses of 150–200 HOXU/kg of food (typical) to 500–2500 HOXU/kg of food (maximum). The commercial preparation contains 0.2 mg of total organic solids (TOS) per HOXU. The technological functions of hexose oxidase are dough strengthening, curd formation, oxygen scavenging, and decreasing the formation of the products of the Maillard reaction.

1.1 Genetic modification

The gene encoding hexose oxidase was derived from the red alga *C. crispus*, which is not known to be pathogenic or toxigenic. *C. crispus* has a long history of use in food in Asia and is one of the sources of carageenan, which has been evaluated previously as a food additive by the Committee (Annex 1, references *32*, *137*). A synthetic gene was constructed, based on the hexose oxidase cDNA from *C. crispus*, that was adapted for expression in yeast. The synthetic gene encodes a hexose oxidase with the same amino acid sequence as that of the native *C. crispus* enzyme. The synthetic gene was combined with regulatory sequences, promoter and terminator, derived from *H. polymorpha*, and inserted into the commonly-used plasmid pBR322. The *URA3* gene from *Saccharomyces cerevisiae* and the *HARS1* sequence from *H. polymorpha* were also inserted into the plasmid. The *URA3* gene serves as a selectable marker to identify cells containing the transformation vector. The native pBR322 plasmid contains genes encoding proteins that confer resistance to ampicillin and tetracycline. These genes were removed during the construction of the transformation vector.

In order to develop the *H. polymorpha* production strain, the wild-type strain ATCC 34438 was subjected to chemical mutagenesis. A strain requiring uracil for growth (uracil auxotroph) was used as a host strain. The strain was transformed with the hexose oxidase transformation vector. The transformed strain was further improved by mutagenesis using ultraviolet light (UV mutagenesis) and used as the hexose oxidase production strain. All the introduced DNA is well-characterized and would not result in the production of any toxic or undesirable substances. The production strain is stable with respect to the introduced DNA.

1.2 Product characterization

Hexose oxidase is produced by submerged fermentation of a pure culture of the *H. polymorpha* production strain. The enzyme is produced intracellularly and, upon cell disruption with lauryl trimethyl ammonium bromide (LTAB), is released into the fermentation broth and is subsequently separated from the yeast cells and subjected to ultrafiltration and diafiltration to obtain concentrated hexose oxidase. It is then spray-dried onto a suitable food-grade carrier, such as wheat starch, to form microgranules that are off-white to brownish in colour. Small amounts of LTAB may be present in the final product. The enzyme is typically denatured during heat treatment, and thus is no longer active in the final food product as eaten. The enzyme preparation conforms to the *General specifications for enzyme preparations in food processing* (Annex 1, reference *156*).

2. BIOLOGICAL DATA

2.1 Biochemical aspects

No information was available.

2.2 Toxicological studies

Toxicological studies have been performed with the enzyme preparations Ferm sample I, Ferm sample II, HOX-TOX-3-99, HOX-TOX-1 and HOX-TOX-4, all of which are yellow, water-soluble turbid liquid concentrates produced from fermentation of the recombinant production organism. As hexose oxidase is produced intracellularly in the host, cells are made permeable after fermentation to release the enzyme. In the enzyme preparations tested this was done by mechanical disruption (Ferm sample I), treatment with cetyl trimethyl ammonium bromide (CTAB; Ferm sample II), treatment with LTAB (HOX-TOX-3-99 and HOX-TOX-1), or treatment with hexanol (HOX-TOX-4). The preferred treatment was with LTAB. Owing to carry-over of LTAB into the enzyme preparation, it is possible that small quantities of this quaternary ammonium compound might be present in the final food product.

2.2.1 Acute toxicity

Studies of acute toxicity have been performed with two enzyme preparations, designated as Ferm sample I and Ferm sample II, with enzyme contents of 300 and 400 HOXU/ml, respectively. The studies followed OECD test guideline 420 (fixed dose method, 1992), and were certified for compliance with good laboratory practice (GLP) and quality assurance (QA). The results are summarized in Table 1.

2.2.2 Short-term studies of toxicity

Rats

In a range-finding study that was certified for compliance with GLP and QA, groups of five male and five female Sprague Dawley rats (aged 5–6 weeks) received enzyme preparation HOX-TOX-1 (enzyme content, 500 HOXU/g; TOS content, 8.57%) at a dose equivalent to 0, 500, 1250, or 5000 HOXU/kg bw per

Table 1. Acute toxicity of hexose oxidase

Enzyme preparation	Species	Sex	Route	LD_{50} (mg/kg bw)	References
Ferm sample I	Rat	M, F	Oral	>2000	Kaaber (2000a)
Ferm sample II	Rat	M, F	Oral	>2000	Kaaber (2000b); Cook & Thygesen (2003)

F, female; M, male.

day by oral gavage for 2 weeks. The vehicle was sterile water. All visible signs of ill health and behavioural changes were recorded daily. Body weight and food consumption were recorded once per week. At termination of treatment, the animals were weighed and macroscopic examinations were performed.

One male rat receiving the intermediate dose and one female receiving the highest dose showed haemorrhages in the thymus. These were considered to be incidental findings. No adverse effects were observed at up to and including the highest dose of 5000 HOXU/kg bw per day (Glerup, 2000; Cook & Thygesen, 2003).

Groups of ten male and ten female Sprague Dawley rats (aged 5–6 weeks) were given enzyme preparation HOX-TOX-3-99 (enzyme content, 500 HOXU/g; TOS content, 9.55%[1], LTAB content, 1130 μg/g) at a dose equivalent to 0, 500, 1250, or 5000 HOXU/kg bw by gavage (in sterile water), daily for 13 weeks. The study was performed according to OECD test guideline 408 (1998), and was certified for compliance with GLP and QA. All visible signs of ill health or behavioural changes were recorded daily, as were morbidity and mortality. Once per week, body weight and food consumption were recorded, and detailed clinical observations were performed outside the cage. All animals were examined by ophthalmoscopy at the start of the experiment, and animals in the group receiving the highest dose and in the control group were re-examined before termination, as were animals in the groups receiving the lowest and the intermediate dose when this was indicated. In week 11 or 12, all animals were examined for sensory reactivity to different types of stimuli, grip strength, and motor activity. At termination of treatment, blood samples were collected from all animals for haematology and clinical chemistry determinations. At necropsy, a macroscopic examination was performed on all animals, and absolute and relative (to body weight) weights of 11 organs were determined. Microscopy was carried out on about 35 organs and tissues from all animals in the control group and at the highest dose, on all organs and tissues from animals dying or sacrificed during the study, and on all gross lesions from all animals.

A few incidences of haemorrhages in the thymus and mandibular lymph nodes were attributed to blood sampling before necropsy and were not considered to be related to treatment. No adverse effects were noted on any other parameter examined. The no-observed-effect level (NOEL) was 5000 HOXU (equivalent to an intake of TOS of 955 mg/kg bw per day), the highest dose tested (Glerup, 2001; Cook & Thygesen, 2003).

2.2.3 Long-term studies of toxicity and carcinogenicity

No information was available.

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[1] Theoretical value; since ash content was not known, it was assumed that all dry matter was organic material.

2.2.4 Genotoxicity

The results of two studies of genotoxicity with hexose oxidase in vitro are summarized in Table 2.

In the first study, which followed OECD test guideline 471 (1997) and was certified for compliance with GLP and QA, the enzyme preparation tested was designated as HOX-TOX-3–99 (enzyme content, 500 HOXU/g; TOS content, 9.55%; LTAB content, 1130 μg/g). In the second study, which followed OECD test guideline 473 (1997), also certified for compliance with GLP and QA, the enzyme preparation tested was HOX-TOX-1 (enzyme content, 500 HOXU/g; TOS content, 8.57%).

Table 2. Studies of genotoxicity with hexose oxidase in vitro

Enzyme preparation	End-point	Test system	Concentration	Results	References
HOX-TOX 3–99	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	50–5000 μg/plate, ± S9. Solvent: sterile distilled water.	Negative[a]	Edwards (2001a); Cook & Thygesen (2003)
HOX-TOX 1	Chromosomal aberration	Human lymphocytes	First experiment: 75, 150, and 300 μg/ml, −S9; 150, 300, and 600 μg/ml, +S9. Second experiment: 9.4, 18.8, and 37.5 μg/ml −S9; 150, 300, and 600 μg/ml +S9. No solvent used	Negative[b]	Edwards (2001b); Cook & Thygesen (2003)

[a] With and without metabolic activation from S9 (9000 × *g* supernatant of rat liver), using the 'treat-and-plate' method (to avoid any problems that might have been caused had the test substance contained significant concentrations of bioavailable histidine). Cytotoxicity was observed at the highest or two higher doses.

[b] With and without metabolic activation from S9. In the first experiment, the cell cultures were treated for 3 h with and without S9 and were harvested 17 h later. Reductions in mean mitotic index were observed without S9 (to 75, 83 and 46% of values for the negative control at 75, 150 and 300 μg/ml, respectively) and with S9 (to 81 and 47% of values for the negative control at 300 and 600 μg/ml, respectively, but not at 150 μg/ml). In the second experiment, the cells were exposed continuously for 20 h without S9 and then harvested; with S9, cells were treated for 3 h and harvested 17 h later. Without S9, reductions in the mean mitotic index of 61, 58 and 26% that for the negative control were observed at 9.4, 18.8 and 37.5 μg/ml, respectively. With S9, the mean mitotic index was 95, 93 and 57% that of the negative control at 150, 300 and 600 μg/ml, respectively.

2.2.5 Reproductive toxicity

No information was available.

2.2.6 Special studies

(a) Dermal irritation

A study of primary dermal irritation in rabbits was performed with the enzyme preparation designated HOX-TOX-4 (enzyme content, 360 HOXU/g; dry matter content, 3.6%). The study followed OECD test guideline 404 (1992), and was certified for compliance with GLP and QA. An occluded application of 0.5 ml of HOX-TOX-4 was applied to two test sites on the closely clipped dorsal skin of three female New Zealand white rabbits for 4 h. Two other clipped dorsal skin sites remained untreated. After removal of the test substance, the treated skin was washed with lukewarm water and mild soap, and skin reactions were assessed 1, 24, 48 and 72 h later. No skin reactions were observed in any of the animals at any time-point (Bollen, 2002a).

(b) Ocular irritation

A study of acute ocular irritation in rabbits was performed with HOX-TOX-4. This study followed OECD test guideline 405 (1987), and was certified for compliance with GLP and QA. Three female New Zealand white rabbits received a single ocular instillation of 0.1 ml of HOX-TOX-4 in the left eye. The right eye remained untreated and served as a control. No ocular reactions were observed in any of the animals at 1, 24, 48 and 72 h after instillation (Bollen, 2002b).

(c) Toxicological data on LTAB and CTAB

A literature search did not reveal any toxicological data on LTAB (CETOX, 1999). The only data available on LTAB were on LTAB as a residue in the enzyme preparation HOX-TOX-3-99. As such, it was implicitly tested in an Ames test (Edwards, 2001a) and in a 13-week study of toxicity (Glerup, 2001), and did not cause adverse effects. More information was available on the closely related quaternary ammonium compound CTAB. CTAB is poorly absorbed from the gastrointestinal tract. CTAB did not show mutagenic activity in the Ames test, and the very similar compound cetyl trimethyl ammonium chloride (CTAC) also tested negative in the Ames test, as it did in assays for chromosomal aberrations in Chinese hamster V79 cells in vitro and cell transformation in Syrian golden hamster embryo cells in vitro. In pregnant rats given CTAB orally at a dose of 50 mg/kg bw per day (the highest dose) on days 5–14 of gestation, reduced fetal survival and increased incidence of resorption sites were observed. The NOEL was 25 mg/kg bw per day (Anonymous, 1997; CETOX, 1999).

In a 1-year study of toxicity, rats given drinking-water containing CTAB at a dose of 45 mg/kg bw per day (the highest dose) only showed a reduction in body weight. The NOEL was 20 mg/kg bw per day (Isomaa et al., 1976; Anonymous, 1997; CETOX, 1999).

2.3 Observations in humans

No information was available.

3. INTAKE

Hexose oxidase can be used as an alternative to glucose oxidase in the baking industry to strengthen dough and, in a similar way, in the pasta and noodle industries to produce a firmer structure. Hexose oxidase can also be used in foods for which the browning Maillard reactions that normally occur with heating are not desirable, and in cheese and tofu manufacture to improve curd formation. It is claimed that the enzyme can also function as an oxygen scavenger in sauces and dressings to improve appearance and shelf-life (Cook & Thygesen, 2003). Hexose oxidase may therefore be used in the production of a broad range of foods, including milk and milk products (e.g. cheese, yoghurts, creams, whey protein concentrate), cheese and cheese products (e.g. tofu), grain products (e.g. bread, pasta), potatoes (e.g. fried potatoes), eggs (e.g. powder of egg white), condiments, and salad dressings (e.g. mayonnaise, ketchup). The typical use levels of hexose hoxidase range from a minimum of 150–200 HOXU/kg of food, to a maximum of 500–2500 HOXU/kg of food, according to food applications.

The enzyme is typically denatured during heat treatment of foods, such as baking or pasteurization (data provided suggest that it is not stable at temperatures above 30 °C) and is therefore no longer active in the final product as eaten. It can thus be regarded as a processing aid.

The daily intake resulting from the combined consumption of several foods in a 'worst-case' situation was estimated. Based on the addition of high-level (90th percentile) intakes for each separate food category, the estimated combined intake levels were 42 and 43 HOXU/kg bw per day on the basis of food consumption data from the USA and Denmark, respectively. This approach assumes that high-level consumers of large quantities of one food are also high-level consumers of all the others. It is, however, very unlikely that an individual has a high intake of many food categories. In order to refine the intake estimates, an alternative approach was used, which was developed within SCOOP Task 4.2 (European Commission, 1998). This approach is applicable when the number of food categories under consideration is limited. It assumes that an individual might have a high consumption of food in two categories and an average consumption of foods in other categories. Average and high potential intakes of intake were calculated using data from the North/South Ireland Food Consumption Survey (Irish Universities Nutrition Alliance, 2001) and the maximum recommended dosages per food category (see Table 3).

The two highest (95th percentile) potential intakes estimated were 482.5 HOXU/person from white breads and rolls and 380 HOXU/person from wholemeal and brown breads, corresponding to a total of 14.4 HOXU/kg bw per day for a 60 kg person ([482.5 + 380]/60). The mean potential intake from the rest of the food categories was 437.5 HOXU/person, equivalent to 7.3 HOXU/kg bw per day for a 60 kg person. The combined intake for all food categories is thus

Table 3. Average and high potential intake of hexose oxidase (in HOXU) per food category based on data on food consumption from Ireland

Food group	Average food intake (kg/person)	95th percentile food intake (kg/person)	Maximum dosage (HOXU/kg of food)	Average intake of HOXU/ person	95th percentile intake of HOXU/ person
Rice, pasta, flours, grains, starches	0.020	0.086	2500	50.0	215.0
Savouries (e.g. pizzas)	0.024	0.094	2500	60.0	235.0
White breads, rolls	0.078	0.193	2500	195.0	482.5[a]
Wholemeal, brown breads, rolls	0.045	0.152	2500	112.5	380.0[a]
Other breads (e.g. scones, croissants)	0.015	0.061	2500	37.5	152.5
Ready-to-eat breakfast cereals	0.019	0.064	2500	47.5	160.0
Biscuits	0.014	0.047	2500	35.0	117.5
Cakes, pastries and buns	0.017	0.064	2500	42.5	160.0
Other milks (e.g. processed milks)	0.005	0.010	2000	10.0	20.0
Creams	0.002	0.009	2000	4.0	18.0
Cheeses	0.012	0.039	2000	24.0	78.0
Yoghurts	0.016	0.089	2000	32.0	178.0
Ice-creams	0.007	0.034	2000	14.0	68.0
Milk puddings (e.g. rice pudding, custards)	0.006	0.036	2000	12.0	72
Eggs, egg dishes	0.017	0.054	500	8.5	27.0
Low fat spreads	0.004	0.027	500	2.0	13.5
Other spreading fats	0.012	0.004	500	6.0	20.0
Chipped, fried and roasted potatoes	0.059	0.178	500	29.5	89.0
Soups, sauces, miscellaneous foods	0.046	0.151	500	23.0	75.5

From Irish Universities Nutrition Alliance (2001)
HOXU, hexose oxidase units.
[a] The values in bold correspond to the category leading to the highest intake of HOXU at the 95th percentile of food intake.

estimated to be 14.4 + 7.3 = 22 HOXU/kg bw per day, equal to an intake of TOS of 4 mg/kg bw per day.

LTAB is used as a processing aid during the production of the enzyme and thus may be carried over in the enzyme preparation and be present in the final food product. LTAB may be present in the final enzyme preparation at a concentration of 0.005–0.05 mg/g. Based on the addition of high levels (90th percentiles) of intake for each separate food category and taking the maximum recommended enzyme dosage and maximum content of LTAB residue, the combined intake of LTAB was conservatively estimated to be 5.35 μg/kg bw per day, on the basis of food consumption data from Denmark. On the basis of the SCOOP approach, described above, the estimated intake of enzyme, 22 HOXU/kg bw per day, results in an intake of LTAB of 2.7 μg/kg bw per day ($22 \times 1000/400 \times 0.05$) for an enzyme preparation with a specific activity of 400 HOXU/g.

4. COMMENTS

Toxicological studies were performed with water-soluble turbid liquid enzyme test concentrates, designated Ferm sample I, Ferm sample II, HOX-TOX-3-99, HOX-TOX-1 and HOX-TOX-4. These enzyme preparations were not acutely toxic when tested in rats, nor irritating to the skin or eye of rabbits, nor mutagenic in an assay for mutations in bacteria in vitro nor clastogenic in an assay for chromosomal aberrations in mammalian cells in vitro. In a 2-week range-finding study in rats treated with HOX-TOX-1 by gavage and in a 13-week study in rats treated by gavage with HOX-TOX-3–99 (containing not only hexose oxidase but also LTAB), no significant treatment-related effects were seen at up to and including the highest dose of 5000 HOXU/kg bw per day (equivalent to an intake of TOS of 955 mg/kg bw per day). This highest dose, which also represents an exposure to LTAB at 11.3 mg/kg bw per day, is therefore considered to be the NOEL. No toxicological data on LTAB only were available. The closely-related quaternary ammonium compound CTAB was not mutagenic in an assay for mutations in bacteria in vitro. In a 1-year study of toxicity with CTAB in rats, the only effect observed was reduced body-weight gain; the NOEL was 20 mg/kg bw per day.

Neither *H. polymorpha* nor *C. crispus* have been associated with allergenicity.

A conservative estimate of the intake of hexose oxidase when used at maximum dosage in the production of all potential food categories is 22 HOXU/kg bw per day (equivalent to an intake of TOS of 4 mg/kg bw per day). When this intake is compared with the NOEL of 5000 HOXU (equivalent to an intake of TOS of 955 mg/kg bw per day), the highest dose tested in the 13-week study of oral toxicity, the margin of safety exceeds 200. The concomitant intake of LTAB present at maximum concentrations of residue in all potential food categories was estimated to be 2.7 μg/kg bw per day. When this intake is compared with the NOEL for LTAB of 11.3 mg/kg bw per day in the 13-week study of oral toxicity and with the NOEL for the closely-related substance CTAB of 20 mg/kg bw per day in a 1-year study of toxicity in rats, the margin of safety is at least 4000.

5. EVALUATION

The Committee allocated an ADI 'not specified' to hexose oxidase from the recombinant strain of *Hansenula polymorpha* when used in the applications specified and in accordance with good manufacturing practice. The Committee concluded that the presence of LTAB at the concentrations observed in the enzyme preparation would not pose a safety concern to consumers.

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LUTEIN FROM TAGETES ERECTA *L.*

First draft prepared by

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1. EXPLANATION

Lutein ((all-*E*,3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol), a naturally occurring xanthophyll pigment, is an oxygenated carotenoid that has no provitamin A activity. It occurs with the isomeric xanthophyll zeaxanthin in many foods, particularly

Figure 1. Chemical structure of lutein

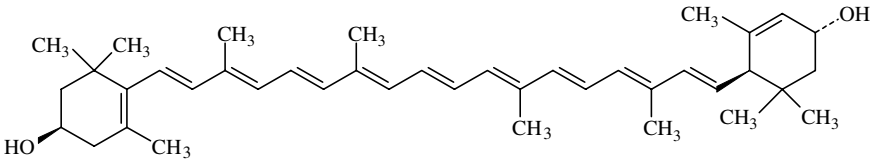
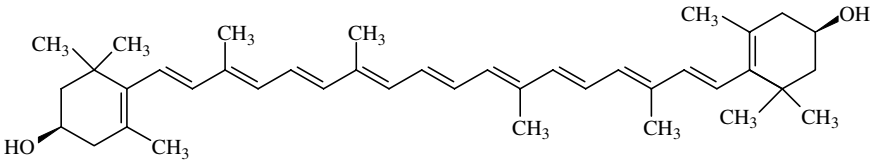


Figure 2. Chemical structure of zeaxanthin



vegetables and fruit. It is used as a food colour and nutrient supplement in a wide range of applications at concentrations ranging from 2 to 330 mg/kg.

Xanthophylls obtained from *Tagetes erecta* L. (marigold) petals were considered by the Committee at its thirty-first meeting (Annex 1, reference *77*). At that time, tentative specifications were prepared, but no toxicological data were available and no toxicological evaluation was made. *Tagetes* extract, containing xanthophylls at low concentrations, was again considered by the Committee at its fifty-fifth and fifthy-seventh meetings (Annex 1, references *149*, *154*) and the revised tentative specifications (Annex 1, reference *151*) were superseded by full specifications (Annex 1, reference *156*). At the present meeting, information was received on *Tagetes* preparations with a high lutein content (>80%), which had been used in a number of toxicological studies. These studies were reviewed in the safety assessment and allocation of an acceptable daily intake (ADI) for this product.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution, and excretion

(a) Absorption

Xanthophylls may be ingested in either free or esterified forms, although non-esterified lutein is the subject of the present evaluation. Before absorption, the esters are hydrolysed by pancreatic esterases and lipases such that only the free forms are found in the circulation (Wingerath et al., 1995). Once released from the food matrix as a lipid emulsion, like other non-polar lipids, these compounds must be solubilized within micelles in the gastrointestinal tract to permit absorption by

mucosal cells (Erdman et al., 1993). The transfer of carotenoids from lipid emulsion droplets to mixed micelles depends on their hydrophobicity, as well as pH and concentration of bile acid (Tyssandier et al., 2001). Other carotenoids, such as lycopene and xanthophylls, can impair the transfer of β-carotene, but neither β-carotene nor other xanthophylls affect the transfer of lutein (Tyssandier et al., 2001). The more polar carotenoids, such as the xanthophylls, are preferentially solubilized on the surface of lipid emulsion droplets and micelles, while the less polar carotenoids are incorporated into the core area (Borel et al., 1996). This facilitates the transfer of compounds like lutein and zeaxanthin from the lipid droplets to the aqueous phase. Indeed, it has been demonstrated that xanthophylls are more readily incorporated into micelles than are other carotenoids (Garrett et al., 1999, Garrett et al., 2000).

The absorption of carotenoids, including lutein, is potentially affected by the food matrix in which the carotenoids are consumed, dietary fat, and the presence of other carotenoids in the diet (reviewed in Castenmiller & West, 1998; Zaripheh & Erdman, 2002).

The absorption of carotene is higher when fat is present in the diet (Roodenburg et al., 2000) and lower in disease-induced cases of malabsorption of fat (Erdman et al., 1993). The presence of fat in the small intestine stimulates the secretion of bile acids from the gall bladder and improves the absorption of carotenoids by increasing the size and stability of micelles, thus allowing a greater amount of carotenoids to be solubilized. Absorption of carotenoids by mucosal cells is believed to occur by passive diffusion (Hollander & Ruble, 1978).

After uptake into mucosal cells, carotenoids are incorporated into chylomicrons and released into the lymphatics. When mucosal cells are sloughed off, carotenoids that have been taken up by the cells but not yet incorporated into chylomicrons are lost into the lumen of the intestine. The carotenoids within the chylomicrons are transported to the liver where they are distributed between the lipoprotein fractions. In contrast to the less polar carotenoids, a significant fraction of the xanthophylls is carried in the blood stream by high-density lipoprotein (HDL) (Romanchik et al., 1995).

The availability of lutein from a diet of mixed vegetables has been shown to be 67% relative to a that from a diet supplemented with crystalline lutein (van het Hof et al., 1999a). In another study, the relative bioavailability of lutein and β-carotene from various spinach products was compared with that from supplements containing 6.6 mg of lutein plus 9.8 mg of β-carotene. The values ranged from 45–54% for lutein to only 5.1–9.3% for β-carotene (Castenmiller et al., 1999). Processing, such as mechanical homogenization or heat treatment, has been shown to increase the availability of β-carotene in vegetables by 18 to 600% (van het Hof et al., 2000). There is evidence, however, that disruption of the matrix affects the bioavailability of carotenoids differentially, possibly because of differences in their lipophilic character. For example, the plasma concentration of lutein was increased by about 14% when spinach was consumed chopped rather than whole, while that of β-carotene was not affected (van het Hof et al., 1999b). The matrices of formulated natural or synthetic carotenoids (e.g. water-dispersible beadlets, crystalline powders, oils suspensions etc.) and whether

the compounds are esterified or non-esterified may clearly affect availability (Swanson et al., 1996; Boileau et al., 1999).

The presence of dietary fibre may explain, at least in part, the low availability of carotenoids from plant foods. It has been suggested that fibre interferes with micelle formation by partitioning bile salts and fat in the gel phase of the fibre. Riedl et al. (1999) tested the effects of pectin, guar, alginate, cellulose or wheat bran on the availability of lutein in six healthy female volunteers. All the fibres significantly reduced the plasma concentrations of lutein, with a range of 40 to 74%. Another study, however, indicated that pectin had no effect on serum concentrations of lutein after administration of a diet supplemented with liquefied spinach (Castenmiller et al., 1999).

Because the absorption of carotenoids occurs via incorporation into mixed micelles, ingestion of fat affects their availability. The amount of dietary fat required to ensure absorption of carotenoids seems to be low (3–5 g/meal), although it depends on the physico-chemical characteristics of the carotenoids ingested. In one experiment, the plasma concentration of lutein, added as esters, was about 100% higher when lutein was consumed with 35 g of fat than with 3 g of fat (van het Hof et al., 2000). The low amount of fat may have limited the solubilization of lutein esters and/or the release of esterases and lipases (Roodenburg et al., 2000). Bioavailability of carotenoids is also affected by the absorbability of the dietary fat (Borel et al., 1998). Sterol and stanol esters apparently have no effect on absorption of lutein (Raeini-Sarjaz et al., 2002). Egg yolk is a source of highly bioavailable zeaxanthin and lutein. The lipid matrix of the egg yolk, containing cholesterol, triacylglycerols and phospholipids, provides a vehicle for the efficient absorption of xanthophylls (Handelman et al., 1999).

Interactions between carotenoids may decrease absorption. Competition for absorption may occur at the level of micellar incorporation, intestinal uptake, lymphatic transport or at more than one level. Alternatively, simultaneous ingestion of various carotenoids may induce an antioxidant-sparing effect in the intestinal tract, resulting in increased levels of uptake of the protected carotenoids. It has been demonstrated that in the presence of large amounts of β-carotene, chylomicrons preferentially take up xanthophylls rather than β-carotene from the intestinal lumen (Gärtner et al., 1996). An inhibitory effect of dietary lutein on the absorption of β-carotene has been observed when the carotenoids were measured in plasma lipoproteins (van den Berg, 1998; van den Berg & van Vliet, 1998). In another study, healthy volunteers were given single oral doses of 15 mg of lutein derived from marigold extract either alone or together with 15 mg of β-carotene derived from palm oil. The inclusion of β-carotene reduced the area under the curve of concentration–time (AUC) for lutein to 54–61% of that for lutein administered alone (Kostic et al., 1995). In the same study, while lutein appeared to slow the initial absorption of β-carotene, lutein did not have any significant effect on the plasma concentration of β-carotene at the main peak or on the AUC value for β-carotene. Indeed, lutein enhanced the AUC value for β-carotene in subjects whose AUC value for β-carotene only was the lowest. In a similar study to investigate the interactions between β-carotene and dietary lutein, healthy male subjects on controlled diets were given capsules containing purified β-carotene at a high daily dose

(12 or 30 mg/day, corresponding to 0.2 or 0.5 mg/kg bw per day) for 6 weeks. Plasma concentrations of lutein in the group receiving β-carotene were decreased compared with baseline and were significantly lower than the levels reported in control groups given a placebo (Micozzi et al., 1992). Another study showed that the post-prandial appearance of vegetable-borne carotenoids in chylomicrons is competitive, but that this did not affect the plasma concentrations of the carotenoids after 3 weeks of feeding (Tyssandier, et al., 2002). Van den Berg (1999) has concluded that, in general, long-term supplementation with β-carotene has limited or no effect on plasma or serum concentrations of other carotenoids. However, in the α-Tocopherol and β-Carotene Cancer Prevention Study Grou (ATBC Study), a total of 29 133 male Finnish smokers aged 50–69 years were given daily supplements of 20 mg of β-carotene (0.3 mg/kg bw per day) for an average of 6.7 years. Significantly decreased serum concentrations of lutein (no changes in concentrations of zeaxanthin) were observed in comparison with groups that did not receive supplements containing β-carotene (Albanes et al., 1997).

In contrast to the interactions observed between lutein and β-carotene during absorption, supplementation with lycopene (5 mg/day from whole tomatoes, tomato juice, or gel capsules containing tomato oleoresin) reportedly had no effect on the plasma concentrations of lutein or zeaxanthin in a 6-week intervention study in 22 healthy female volunteers (Böhm & Bitsch, 1999).

In addition to the factors already described, the isomeric form (*cis* versus *trans*) of the carotenoids may affect their absorption. Lutein and zeaxanthin occur in nature predominantly in the all-*trans* configuration. Small amounts of *cis* isomers of each carotenoid, however, have been isolated from human serum (Krinsky et al., 1990; Khachik et al., 1999). It is not known whether the presence of *cis* isomers in human serum is exclusively due to their selective uptake and absorption from the diet, or whether they are the product of in vivo isomerization of all-*trans* lutein/zeaxanthin in the presence of gastric acids. Snodderly et al. (1990) investigated the major carotenoid pigments in the plasma and in a common, non-purified diet for macaques and squirrel monkeys. In the diet, both lutein and zeaxanthin were abundant in the all-*trans*, the 9-*cis*, and the 13-*cis* isomers. In the plasma, however, the 9-*cis* isomer was rarely detectable, while the 13-*cis* isomer was found in higher proportions than in the diet. These results suggest that either the isomers are absorbed selectively, or that isomerization processes occur in the animal gut.

A number of non-dietary factors also affect the availability of carotenoids, including exposure to tobacco smoke, alcohol consumption, intestinal parasites, malabsorption diseases, liver and kidney diseases, hormone status, poor intake of iron, zinc and protein, gastric pH and hyperthyroidism (Albanes et al., 1997; Williams et al., 1998; Patrick, 2000; Alberg, 2002).

(b) Pharmacokinetic studies

Pharmacokinetic studies with lutein have been performed in mice, rats, cows and humans.

Mice

In a study designed to investigate the uptake of lutein and zeaxanthin, groups of 36 BALB/c mice received diets containing an extract of marigold petals for up to 28 days (Park et al., 1998a). Based on data on food intake and body weight, intakes of lutein for each group corresponded to approximately 0, 75, 150, 300, or 600 mg/kg bw per day, while intakes of zeaxanthin were approximately 0, 1.0, 2.0, 4.0, or 8.0 mg/kg bw per day, respectively. Six mice per group were killed on days 0, 3, 7, 14, 21 or 28. Body, liver and spleen weights did not differ between the treated group throughout the experiment. Plasma uptake of lutein and zeaxanthin (analysed together in all cases) was rapid and reached a maximum (about 3 µmol/l) by day 3 of dosing (the first time-point examined after the start of dosing) and did not differ between groups thereafter. Until day 3 there was also a rapid increase in concentrations of lutein and zeaxanthin in the liver and spleen, with continued, although small, increases to day 28. The liver was considered to be the major storage organ for lutein and zeaxanthin.

Rats

The absorption, distribution and excretion and plasma kinetics of [^{14}C]lutein given as a single oral dose at 2 mg/kg bw were investigated in groups of three female RoRo SPF rats per time-point (Wendt et al., 2000). The synthetic radiolabelled compound was diluted with nonradiolabelled lutein purified from marigold petals, and was formulated as a beadlet containing an emulsion of gelatin and vegetable oil. Lutein was rapidly absorbed from the intestinal tract, resulting in peak plasma concentrations within 4 h after dosing. About 80% of the dose was recovered from the faeces and 11% from the urine within 96 h after dosing. Of the radiolabel excreted, 80% was recovered within 24 h. Low tissue concentrations of radiolabel indicated that lutein and/or its metabolites did not accumulate. With the exception of the intestinal tract, kidneys and liver, radiolabel was present in all tissues at all times at <0.01% of the administered dose. Residual radioactivity in the carcass and dissected tissues was negligible (0.23%). From the data on excretion, absorption was estimated to have been 11.3%.

The pharmacokinetics and tissue distribution of [^{14}C]lutein were investigated in groups of five male and five female Wistar rats, following a single dose of 2 or 20 mg/kg bw administered by gavage (Froescheis et al., 2001). Before administration of [^{14}C]lutein, the rats had been maintained on a diet containing 2 or 20 mg/kg bw per day nonradiolabelled lutein for 2 weeks to establish steady-state conditions. The nonradiolabelled lutein was administered in the diet as a beadlet formulation. The absorption of the [^{14}C]lutein was rapid, with peak plasma concentrations reached within 3 to 4 h at either dose. The pharmacokinetics of lutein were not linear with a tenfold increase in dose resulting in an increase of approximately twofold in the maximum plasma concentration of radioactivity. Elimination from plasma was not complete by 48 h. At 4 h after dosing, the majority of tissues had been exposed to low levels of radiolabelled lutein, maximum tissue concentrations having been reached at this time-point. Highest concentrations were found in the liver and gastrointestinal mucosa. Concentrations of lutein at 96 h after dosing were

below the limit of detection in all tissues examined except the liver. There was no evidence for accumulation of lutein in any tissue. Most radiolabelled lutein was eliminated in the faeces (>90% and >65% of the administered dose for males and females, respectively, within 48 h of dosing), with urinary and biliary excretion accounting for <6% and <2% of the administered dose. There was negligible (<0.1%) recovery of radiolabel from expired air. Excretion was slightly more prolonged in females than males. Increasing the dose had minimal effects on the absorption or the rates and routes of excretion. There were no significant differences between males and females.

Plasma and liver concentrations of lutein were assessed as part of a 4-week study of toxicity, which complied with good laboratory practice (GLP). Groups of six male and six female Wistar rats received crystalline lutein formulated as beadlets (actual doses achieved were 85–115% of target doses) at dietary doses of 0, 2, 6, 20, 60, 200, or 600 mg/kg bw per day (Buser et al., 1999; Simpson, 1999). The lutein was extracted from marigold petals. There was a dose-dependent, almost linear increase in plasma concentrations of lutein. A tenfold increase in dose between 20 and 200 mg/kg bw resulted in a two- to threefold increase in plasma concentrations. Plasma steady state conditions were reached by day 3 (plasma concentrations were below the limit of detection at the lowest dose, and there were insufficient data at the next higher dose of 6 mg/kg bw per day). Liver tissue determinations revealed a dose-dependent increase in concentrations of lutein, and suggested that saturation was reached at 200 mg/kg bw per day. There were no relevant sex differences in plasma concentrations, but the liver content of lutein was 1.5–3-fold higher in females than in males in the three groups receiving the highest dose.

The absorption of lutein was investigated in groups of 15 male weanling Fischer 344 rats maintained on diets supplemented with lutein for 8 weeks (Jenkins et al., 2000). Lutein (extracted from marigolds) was formulated with 2.2% vitamin E (α- and γ-tocopherols) in beadlets delivering lutein at a dose of 0, 2.1 4.3, 8.6, 17.8 or 34.7 mg/day. The apparent absorption of lutein was estimated to range from 28.7% at highest dose to 43.1% at the lowest dose, based on intake and the faecal excretion of lutein. The limited absorption at higher intakes was reportedly due, in part, to factors such as solubility (i.e. limited capacity for micellar incorporation). There were significantly increased plasma concentrations of lutein in animals fed the two higher doses, and increases in liver and spleen concentrations of lutein with increasing dietary intake. The relative distribution of lutein between the liver and spleen was approximately 95 and 5%, respectively, with no lutein detected in the heart, lung, kidney, testes, or brain, the only other organs examined.

Cows

Six calves were fed milk replacer for 1 week, and then were given a single oral dose of 20 mg (about 0.4 mg/kg bw) of crystalline lutein from marigold petals (containing small amounts of zeaxanthin) in olive oil (Bierer et al., 1995). The calves showed increased plasma concentrations of lutein that peaked at 12 h after dosing, declined rapidly thereafter, and levelled out at approximately 72 h.

Humans

Concentrations of lutein and zeaxanthin in serum and tissues have been shown to be quite variable (Boileau et al., 1999), but to increase, as expected, with increased intake either from dietary sources or from supplements (e.g. Hammond et al., 1997; Landrum et al., 1997a, 1997b; Carroll et al., 1999; Müller et al., 1999; Tucker et al., 1999; Berendschot et al., 2000; Johnson et al., 2000; Curran-Celantano et al., 2001; Olmedilla et al., 2001; Schalch et al., 2001; Bone et al., 2003). In a population-based study, Brady et al. (1996) reported that lower serum concentrations of lutein and zeaxanthin are generally associated with males, smoking, younger age, lower HDL cholesterol, greater consumption of ethanol and higher body mass index. Carotenoids are present in variable amounts in many tissues such as kidneys, buccal mucosal cells and adrenal glands, but the main sites of storage are adipose tissue and liver (Parker, 1996). As in serum, β-carotene, lutein and lycopene are the main carotenoids found in tissues, although β-cryptoxanthin and zeaxanthin are also present in significant amounts (Boileau et al., 1999). The eye in general, and the retina in particular, contain extraordinarily high concentrations of zeaxanthin and lutein (Bone et al., 1993). Other carotenoids are present in only trace amounts in the retina and lens (Khachik et al., 1997a, 1997b, Yeum et al., 1999, Bernstein et al., 2001). Zeaxanthin and lutein are the pigments responsible for the colouration of the macula lutea (yellow spot) (Landrum & Bone, 2001). In humans, the administration of lutein or lutein esters extracted from marigold petals at doses of 0.2 to 0.5 mg/kg bw has been shown to result in accumulation of lutein in the macula, as evidenced by an increase in the macular pigment density (Landrum et al., 1997a, 1997b; Berendschot et al., 2000; Duncan et al., 2002).

Plasma concentrations of lutein and zeaxanthin were measured in a small pilot study in groups of eight volunteers (four men and four women) after daily supplementation with capsules containing either 4.1 mg of crystalline lutein (with 0.34 mg of zeaxanthin) or 20.5 mg of lutein (with 1.7 mg of zeaxanthin) for 42 days (Cohn et al., 2001). Subjects were monitored for a further 25 days after the dosing phase. Steady-state concentrations of xanthophylls were reached between days 38 to 43 (0.06 μmol/l and 0.13 μmol/l for the lowest and highest doses, respectively). Dose-normalized incremental maximum and average steady-state concentrations of lutein and zeaxanthin were found to be comparable, indicating that they have similar bioavailability. The elimination half-life was calculated to be approximately 5–7 days for either compound.

In studies of the absorption of lutein administered as a supplement in single doses, a number of investigators have noted considerable interindividual variability in the efficiency of absorption, on the basis of plasma concentrations of lutein following supplementation (Kostic et al., 1995; Burri & Neidlinger, 2000; Torbergsen & Collins, 2000).

When absorption of lutein was measured in the triacylglycerol-rich fraction of the blood of three men and three women fed with a standard meal after an overnight fast and given lutein at a dose of 31.2 mg (about 0.4 mg/kg bw, based on the mean body weight of 75.4 kg in these individuals), peak concentrations of lutein were observed 2 h after dosing (O'Neill & Thurnham, 1998). This peak concentration occurred earlier than for β-carotene or lycopene.

Serum levels of lutein were measured in eight adult subjects (males and females) given single doses of 0.5 μmol/kg bw (about 0.3 mg/kg bw, or about 17 mg per 60 kg adult) of crystalline lutein from marigold extract in corn oil (Kostic et al., 1995). A mean peak serum concentration of about 0.7 μmol/l was reached at 16 h after dosing, followed by a moderate decline to about 50% of the peak in the subsequent 120 h, then a slow decline to baseline levels at 440 h. β-Carotene was shown to reduce the rate of absorption of lutein. Similarly, plasma concentrations of radiolabelled lutein from an algal source measured in four women given 3 mg of [^{13}C]lutein (0.05 mg/kg bw for a 60 kg adult) showed a mean peak concentration of about 0.007 μmol/l that was reached between 11 and 16 h after dosing, followed by a moderate decline to about 50% of the peak concentration in the subsequent 100 h, then a slow decline to baseline levels within about 500 h (Yao et al., 2000).

In three studies, repeated dosing of lutein from marigold petals formulated either in capsules or mixed in oil resulted in dose-dependent increases in plasma concentrations of lutein that were observed at least 7 days after dosing at 10 to 20 mg/day. In one of these studies, doses of 4 or 20 mg were administered to 16 healthy volunteers for 42 days (based on mean body weights for each group, the doses were about 0.1 and about 0.3 mg/kg bw per day, respectively) (Schalch et al., 2001). Plasma concentrations of lutein were observed to be about three and about eight times greater than those in untreated controls. Plasma concentrations were nearly at baseline levels 25 days after the end of dosing for the group receiving the lower dose, but were still noticeably greater than baseline in the group receiving the higher dose. At the lower dose, subjects continued to show increasing plasma concentrations of lutein up to day 42, but many subjects at the higher dose showed peak concentrations at earlier time-points. Similarly, three males receiving doses of 10 mg lutein/day for a total of 18 days (about 0.2 mg/kg bw per day based on a 60 kg adult) showed four- to fivefold increases in plasma concentrations of lutein compared with baseline by day 7 of dosing (Khachik et al., 1995a). In another study, five male and three female subjects received 15 mg of lutein/day for 7 days (about 0.25 mg/kg bw per day, based on a 60 kg adult) (Torbergsen & Collins, 2000). The lutein was in capsule form and contained 80% all-*trans*-lutein and 20% *cis*-luteins. By day 7, plasma concentrations of lutein had increased two- to threefold compared with baseline levels, decreasing to near-baseline levels after 3 weeks of wash-out.

The kinetics of carotenoid depletion and elimination have been investigated in 19 healthy women (Burri et al., 2001) and 12 healthy men (Rock et al., 1992) fed controlled low-carotenoid diets for approximately 10 and 13 weeks, respectively. In females, the decline in serum concentrations of all carotenoids (including lutein and zeaxanthin) followed apparent first-order kinetics, and approached a steady-state level after about 30 days, indicating an elimination half-life of about 6 days. The authors analysed these data and derived half-lives for lutein and zeaxanthin of 76 and 38 days, respectively (Burri et al., 2001). In males, there were significant decreases in concentrations of all carotenoids, including lutein and zeaxanthin, up to days 14–15, followed by a slower decline to days 63–64 that may be indicative of two pools, with one pool having a more rapid rate of turnover. Concentrations of lutein and zeaxanthin in the final sample of plasma (days 63–64) averaged 40%

of the initial concentration, and the mean plasma depletion half-life for lutein and zeaxanthin (combined) was estimated by the authors to be between 33 and 61 days.

In a study of 41 patients with biliary and pancreatic diseases and 14 healthy controls, there was a non-statistically significant trend toward decreased concentrations of lutein in plasma and bile in diseased patients compared with controls (Leo et al., 1995). It is clear, however, that carotenoids, including lutein/zeaxanthin, undergo appreciable biliary secretion. In addition, it was noted that biliary concentrations of carotenoids reflect plasma concentrations in both normal and pathological conditions. Interference with biliary secretion did not result in plasma retention of carotenoids.

2.1.2 Biotransformation

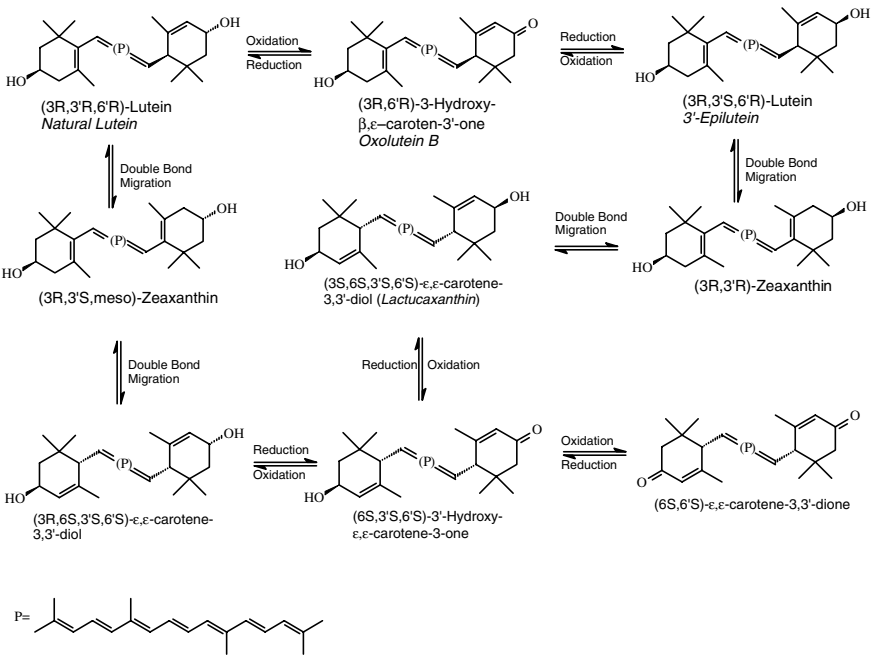
A number of compounds derived from lutein and zeaxanthin have been identified in human serum (Figure 3) (reviewed by Khachik et al., 1995a). These are called 'metabolites', but they are undoubtedly formed by chemical rather than enzymic reactions. The metabolites result principally from three types of reactions involving the end groups of these carotenoids — oxidation, reduction and double-bond migration (Figure 3). Lutein and zeaxanthin can exist in an equilibrium involving the intermediate carotenoid 3′-epilutein. Allylic oxidation of lutein at C3 results in the formation of oxolutein B that can exist in equilibrium with lutein and 3′epilutein through reduction reactions. 3′-Epilutein and zeaxanthin can also exist in equilibrium through reversible double-bond migration. Thus, presence of 3′-epilutein in human serum may be due to conversion of lutein and/or zeaxanthin. Acid-catalysed dehydration is another reaction of carotenoids with 3-hydroxy-ε end groups. Lutein is believed to undergo dehydration in stomach acid to form 3-hydroxy-3′,4′-didehydro-β,γ-carotene and 3-hydroxy-2′,3′-didehydro- β,ε-carotene (anhydroluteins) that have been isolated from serum. In addition to their presence in human serum, these metabolites have also been detected in breast milk as well as retinal extracts (Khachik et al., 1995b; Khachik et al., 1997a, 1997b, 1997c). The toxicological importance of these compounds is not known.

2.1.3 Effects on enzymes and other biochemical parameters

The xanthophylls are precursors of retinol. Indeed, they have been shown to have little or no activity as substrates of β-carotene-15,15′-dioxygenase, although they are able to inhibit the conversion of β-carotene to retinol (Ershov et al., 1993; van Vliet et al., 1996; Grolier et al., 1997). However, in a rat model, Weiser & Korman (1993) showed that the xanthophylls have small but significant provitamin A activity (4–5% of the activity of β-carotene), probably via a vitamin A-sparing effect. Furthermore, Weiser & Korman reported that dietary zeaxanthin is able to induce duodenal 15,15′-dioxygenase activity in 1-day-old chicks.

The effects of lutein (oleoresin extracted from marigold petals) on cytochrome P450 and glutathione-*S*-transferase enzyme activity in the liver, lung, kidney, and small intestine were measured in male Wistar rats after 16 days of dietary supplementation (Jewell & O'Brien, 1999). Groups of eight animals received (i) a basal

Figure 3. Proposed reactions of lutein and zeaxanthin in humans



Adapted from Khachik et al. (1997a)

diet that had not been supplemented with lutein (negative controls); or (ii) a basal diet supplemented with lutein to provide a dose of approximately 45 mg/kg bw per day; or (iii) a basal diet supplemented with 3-methylcholanthrene (3-MC) (positive controls). There were no changes in feed intake, body-weight gains, or organ weights after dietary supplementation with lutein. As expected, lutein content was increased in the tissues examined. However, cytochrome CYP450 activities — ethoxyresorufin-*O*-deethylation (EROD), methoxyresorufin-*O*-demethylation (MROD), benzyloxyresorufin-*O*-dearylation (BROD) and pentoxyresorufin-*O*-dependentylation (PROD) — were not induced in the liver, kidneys, or lungs after supplementation with lutein, and BROD activity was significantly decreased in the lung. CYP450 enzyme activities were undetectable in the small intestine. Supplementation with lutein had no effect on glutathione-*S*-transferase activity or glutathione levels in any of the tissues examined.

In another study, the effects of dietary exposure to lutein (extracted from marigold petals) on xenobiotic metabolizing enzymes in the liver were investigated in male SPF Wistar rats (Gradelet et al., 1996). Two groups of six animals received diets containing corn oil (control) or 10% lutein oleoresin (mixed with corn oil) at 300 mg/kg of diet for 15 days. Based on reference body weight and data on feed intake (Blackburn, 1988), this corresponded to a dose of lutein of approximately

2.8 mg/kg bw per day. Total microsomal P450 and associated cytochrome *c* reductase activities, as well as EROD, MROD, PROD, BROD, erythromycin *N*-demethylase (ERDM) and *N*-nitrosodimethylamine *N*-demethylase (NDMAD), *p*-nitrophenol- and 4-hydroxybiphenyl UDP glucuronosyl transferases (4NP-UGT and 4-HBT-UGT), and total cytosolic glutathione-*S*-transferase activity were measured. There were no changes in feed intake, body weights, or organ weights throughout the 15-day feeding period. Lutein was present in liver microsomes (approximately 0.3 nmol/mg of protein), but did not induce any significant changes in any of the phase I or phase II enzymes.

In humans, plasma concentrations of lutein have been negatively associated with the activity of CYP1A2 (caffeine test), a liver enzyme involved in the metabolic activation of a number of human carcinogens (Le Marchand et al., 1997).

2.2 Toxicological studies

2.2.1 Acute toxicity

In a study of acute toxicity, which complied with GLP, groups of three female or three male HanIbm:WIST rats were given lutein (purified plant extract containing 70–85% lutein) at a dose of 2000 mg/kg bw by oral gavage in a polyethylene glycol (PEG 300) vehicle. No deaths occurred during the study and no other treatment-related effects were observed. On the basis of the lack of lethality observed in this study, the median lethal dose (LD_{50}) for orally-administered lutein in rats was estimated to be >2000 mg/kg bw (Pfannkuch et al., 1999).

2.2.2 Short-term toxicity

Mice

In a study designed to investigate the absorption of dietary lutein, groups of 36 BALB/c mice were given diets containing lutein esters from marigold extract (37% lutein esters and 0.5% zeaxanthin esters) for up to 28 days. Based on reported food intake and body weight data, daily intakes of lutein corresponded to approximately 0, 75, 150, 300, or 600 mg/kg bw respectively, and, taking into account the composition of the marigold extract, daily intakes of zeaxanthin corresponded to approximately 0, 1.0, 2.0, 4.0, or 8.0 mg/kg bw, respectively. Six mice per group were killed on days 0, 3, 7, 14, 21 and 28. No differences in body, liver, or spleen weights among treatment groups were reported, and there were no significant differences in feed intake throughout the experimental period (Park et al., 1998a).

Rats

In a study of oral toxicity, which complied with GLP, groups of six male and six female Wistar rats were given diets containing lutein from marigold petals at target doses of 0, 2, 6, 20, 60, 200, or 600 mg/kg bw per day in a beadlet formulation containing gelatin and vegetable oil for 28 days. Actual doses based on food consumption were 85–115% of the target doses. Furthermore, on the basis of accompanying analyses of plasma concentrations at weeks 2 and 4 and determinations

of lutein content in the liver at termination, which showed dose-dependent increases in concentrations of lutein, it was considered that rats had been adequately exposed to lutein in this study (Buser et al., 1999). No exposure-related mortality was reported. There were no effects of dietary lutein on body weights, feed consumption, haematology, clinical chemistry parameters, organ weights (selected organs), or gross pathology at necropsy. Histopathological findings were limited to histiocyte foci in the mesenteric lymph nodes of some animals (particularly females) at the highest dose. The foci were characterized as discrete subcapsular aggregations of macrophages with abundant cytoplasm containing varying levels of red granular material. These foci were considered to be related to physiological uptake of lutein, and were not considered by the investigators to be reflective of specific target organ toxicity. The mesenteric lymph nodes from animals in the other treated groups were comparable to those of animals in the control group. The no-observed-effect level (NOEL) in this 28-day study in rats was 600 mg/kg bw per day, the highest dose tested (Simpson, 1999).

The oral toxicity of lutein was evaluated in Wistar rats in a 13-week study that complied with GLP. Rats received target doses of lutein (from marigold petals containing about 79 and 5% lutein and zeaxanthin, respectively) of 0, 2, 20, or 200 mg/kg bw per day formulated as beadlets incorporated into the diet. Actual doses were 99–101% of target doses for placebo and treated animals. Background concentrations of lutein and zeaxanthin in the diet were 0.5–1.4 and 0.2–0.4 mg/kg, respectively. There were 10 animals of each sex per group at the lowest and intermediate doses, and 15 animals of each sex per group for the control group and at the highest dose, owing to the inclusion of 5 animals of each sex per group for a 4-week recovery phase. There were reported to be no treatment-related deaths, clinical signs, or adverse effects. There were no significant changes in body-weight gain, feed intake, or haematology or clinical chemistry parameters. A battery of tests for neurotoxicity and ophthalmoscopic observations did not indicate any changes attributable to treatment. Mean absolute organ weights were not different between treatment and control groups in either the main study or in the animals in the recovery phase. There were no treatment-related, biologically significant macroscopic or microscopic abnormalities reported in any of the animals, except for occasional incidences of vacuolation in the liver and tubular degeneration/regeneration in the kidney of some control and treated females. The incidence and severity of these lesions in treated females was not dose-related, and they were considered to be associated with blood sampling. Analyses of blood samples showed the presence of lutein (and zeaxanthin) at dose-related concentrations in the plasma and liver, and it was considered that animals were adequately exposed to the xanthophylls under the conditions of the study. The NOEL in this 13-week study in rats was 200 mg/kg bw per day (208 mg of lutein + zeaxanthin/kg bw per day), the highest dose tested (Pfannkuch et al., 2000a; Pfannkuch et al., 2001; Kruger et al., 2002).

Monkeys

In a study that complied with GLP and that was designed primarily to investigate the ocular effects of long-term exposure to lutein, cynomologus monkeys

aged 4–7 years were given lutein (extracted from marigold petals) at doses of 0, 0.2, or 20 mg/kg bw per day by gavage for 52 weeks. The solutions for gavage were prepared by dispersing beadlets in water. The control was prepared using beadlets that contained no lutein. There were two males and two females in each group, with an additional male and female included in the group receiving the highest dose, which were designated for examination at 6 months. All animals survived the treatment period. All animals at 20 mg/kg per day showed orange/yellow discolouration of the faeces from day 2 of the study onwards (attributed to treatment with lutein). There was no effect on overall mean body-weight gain and on overall group mean feed intake in either of the treatment groups. There were no treatment-related changes in haematology, blood chemistry, or urine analysis measurements. There were no changes in data on electrocardiogram waveform or blood pressure that could be regarded as being related to the administration of lutein. There were no treatment-related organ weight changes. Most of the animals showed dark yellow-coloured mesenteric fat at interim sacrifice and golden yellow mesenteric fat at terminal sacrifice (attributed to treatment with lutein). Histopathological examinations revealed no treatment-related findings. Comprehensive ophthalmic examinations showed no evidence of adverse changes related to treatment. Animals showed a dose-related increase in plasma and liver concentrations of lutein. Lutein was thus considered to be well tolerated. The NOEL in this 52-week study in cynomologus monkeys was 20 mg/kg bw per day, the highest dose tested (Pfannkuch et al., 2000b, 2000c; Pfannkuch, 2001).

2.2.3 Long-term studies of toxicity and carcinogenicity

No experimental lifetime bioassays or studies of carcinogenicity have been conducted with lutein. However, lutein has been reported to have chemopreventive effects in a number of models of tumours in mice and rats (reviewed by Nishino et al., 2000). The dosing period in all these studies was of shorter duration than that in the 13-week study of oral toxicity in rats, described above.

The chemopreventive effects of lutein have been examined in models of tumours in mice and rats given lutein in the diet or by gavage, respectively. In $B6C3F_1$ mice initiated with the colon carcinogen 1,2-dimethylhydrazine and then given diets containing 0.05% lutein (purity, 95%) for 8 weeks, the number of putative preneoplastic aberrant crypt foci was significantly decreased compared with mice not given lutein (Kim et al., 1998).

In another study on formation of aberrant crypt foci, Sprague-Dawley rats were given three intrarectal doses of methylnitrosourea in week 1 and lutein (unspecified source) at a daily dose of 6, 1.2, or 0.24 mg by gavage during weeks 2 and 5. The formation of aberrant crypt foci was significantly reduced at all doses (Narisawa et al., 1996).

In a model of breast cancer in which BALB/c mice were inoculated with mouse mammary tumour cells, then given diets containing lutein esters from marigold petals for 70 days, the incidence of tumours was decreased and tumour latency was increased in animals fed with lutein at low levels (0.002 or 0.02%) while higher levels (0.2 or 0.4%) were less effective (Park et al., 1998b).

In a similar experiment using a transplantable murine mammary tumour, it was demonstrated that mice consuming diets containing lutein esters from marigold petals at a level of 0.1 or 0.4% for 3 weeks had significantly fewer tumours and increased tumour latency than did controls, and the effect was dose-dependent (Chew et al., 1996).

In all these studies, lutein showed no effect on body weight or feed intake. One study showed that lutein did not affect spleen weights (Park et al., 1998b), although decreased liver weights without accompanying histopathological changes were reported in another study (Kim et al., 1998).

2.2.4 Genotoxicity

A number of studies that evaluated the potential genotoxicity of lutein in vitro and in vivo are summarized in Table 1. In these studies, there was no evidence of genotoxicity. The concentrations and doses in some of the studies were considered to be low, but the maximum feasible doses were used.

In addition to the tests reported in Table 1, Lutein was evaluated for mutagenic activity in (Ames) assays for reverse mutation using both the plate incorporation and the preincubation methods (Kruger et al., 2002). *Salmonella typhimurium* strains TA1535, TA97, TA98, TA100, and TA102, with and without metabolic activation (S9 fraction from rat liver) were used. The dose range of lutein (from marigold petals containing 79% lutein and 5% zeaxanthin) tested was 15.8–1580 μg/plate in the preincubation and the plate incorporation assays. In addition, lutein in a beadlet formulation (10%) was tested at concentrations of 158–15 800 μg/plate in both the plate incorporation and preincubation assays. No increases in the number of mutant colonies were observed for any of the five tester strains after treatment with lutein or lutein as a 10% beadlet, demonstrating that neither formulation is mutagenic in *S. typhimurium* strains. Positive controls verified the sensitivity of the strains and the activity of the S9 mix. No cytotoxic effects as evidenced by a reduction of background colony numbers were apparent for any of the strains with the possible exception of TA102 in the absence of the metabolic activation system. However, it should be noted that precipitation of the test compound frequently occurred at concentrations of 50 μg/plate or higher. Thus, the 10% beadlet formulation was not evaluated at the highest concentration of 15 800 μg/plate due to precipitation and the 1580 μg/plate dose was discarded for the plate incorporation test plates.

The ability of lutein to modulate the genotoxic effects of known mutagens has also been investigated in various studies that are summarized in Table 2.

It is clear from Table 2 that lutein inhibits the activity of known mutagens, both in vitro (bacterial test systems), and in vivo (micronucleus assay). Rauscher et al. (1998) proposed that the antimutagenic effects of lutein may be caused by inhibition of metabolic activation of the mutagens.

Table 1. Studies of genotoxicity with lutein

End-point	Test system	Concentration/dose	Results	Reference
In vitro				
Bacterial reverse mutation (Ames) assay with metabolic activation from S9	*S. typhimurium* TA98	0.1 ml of diluted eggplant fruit juice extracts containing lutein in the methanol layer	Negative	Yoshikawa et al. (1996)
Bacterial reverse mutation (Ames) assay with metabolic activation from S9	*S. typhimurium* TA98 and TA100 Human peripheral blood lymphocytes	25, 50, 75, or 100 µl of solvent extracts from fruits and vegetables containing lutein; isolated lutein also tested	Negative	Rauscher et al. (1998)
Chromosome aberration		10% lutein in a beadlet formulation: 3.9–125 µg/ml for 3 or 24 h in the absence of metabolic activation, or for 3 and 5 h in the presence of microsomes from phenobarbital- and 5,6-benzoflavone-ore-treated rats, respectively	Negative	Chételat et al. (2002a)
In vivo				
Micronucleus formation	Male NMRI mice	180 mg/kg bw per os	Negative	Rauscher et al. (1998)
Micronucleus formation	Rat	45, 89, or 178 mg 10% lutein beadlets/kg bw on 2 consecutive days	Negative	Chételat et al. (2002b)
Comet assay	Human lymphocytes	Subjects from whom lymphocytes were obtained had consumed 15 mg supplemental lutein/day for 12 weeks with measurement at 16 weeks	Negative	Collins et al. (1998)

S9, 9000 × *g* supernatant from rat liver.

Table 2. Summary of studies evaluating the effect of lutein on modulation of induced genotoxic effects

End-point	Test system	Concentration/dose	Genotoxicant	Results	Reference
In vitro					
Reverse mutation	*Salmonella typhimurium* (TA98)	0.005, 0.01, 0.05, 0.1, and 0.2 ml/plate (lutein-rich methanol extract of eggplant)	Trp-P-2	Inhibitory	Yoshikawaet al. (1996)
Reverse mutation	*S. typhimurium* (TA98, TA100)	38, 40 nmol of lutein/ml	AFB_1, BaP or CP (TA100), or IQ (TA98)	Inhibitory for AFB_1 and BaP	Rauscher et al. (1998)
Reverse mutation	*S. typhimurium* (tester strain YG1024)	0.0, 0.02, 0.09,0.20, 0.90, 2.00, or 10.00 μg of lutein/plate (isolated from Aztec Marigold)	AFB_1	Inhibitory	González de Mejía et al. (1997a)
Reverse mutation	*S. typhimurium* (tester strain YG1024)	0.002, 0.02, 0.20, 2.00, or 10.00 μg of lutein/plate (isolated from Aztec Marigold)	1-nitro-pyrene	Inhibitory	González de Mejía et al. (1997b)
Gene expression	*S. typhimurium* (TA1535/pSK 1002)	2 or 20 μg of lutein/ml (extracted from red alga, *Porphyra tenera*)	Trp-P-2	Inhibitory	Okai et al. (1996)
In vivo					
Micronucleus formation (chromosome aberration)	Mouse bone-marrow cells	180 mg of lutein/kg bw (single oral gavage dose administered to male NMRI mice)	BaP and CP	Inhibitory	Rauscher et al. (1998)
Comet assay (DNA strand breakage)	Human lymphocytes	15 mg of lutein/day for 1 week (encapsulated lutein)	H_2O_2 (exposure in vitro)	No effect	Torbergsen & Collins (2000)

AFB_1, aflatoxin B_1; BaP, benzo[*a*]pyrene; CP, cyclophosphamide; H_2O_2, hydrogen peroxide; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; Trp-P-2, 3-amino-1-methyl-5*H*-pyridol[4,3-*b*]indole.

2.2.5 Reproductive toxicity

(a) Multigeneration studies

No multigeneration studies were available.

(b) Developmental toxicity

In a GLP-compliant study of developmental toxicity, three groups of female Sprague-Dawley rats (mated when they were aged 10–13 weeks) were given diets mixed with beadlets containing 10% lutein (from marigold extract; 79% lutein, 5% zeaxanthin) from day 6 to day 20 of gestation (Edwards et al., 2002). The target doses of lutein were 250, 500, and 1000 mg/kg bw per day, while the actual doses administered as measured from data on dietary intake were 252, 535, and 1118 mg/kg bw per day. A fourth group of 25 mated females received diet containing placebo beadlets (no zeaxanthin or lutein). Animals at the lowest and intermediate doses received placebo beadlets in addition to the beadlets containing lutein, to ensure similarity in the total concentration of beadlets received by all treatment groups. There was no evidence of an effect of lutein in the dams, but there was an inverse dose-related reduction in food consumption and in both maternal and fetal body weight at the lowest and intermediate doses. Study investigators attributed these findings to decreased palatability of the diet at these doses (the lowest dose would have contained the highest quantity of the placebo beadlets which were considered to be less palatable than the beadlets containing lutein). In keeping with this hypothesis, maternal and fetal body weights in the group receiving the highest dose (no placebo beadlets) were comparable to those for historical controls. There were no effects on pre- or postimplantation, embryo-fetal survival, or sex ratios.

Fetuses were examined for visceral and skeletal abnormalities and soft tissue changes. There was an inverse dose-related increase in forms of reduced ossification, but the degree of ossification in the fetuses at the highest dose was similar to that for historical controls. These findings were considered to be in keeping with the maternal findings of decreased food consumption in the control group and at the lowest dose.

There were no adverse effects of treatment with lutein on the incidence of external or skeletal abnormalities in any group. Minor visceral abnormalities were observed in one or two fetuses in each of the treated groups, but the incidence of these changes was similar to that for historical controls and was therefore not considered to be treatment-related. There was a slight, dose-related increase in the incidence of rudimentary extra lumbar ribs in the groups receiving the intermediate and highest doses. However, these findings were not considered to be of toxicological significance owing to the known reversibility of this minor skeletal finding. Analyses of blood samples showed dose-dependent increases in mean total plasma concentrations of lutein on days 7 and 16 of gestation. Mean plasma concentrations were approximately 80% higher on day 16 of gestation than on day 7. These data indicate that animals were adequately exposed to lutein throughout the experimental period. The NOEL in this study of embryotoxicity/teratogenicity in rats was 1000 mg/kg bw per day, the highest dose tested.

2.2.6 Special studies

(a) Cardiovascular effects

The effect of supplementation with crystalline lutein (0.2% by weight in the diet) on atherosclerotic lesion formation has been assessed in apolipoprotein E (ApoE)-null mice and low-density lipoprotein (LDL) receptor-null mice (Dwyer et al., 2001). Groups of 10 females of each strain received either diet supplemented with lutein or basal diet for 8 weeks. Based on reported data on body weight and reference intake (Blackburn, 1988), the administered dose of lutein corresponded to approximately 500 mg/kg bw per day. Supplementation with lutein was well tolerated, and there were no adverse effects on body-weight gain throughout the 8 weeks of exposure. Supplementation with lutein significantly reduced the size of atherosclerotic lesions in ApoE- and LDL receptor-null mice, and significantly reduced the level of lipid hydroperoxides, the levels of very-low-density lipoprotein (VLDL) plus intermediate-density lipoprotein (IDL), and lysis of erythrocytes in ApoE-null mice. No significant changes were observed in LDL or HDL levels. On the basis of these findings, it was concluded that increased intake of lutein protected against the progress of early atherosclerosis, possibly via an pathway involving antioxidants.

(b) Immune responses

Mice

The effects of lutein on mitogen-induced lymphoproliferation, cytotoxicity, and interleukin-2 (IL-2) production) were investigated in BALB/c mice given diets containing 0.1 or 0.4% lutein esters from marigold extracts (37% lutein esters and 0.5% zeaxanthin esters) for 2 or 4 weeks (Chew et al., 1996). Based on reported data on body weight and feed intake, as well as the composition of the marigold extract, these doses corresponded to approximately 200 and 803 mg/kg bw per day for lutein, and 2.7 and 10.9 mg/kg bw per day for zeaxanthin. No significant treatment-related differences in body-weight gain or feed intake were reported. Dietary lutein enhanced phytohaemagglutinin-induced lymphocyte proliferation, but had no effect on IL-2 production or lymphocyte cytotoxicity.

The effects of lutein on antibody production were investigated in groups of 8–12 young C57B/6J mice primed with T-dependent antigens (sheep erythrocytes) (Jyonouchi et al., 1994). Mice were injected intraperitoneally with 0.5 ml of lutein in RPMI cell culture medium supplemented with calf serum (10^{-6} mol/l; purity, >97%; 5×10^{-10} mol/mouse) 1 h before priming, and were euthanized 5 days later. Based on reference data on body weight (Blackburn, 1988), this corresponded to a dose of lutein of approximately 31.5 μg/kg bw. Spleen cells were isolated and prepared for the plaque formation cell assay, and spleen weights and cell numbers were recorded. Exposure to lutein via intraperioneal injection had no effect on spleen weights. However, compared with control animals, mice exposed to lutein had significantly enhanced plaque formation (i.e. enhanced antibody formation), and a significantly increased number of immunoglobulin M (Ig-M)- and Ig-G-secreting cells, suggesting that lutein may modulate humoral immune responses to T-dependent antigens.

The possible effects of lutein on the expression of the *pim-1* gene, which is involved in early activation of T cells and cells of other lineages, were investigated in BALB/c mice fed diets containing 0, 0.02, or 0.4% lutein for 14 days (Park et al., 1999). Based on reference data on body weight and feed intake (Blackburn, 1988), lutein was received at a dose of approximately 0, 40, or 780 mg/kg bw per day, respectively. Analyses of samples of plasma and liver collected after 14 days revealed dose-dependent increases in concentrations of lutein. No external signs of toxicity were noted in any of the treated mice. Splenocytes isolated from mice fed with lutein were cultured in the presence of concanavalin A (Con A), showed a dose-dependent increase in steady-state levels of *pim-1* mRNA. This is a potential mechanism through which lutein may modulate immune function.

Cats and dogs

In similar experiments, the effects of diets containing lutein (crystalline, from marigold petals containing about 77% lutein and about 5% zeaxanthin) on humoral and cell-mediated immune responses were investigated in female tabby cats (Kim et al., 2000a) and female beagle dogs (Kim et al., 2000b). In each study, animals (56 per species) received basal diets supplemented with lutein at 0, 1 (cats only), 5, 10, or 20 (dogs only) mg/day for 12 weeks. In the cats, these intakes corresponded to doses of lutein of approximately 0, 0.7, 3.5, or 7.1 mg/kg bw per day, and of zeaxanthin of approximately 0, 0.05, 0.25, and 0.5 mg/kg bw per day. In dogs, these intakes corresponded to doses of lutein of 0, 0.4, 0.9, or 1.75 mg/kg bw per day, and of zeaxanthin of 0, 0.03, 0.06, and 0.12 mg/kg bw per day. In dogs only, blood was collected from weeks 13 to 17 to determine changes in plasma concentrations of immunoglobulin following second and third challenges. Dietary supplementation with lutein increased plasma concentrations of lutein in each experimental animal model, but did not significantly affect changes in body weights. In cats, there was a significant dose-related increase in delayed-type hypersensitivity response to vaccine, but not to concanavalin A (Con A), and significantly enhanced Con A- and pokeweed mitogen (PWM)-stimulated proliferation of peripheral blood mononuclear cells. At the highest dose (10 mg/day), the percentages of $CD4^+$ and $CD21^+$ cells were significantly elevated at week 12, and in the groups fed lutein at 1 and 10 mg/day, concentrations of IgG were significantly higher from weeks 8 to 12. In dogs, supplementation with lutein significantly increased the delayed-type hypersensitivity response to vaccine and phytohaemagglutinin (PHA), and significantly increased mitogen (PHA, Con A, and PWM)-stimulated proliferation of peripheral blood mononuclear cells. The percentages of cells expressing CD5, CD4, CD8 and major histocompatibility complex class II molecules were significantly increased, and the production of IgG significantly increased after the second antigenic challenge. There were no differences in IL-2 production in cats or dogs throughout the experimental periods. These results suggest that dietary lutein stimulated both cell-mediated and humoral immune responses in cats and dogs.

(c) Ocular toxicity

The long-term ingestion of canthaxanthin at high doses has been shown to lead to accumulation and crystallization in the retina of humans (Arden & Barker, 1991) and monkeys (Goralczyk et al., 1997), and the question has therefore arisen as to whether lutein behaves similarly. The effects of lutein on the eye were investigated in a GLP-compliant study in cynomolgus monkeys treated via oral gavage with daily doses of lutein (from marigold petals) as a 10% beadlet formulation for 52 weeks (Pfannkuch et al., 2000c; Pfannkuch, 2001). The cynomolgus monkey was chosen since it was shown to be an excellent model for investigating the induction and dose-dependency of carotenoid crystal formation in the retina (Goralczyk et al., 1997; Goralczyk, 2000; Goralczyk et al., 2002). Groups of two monkeys of each sex were given lutein at a dose of 0 (placebo beadlet), 0.2, or 20 mg/kg bw per day; an additional male and female were included in the group receiving the highest dose. One male and one female at the highest dose were killed after 26 weeks (6 months). Occasional retinal changes, such as inclusions in the macula, were observed in some groups of animals, including controls, and were considered to be unrelated to treatment. Overall, comprehensive ophthalmic examinations (ophthalmoscopy and biomicroscopy examinations, fundus photography, electroretinography (considered to be a very sensitive procedure to detect early signs of generalized retinal degeneration), and post-mortem examinations of the right retina (including macroscopic inspection, microscopic pathology under polarized and bright light for peripheral retina and macula, confocal microscopy of the macula and histopathological examination of the peripheral retinal) showed no evidence of treatment-related adverse changes, including no evidence for formation of crystals in the eyes during or after 52 weeks of treatment with lutein. Dose-dependent increases in concentrations of lutein were reported in the peripheral retina. In the central retina and lens, lutein content was markedly increased in animals at the highest dose, but there was no evidence for crystalline deposits. It was concluded that cynomolgus monkeys treated with lutein for 52 weeks in at doses of 0.2 and 20 mg/kg bw per day did not show toxic effects on the eye.

(d) Dermal and ocular irritation

Crystalline lutein (76%) extracted from marigold petals was tested in a study of primary skin irritation in three adult New Zealand white rabbits (Csato & Braun, 1999). The primary irritation score for lutein was 0.00 (maximum potential score is 8.0) and it was classified as ‘not irritating’ to rabbit skin in this study, which complied with GLP.

Crystalline lutein (76%) extracted from marigold petals was also tested in a study of primary eye irritation in three adult New Zealand white rabbits (Csato & Arcelin, 1999). The primary irritation score for lutein was 0.11 (maximum potential score is 13.0) and it was classified as ‘not irritating’ to the rabbit eye in this study, which complied with GLP.

2.3 Observations in humans

2.3.1 Clinical studies

There have been a number of studies designed to investigate the pharmacokinetics of lutein and zeaxanthin that did not necessarily include safety end-points, but also did not report any adverse effects of the xanthophylls (see sections 2.1.1 and 2.1.2). Furthermore, a relatively large number of studies in humans has examined correlations between dietary intake of lutein or zeaxanthin, the effects of dietary supplements, or serum concentrations of lutein or zeaxanthin and the incidence of age-related macular degeneration, macular pigment density or cataractogenesis with varying results (Eye Disease Case–Control Study Group, 1993; Seddon et al., 1994; Mares-Perlman et al., 1995a, 1995b; Khachik et al., 1997b; Beatty et al., 1999; Chasan-Taber et al., 1999; Lyle et al., 1999a, 1999b; Pratt, 1999; Richer, 1999; Bone et al., 2000; Johnson et al., 2000; Gale et al., 2001; Schalch et al., 2001; Bone et al., 2003; Gale et al., 2003). These studies will not be reviewed here, but in many cases, weak inverse associations were found, although it is apparent that the protective effect of lutein or zeaxanthin against age-related macular degeneration or cataract formation remains unproven. Of importance here, however, is that none of these studies reported adverse effects of lutein/zeaxanthin, including ocular toxicity, although in some cases diets or supplements containing lutein/zeaxanthin at high concentrations were consumed.

A few other clinical studies have been performed that are relevant to the safety evaluation of lutein in humans.

In a multicentre trial, 90 healthy volunteers were given lutein (mixed ester forms extracted from marigold petals) at 15 mg/day, corresponding to a dose of 0.25 mg/kg bw per day, for 20 weeks (Olmedilla et al., 2002). After 20 weeks, there was an elevation in serum concentrations of lutein (about fivefold) and serum zeaxanthin (about twofold). Blood samples taken after fasting (collected from a subpopulation of approximately 16 male and female Spanish subjects at baseline, each month during the supplementation period, and 3 months after supplementation) showed no changes in haematological or biochemical parameters or in total cholesterol, HDL-cholesterol, or LDL-cholesterol levels. Carotenodermia, a yellowish discolouration of the skin that is considered to be a harmless, reversible effect of high intake of carotenoids (Institute of Medicine, 2000), was observed in 40% of the Spanish cohort following the intervention period. Carotenodermia was not observed in the other cohorts from the Netherlands, Northern Ireland, or the Republic of Ireland (Olmedilla et al., 1997; Granado et al., 1998; Olmedilla et al., 2001).

In a study of the effects of 2-week exposures to the different carotenoids, including lutein, from foods (tomato juice, carrot juice, spinach) (Müller et al., 1999), it was found that daily ingestion of 11.3 mg of lutein in a liquid spinach powder preparation administered daily with meals was well tolerated by all subjects (23 healthy volunteers). There were no significant changes in blood concentration of haemoglobin, leukocytes, or serum electrolytes (sodium, potassium, chloride).

In a study designed to examine the effect of the food matrix on the bioavailability of carotenoids, measurements of serum concentrations of cholesterol and triacylglycerol were included (Castenmiller et al., 1999). After administration of diets containing crystalline lutein from marigold petal extracts (suspension in vegetable oil with β-carotene) at 6.6 mg/day for 3 weeks, no significant differences in serum concentrations of cholesterol or triacylglycerol were reported in non-obese, nonsmoking, normolipidaemic men and women (aged 18–58 years) compared with values for controls.

In a double-blind, parallel, placebo-controlled intervention study to investigate the effects of significant elevations in plasma concentrations of lutein on fasting plasma fatty acid profiles, healthy nonsmoking males received daily supplementation with lutein (lutein-rich marigold extract, encapsulated) at 15 mg/day (10 subjects) or placebo (encapsulated corn oil) (11 subjects) for 26 days (Wright et al., 1999). Blood samples were taken before treatment (baseline) and on day 28 for analysis of concentrations of long-chain fatty acid. Supplementation with lutein for 4 weeks had no effect on individual fatty acids (14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 18:3, 20:3, 20:4, 20:5, 22:6), total fatty acids, total saturated (S), total unsaturated (U), monounsaturated (M), or polyunsaturated (P) fatty acids, or on ratios of U:S, P:S, and M:S fatty acids.

2.3.2 Epidemiological studies

Most epidemiological studies on xanthophylls have addressed the hypothesis that intake of these compounds is inversely related to development of cancer. A number of such studies has suggested that dietary xanthophylls may protect against the development of a variety of cancers including those of the oesophagus, colon, breast, prostate and lung (e.g. Le Marchand et al., 1995; Freudenheim et al., 1996; Zhang et al., 1997; Franceschi et al., 2000; Levi et al., 2000; Lu et al., 2001; Nkondjock & Ghadirian, 2004), although recent studies on breast and lung cancer have indicated that these compounds are not protective (Terry et al., 2002; Mannisto et al., 2004).

A recent large prospective study examined the relationship between serum concentrations of carotenoids and subsequent risk of developing cancers of the stomach and upper digestive tract in a region of China with epidemic rates of oesophageal and gastric cancer (Abnet et al., 2003). There was an association between the incidence of gastric non-cardia cancer and the serum concentrations of lutein/zeaxanthin derived from normal dietary sources. These observations, however, are only correlative and, indeed, the results of a Dutch cohort study have suggested that dietary intake of lutein/zeaxanthin is not associated with risk of gastric cancer (pathological type not specified), although intakes of retinal and β-carotene were positively associated with risk of this cancer (Botterweck et al., 2000).

In view of the structural similarities between xanthophylls and β-carotene, the Committee considered the outcome of two trials that showed that supplementation with β-carotene increases risk of lung cancer in heavy smokers; one study involved the administration of β-carotene at 30 mg/day plus 25 000 IU of retinyl palmitate in

18 314 smokers, former smokers and workers exposed to asbestos (Omenn et al., 1996), while in the second study, β-carotene at 20 mg/day with or without 50 mg of α-tocopherol was given to 29 133 male smokers (The α-Tocopherol and β-Carotene Cancer Prevention Study Group, 1994). However, in the light of the negative results in studies of genotoxicity and the absence of tumour-promoting activity of lutein, it was considered that these intervention studies with β-carotene were not appropriate for the risk assessment for lutein.

The results of a number of epidemiological studies, including descriptive, cohort and case–control studies, suggest that carotenoid-rich diets are associated with reduced risk of cardiovascular disease (reviewed in Institute of Medicine, 2000). Furthermore, no adverse outcomes have been reported between increased serum levels of lutein and zeaxanthin and risk of subsequent myocardial infarction (Street et al., 1994). Recent epidemiological findings, as well as those from studies in vitro and in mouse models, support the hypothesis that increased dietary intake of lutein protects against the development of early atherosclerosis (Dwyer et al., 2001).

3. INTAKE

3.1 Concentrations in foods

A database of concentrations of carotenoids, including lutein and zeaxanthin, in 120 foods was assembled by Mangels et al. (1993), and was updated by Holden et al. (1999). It should be noted that the carotenoid content of food is highly variable and depends on a number of factors, including geographical area and growing conditions, cultivar or variety, processing techniques, preparation and length and conditions of storage (Holden et al., 1999, and references therein). Major sources of lutein/zeaxanthin are leafy green vegetables (e.g. raw spinach, 11.9 mg/100 g), corn (boiled, 1.8 mg/100 g) and green vegetables such as broccoli (raw, 2.4 mg/100 g), Brussels sprouts (boiled, 1.3 mg/100 g), green beans (boiled, 0.7 mg/100 g), and peas (canned, 1.3 mg/100 g). Although it is not a major part of the diet in western Europe and North America, kale has the highest lutein/zeaxanthin content of all foods analysed (raw, 39.5 mg/100 g).

3.2 Dietary intake

Dietary recall data from 1102 adult women participating in the 1986 Continuing Survey of Food Intake by Individuals indicate mean intakes of lutein/zeaxanthin of 1.3 mg/day with a total carotenoid intake of 6 mg/day (Chug-Ahuja et al., 1993). Food frequency data from 8341 adults participating in the 1992 National Health Interview Survey indicate that mean intakes of lutein for men were 2.2 mg/day and for women 1.9 mg/day (Nebeling et al., 1997). The Nutritional Factors in Eye Disease Study reported mean dietary intakes of lutein/zeaxanthin of 0.7–0.8 mg/day (VandenLangenberg et al., 1996). In a pooled analysis of seven cohort studies designed to assess the effect of dietary carotenoids on risk of lung cancer, intakes of lutein/zeaxanthin were energy-adjusted using the predicted intake of 2100 kcal/day for men and 1600 kcal/day for women (Mannisto et al., 2004). Food consumption was assessed at baseline using a validated dietary questionnaire for each

study population. For these seven populations, the mean intake of lutein/zeaxanthin for men and women combined was 3.7 mg/day (range, 1–6 mg/day).

The estimated mean and 90th-percentile consumption of lutein and zeaxanthin in a survey of sample foods were 1.71 and 3.01 mg/day respectively in the United States of America (USA) (DSM Nutritional Products, 2004) (Table 3). Simulations considering proposed food use levels in the total population of the USA resulted in estimated mean and 90th-percentile intake of lutein by all users of 7.3 and 13.4 mg/day respectively (DSM Nutritional Products, 2004) (Table 3). Kruger et al. (2002) estimated the intake of lutein/zeaxanthin in the USA using dietary records. The mean and 90th-percentile intakes of lutein/zeaxanthin were 3.83 and 7.29 mg/day respectively, and 0.91 and 1.77 mg/day respectively from crystalline lutein product (Table 3). Intake of lutein in 1543 Canadians (aged 18–65 years), estimated by 24 h recall, was 1.41 and 0.57 mg/day (mean and median, respectively) (Johnson-Down, 2002) (Table 3). Intake of lutein in 76 women (aged 50–65 years) from the United Kingdom (UK), estimated by the determination of both food intake and concentrations of lutein was 0.92 mg/day (Scott et al., 1996) (Table 3).

Formulations of lutein/zeaxanthin are also available as dietary supplements, but there are no reliable estimates of intake from these sources.

4. COMMENTS

In rats, peak concentrations of radiolabel in the plasma and tissues occurred about 4 h after a single oral dose of [^{14}C]lutein. Most of the radiolabel was eliminated via the faeces within about 2 days; very low urinary and biliary excretion indicated that there was poor absorption from the intestinal tract. Based on data on faecal excretion, the absorption of lutein was about 30–40% when administered to rats in the diet as beadlets containing vitamin E (the beadlet formulation was used to enhance the stability of lutein). Tenfold increases in dose, in the range of 2 to 200 mg/kg bw, resulted in two- to threefold increases in plasma concentrations, indicating reduced absorption at higher doses. Steady-state plasma concentrations of lutein were reached by about 3 days after the start of dietary administration of lutein to rats, indicating that the half-life of lutein is about 1 day.

In humans, peak plasma or serum concentrations of lutein occurred at 11–16 h after administration of a single oral dose. During daily supplementation with 20 mg of lutein/day, steady-state plasma concentrations were reached within about 30 days. This is consistent with an elimination half-life of about 5–7 days.

The food matrix, including its fibre and lipid contents, and the concentrations of other carotenoids in the diet may influence the extent of absorption of carotenoid compounds. The relative absorption of lutein from a mixed vegetable diet was lower than from a diet containing pure lutein. A mixed preparation of lutein and zeaxanthin did not influence the absorption of β-carotene.

Lutein has an oral LD_{50} of >2000 mg/kg bw in rats. In a 13-week study in rats, lutein administered at oral doses of up to 200 mg/kg bw, the highest dose tested, caused no treatment-related effects. In a 52-week study designed primarily to investigate possible adverse effects on the eye in monkeys, lutein was adminis-

Table 3. Estimated daily intake of lutein **(Tagetes extract)**

Country	Estimated daily intake (mg/person per day)	Target year	Method/ compound	Standards for use	Reference
New Zealand	No data	1997	As lutein, GMP*food consumption	Food colour, GMP/lowest possible level	submitted by New Zealand for the Committee at its 63rd meeting
USA	1.71 (mean), 3.01 (90th[a])	1994–1996, 1998	Food-uses and food consumption amount, all person, including zeaxanthin		DSM Nutritional Products and Nemin Foods, LC, for the Committee at its 63rd Meeting (from: Dietary Reference Intakes, Institute of Medicine, 2000)
	7.3 (mean), 13.4 (90th)	1994–1996, 1998	Food-uses and food consumption amount, all users, including zeaxanthin		
USA	0.91 (mean), 1.77 (90th)	1994–1996	From 84% pure crystalline product, lutein + zeaxanthin		Kruger et al. (2002)
	3.83 (mean), 7.29 (90th)	2000	Dietary guidelines, natural lutein and zeaxanthin		
Canada	1.41 (mean), 0.57 (median)	Sept 1997–Jul 1998	24-h food recall, lutein, for adults (aged 18–65 years)		Johnson-Down et al. (2002)
UK	0.92 (women aged 50–65 years)	Nov 1988–Oct 1989	Determination of both food intake and concentration of lutein		Scott et al. (1996)

EU: European Union; GMP: Good manufacturing practice.
[a] 90th percentile.

tered at a dose of 0.2 or 20 mg/kg bw per day by gavage. This study was performed because adverse ocular effects had been seen with canthaxanthin (Annex 1, references *78*, *89*, *117*). There were no treatment-related effects on a wide range of toxicological end-points. Furthermore, comprehensive ophthalmic examinations, including electroretinography, showed no evidence of treatment-related adverse changes.

No long-term studies of toxicity or carcinogenicity were undertaken.

Lutein gave negative results in several studies of genotoxicity in vitro and in vivo. Although the Committee noted that the doses used in these tests were low, it recognized that maximum feasible doses were used. There was no evidence for tumour promoting activity in animal models.

In a study of developmental toxicity with lutein in rats, there was no evidence for toxicity at doses of up to 1000 mg/kg bw per day, the highest dose tested.

In a 20-week multicentre intervention trial with lutein in healthy human subjects, there were no changes in haematological or biochemical parameters after continuous daily doses of lutein of 15 mg (0.25 mg/kg bw, assuming a body weight of 60 kg). There has been a relatively large number of studies in humans that have examined correlations between macular degeneration and dietary intake of lutein or zeaxanthin, intakes via dietary supplements, or serum concentrations. Although these studies were designed to look for ocular effects, where clinical or biochemical parameters were also examined, no adverse effects of these xanthophylls were reported.

Intake

Data on dietary intake from a number of studies in North America and the UK indicate that intake of lutein from natural sources is in the range of 1–2 mg/day (approximately 0.01–0.03 mg/kg bw per day). Simulations considering proposed levels of use as a food ingredient resulted in an estimated mean and 90th-percentile intake of lutein plus zeaxanthin of approximately 7 and approximately 13 mg/day, respectively. Formulations containing lutein and zeaxanthin are also available as dietary supplements, but there were no reliable estimates of intakes from these sources.

5. EVALUATION

In several studies of toxicity, including developmental toxicity, no adverse effects were documented in animals, including monkeys, or humans. Taking into account data showing that lutein was not genotoxic, had no structural alert, did not exhibit tumour-promoting activity, and is a natural component of the body (the eye), the Committee concluded that there was no need for a study of carcinogenicity.

Lutein has some structural similarities to β-carotene, which has been reported to enhance the development of lung cancer when given as a supplement to heavy smokers. The available data indicated that lutein in food would not be expected to have this effect. The Committee was unable to assess whether lutein in the form of supplements would have the reported effect in heavy smokers.

The 52-week study in monkeys was designed to evaluate ocular effects, and although there were no adverse toxicological effects at the highest dose tested (20 mg/kg bw per day), this study was considered to be inappropriate for the establishment of an ADI, in view of the much higher doses used in several other studies and found to be without effect. The available comparative toxicokinetic data for humans and rats indicated that the studies of toxicity in rats could be used to derive an ADI. The Committee concluded that the best study for this purpose was the 90-day study in rats. An ADI of 0–2 mg/kg bw was established based on the NOEL of 200 mg/kg bw per day (the highest dose tested in this study) and a safety factor of 100.

Although the ADI was based on the results of a short-term study, the supporting data and lack of effects at much higher doses in some studies (e.g. a study of developmental toxicity), indicated that the safety factor of 100 was appropriate.

In view of the toxicological data and structural and physiological similarities between the xanthophylls lutein and zeaxanthin, the Committee decided to include zeaxanthin in the ADI (0–2 mg/kg bw) for lutein, which had a stronger toxicological database, and to make this a group ADI for the two substances. This group ADI does not apply to other xanthophyll-containing extracts with a lutein or zeaxanthin content lower than that cited in the specifications.

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PEROXYACID ANTIMICROBIAL SOLUTIONS CONTAINING 1-HYDROXYETHYLIDENE-1,1-DIPHOSPHONIC ACID (HEDP)

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1. EXPLANATION

The Committee considered the safety of antimicrobial solutions that are prepared from acetic acid and octanoic acid (singly or in combination), together with hydrogen peroxide, and using 1-hydroxyethylidene-1,1-diphosphonic acid (HEDP) as a sequestrant or stabilizer. Preparations that are ready for use also contain as active compounds the peroxy forms of both acids. Before use, concentrated solutions are diluted to achieve target concentrations of total peroxyacid ranging from 80 to 200 mg/kg. These antimicrobial solutions are intended for use as components of wash solutions on fresh poultry and meat and in wash water for fresh and processed fruits and vegetables. After being applied in process water, they are largely eliminated by drainage, further washing and trimming of products, and evaporation. The safety of the antimicrobial solutions was therefore assessed on a component-by-component basis, considering the potential residue of each component or its breakdown products in food as consumed.

At its seventeenth meeting (Annex 1, reference *32*), the Committee allocated an acceptable daily intake (ADI) 'not limited'[1] to acetic acid and its potassium and sodium salts. This ADI was retained at the forty-ninth meeting (Annex 1, reference *131*) when the Committee evaluated a group of flavouring agents (saturated aliphatic acyclic linear primary alcohols, aldehydes, and acids) that included acetic acid.

At its forty-ninth meeting, the Committee evaluated octanoic acid for use as a flavouring agent as part of the group of saturated aliphatic acyclic linear primary alcohols, aldehydes, and acids, and concluded that octanoic acid posed no safety concerns at intakes of up to 3800 μg/person per day (or 63 μg/kg bw per day, assuming a body weight of 60 kg).

At its twenty-fourth meeting (Annex 1, reference *53*), the Committee evaluated hydrogen peroxide as a preservative and sterilizing agent for use in milk. While an ADI was not allocated, the Committee noted that hydrogen peroxide should be used only when better methods of milk preservation were not available.

Peroxyacetic acid and peroxyoctanoic acid, and HEDP have not been previously evaluated by the Committee.

At its present meeting, the Committee considered a number of studies on the antimicrobial efficacy of peroxyacid solutions, the toxicity of HEDP, and the effects

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[1] A term no longer used by the Committee, which has the same meaning as ADI 'not specified'.

of peroxyacid solutions on food quality and nutritional value. The Committee also evaluated estimates of the intake of the individual components in these solutions for consideration in the safety evaluation.

1.1 Composition of antimicrobial solutions

The composition of four antimicrobial solutions, A–D, are described in Table 1. The concentrated solutions are diluted before use to achieve target concentrations of total peroxyacid ranging from 80 to 200 mg/kg.

In manufacturing each antimicrobial solution, measured quantities of each component are added in a specific order. Hydrogen peroxide reacts with acetic acid to form peroxyacetic acid, which it thus helps to stabilize. Hydrogen peroxide also reacts with octanoic acid, when present, to form peroxyoctanoic acid. The result is an equilibrium solution containing peroxyacetic acid, acetic acid, hydrogen peroxide, HEDP, and in some cases, octanoic acid and peroxyoctanoic acid. The concentration of peroxyacids continues to increase for 7–13 days after manufacture. HEDP is needed to ensure the stability of the solution since peroxy compounds are inherently unstable. Once equilibrium is achieved, the solution remains relatively stable at room temperature for up to 1 year. The main chemical reactions that occur in the equilibrium solutions are shown in Figure 1.

1.2 Residues of components of antimicrobial solutions

After application, the antimicrobial solutions and their components are largely lost due to drainage, further washing, trimming and evaporation. Residues of hydrogen peroxide, peroxyacetic acid, or peroxyoctanoic acid on food rapidly decompose into water, oxygen, acetic acid and octanoic acid (Figure 1). Small amounts of acetic acid, octanoic acid and HEDP will remain on the treated commodities. Intake assessments for the components of the antimicrobial solutions are described in section 3.

2. BIOLOGICAL DATA

Antimicrobial mixtures are equilibrium mixtures that are diluted in water prior to their use in processing food. Hydrogen peroxide in these mixtures will dissociate into water and oxygen. Although their stability is enhanced by HEDP, both peroxyacetic acid and peroxyoctanoic acid are also inherently unstable and will break down into acetic acid and octanoic acid, respectively. Low residual levels of these simple organic acids present on food would pose no concern. No residues of peroxyacetic acid or peroxyoctanoic acid in these mixtures were expected to remain on treated foods. Thus, the peroxide components of the peroxyacid antimicrobial mixtures did not pose toxicological concerns for the uses being considered at present and the focus of the biochemical and toxicological aspects of this safety evaluation was HEDP.

Table 1. Composition of four antimicrobial solutions (A–D) and maximum concentration of components in ready-to-use solutions (after dilution)

Component	Weight of each component in the solution at equilibrium[a] (%)				Maximum concentration of each component in the solution after dilution[b] (mg/kg)			
	A	B	C	D	A	B	C	D
Acetic acid	40.6	49.4	32.0	42.0	985	2000	208[d]	NS
Peroxyacetic acid	12	12.2	15.0	12.0	213[c]	220[c]	80	80
Hydrogen peroxide	6.2	4.5	11.1	4.0	110	150	59	59
Octanoic acid	3.2	8.8	0.0	10.0	74	300	0	NS
Peroxyoctanoic acid	0.8	1.4	0.0	3.4	14[c]	25[c]	0	NS
1-Hydroxy-ethylidene-1, 1-diphosphonic acid (HEDP)	0.6	0.6	0.9	0.6	13	13	4.8[d]	4.8
Water	36.6	23.1	41.0	28.0	—	—	—	—

NS, not stated.

[a] At equilibrium, which occurs 7–13 days after manufacture, depending on the temperature at which the solution is stored.

[b] Solutions A and B are diluted to achieve a target concentration of total peroxyacid of 200 mg/kg; to account for variations, maximum values assume that use will result in a concentration of total peroxyacids of 220 mg/kg. Solutions C and D are diluted to achieve a target concentration of total peroxyacid of 40 mg/kg; to account for variations, maximum values assume that use will result in a concentration of total peroxyacids of 80 mg/kg.

[c] Concentration of total peroxyacid as peroxyacetic acid = [220] + [(22/160) × 76]; there is variation of up to 10% in the measured concentration of peroxyacetic acid, due to differences in equipment for measurement and dispensing.

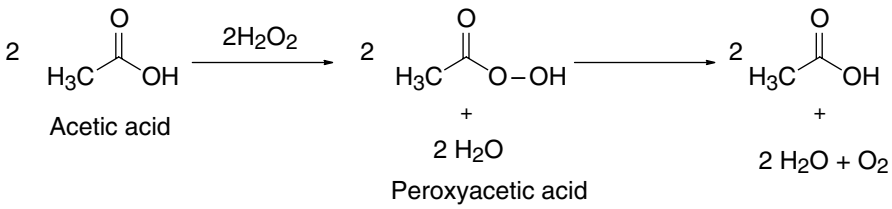
[d] Theoretical value (not based on analysis).

2.1 Biochemical aspects

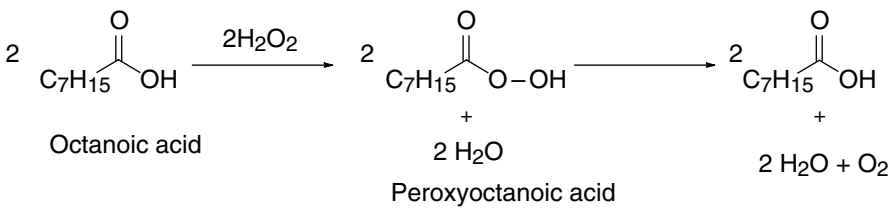
2.1.1 Absorption, distribution and excretion

Caniggia & Gennari (1977) published a concise report on the intestinal absorption and kinetics of ^{32}P-labelled EHDP (disodium ethane-1-hydroxy-1, 1 diphosphonate; disodium etidronate; referred to as HEDP in this monograph) in humans. Ten volunteers were given HEDP at an oral dose of 20 mg/kg (the carrier dose) together with 40 μCi (1480 kBq) of [^{32}P]HEDP. After 6 days, 70–90% of the administered dose was found in the faeces. Seven other subjects were given EHDP at an oral dose of 100 mg (the carrier dose) together with 20 μCi (740 kBq) of [^{32}P]HEDP intravenously. Six days after intravenous administration, 35–50% of the radiolabel administered was excreted unchanged in the urine, with negligible [^{32}P]HEDP found in the faeces. There was a rapid decline in the concentration of [^{32}P]HEDP in the plasma; after 6 days, <0.03% of the administered dose remained in the plasma. Although only limited information was published in this report, the results suggested that orally administered HEDP is poorly absorbed in humans, and that HEDP may accumulate outside of the blood.

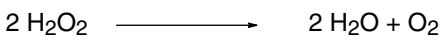
Figure 1. Main chemical reactions in the equilibrium solutions



Acetic acid



Octanoic acid



Hydrogen peroxide

2.1.2 Biotransformation

Michael et al. (1972) studied the absorption, distribution, and metabolism of HEDP in rats ($n = 3$ or 4), rabbits ($n = 3$), dogs ($n = 2$ or 3) and monkeys ($n = 3$) after oral administration of ^{14}C-labelled HEDP via intragastric cannula. The doses administered ranged from 10 to 50 mg/kg bw. The authors found that about 90% of the administered dose was excreted in the faeces of the adult animals. Absorption, which occurred in the stomach, was <10% in rats, rabbits and monkeys, and ranged from about 14% in older dogs to 21% in young dogs. Consistent with a possible age-dependent effect, absorption was somewhat greater in weanling rats. Some rats had been previously fed HEDP as part of their diet, but such preconditioning did not affect absorption. The authors attempted to identify metabolites in biological samples obtained from rats and dogs, but they reported that there was no metabolism of HEDP in either species. In all species, about half the absorbed dose was excreted unchanged in the urine and the rest was deposited in the bone, where its half-life in rats was demonstrated to be about 12 days. The results of these studies, conducted in a variety of species with small numbers of test animals, were consistent with the data obtained from a small sample of human volunteers. Collectively, the data indicated that absorption of HEDP from the gastrointestinal tract is very limited and its metabolism is negligible. Negligible metabolism of

systemic HEDP could have been due to the fact that carbon–phosphorus (C–P) bonds are difficult to break.

2.2 Toxicological studies

2.2.1 Acute toxicity

The available median lethal dose (LD_{50}) values for HEDP administered orally are summarized in Table 2. Two of these studies, in which high doses of HEDP were associated with kidney damage in rats and rabbits, are described more fully below.

Rats

Groups of 10 male and 10 female fasted Charles River CD rats were given HEDP (as disodium etridronate, the disodium salt of HEDP) by stomach tube, at one of four doses selected on the basis of an assumed toxicity and dose–response curve. The LD_{50} was determined to be 1.34 g/kg bw. Among the surviving animals that had received a higher dose (1.60 or 1.14 g/kg bw) of disodium etidronate, 3 out of 10 were found to have light grey, granular kidneys. Microscopic evaluation revealed damage to the nephritic tubules. The kidneys of all animals receiving the lowest dose (0.814 g/kg bw) also showed mild tubular changes. The kidney : body weight ratios of the animals at the two higher doses were significantly higher than those of animals at 0.814 g/kg bw, as well as being higher than the normal range (Nixon et al., 1972).

Rabbits

The same procedure was used to determine LD_{50} values in New Zealand rabbits. It was found that the susceptibility of rabbits to the acute effects of HEDP (as disodium etridronate, the disodium salt of HEDP) is a function of age, weight, and sex. The LD_{50} values ranged from 0.581 to 1.14 g/kg bw, and were lower in males than in females, and lower in mature animals (body weight, >3300 g) than immature animals (body weight, about 2500 g). No unusual lesions were reported upon microscopic analysis. About 50% of the surviving animals (in all groups) were found to have kidney lesions indicative of chronic interstitial nephritis; however,

Table 2. Acute toxicity of HEDP administered orally

Species	Sex	LD_{50} (g/kg bw)	Reference
Rat	Not specified	2.40	Monsanto MSDS
Rat	M, F	1.34	Nixon et al. (1972)
Rabbit	M, F	0.581–1.14	Nixon et al. (1972)
Dog	M, F	84.80[a]	Nixon et al. (1972)
Rat	M, F	3.13	Younger Laboratories (1965)

F, female; M, male.
[a] Value was estimated, due to emesis in some dogs.

chronic interstitial nephritis is commonly found in the rabbit and it is thus hard to determine whether the finding was related to treatment (Nixon et al., 1972).

Dogs

The administration of HEDP (as disodium etridronate, the disodium salt of HEDP) produced an immediate emetic response in some dogs, and it was thus not possible to clearly define an LD_{50} value. On the basis of early deaths and necropsy of animals found in a moribund condition at higher doses (1.0–10.0 g/kg), however, the LD_{50} was estimated to be about 1.0 g/kg (Nixon et al. 1972).

2.2.2 Short-term studies of toxicity

Rats

In a 91-day feeding study, groups of 20 male and 20 female Charles River CD rats were fed diets containing HEDP (disodium monohydrate salt) at 0, 0.2, 1.0, or 5.0% (equivalent to doses of 0, 100, 500, and 2500 mg/kg bw per day). Owing to severe mortality and weight loss observed at 5.0%, the study of that group was terminated after 1 week. After conclusion of the study (91 days for groups at 0, 0.2, and 1.0%, and 1 week for the 5.0% group), five males and five females from each group were randomly selected for necropsy. Histopathological lesions and/or alterations of blood parameters were observed and appeared to be associated with gastritis. At 5.0%, gastrointestinal erosions were observed and the kidney : body weight ratio was high (1.48% and 1.55% for females and males, respectively) compared with controls (1.11%). No treatment-related changes were observed in histopathological lesions or blood haematological values at 0.2% or 1.0%. The kidney : body weight ratio in females at 1.0% was slightly higher (at 0.82%) than controls (0.64%). All other parameters measured in the study were normal and similar to those in the controls. Hence, the no-observed-effect level (NOEL) was 1.0%, equivalent to a dose of 500 mg/kg bw per day (Nixon et al., 1972).

A 90-day feeding study in rats was designed to assess the toxicity of HEDP (crystalline sodium salt characteristic of DEQUEST®2010 phosphonate in the nature and amount of by-products and impurities). Groups of 15 male and 15 female rats were given HEDP at a dietary concentration of 0, 3000, 10 000 or 30 000 mg/kg of feed (equivalent to 0, 150, 500, or 1500 mg/kg bw per day) of HEDP. Body weights, food consumption and mortality were determined weekly. At 45 and 90 days, haematology and clinical chemistry parameters were assessed and urine analysis was performed. The animals were necropsied at the end of the study and histopathological examinations were performed on tissues from animals treated at the highest dose only. A high level of mortality was observed at 30 000 mg/kg. This finding might be related to the ingestion of HEDP, although it was possibly the result of trauma induced by blood collection. At 30 000 mg/kg, body-weight gain was inhibited in males. At the highest dose tested, haematology revealed significant changes, including increased erythrocyte counts in males, decreased haemoglobin concentration and erythrocyte volume fractions in males and females, and decreased leukocyte counts at the end of the study in females only. The lesions observed in histopathological examinations, which were con-

ducted on animals treated at the highest dose only, were described by the pathologist as being typical of the controls. At 10 000 mg/kg (500 mg/kg bw per day), no adverse effects were noted in any parameters measured in this study; however, histopathological examinations were not conducted on rats in the groups given the two lower doses. The NOEL was 500 mg/kg bw per day (Industrial Bio-Test Laboratories, Inc., 1975a).

Dogs

In a 90-day study of toxicity designed to test the effects of HEDP (crystalline sodium salt characteristic of DEQUEST® 2010 phosphonate in nature and amount of by-products and impurities), groups of four male and four female beagle dogs (aged 5 months at the start of the study) were given HEDP at a concentration of 0, 1000, 3000, or 10 000 mg/kg of diet (equivalent to a dose of 0, 25, 75, or 250 mg/kg bw per day). Food and water were available ad libitum. Body weights and food consumption were recorded weekly. Haematology and blood chemistry parameters were assessed and urine analysis was conducted at the beginning of the study and at 56 and 85 days. At the end of the study, organ weights were determined and gross and histopathological examinations were conducted. There were no adverse effects of the test material on body weight, although food intake in females at the intermediate and highest doses was decreased compared with that of the controls. No deaths were reported. Small changes in haematological parameters (increased erythrocyte counts and decreased mean corpuscular volume) and variations in blood chemical parameters (serum potassium and magnesium concentrations in males and females, respectively) were noted, but the effects were inconsistent and were not attributed to treatment. Increased numbers of leukocytes and crystals were found in the urine of dogs from all treatment groups at the final analysis. However, this was not considered to be a significant finding since no changes in the genitourinary system were observed on microscopy. Some differences were noted in organ weights, including increased brain weights in females at the intermediate and highest doses and increased thyroid weights in males at the highest dose. These differences, which were not associated with microscopic changes in these organs, were not considered to be related to treatment. There were no gross or histopathological changes reported for any of the tissues or organs examined in this study. The NOEL was 250 mg/kg bw per day (Industrial Bio-Test Laboratories, Inc., 1975b).

2.2.3 Long-term studies of toxicity and carcinogenicity

Long-term studies to address the toxicity or carcinogenic potential of HEDP were not available for this evaluation. The publication by Nixon et al. (1972) refers to data obtained in chronic tests that were “to be published elsewhere”. However, a literature search failed to reveal any data resulting from traditional long-term tests of toxicity in animals. One study on the skeletal effects of HEDP administered subcutaneously to beagle dogs for approximately 1 or 2 years is described below (see 2.2.6, special study).

2.2.4 Genotoxicity

The potential genotoxicity of HEDP was assessed using an assay for reverse mutation in which five strains of *S. typhimurium* (TA98, TA100, TA1535, TA1537 and TA1538), were tested, with and without metabolic activation provided by rat liver microsomes, at doses of 0.001, 0.01, 0.1, 1, 5 or 10 µl/plate (in water). The test article was a commercial product that contained 60% HEDP in aqueous solution. Aberrations in the background lawn were observed at concentrations of 5 and 10 µl/plate, indicating toxicity at the two highest concentrations tested for all five strains of *Salmonella*. The tester strains responded as expected to solvent and positive controls. Under the conditions of the assay, the test article was not mutagenic (Monsanto Co., 1977).

The potential genotoxicity of HEDP was also assessed by thymidine kinase (*Tk*) gene forward mutation assay in mouse lymphoma L5178Y cells, with and without metabolic activation provided by rat liver microsomes; at doses of 0.064, 0.125, 0.250, 0.500, or 0.600 µl/ml in the absence of microsomal enzymes and 0.125, 0.250, 0.500, 0.600 and 0.800 µl/ml in the presence of microsomal enzymes. The test article, a commercial product that contained 60% HEDP in an aqueous solution, was the same as that tested in the assay for reverse mutation, but was in this case diluted in dimethyl sulfoxide (DMSO). At concentrations of ≥0.5 µl/ml, cytotoxicity was observed that was greater in the presence of microsomal enzymes. In the first of the two trials, although control values for spontaneous mutagenesis were higher than expected, the incidence of mutagenesis caused by HEDP at the highest concentration tested was more than 2.5 times that for the controls for spontaneous mutagenesis with microsomal activation. In the second trial, the incidence of spontaneous mutagenesis was not elevated and the incidence of mutagenesis caused by HEDP at the highest concentration tested was about twice that for the controls for spontaneous mutagenesis. The positive controls gave the expected results. Under the conditions of the assay, the test article did not induce forward mutation in the mouse lymphoma assay (Litton Bionetics, Inc., 1978).

2.2.5 Reproductive toxicity

Rats

In a combined two-generation study of reproductive toxicity and teratogenicity, five groups of 22 female and 22 male weanling Charles-River rats were given the disodium salt of HEDP (disodium etidronate) at a dietary concentration of 0, 0.1 or 0.5% (equivalent to a dose of 0, 50 or 250 mg/kg bw per day), either continuously or only on days 6–15 of gestation for two generations. Reproductive endpoints and offspring parameters were analysed in the F_{1a}, F_{1b}, and F_{2a} litters. The third litter of the F_1 generation (F_{1c}) and the second litter of the second generation (F_{2b}) were used in teratological examinations. During the teratology phase, half of the animals in each group were sacrificed at day 13 and the others at day 21 of gestation. Body-weight gains were similar for all groups in both generations, and the overall conception rate was 90%, indicating that the compound did not interfere with spermatogenesis or ovulation. In the first generation, at the highest dose, the number of pubs born in the first litter (F_{1a}) was reduced and there was an increase

in stillborn pups in the second litter (F_{1b}). The rate of mortality in pups after birth was low and the weights of pups at day 4 and at weaning were the same. Teratological examination of the third litter (F_{1c}) showed no differences in resorptions or implantations in females sacrificed at day 13 (corpora lutea were not counted) and no differences in live fetuses, corpora lutea, or implantation at 21 days. At day 21, however, significant resorptions were reported in the controls. In the second generation, the first litters (F_{2a}) were smaller than the litters in the first generation, but there were no other differences in reproductive parameters. During the teratology phase, no differences in corpora lutea, implantations, or resorptions were noted in rats sacrificed at 13 days. In continually fed rats sacrificed at 21 days, the number of implantations was reduced and corpora lutea formation was depressed at the highest dose. A decrease in the number of live fetuses at the highest dose, significant only in rats fed during gestation, was also observed. The incidence of defective pups was similar to that in control animals and the study authors concluded that disodium etidronate was not teratogenic in rats at either dose tested. The NOEL, based on reduced litter size and decreased number of pups at the highest dose, was 50 mg/kg bw per day (Nolen & Buehler, 1971).

Rabbits

In a combined study of reproductive toxicity and teratogenicity in rabbits, two separate experiments were conducted. In the first experiment, four groups of 25 virgin New Zealand white rabbits were given HEDP (as an aqueous solution of disodium etidronate) at a dose of 0, 100, 250 or 500 mg/kg bw per day via intubation on days 2–16 of gestation. The rabbits were inseminated (day 1) and dosing commenced before implantation (day 7). After 4–5 consecutive doses of HEDP at 500 mg/kg bw per day, the pregnant rabbits died. Four rabbits survived three daily doses of HEDP at 500 mg/kg bw per day and these animals subsequently received HEDP at 250 mg/kg for the rest of the study. At a dose of 100 mg/kg bw per day, HEDP caused a 68% reduction in the conception rate in rabbits. Owing to toxicity at 500 mg/kg bw per day and a reduced conception rate at the lowest dose tested (i.e.100 mg/kg bw per day), a second experiment was performed in which the highest dose tested was 100 mg/kg bw per day.

In the second experiment, four groups of 25 virgin New Zealand white rabbits were given HEDP (disodium etidronate) at a dose of 0 (water), 25, 50, or 100 mg/kg bw per day in the diet or 100 mg/kg by gavage, on days 2–16 of gestation. An additional untreated control group was also used in this study. Reproductive parameters and offspring malformations were analysed. The authors reported no statistical differences in food consumption or body-weight gain, although these data were not presented. However, the authors noted that rabbits given the highest dose (100 mg/kg bw per day) consumed the least amount of food and gained the least weight. The conception rate in dams given disodium etidronate at a dose of 100 mg/kg bw per day by gavage or in the diet was 90% or 95%, respectively, indicating no effect on conception or nidation. No differences were observed in the numbers of corpora lutea, resorptions, or live fetuses. Fetuses from dams given disodium etidronate at a dose of 100 mg/kg bw per day by gavage were significantly smaller than those from untreated controls. No differences in the defective

fetuses in treated groups compared with the controls were reported. Very few skeletal defects were seen, although variations in the number of ribs and sternebrae occurred in up to 50% of the rabbit fetuses. The study authors stated that these variations in the ribs and sternebrae were not teratogenic effects (Cozens, 1965). They thus concluded that there were no treatment-related adverse effects on reproduction parameters and that disodium etidronate is not teratogenic in rabbits (Nolen & Buehler, 1971).

Nolan & Buehler (1971) speculated that the effects on conception rate in rabbits given HEDP (disodium etidronate) at a dose of 100 mg/kg per day in their first experiment may have resulted from stress caused by gavage, because a reduction in conception rate was not observed in their second experiment. In the second experiment, however, the authors reported a reduction in fetal weights with HEDP at a dose of 100 mg/kg bw per day administered by gavage, which they attributed to slightly larger litters. Although, not statistically significant, decreases in food consumption and body-weight gain in dams at the same dose were reported in the second experiment. On the basis of decreased fetal weights, the NOEL was set conservatively at 50 mg/kg bw per day (Nolan & Buehler, 1971).

2.2.6 Special study: skeletal effects in dogs

In a long-term study to determine the skeletal effects of HEDP, adult female beagle dogs (aged 3–4 years at the start of the study) were given HEDP at a dose of 0, 0.1, 0.5, 2, 5 or 10 mg/kg bw per day via subcutaneous injection for different times ranging from 1 to 2 years. There were 10 dogs in the control group and five dogs in each treatment group. Dogs at the two lower doses (0.1 and 0.5 mg/kg bw per day) were treated for 2 years. Dogs at 5 mg/kg bw per day were sacrificed after 13.5 months, while dogs at 2 and 10 mg/kg bw per day were sacrificed after 12 months. At the two lower doses, there was a slight reduction in osteoblastic activity, reduction in the percentage of bone surfaces with active mineralization, a reduction in mineralization rates, and a reduction in resorption spaces, but no change in osteoid seam width. There were no treatment-related fractures at 0.1 mg/kg bw, but radiographic studies indicated that the incidence of fractures was slightly increased at 0.5 mg/kg bw per day. Profound effects on bone parameters were observed at doses of 2–10 mg/kg bw per day. The number of resorption spaces was reduced and mineralization activity was blocked to the extent that osteoid seams became thickened. At these higher doses, the incidence of fractures was markedly increased and fractures were radiologically apparent after 9–12 months. Healing of fractures, when they occurred, was inhibited at doses of HEDP of >0.5 mg/kg bw per day. The authors suggested that high doses of HEDP did not cause any permanent change in the skeleton that would interfere with fracture healing. This study indicates that HEDP caused profound effects on the skeletal system that are dose-related and dependent on the period of treatment, but that the effects are reversible (Flora et al., 1981).

Flora et al. (1981) also pointed out that oral administration of the disodium salt of HEDP at a dose of 5 mg/kg bw per day for up to 6 months is recommended for the treatment of Paget disease in humans. The authors indicated that the dose of HEDP that resulted in the development of spontaneous fractures in dogs was

about 10 times higher than the dose recommended for extended use in humans. This is based on the assumption that gastrointestinal absorption of orally administered HEDP would occur at a rate of 1% in humans. Thus, a orally administered dose of HEDP of 5 mg/kg bw per day would be expected to lead to a systemic dose of 0.05 mg/kg bw per day in humans, or 3 mg/day for an adult with a body weight of 60 kg.

2.3 Environmental studies

HEDP can also undergo photolysis to acetate and phosphate within a few days (Steber & Wierich, 1986). In distilled water and in the presence of calcium, no photodegradation of HEDP was observed, but the addition of Fe(III) and Cu(II) resulted in rapid photodegradation (Fischer, 1993). Thus, after the use of the antimicrobial solutions, residual HEDP in foods may undergo photolysis before the treated foods are consumed.

2.4 Microbiological aspects

2.4.1 Role of components in antimicrobial solutions

Different antimicrobial wash solutions are added to water to spray, wash, rinse, dip, cool or otherwise process meat, poultry, and fresh as well as processed fruits and vegetables. The solutions are used to inhibit the growth of *Salmonella* sp., *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7, and spoilage and decay organisms on the product or surface to be treated (Table 3).

Peroxyacetic acid (also referred to as peracetic acid) is the major active ingredient in all of the antimicrobial wash solutions. The effect of peroxyacetic acid is

Table 3. The intended uses of four antimicrobial wash solutions[a]

Solution	Product/surface to be treated	Function
A	Poultry carcasses, parts, and organs	Antimicrobial efficacy against *Salmonella* sp., *C. jejuni*, *L. monocytogenes*, *E. coli* O157 : H7 and spoilage organisms on poultry
B	Meat carcasses, parts, trims, and organs	Antimicrobial efficacy against *Salmonella sp.*, *L. monocytogenes*, *E. a coli O157 : H7* and spoilage organisms on meat
C	Post-harvest, fresh-cut, and further processed fruits and vegetables, including process water	Antimicrobial efficacy against spoilage and decay organisms on treated fruits and vegetables and in process water
D	Further processed fruits and vegetables	Antimicrobial efficacy against S*. a javiana*, *L. monocytogenes*, *E. coli O157 : H7*, spoilage and decay organisms on further processed fruit and vegetable surfaces.

[a] See Table 1 for the composition of these solutions.

similar to that of other antimicrobial agents that function as oxidizing agents, and which attack multiple cell sites and can disrupt the chemiosmotic balance. A recently published summary (Kitis, 2004) stated that peracetic acid was identified to have antimicrobial properties as early as 1902; that it had often been used in 'cold sterilization' procedures for medical instruments and had been found to be bactericidal at 0.001%, fungicidal at 0.003% and sporicidal at 0.3%; and that it had been used in the production of gnotobiotic (germ-free) animals. This publication also proposed that the antimicrobial action of peracetic acid may result from the oxidation of proteins and, in particular, their sulfhydryl bonds. Alternatively, peracetic acid may disrupt the chemosmotic functions of outer membrane lipoproteins and oxidize nitrogenous bases in DNA, resulting in cell death. Peracetic acid was compared favourably with chlorine-based compounds; it was proposed that its antimicrobial efficacy was similar and its decomposition to the environmentally safe products acetic acid, water and oxygen provides an advantage over chlorine-based products.

Octanoic acid also contributes to the efficacy of these antimicrobial solutions. A publication by Sun et al. (2002) concludes that at lower pH, caproate (C6:0) and caprylic acid (C8:0, the alternative name for octanoic acid) inhibit microbial growth. In addition, octanoic acid functions as a surfactant to aid in wetting hydrophobic surfaces, particularly on meat.

While acetic acid and hydrogen peroxide are known to have antimicrobial effects, their effects within these solutions are minimal. Acetic acid and hydrogen peroxide are, however, in equilibrium with the peroxyacetic acid, so their presence is critical for the antimicrobial effects of the peroxyacetic acid. Peroxyoctanoic acid does not have antimicrobial activity. It is present in the solution because it is produced when octanoic acid reacts with hydrogen peroxide. HEDP has no antimicrobial effects. It functions as a stabilizer in these solutions by preventing metal ions from catalysing the breakdown of peroxyacetic acid and hydrogen peroxide.

2.4.2 Studies of antimicrobial efficacy

Laboratory and in-plant studies were done on four antimicrobial wash solutions, described as solutions A, B, C and D in Tables 1 and 3, to demonstrate the reduction of microbes for the intended use of each solution. Overall, the results of these tests indicate modest reductions in the number of surface microbes on poultry and meat. In wash water for fresh and processed fruits and vegetables, greater reductions in concentrations of microbes were observed. The results of studies that were available for this evaluation are described below.

(a) Solution A

The proposed use of antimicrobial wash solution A is for addition to water used for spraying or submerging, or spraying followed by submerging eviscerated poultry carcasses. Tests were done to compare specimens treated with water with those treated with the test substance. Thus, the key result is the net reduction beyond that found with water only. There were three groups, a group that was submersion-chilled, a group that was sprayed, and a group that was sprayed, then

submerged. The mean $\log_{10}$ reductions using United States Department of Agriculture procedures for carcass processing are listed in Table 4. These results indicate that a modest net reduction of up to about $\log_{10}$ 0.8 can be achieved from these treatments (unpublished data from the submitter).

A second set of tests was performed on pathogens (*Listeria monocytogenes*, *Salmonella typhimurium*, and *Escherichia coli* O157:H7) on different chicken parts (carcasses, wings, and livers). Net $\log_{10}$ reductions in pathogens varied from a modest to a considerable amount (from $\log_{10}$ 0.32 to 0.75 for *S. typhimurium*, from $\log_{10}$ 1.13 to 2.11 for *L. monocytogenes*, and from $\log_{10}$ 0.82 to 3.17 for *E. coli* O157:H7).

(b) Solution B

The proposed use of antimicrobial wash solution B is for adding to water used for spraying beef carcasses. The solution was diluted appropriately and added to water for spraying beef. Three separate test runs were conducted. In the first test, 10 randomly selected carcasses were selected; in the second test, 29–30 randomly selected carcasses were selected, and in the third, 128 carcasses were selected in-plant. In all tests, the carcasses were aseptically sampled by tissue excision, either before treatment, after treatment, or at final inspection, then serially diluted and plated. The number of colonies formed (CFU/cm^2) for all aerobic bacteria (total aerobic plate counts), coliforms, and *E. coli* were determined. For these trials, reductions ranged from $\log_{10}$ 0.434 (standard deviation (SD), 1.083) to 1.05 (SD, 0.495) for samples taken immediately after treatment and from $\log_{10}$ 0.246 (SD, 1.221) to 0.573 (SD, 0.567) at the final inspection. In essence, the values indicated that a modest, but highly variable, initial reduction of microorganisms was followed by some renewed microbial growth or acquisition of more microbes.

When pathogens were inoculated onto beef, reductions in the numbers of microbes were modest, approximately $\log_{10}$ 0.5 to 1.0 more than reductions after washing with water only. The specific results are summarized in Table 5. The relative reductions reported were modest, ranging from $\log_{10}$ 0.5 to 1.3 (unpublished data from the submitter).

Microbial contamination primarily occurs on the surface of meats. Various spraying and dipping methods, usually transient in nature, are employed to remove surface bacteria. Although several chemicals are employed in these methods, the levels of reduction of microbes, with respect to resident bacteria and specific pathogens, are typically low. In a recent publication, the use of one of these products was compared with other methods (Gill & Badoni, 2004); the results indicated that use of a solution containing 0.02% peroxyacetic acid was associated with modest reductions in the number of pathogens from $\log_{10}$ 0.5 to 1.0 l compared with meat treated with water only, but reductions after treatment with lactic acid were $\log_{10} > 1$.

Table 4. Mean log_{10} reductions of microbes on poultry carcasses treated with water or with antimicrobial wash solution A

Poultry process	Aerobic plate counts (log_{10} reduction)			*E. coli* (log_{10} reduction)			Coliforms (log_{10} reduction)		
	Water	Solution A	Net reduction	Water	Solution A	Net reduction	Water	Solution A	Net reduction
Submerged	0.53	1.21	0.68	0.56	1.37	0.81	0.6	1.27	0.67
Sprayed only	0.46	0.62	0.16	0.46	0.84	0.38	0.33	0.64	0.31
Submerged then sprayed	0.84	1.33	0.49	0.85	1.44	0.59	0.78	1.31	0.53

From unpublished data from the submitter.

Table 5. Mean log_{10} reductions in specific pathogens inoculated onto beef washed with water or with antimicrobial wash solution B

Pathogen	Average log_{10} reduction		Relative log_{10} reduction, solution B relative to water
	Water	Solution B	
L. monocytogenes	0.7	1.22	0.52
S. typhimurium	0.32	1.62	1.3
E. coli	0.4	1.48	1.08

From unpublished data from the submitter.

Table 6. Mean log_{10} reductions in microorganisms found in water treated with antimicrobial wash solution C relative to untreated water

Residual peroxyacetic acid (mg/kg)	Log_{10} reduction (log_{10}CFU)
<3	≤2
10–30	2–4
40–50	5–6

From unpublished data from the submitter.
CFU, colony-forming units.

(c) Solution C

The proposed use of antimicrobial wash solution C is for addition to water used for processing vegetables for post-harvest, fresh-cut, and further processed fruit and vegetables. Peroxyacetic acid at a concentration of 10–30 mg/kg was added to water for processing vegetables. There was a reduction of up to 4-log (log_{10} 4) in the relative concentrations of microorganisms found in the treated wash water, compared with the untreated wash water; this correlated with the amount of residual peroxyacetic acid (Table 6) (unpublished data from the submitter).

(d) Solution D

The proposed use of antimicrobial wash solution D is for reduction of contamination, either for organisms on surfaces or for cross-contamination in wash water, on the surface of processed fruit and vegetables. To test the reduction of contamination, tomato surfaces were inoculated with *E. coli O*157:H7, *L. monocytogenes*, and *S. javiana* and treated with either tap water or Tsunami 200. The results indicated that solution D effectively reduced numbers of these pathogens (Table 7).

To test for the elimination of cross-contamination, cherry tomatoes were inoculated with the same target pathogens, which were allowed to attach for 24 h. Inoculated and non-inoculated cherry tomatoes were then submerged in solution D (Tsunami 200) or in tap water. The non-inoculated tomatoes were removed to a

Table 7. Mean log_{10} reductions in pathogens on tomato surfaces treated with water only or with antimicrobial wash solution D

Pathogen	Pathogens on tomato surfaces (log_{10} CFU)		
	Water	Solution D	Log_{10} reduction
L. monocytogenes	4.73	0.00	4.73
E. coli	5.00	0.87	4.13
S. javiana	2.62	0.00	2.62

From unpublished data from the submitter.

neutralizing solution, which was vortexed to remove bacteria from the tomatoes, then diluted and plated. There was a reduction of greater than 2-log (log_{10} 2) in all three pathogens transferred by cross-contamination (unpublished data from the submitter).

2.5 Observations in humans

The disodium salt of HEDP, which is known clinically as sodium etidronate, is used to treat Paget disease, which is an idiopathic disease characterized by accelerated bone metabolism. Fractures and other abnormalities of bone are common in patients with Paget disease. Due to its high affinity for solid-phase calcium phosphate, HEDP prevents hydroxyapatite crystal growth and dissolution on crystal surfaces of bone. Its mechanism of action, however, is not fully understood.

The recommended dose of sodium etidronate is 5–10 mg/kg bw given orally once daily for 6 months or less, or 11–20 mg/kg bw per day for 3 months or less. Doses in excess of 20 mg/kg bw per day are not recommended. The dose must be reduced in cases of renal insufficiency. Sodium etidronate is generally well tolerated and the incidence of side-effects is low (Center for Drug Evaluation and Research, 2001; Physician's Desk Reference, 2004). Initial therapy with a dose of 5 mg/kg bw per day of sodium etidronate appears to maximize benefits for patients with Paget disease while minimizing possible adverse effects (Canfield et al., 1977).

Numerous abstracts/citations addressing the use of HEDP in cancer therapy, osteoporosis, nuclear imaging, and hypercalcaemia associated with malignancy, and other disorders of calcium and phosphorus balance have been published. Such studies were not considered to be relevant to food safety and are beyond the scope of this assessment.

3. INTAKE

3.1 Residues on foods

The use of the four solutions of peroxyacid in antimicrobial water washes for the processing of meat, poultry, fruits, and vegetables results in predictable

residues on treated foods. The hydrogen peroxide in the solution and the peroxyacetic and peroxyoctanoic acids formed in situ are inherently unstable, especially in the presence of oxidizable organic material. Therefore, there would be no expected residues of these substances on treated foods. Acetic and octanoic acid present in the solution and as by-products from the corresponding peroxyacids would be expected to remain on any treated foods that are not washed or further processed after treatment, as would HEDP, which is stable and non-reactive under the conditions of use.

Acetic and octanoic acids are components of many foods and are also used as flavouring agents in foods. The Committee has previously evaluated both of these substances. The minor residues of these substances remaining on treated foods result in exposures that are insignificant in comparison to those from consumption of foods containing the substances naturally, or as added flavouring agents. The mean intake of octanoic acid from foods consumed as part of the diet in the USA was estimated to be approximately 200 mg/day. A highly conservative estimate of exposure for octanoic acid of 1.9 mg per day resulting from the use of the antimicrobial solutions was noted by the Committee. This estimate was prepared employing WHO Global Environment Monitoring System — Food Contamination Monitoring and Assessment Programme (GEMS/Food) international diets. Intake of acetic acid was not explicitly analysed, but its use in and on foods (vinegar) would result in a greater food exposure than that from octanoic acid. Exposure to these common food acids was not further considered in this evaluation.

HEDP is expected to remain on foods that are treated with the antimicrobial washes and not further washed, processed, or cooked. The Committee considered submitted information concerning residues of HEDP on foods. Studies were conducted with meats, poultry, fruits, and vegetables, each treated with one of the solutions under typical conditions of use. The foods were allowed to drain, but were not further processed or cooked. It was assumed that all additional weight in the meats treated was attributable to the antimicrobial wash; the concentration of HEDP in the solutions was used to determine the residual concentration of HEDP present in the meat. Poultry was further treated to recover any HEDP present. Fruit and vegetables were washed with deionized water to recover the residual HEDP. For vegetables, lower- and upper-bound estimates of intake were made based on the differing surface areas of the treated foods. Broccoli, a vegetable with a high surface area, provided the data for the upper-bound estimates, while tomato was used to provide the lower-bound estimates. Furthermore, for processed fruit and vegetables, it was assumed that each would be treated twice; before cutting or processing and again afterwards. Thus, the measured residues were doubled, assuming no loss from either treatment. The results are reported in Table 8 (unpublished data from the submitter).

3.2 International estimates of intake

The Committee considered international estimates of intake of HEDP, prepared using food information taken from the GEMS/Food regional diets and the data on HEDP residues from Table 8. The intake of every food that could be treated with

Table 8. Residues of HEDP in treated foods

Type of food treated	Residue of HEDP (μg/kg, ppb)
Meats	
Carcasses	58
Parts/trim	161
Poultry	198
Fruit and vegetables (single treatment)	
Low surface area	4.2
High surface area	67.5
Fruits and vegetables (double treatment)	
Low surface area	8.4
High surface area	135

From unpublished data from the submitter.

HEDP was combined with the appropriate residue concentration for each of the five regional diets. Two estimates were prepared for each region; one using the residue concentration for a vegetable with a low surface area and the other using the residue concentration for a vegetable with a high surface area. It was assumed that there would be no reduction in HEDP residues after washing or cooking. Further, it was assumed that all fruit and vegetables would be treated three times with the antimicrobial solution with no loss; once on the raw commodity and twice during further processing.

The highest estimate of intake was from the European diet; 3.6 μg/kg bw per day for the upper-bound estimate using a model for a vegetable with a high surface area. All the estimates are summarized in Tables 9 and 10.

3.3 National estimates of intake

The Committee considered three national estimates of intake. The first was a total diet study from the Czech Republic. The two remaining studies were based on individual dietary records in the USA and the UK, respectively.

Completed in 1995, the Czech total diet study considered 160 foodstuffs. The foods were prepared using standard recipes. Each food that might be treated with the antimicrobial solution was considered, with a food intake matched to an HEDP concentration from Table 8. Lower-bound and upper-bound estimates were made using the data for vegetables with low surface area and data for the vegetables with high surface area separately. Average daily food consumption values for the Czech Republic were used. The lower-bound estimate of exposure was 0.405 μg/kg bw per day and the upper-bound estimate was 2.224 μg/kg bw per day.

The estimates from the USA and the UK were made in a similar manner. For each individual surveyed, all foods that could have been treated with the antimicrobial solution were considered. The appropriate concentration of HEDP residue was multiplied by the intake of each food and the total intake of HEDP for each

Table 9. International estimates of intake of HEDP (lower bound)

GEMS/ Food code	Food	HEDP residue (μg/kg, ppb)	Intake of HEDP (μg/kg bw per day) in GEMS/ Food regional diet				
			Middle East	Far East	Africa	Latin America	Europe
VR75	Roots	12.6	0.013	0.023	0.067	0.033	0.051
VD70	Pulses	12.6	0.005	0.004	0.004	0.005	0.003
VD70	Nuts	12.6	0.003	0.011	0.007	0.012	0.006
VD70	Vegetable fat	12.6	0.008	0.003	0.005	0.005	0.008
HS93	Spices	12.6	0.001	0.001	0.000	0.000	0.000
HS93	Vegetables	12.6	0.049	0.038	0.016	0.032	0.078
PE112	Fruit	12.6	0.043	0.018	0.020	0.057	0.045
MO105	Offal	68	0.005	0.002	0.003	0.007	0.014
MO105	Meat	68	0.042	0.037	0.027	0.053	0.176
PM110	Poultry	198	0.102	0.044	0.018	0.083	0.175
PO111	Poultry offal	198	0.000	0.000	0.000	0.001	0.001
PF111	Poultry fat	198	0.010	0.004	0.002	0.008	0.017
MF95	Mammalian fat	68	0.001	0.002	0.001	0.005	0.009
Total intake			0.321	0.222	0.114	0.211	0.753

Table 10. International estimates of intake of HEDP (upper bound)

GEMS/ Food code	Food	HEDP residue (μg/kg, ppb)	Intake of HEDP (μg/kg bw per day) in GEMS/ Food regional diet				
			Middle East	Far East	Africa	Latin America	Europe
VR75	Roots	202.4	0.208	0.366	1.084	0.537	0.816
VD70	Pulses	202.4	0.083	0.067	0.060	0.078	0.041
VD70	Nuts	202.4	0.043	0.169	0.115	0.194	0.101
VD70	Vegetable fat	202.4	0.136	0.048	0.079	0.074	0.130
HS93	Spices	202.4	0.008	0.010	0.006	0.002	0.002
HS93	Vegetables	202.4	0.786	0.604	0.260	0.508	1.254
PE112	Fruit	202.4	0.689	0.288	0.319	0.915	0.716
MO105	Offal	68	0.005	0.002	0.003	0.007	0.014
MO105	Meat	68	0.042	0.037	0.027	0.053	0.176
PM110	Poultry	198	0.102	0.044	0.018	0.083	0.175
PO111	Poultry offal	198	0.000	0.000	0.000	0.001	0.001
PF111	Poultry fat	198	0.010	0.004	0.002	0.008	0.017
MF95	Mammalian fat	68	0.001	0.002	0.001	0.005	0.009
Total intake			2.153	1.676	1.994	2.515	3.623

Table 11. Estimates of intakes of HEDP used in antimicrobial wash solutions

Estimates	Exposure (μg/kg bw per day)
GEMS/Food	3.6 (European regional diet)
Czech Total Diet Study	2.224 (mean)
USA dietary records	4.706 (90th percentile, upper bound)
UK dietary records	3.263 (90th percentile, upper bound)

food was calculated for each individual. The mean and 90th-percentile intakes for the whole population were computed from the individual records. The data on food intake from the USA were taken from the USA Department of Agriculture Continuing Survey of Food Intakes by Individuals, 1994–6, 1998. The data on food intake from the UK were taken from the Ministry of Agriculture, Food, and Fisheries Dietary and Nutritional Survey of British Adults, 1986–7. Here again, lower-bound and upper-bound estimates were made using the data for vegetables with low surface area and vegetables with high surface area separately.

The mean estimate of intake for the USA was 0.357 (lower bound) or 2.235 (upper bound) μg/kg bw per day. The corresponding intakes for individuals at the 90th percentile of consumption were 0.740 and 4.706 μg/kg bw per day, respectively. The mean estimate of intake for the UK was 0.243 (lower bound) or 1.795 (upper bound) μg/kg bw per day. The corresponding intakes for individuals at the 90th percentile of consumption were 0.458 and 3.263 μg/kg bw per day, respectively.

Table 11 summarizes the estimates of intake of HEDP used in antimicrobial wash solutions.

3.4 Non-food uses of HEDP

HEDP is used as an anti-scaling agent for water treatment and in boilers. The regulatory limit for this use in the USA is 25 μg/l. However, HEDP is known to be used in the rest of the world, including China. It is also used as a drug for treatment of Paget disease (a disease of excessive bone turnover) and in some over-the-counter cosmetic and pharmaceutical formulations. The Environmental Protection Agency in the USA has estimated exposure to HEDP from these uses to be no more than 6 μg/kg bw per day, including 0.04 μg/kg bw per day from its use on food (Environmental Protection Agency, 1998). The Committee noted that this estimate of exposure for food uses of HEDP was much less conservative than that evaluated herein, assuming that cooking and further processing of treated foods would result in concentrations of HEDP of no greater than 1 μg/kg on food as consumed.

4. STUDIES ON THE QUALITY, NUTRITIONAL VALUE, OR OTHER PROPERTIES OF FOOD TREATED WITH ANTIMICROBIAL SOLUTIONS

4.1 Thiobarbituric acid and fatty acid profiles of meat and poultry products

The antimicrobial wash solution identified as solution A was added to water used for spraying and dipping poultry carcasses in a study to determine whether the treatment resulted in significant differences in thiobarbituric acid (TBA) and fatty acid profiles of raw or cooked poultry products. No differences were found when compared with treatment with water (Ecolab, Inc., 2000).

Samples of fresh beef were exposed to antimicrobial wash solution B, which contains total peroxyacids at a concentration of 200 mg/kg, to determine whether the treatment resulted in significant differences in TBA and fatty acid profiles of cooked and uncooked meat (Ecolab, Inc., 1999a, 1999b). Cooking to an internal temperature of 175 °F increased the TBA value by eightfold relative to uncooked samples. No differences in TBA or fatty acid profiles compared with treatment with water were found. There was a slight difference ($p = 0.54$) in values for myristic acid between raw meat and cooked meat treated with peroxyacid; this was attributed to cooking or variation in the meat samples tested.

The reagent TBA is commonly used to determine the extent to which animal and vegetable fats and oils (including fatty acids, their esters, and related substances) are oxidized. Thus TBA values provide a measure of rancidicity. The results of testing for TBA and the determinations of fatty acid profiles described above suggested that treating poultry or meat with solutions A or B did not adversely impact the quality of treated poultry or meat products, respectively.

4.2 The effect of the potential reactivity of hydrogen peroxide and peroxyacetic acid on meat and poultry products

In a study by Upendraroa et al. (1972), vegetable oils placed in contact with 30% or 60% hydrogen peroxide and 5% or 17% peroxyacetic acid for 2–10 h underwent epoxidation. Other studies that used high concentrations of hydrogen peroxide and long periods of contact were found in the literature and mainly indicated potential reactions of hydrogen peroxide with other food components. The low concentrations of the components of antimicrobial solutions in ready-to-use wash solutions and sprays, and the transient nature of their contact with food is expected to prevent potential oxidation reactions from occurring on food. The low reactivity potential of solutions C and D has been confirmed in a study of their effects, under the intended condition of use, on fruit and vegetables (Ecolab, Inc., 1995).

4.3 Nutritional tests to determine the effects of peroxyacetic acid and hydrogen peroxide on fruit and vegetables

A study was conducted to determine the effects of peroxyacetic acid and hydrogen peroxide on the nutrient content of fruit and vegetables (Ecolab, Inc.,

1995). Tomatoes, potatoes, and broccoli were prepared for consumption, exposed to solution C (containing peroxyacetic acid at 80 mg/kg and hydrogen peroxide at 59 mg/kg) for 5 min ('worse-case' conditions of exposure), then rinsed. Control and treated samples were analysed for effects on β-carotene and vitamin C, nutrients chosen for analysis because of their susceptibility to oxidation and other degradation reactions. There was no effect on β-carotene content in tomatoes or broccoli. There was no effect on vitamin C in potatoes or broccoli. There was a treatment-related decrease of 37% in the ascorbic acid content of tomatoes, which occurred in conjunction with an equivalent increase in dehydroascorbic acid content. Thus, the active content of vitamin C (Sabry et al., 1958) in tomatoes was unchanged (unpublished data from the Pillsbury Company). These results indicated that the use of antimicrobial wash solution C on fresh fruits and vegetables would not be expected to adversely affect their nutrient content.

5. COMMENTS

Antimicrobial solutions are equilibrium solutions that are diluted in water before use in food processing. Hydrogen peroxide in these solutions will dissociate into water and oxygen. Both peroxyacetic acid and peroxyoctanoic acid are also inherently unstable and will break down into acetic acid and octanoic acid, respectively, although their stability is enhanced by HEDP. Low residual amounts of these simple organic acids present on food at the time of consumption would pose no safety concern. It is not expected that residues of peroxyacetic acid or peroxyoctanoic acid from these solutions will be present on treated foods at the time of consumption. The peroxide components of the peroxyacid antimicrobial solutions thus pose no toxicological concerns with regard to the uses considered by the Committee. The Committee concluded that HEDP, which sequesters metal ions, thereby stabilizing the peroxy compounds in peroxyacid antimicrobial solutions, is the only component of potential toxicological concern.

Data reviewed by the Committee indicated that absorption of HEDP from the gastrointestinal tract is very limited and that its metabolism is negligible. The limited amount of data available to the Committee suggested that absorption may be related to age and species. The skeleton is the target site for the disposition of HEDP in all species.

HEDP did not show evidence of mutagenic activity in assays in five strains of *Salmonella* or in an assay for mutation in mouse lymphoma L51718 $Tk^{+/-}$ cells, with and without metabolic activation from mammalian microsomes.

In two 90-day studies of toxicity, rats were fed diets containing HEDP at doses ranging from 100 to 2500 mg/kg bw per day. The highest dose tested in each study (i.e. 1500 or 2500 mg/kg bw per day) caused mortality and signs of toxicity, but no effects were reported at lower doses in either study. The NOEL was 500 mg/kg bw per day in both studies.

In a 90-day study of toxicity in dogs, HEDP was administered orally at a dose equivalent to 0, 25, 75, or 250 mg/kg bw per day. No adverse effects attributable to treatment were reported and the NOEL for HEDP was 250 mg/kg bw per day. The Committee also evaluated the results of a long-term study to determine the

skeletal effects of daily subcutaneous injections of HEDP administered to adult female dogs for varying periods ranging from 1 to 2 years. Some effects on bone parameters were observed at all doses. Profound skeletal effects were associated with the administration of daily subcutaneous doses of HEDP of 2–10 mg/kg bw per day for 1 year. Spontaneous bone fractures were slightly increased in dogs given daily subcutaneous doses of 0.5 mg/kg bw for 2 years, but no permanent skeletal changes were observed at this dose and healing was normal. No fractures were observed at a daily subcutaneous dose of 0.1 mg/kg bw after 2 years. Assuming that 10–20% of the administered dose were absorbed from the gut in dogs, a subcutaneous dose of 0.1 mg/kg bw per day would correspond to an oral dose of 0.5–1 mg/kg per day. In considering these studies, the Committee noted that 90 days might not be long enough to observe skeletal effects in dogs and that there might be differences in the disposition of HEDP in bone that are related to the route of administration.

In a combined two-generation study of reproductive toxicity and teratogenicity, rats were given HEDP (disodium salt) in the diet at concentrations equivalent to 0, 50 or 250 mg/kg bw per day either during their lifetime or only on days 6–15 of gestation, for two generations. No fetal abnormalities indicative of a teratogenic effect were reported at either dose tested. HEDP was embryotoxic when administered at a dose of 250 mg/kg bw per day during organogenesis. The NOEL for HEDP was 50 mg/kg bw per day.

The effects of HEDP (disodium salt) were determined in a combined study of reproductive toxicity and teratogenicity in rabbits. Two experiments were performed because of the observation of toxicity at the lowest and highest doses, administered by gavage, in the first experiment. In the second experiment, rabbits received HEDP at a dose of 0, 25, 50, or 100 mg/kg bw per day in the diet, or 100 mg/kg bw per day by gavage. Fetuses from dams receiving HEDP at a dose of 100 mg/kg bw per day by gavage were significantly smaller than those from untreated controls. No fetal abnormalities indicative of a teratogenic effect in rabbits were observed in either experiment. The NOEL was 50 mg/kg bw per day.

Use of HEDP to treat Paget disease

The disodium salt of HEDP, known clinically as sodium etidronate, is administered orally at a starting dose of 5 mg/kg bw per day, for not longer than 6 months, to treat patients with Paget disease. Paget disease is an idiopathic disease characterized by accelerated bone metabolism; fractures and other abnormalities of the bone are common in patients with Paget disease. Owing to its high affinity for solid-phase calcium phosphate, HEDP prevents the growth and dissolution of hydroxyapatite crystals on crystal surfaces of bone. The mechanism of action, however, is not fully understood.

Antimicrobial efficacy

Information available to the Committee indicated that solutions of peroxyacetic acid enhance the action of water sprayed on food surfaces to reduce numbers of bacteria. While reductions in numbers of microbes were demonstrated, some of

the data provided suggest that the results of replicate tests were rather inconsistent, with standard deviations close to or greater than the value of the reductions themselves. Testing of food surfaces showed modest reductions in numbers of microbes, when either endogenous microorganisms (represented by total aerobic plate counts) or inoculated ('spiked') pathogens (commonly *L. monocytogenes*, *E. coli* O157:H7, and some *Salmonella* serotypes) were monitored. Data from laboratory and in-plant tests indicated that the use of these solutions would minimize the possibility of cross-contamination, although they are unable to remove all adherent viable bacteria from food surfaces.

The Committee did not further consider the antimicrobial efficacy of peroxyacid antimicrobial solutions containing HEDP.

Intake

The Committee evaluated estimates of intake of each component used in the peroxyacid solutions on the basis of residual amounts anticipated to be present on treated food at the time of consumption. Consistent with what was known about the chemistry of peroxy compounds, no residues of hydrogen peroxide, peroxyacetic acid, or peroxyoctanoic acid were anticipated to be present on foods that have been washed in, sprayed with, or otherwise treated using these solutions.

Acetic and octanoic acid present in the solutions and as by-products from the corresponding peroxyacids would be expected to remain on any treated foods that are not washed or further processed after treatment. The Committee noted that the estimate of exposure to octanoic acid resulting from the use of the antimicrobial solutions, 1.9 mg/day, was highly conservative. The mean intake of octanoic acid from foods consumed as part of the diet in the USA was estimated to be approximately 200 mg/day. Intake of acetic acid was not explicitly analysed, but its use in and on foods (vinegar) would result in a greater exposure than that from the use of peroxyacid antimicrobial solutions. The Committee did not further consider exposure to these common food acids.

HEDP is expected to remain on foods that are treated with antimicrobial solutions and that are not further washed, processed, or cooked. The highest estimate of intake of HEDP prepared using GEMS/Food diets was that for the European diet: 3.6 μg/kg bw per day for the upper-bound estimate using a model for vegetables with a high surface area. The Committee also considered national estimates of intake from the Czech Republic, the USA, and the UK. The upper-bound estimate of intake was 2.2 μg/kg bw per day for the Czech Republic. The mean and 90th percentile upper-bound estimates of intake for the USA were 2.2 and 4.7 μg/kg bw per day, respectively. The mean and 90th percentile upper-bound estimates of intake for the UK were 1.8 μg/kg bw per day and 3.3 μg/kg bw per day, respectively.

The Committee was aware of the non-food uses of HEDP. It is used as an anti-scalant for water treatment and in boilers worldwide (the regulatory limit for this use is 25 μg/l in the USA). HEDP is also used as a drug to treat Paget disease, and in some over-the-counter cosmetic and pharmaceutical formulations. The USA Environmental Protection Agency estimated that exposure to HEDP from all these

uses was not more than 6 μg/kg bw per day, including 0.04 μg/kg bw per day from its use on food (Environmental Protection Agency, 1998). The Committee noted that this estimate of exposure resulting from food uses of HEDP was much less conservative than that used in the present evaluation.

Assessment of the effects on food quality and nutritional value

Limited data on the quality and nutritional value of foods treated with peroxyacid antimicrobial solutions were provided to the Committee. Studies were conducted to determine whether treatment of foods with peroxyacid antimicrobial solutions resulted in significant differences in concentrations of thiobarbituric acid (a measure of rancidity), or in fatty-acid profile testing of raw or cooked poultry products and fresh beef samples, when compared with treatment with water only. No differences were found.

The Committee was aware that studies in the literature indicated potential reactions of hydrogen peroxide with components of food. The Committee noted that such studies are typically conducted using high concentrations and long periods of exposure and that, under the conditions of their intended use, the potential reactivity of peroxyacid antimicrobial solutions is expected to be limited. Studies available to the Committee confirmed the low potential reactivity of two peroxyacid antimicrobial solutions in dilute ready-to-use solutions that are in brief contact with fruits and vegetables.

A study was conducted to determine the effects of peroxyacetic acid and hydrogen peroxide on the content of β-carotene and vitamin C in tomatoes, potatoes and broccoli. These foods were prepared for consumption using 'worst-case' exposure conditions, i.e. peroxyacetic acid at 80 mg/kg and hydrogen peroxide at 59 mg/kg for 5 min, and then rinsed. When treated samples were compared with controls, there were no effects on the β-carotene content of tomatoes or broccoli, on the vitamin C content of potatoes or broccoli, or on the active vitamin C content of tomatoes.

On the basis of the available data, the Committee concluded that peroxyacid antimicrobial solutions are unlikely to have an adverse effect on food quality or nutritional value, with regard to the uses considered by the Committee.

6. EVALUATION

The Committee considered the safety, on a component-by-component basis, of antimicrobial solutions containing HEDP and three or more of the following components: peroxacetic acid, acetic acid, hydrogen peroxide, octanoic acid and peroxyoctanoic acid. These solutions are intended to be diluted before use to achieve peroxyacid concentrations in the range of 80 to 220 mg/kg. The Committee concluded that the peroxy compounds in these solutions (hydrogen peroxide, peroxyacetic acid and peroxyoctanoic acid) would break down into acetic acid and octanoic acid, and that small residual quantities of these acids on foods at the time of consumption would not pose a safety concern. Therefore, the Committee focused its evaluation on the residues of HEDP that are expected to remain on

foods treated, in accordance with manufacturers instructions, with peroxyacid antimicrobial solutions that contain HEDP at up to <1%.

The Committee compared the highest estimate of intake of HEDP from the uses of peroxyacid antimicrobial solutions considered by the Committee (i.e. 0.004 mg/kg bw per day) with the starting oral dose used to treat Paget disease (i.e. 5 mg/kg bw per day) and noted that the margin of exposure is >1000. On the basis of this margin of exposure, the conservative nature of the estimates of intake of HEDP, and the available toxicity data, the Committee concluded that HEDP does not pose a safety concern at the concentrations of residue that are expected to remain on foods.

The Committee noted that the use of peroxyacid antimicrobial solutions does not replace the need for good hygienic practices in handling and processing of food.

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STEVIOL GLYCOSIDES

First draft prepared by

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1. EXPLANATION

Steviol glycosides are natural constituents of the plant *Stevia rebaudiana* Bertoni, a member of the *Compositae* family. The leaves of *S. rebaudiana* Bertoni contain at least ten different glycosides, the major constituents being stevioside and rebaudioside A. The material evaluated at the present meeting contains not less than 95% glycosylated derivatives of steviol, primarily stevioside, rebaudiosides A and C and dulcoside A (Figure 1), with minor amounts of rubusoside, steviolbioside, and rebaudiosides B, D, E and F (Figure 2).

At its fifty-first meeting (Annex 1, reference *149*), the Committee evaluated toxicological data on stevioside and the aglycone steviol. The Committee noted several shortcomings in the available information and requested that specifications should be developed to ensure that the material tested is representative of the material of commerce. Further information was required on the nature of the substance tested, on the metabolism of stevioside in humans and on the activity of steviol in suitable studies of genotoxicity in vivo.

There is no single common or trivial name in common usage for the evaluated mixture of glycosylated derivatives of steviol. At its thirty-third meeting (Annex 1, reference *83*), the Committee developed guidelines for designating titles for specification monographs. According to these guidelines, the title of a monograph should, in such circumstances, be selected from the available scientific, common and trivial names. The name chosen must be nonproprietary and should be a scientifically accurate description of the substance. In addition, the name should communicate to the consumer an accurate description of the substance, within the scope of existing names for food additives. At its present meeting, the Committee established that the evaluated material of commerce for which specifications were developed should be known as 'steviol glycosides'. The Committee reviewed additional biochemical and toxicological data on the major glycosylated derivatives of steviol and on the aglycone, steviol.

This monograph describes the new data on steviol glycosides discussed at the present meeting, together with summaries of the key toxicological data on stevioside evaluated by the Committee at its fifty-first meeting.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

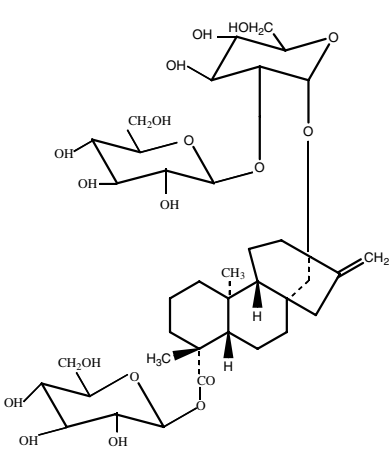
2.1.1 Absorption, distribution and excretion

At its fifty-first meeting (Annex 1, reference *149*), the Committee noted that in rats treated orally stevioside is not readily absorbed from the upper small intestine but is hydrolysed to the aglycone, steviol, before absorption from the gut. New information on absorption in in-vitro models and in rats was available at the present meeting.

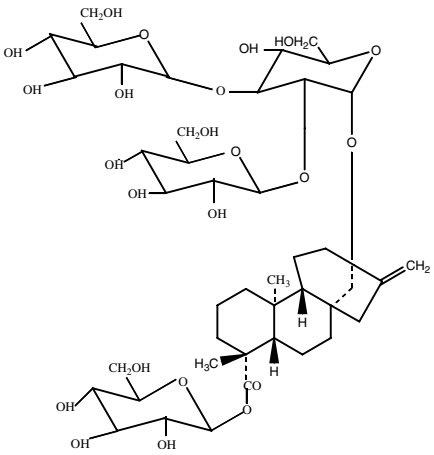
Intestinal transport of stevioside (1 mmol/l), rebaudioside A (1 mmol) and steviol (30 μmol/l – 1 mol/l) has been investigated in a Caco-2 cell monolayer model. The integrity of the monolayer was verified with fluorescein. Transport of stevioside and rebaudioside A was very low (apparent permeability coefficients, 0.16×10^{-6} and 0.11×10^{-6} cm/s, respectively). Steviol was transported more effectively, with a higher apparent permeability coefficient for absorptive transport (44.5×10^{-6} cm/s) than for secretory transport (7.93×10^{-6} cm/s) at a concentration of 100 μmol/l. At concentrations of 300 μmol/l and 1 mol/l, steviol slightly compromised the integrity of the monolayers during transport (Geuns et al., 2003a).

The intestinal absorption of a *Stevia* mixture and the aglycone steviol was investigated using everted gastrointestinal sacs from four male Sprague-Dawley

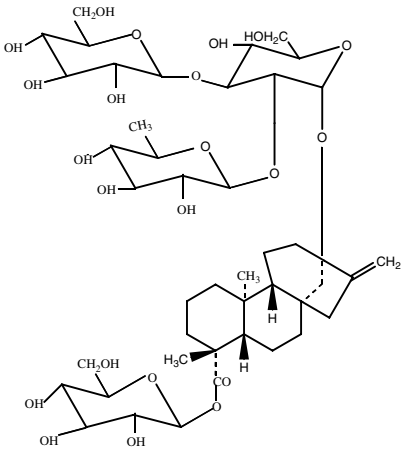
Figure 1. Structures of the major steviol glycosides



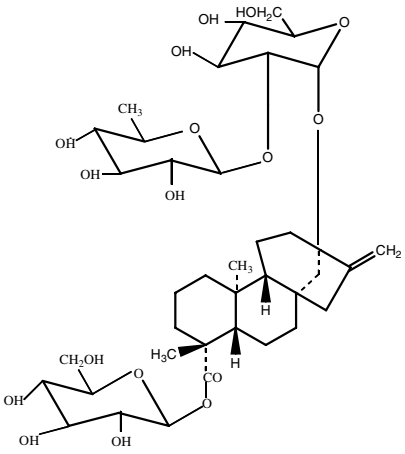
Stevioside



Rebaudioside A

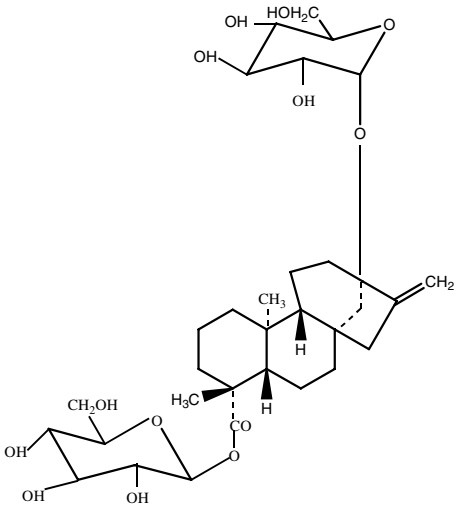


Rebaudioside C

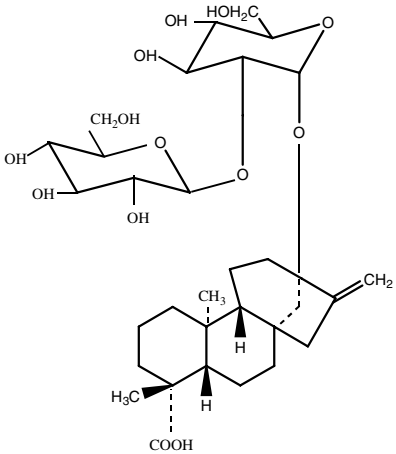


Dulcoside A

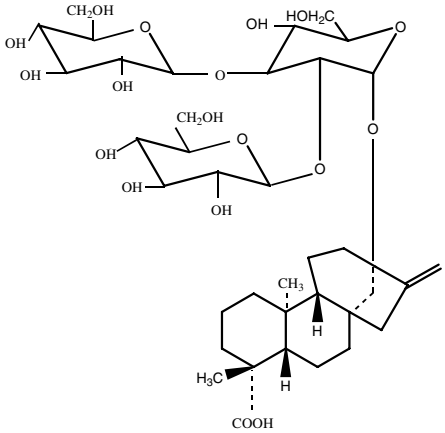
Figure 2. Structures of minor steviol glycosides



Steviolbioside



Rubusoside



Rebaudioside B

rats. The *Stevia* mixture contained rebaudioside A (28.8%), rebaudioside C (25.2%), stevioside (17.0%) and dulcoside A (10.2%). The everted sacs were incubated in *Stevia* mixture (0.5 mg/ml) or steviol (0.1 mg/ml) for 30 min. Transport of salicyclic acid (10 μg/ml) was used to confirm that the sacs were functional. Steviol was transported in both the duodenum–jejunum and the ileum (76% and 95% of salicyclic acid transport, respectively). The steviol glycosides were poorly absorbed from the *Stevia* mixture, with more than 93% remaining in the mucosal fluid (Koyama et al., 2003a).

Absorption of the *Stevia* mixture described above was also investigated in vivo in four male Sprague-Dawley rats. *Stevia* mixture (in 2% gum arabic) was administered at a dose of 125 mg/kg bw. Steviol was not detected in plasma at 1 h, but was detected at increasing concentrations between 2 h and 8 h, when the concentration reached a peak of about 5 μg/ml. In contrast, the peak plasma concentration of steviol (18.31 μg/ml) was observed 15 min after a single oral administration of steviol (45 mg/kg in corn oil). These doses were approximately equimolar for steviol (Koyama et al., 2003a).

Similarly, in male Sprague-Dawley rats given a single oral dose of stevioside (purity, 95%) at 0.5 g/kg bw, low concentrations of steviol were detected in plasma for the first 8 h, followed by a rapid increase to a concentration of about 1000 ng/ml at 24 h. This study used a highly sensitive method for detection of steviol, but did not examine levels of stevioside or other metabolites (Wang et al., 2004).

One study has reported detectable levels of stevioside, but not steviol, in plasma after administration of a *Stevia* product. Groups of male Sprague-Dawley rats were given T100 sunstevia 95% (containing 70% stevioside) at a dose of 0.5 or 2 g/kg bw by gavage. Stevioside was detected in plasma 5 min after dosing. There was considerable variation between animals, with the time to maximum plasma concentration varying from 10 to 300 min. Clearance did not differ significantly between the doses. Reported plasma half-lives were 10.6 ± 8.7 and 6.7 ± 3.7 h at 0.5 and 2.0 g/kg bw, respectively. At 48 h, 5.7–16.9% and 1–6.7% of the total administered dose of stevioside was receovered in the faeces and urine, respectively. Steviol was detected in faeces collected up to 48 h, but not in plasma sampled up to 24 h after dosing (limit of detection, 1 μg/ml) (Sung, 2002).

2.1.2. Biotransformation

Incubation of stevioside (purity, >96%; concentration, 50 mg/l) with chicken excreta under anaerobic conditions for 24 h resulted in a 20% conversion of stevioside into steviol (Geuns et al., 2003b).

Faecal bacterial suspensions from eleven healthy volunteers (six men and five women) were incubated under anaerobic conditions with 40 mg of stevioside (purity, 85%) and 40 mg of rebaudioside A (purity, 90%) for 72 h. Stevioside and rebaudioside A were completely hydrolysed to the aglycone steviol within 10 and 24 h, respectively. Among cultures of coliforms, bifidobacteria, enterococci and bacteroides, only the bacteroides were able to hydrolyse these compounds. The data indicated that both glycosides were initially hydrolysed to steviolbioside (this occurred more slowly with rebaudioside A), and the steviolbioside was then rapidly

metabolized to steviol. Steviol remained unchanged during the 72 h incubation, indicating that bacterial enzymes are not able to cleave the steviol structure (Gardana et al., 2003).

Human faecal metabolism of *Stevia* compounds was investigated in pooled faecal homogenates obtained from five healthy Japanese male volunteers. The materials tested were *Stevia* mixture (main components: rebaudioside A, stevioside, rebaudioside C, dulcoside A), its α-glucose derivative, referred to as enzymatically modified *Stevia* (main components: α-glucosylrebaudioside A, α-glucosylstevioside, α-glucosylrebaudioside C, α-glucosyl dulcoside A), rebaudioside A, stevioside, steviol, rebaudioside C, dulcoside A, rebaudioside B, rubusoside, α-monoglucosylrebaudioside A and α-monoglucosylstevioside. After incubation of the faecal homogenates under anaerobic conditions for 24 h, the *Stevia* mixture, glycosides and α-glucose derivatives were all rapidly degraded. Stevioside was hydrolysed, with successive removal of glucose units via rubusoside, to the aglycone steviol. The metabolism of α-monoglucosylstevioside was similar to that of stevioside after α-deglucosylation. For rebaudioside there were two pathways, a major pathway in which rebaudioside A was hydrolysed via stevioside to steviol, and a minor pathway that suggested that rebaudioside A is metabolized via rebaudioside B to steviol. The metabolism of α-monoglucosylrebaudioside A was similar to that of rebaudioside A after α-deglucosylation. No degradation of steviol was observed over the 24 h incubation period. The authors concluded that steviol was the only final product of the metabolism of *Stevia*-related compounds, including enzymatically modified *Stevia* in human intestinal microflora, and that there were no apparent species differences in the intestinal metabolism of *Stevia* mixture between rats and humans (Koyama et al., 2003b).

Metabolism of steviol (purity not specified) in rats and humans has been investigated using pooled human liver microsomal preparations from five male and five female donors, and from rat liver microsomal preparations with the same protein content. Metabolite formation required a nicotinamide adenine dinucleotide phosphate, reduced (NADPH)-generating system, indicating cytochrome P450 (CYP)-dependent metabolism. The metabolic profile obtained with human liver microsomal fractions was similar to that obtained with rat liver microsomal preparations; mass spectrometric analysis indicated the presence of two dihydroxy metabolites and four monohydroxy metabolites. One additional monohydroxy metabolite was detected with the rat preparation. The liver microsomal clearance of steviol was approximately four times lower in humans than in rats (Koyama et al., 2003a).

Hamsters were given stevioside (purity not specified) at a dose of 1 g/kg bw by gavage and metabolites were measured in the plasma, urine and faeces at 3, 24 and 24 h, respectively. The samples were treated with glucuronidase/sulfatase to hydrolyse conjugated metabolites. Steviol-16,17α-epoxide, stevioside, 15 α-hydroxysteviol and steviolbioside were detected in the plasma, urine and faeces. In addition, isosteviol was detected in the urine and faeces, and steviol was detected in the faeces (Hutapea et al., 1999).

Chickens were given stevioside (purity, >96%) at a dose of 643 or 1168 mg/kg bw by intubation. Most of the stevioside was recovered unchanged in excreta in the 24–48 h after administration, and only about 2% was converted to steviol. Neither stevioside nor steviol were detected in the blood. Sixteen broiler chickens and four laying hens were also given stevioside at a dose of 667 mg/kg of feed for 14 and 10 days, respectively. Most of the stevioside was untransformed in the excreta, with about 21.5% and 7.3% being converted to steviol by broiler chickens or laying hens, respectively. No stevioside or steviol was detected in the blood or in the eggs (Geuns et al., 2003b).

Six female pigswere given stevioside (purity, >96%) at a dose of 1.67 g/kg of feed for 14 days (equivalent to approximately 70 mg/kg bw per day). Steviol, but not stevioside, was detected in the faeces, indicating bacterial metabolism of stevioside to steviol. No stevioside or steviol was detected in the blood. The authors concluded that stevioside was completely converted to steviol and suggested that the possible uptake from the colon was very low (Geuns et al., 2003a).

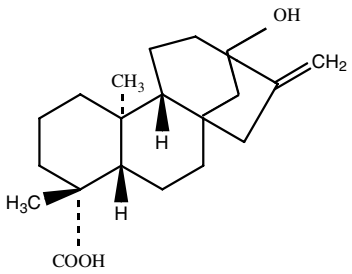
Metabolism of stevioside by human volunteers has been investigated in a collaborative study conducted in Belgium and Italy. In Italy, nine healthy men (aged 20–50 years) were given capsules containing 375 mg of stevioside (purity not specified) after an overnight fast. Low concentrations of stevioside were detected in the plasma of seven of the subjects, with a maximum of 0.1 μg/ml. Peak plasma concentrations occurred at 60 to 180 min after dosing. Steviol glucuronide was detected in five of the men. No free steviol, steviol-16,17α-epoxide, 15α-hydroxysteviol or 15-oxo-steviol was detected. Steviol glucuronide was detected in the urine of all men, and low concentrations of stevioside were also present in the urine of two men. Free steviol or its unconjugated metabolites were not detected. Only free steviol was detected in the faeces. In Belgium, five male and five female volunteers (aged 24 ± 2 years) were each given nine doses of 250 mg of stevioside (purity, >97%; impurities being other *Stevia* glycosides) at 8 h intervals on three successive days. No stevioside or free steviol was detected in the blood. After hydrolysis with β-glucuronidase/sulfatase, steviol was detected at concentrations ranging from 0.7 to 21.3 μg/ml, with peak concentrations occurring at varying times up to 5 h. Similarly, stevioside and conjugated steviol were detected in the urine at 24 h. The only compound detected in the faeces was free steviol. The differences between the two studies were considered to be due to the different doses of stevioside administered and the different detection limits of the analytical method for stevioside. The total recovery of steviol metabolites varied between 22% and 86% of the administered daily dose of stevioside (mean total recovery, 52.1 ± 27%) (Geuns & Pietta, 2004).

The major metabolites of steviol glycosides are shown in Figure 3.

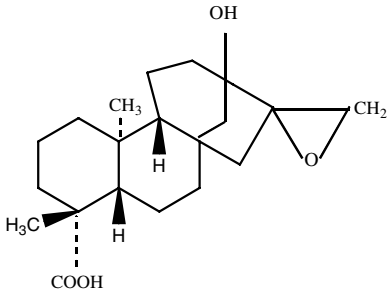
2.1.3 Effects on enzymes and other biochemical parameters in vitro

In isolated aortic rings from normal rats, stevioside (purity not stated) at a concentration of 10^{-8} to 10^{-5} mol/l caused a concentration-dependent relaxation of vasopressin-induced vasoconstriction when incubated in medium containing calcium, but not in calcium-free medium. The results of studies in a rat aortic

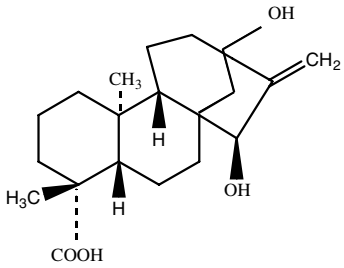
Figure 3. Metabolites of steviol glycosides



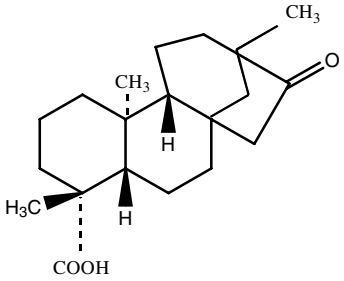
Steviol



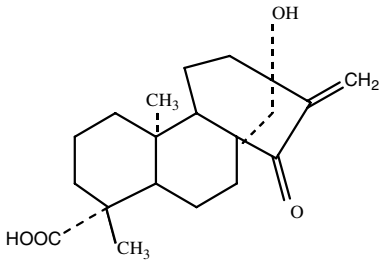
Steviol-16,17α-epoxide



15 α-hydroxysteviol



Isosteviol



15-oxo-steviol

smooth muscle cell line (A7r5) indicated that this was due to inhibition of the stimulatory effects of vasopressin on intracellular calcium ions (Ca^{2+}). Stevioside did not inhibit calcium ionophore (A23187)-induced Ca^{2+} influx. The effects of stevioside were not inhibited by methylene blue. The authors concluded that the vasorelaxation effect of stevioside was mediated mainly through inhibition of Ca^{2+} influx and was not related to nitric oxide (Lee et al., 2001; Liu et al., 2003).

The role of potassium channels in the vasodilator effect of isosteviol (purity not stated) was investigated in isolated aortic rings prepared from Wistar rats. Isosteviol at concentrations of 10^{-8} to 10^{-5} mol/l relaxed the vasopressin-induced vasoconstriction in a concentration-dependent manner. Potassium chloride, and inhibitors specific for the ATP-sensitive potassium channel, inhibited the vasodilator effect of isosteviol. Methylene blue failed to modify the vasodilation produced by isosteviol, suggesting that nitric oxide did not play a role. The authors concluded that vasodilation induced by isosteviol was related to the opening of the calcium-activated and ATP-sensitive potassium channels (Wong et al., 2004).

Stevioside (purity, 95%) and steviol (purity, 90%) at concentrations of 10^{-9} to 10^{-3} mol/l enhanced insulin secretion in isolated mouse pancreatic islets and in a pancreatic-β-cell line (INS-1). The maximal effect was observed with steviol at 10^{-6} mol/l and with stevioside at 10^{-3} mol/l. The insulinotropic effect was dependent on the concentration of glucose (Jeppesen et al., 2000).

Subsequent studies indicated that stevioside at 10^{-3} mol/l enhanced the insulin content of the INS-1 cells, partly by induction of genes involved in glycolysis. Stevioside upregulated the expression of the liver-type pyruvate and acetyl-coenzyme A (CoA)-carboxylase and downregulated the expression of carnitine palmitoyltransferase 1 (CPT-1), long-chain acyl-CoA dehydrogenase, cytosolic epoxide hydrolase and 3-oxoacyl-CoA thiolase. In addition, stevioside improved nutrient sensing mechanisms, increased cytosolic long-chain fatty acyl-CoA and downregulated phosphodiesterase 1 (PDE1). Steviol showed similar effects (Jeppesen et al., 2003).

The effect of stevioside (purity, 95%) on the transepithelial transport of *p*-aminohippurate was investigated in isolated S_2 segments of the rabbit proximal renal tubules. Stevioside (0.70 mol/l) in the tubular lumen had no effect on the transport of *p*-aminohippurate transport, but when present in the bathing medium it inhibited transport by 25–35%; the inhibitory effect was gradually abolished after stevioside was removed. Stevioside had no effect on Na^+/K^+-activated ATPase activity or cell ATP content. The authors concluded that stevioside at a pharmacological concentration of 0.7 mol/l inhibits transepithelial transport of *p*-aminohippurate by interfering with the basolateral entry step, the rate-limiting step for transepithelial transport. The lack of effect of stevioside on transepithelial transport of *p*-aminohippurate on the luminal side and the reversible inhibitory effect on the basolateral side indicated that stevioside did not permanently change *p*-aminohippurate transport and would not be expected to harm renal tubular function at normal levels of intake in humans (Jutabha et al., 2000).

Rats

Groups of normotensive Wistar-Kyoto rats, spontaneously hypertensive rats, deoxycorticosterone acetate-salt sensitive rats and renal hypertensive rats were given stevioside (purity not stated) at a dose of 50, 100, 200 or 400 mg/kg bw per day by intraperitoneal injection for 1 to 10 days. Treatment with stevioside resulted in significantly decreased blood pressure in all strains of rat, and the effect persisted throughout the 10 days of treatment. Decreased blood pressure was also observed in mature, spontaneously hypertensive rats given drinking-water containing 0.1% stevioside. Administration of drinking-water containing 0.1% stevioside also slowed the age-related progressive increase in blood pressure that occurs in rats of this strain (Hsu et al., 2002).

In Goto-Kakizaki rats (which are used as a non-obese animal model of type-2 diabetes), the intravenous administration of stevioside (purity, 96%) at a dose of 200 mg/kg bw resulted in suppressed plasma glucagon, increased insulin response and suppressed the response to a glucose tolerance test (incremental area-under-the-curve: stevioside, 648 ± 50 mol/l × 120 min; control, 958 ± 85 mol/l × 120 min). In normal Wistar rats, insulin concentrations were increased without altering the blood glucose response or glucagon concentrations (Jeppesen, 2002).

In Goto-Kakizaki rats given drinking-water containing stevioside (purity, >99.6%) at a dose of 25 mg/kg bw per day for 6 weeks, an antihyperglycaemic effect was observed, with enhanced insulin response and suppressed glucagon concentrations, and a pronounced suppression of systolic and diastolic blood pressure (Jeppesen, 2003).

Insulin-sensitive lean Zucker rats and insulin-resistant obese Zucker rats were given stevioside (purity not stated) at a dose of 200 or 500 mg/kg bw by oral gavage, 2 h before an oral test for glucose tolerance. There was no effect on plasma glucose, insulin or free fatty acid concentrations in either the lean or obese groups. At the higher dose, stevioside enhanced whole-body sensitivity to insulin in the lean and obese rats, as shown by a decreased insulin incremental area under the curve and glucose–insulin index. No effect was observed after administration of stevioside at 200 mg/kg bw.

In vitro, stevioside at concentrations of 0.01–0.1 mol/l was found to enhance insulin-stimulated glucose transport in type 1 soleus and type IIb epitrochloearis muscle of both lean and obese Zucker rats. Higher concentrations of stevioside inhibited the insulin-stimulated transport of glucose. The authors concluded that one potential site of action of stevioside was the skeletal muscle glucose transport system (Lailerd et al., 2004).

Dogs

In healthy mongrel dogs, nasogastric administration of stevioside (purity not stated) at a dose of 200 mg/kg bw resulted in a lowering of blood pressure that was maximal at 90 min, returning to baseline by 180 min. A more rapid decrease in blood pressure was observed after intravenous injection of stevioside at 50 mg/kg bw, with the maximum decrease at 5–10 min. In dogs with renal hypertension

induced by ligation of the left renal artery, intravenous administration of stevioside at 20–160 mg/kg bw resulted in a dose-dependent decrease in systolic and diastolic blood pressure. No effect was observed at 10 mg/kg bw (Liu et al., 2003).

2.2 Toxicological studies

2.2.1 Short-term studies of toxicity

Chickens

Sixteen broiler chickens and four laying hens were given diets containing stevioside (purity, >96%) at a concentration of 667 mg/kg of feed for 14 and 10 days, respectively. No significant differences were found in feed intake, body-weight gain and feed conversion (Geuns et al., 2003b).

2.2.2 Long-term studies of carcinogenicity

In a study discussed by the Committee at its fifty-first meeting, groups of 50 male and 50 female Fischer 344.DuCrj rats were given access ad libitum to diets containing stevioside (purity, 95.6%; stevioside was added to the powdered diet, which was then pelleted) at a concentration of 0, 2.5 or 5% (equal to doses of 0, 970 and 2000 mg/kg bw per day for males, and 0, 1100 and 2400 mg/kg bw per day for females) for 104 weeks. The doses were selected on the basis of the results of a 13-week study. Thereafter, all of the groups were maintained on basal (0% stevioside) diet for 4 weeks. All surviving rats were killed in week 108. The body-weight gain of the treated animals was slightly depressed, and a dose–response relationship was seen in males (2.3% and 4.4%) and females (2.4% and 9.2%) at the lowest and highest doses, respectively. Food consumption did not differ between the groups. The final survival rate of males receiving diets containing 5% stevioside was significantly decreased (60%) compared with that of the controls (78%). Absolute weights of the kidney were decreased in males and females at the highest dose; however, there was no significant histopathological evidence of neoplastic or non-neoplastic lesions attributable to treatment in any organ or tissue, except for a decreased incidence of mammary adenomas in females and a reduced severity of chronic nephropathy in males. The authors concluded that stevioside was not carcinogenic in Fischer 344 rats under the experimental conditions used (Toyoda et al., 1995, 1997).

The effects of stevioside (purity not stated) have been investigated in models of two-stage skin carcinogenesis in mice. Groups of 15 male ICR mice were initiated by topical application of 7,12-dimethylbenz[*a*]anthracene (DMBA; 100 μg). Promotion treatment commenced 1 week later, and involved topical administration of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; 1 μg) twice per week for 20 weeks. Topical administration of stevioside (68 μg) 1 h before the TPA resulted in a significant decrease in the percentage of animals with papillomas at 10 and 15 weeks, and in the number of papillomas per mouse at 15 and 20 weeks. In a similar study, groups of 15 female SENCAR mice were initiated by administration of peroxynitrite (33.1 μg) followed by promotion with TPA, twice per week for 20 weeks. Administration of drinking-water containing 0.0025% stevioside from 1 week before to 1 week

after initiation inhibited tumour formation. There was a statistically significant decrease in the percentage of animals with papillomas at 10 and 15 weeks, and in the number of papillomas per mouse at 10, 15 and 20 weeks (Konoshima & Takasaki, 2002).

2.2.3 Genotoxicity

Studies of genotoxicity with purified *Stevia* extract and its major components, stevioside and rebaudioside A, reviewed by the Committee at its fifty-first and present meetings, are summarized in Table 1. These compounds gave negative results in vitro and in vivo. Studies of genotoxicity with steviol and other *Stevia*-derived compounds are summarized in Table 2.

Steviol and its oxidative derivatives steviol-16,17-epoxide, 15-oxo-steviol, steviol methylester and 13,16-*seco*-13-oxo-steviol methylester induced forward mutations in *S. typhimurium* TM677 in the presence, but not in the absence, of a metabolic activation system. The metabolizing system decreased the mutagenicity of steviol methylester 8,13-lactone. The results for 15 α-hydroxy-steviol, steviol methylester and 13,16-*seco*-13α-hydroxy-steviol methylester were negative in this assay (Terai et al., 2002).

Steviol gave negative results in assays for cell mutation and DNA damage in cultured cells (Oh et al., 1999; Sekihashi et al., 2002).

Steviol (purity, >99%) has been investigated in two independent studies of DNA damage using the comet assay. In one study, groups of four male BDF1 mice were given steviol at a dose of 0, 250, 500 or 2000 mg/kg bw and the liver, stomach and colon were examined for the presence of comets. In the second study, groups of four male CRJ:CD-1 mice were given steviol at a dose of 0, 500, 1000 or 2000 mg/kg bw and the liver, kidney, colon and testes were examined for the presence of comets. In both studies, groups of animals were sacrificed at 3 h and 24 h after dosing and methylmethanesulfonate (MMS) was used as a positive control. There were no significant differences in DNA migration distance in any of the organs examined. MMS induced a positive response in all organs examined in both studies (Sekihashi et al., 2002).

Steviol (purity, about 90%) has also been tested in assays for induction of micronuclei formation in the bone marrow of Syrian golden hamsters, Wistar rats and Swiss albino mice. Groups of 20 male and 20 female animals were given steviol at a dose of 4000 mg/kg bw (hamsters) or 8000 mg/kg bw (rats and mice) by gavage. Five animals in each group were killed 24, 30, 48 and 72 h after dosing. An additional group, which served as a positive control, was treated with cyclophosphamide and sacrificed at 30 h. There were no significant increases in the frequencies of micronucleated polychromatic erythrocytes (PCEs) in any of the groups treated with stevioside. The ratio of PCEs to normochromatic erythrocytes (NCEs) was significantly reduced in the female hamsters at 72 h after treatment, and in female rats and mice at 48 h and 72 h. The PCE:NCE ratio did not change in male animals. Cyclophosphamide induced a positive response (Temcharoen et al., 2000).

Table 1. Studies of genotoxicity with purified* Stevia *extract and its major components, stevioside and redaubioside A

End-point	Test system	Material	Purity (%)	Concentration or dose	Result	Reference
In vitro						
Reverse mutation	*S. typhimurium* TA98, TA100	Stevioside	99	50 mg/plate	Negative[a]	Suttajit et al. (1993)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100 TA102, TA104, TA1535, TA1537	Stevioside	83	5 mg/plate[e] 1 mg/plate[f]	Negative	Matsui et al. (1996a)
Forward mutation	*S. typhimurium* TM677	Stevioside	83	10 mg/plate	Negative[a]	Matsui et al. (1996)
Forward mutation	*S. typhimurium* TM677	Stevioside	NS	Not specified	Negative[a]	Medon et al. (1982)
Forward mutation	*S. typhimurium* TM677	Stevioside	NS	10 mg/plate	Negative[a]	Pezzuto et al. (1985)
Gene mutation (*umu*)	*S. typhimurium* TA1535/ pSK1002	Stevioside	83	5 mg/plate	Negative[a]	Matsui et al. (1996)
Gene mutation	*B. subtilis* H17 *rec*+, M45 *rec*–	Stevioside	83	10 mg/disk	Negative[a]	Matsui et al. (1996)
Gene mutation	Mouse lymphoma L5178Y cells, $Tk^{+/-}$ locus	Stevioside	NS	5 mg/m	Negative[a,b]	Oh et al. (1999)
Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside	83	8 mg/ml[e] 12 mg/ml[f]	Negative	Matsui et al. (1996)
Chromosomal aberration	Human lymphocytes	Stevioside	NS	10 mg/ml	Negative	Suttajit et al. (1993)
Chromosomal aberration	Chinese hamster lung fibroblasts	Stevioside	85	12 mg/ml	Negative[e]	Ishidate et al. (1984)
Chromosome aberrations	CHL/IU Chinese hamster lung fibroblasts	Rebaudioside A	NS	1.2–55 mg/ml	Negative[a]	Nakajima (2000a)

Table 1. (contd)

End-point	Test system	Material	Purity (%)	Concentration or dose	Result	Reference
In vivo						
Mutation	*D. melanogaster* Muller 5 strain	Stevioside	NS	2% in feed	Negative[b]	Kerr et al. (1983)
DNA damage (comet assay)	Male BDF1 mouse stomach, colon, liver	*Stevia* extract	Stevioside, 52; rebaudioside A, 22	250–2000 mg/kg	Negative[c]	Sekihashi et al. (2002)
DNA damage (comet assay)	Male ddY mouse stomach, colon, liver, kidney, bladder, lung, brain, bone marrow	*Stevia*	NS	2000 mg/kg	Negative[c]	Sasaki et al. (2002)
Micronucleus formation	ddY mouse bone marrow and regenerating liver	Stevioside	NS	62.5–250 mg/kg	Negative[b]	Oh et al. (1999)
Micronucleus formation	BDF1 mouse bone marrow	Rebaudioside A	NS	500–2000 mg/kg bw per day for 2 days	Negative[d]	Nakajima (2000b)

NS, not specified.
[a] With and without metabolic activation (source not specified in original monograph).
[b] Inadequate detail available.
[c] Killed at 3 h and 24 h.
[d] Killed 30 h after second administration.
[e] Without metabolic activation.
[f] With metabolic activation.

Table 2. Studies of genotoxicity with steviol and other* Stevia*-derived compounds

End-point	Test system	Material	Purity (%)	Concentration/dose	Result	Reference
In vitro						
Reverse mutation	*S. typhimurium* TA98 and TA100	Steviol	NS	20 mg/plate	Negative[a]	Suttajit et al. (1993)
Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA102, TA104, TA1535 and TA1537	Steviol	99	5 mg/plate	Negative[a]	Matsui et al. (1996)
Forward mutation	*S. typhimurium* TM677	Steviol	NS	10 mg/plate[h] 0.5–10 mg/plate[f]	Negative Positive	Matsui et al. (1996)
Forward mutation	*S. typhimurium* TM677	Steviol	NS	10 mg/plate[h] 10 mg/plate[f]	Negative Positive	Pezzuto et al. (1985)
Forward mutation	*S. typhimurium* TM677	Steviol	NS	NS	Positive[f]	Terai et al. (2002)
Forward mutation	*S. typhimurium* TM677	Steviol-16,17-epoxide	NS	NS	Positive[f]	Terai et al. (2002)
Forward mutation	*S. typhimurium* TM677	15α-hydroxysteviol	NS	NS	Negative[a]	Terai et al. (2002)
Forward mutation	*S. typhimurium* TM677	15-oxo-steviol	NS	NS	Positive[f]	Terai et al. (2002)
Forward mutation	*S. typhimurium* TM677	Steviol methylester	NS	NS	Positive[f]	Terai et al. (2002)
Forward mutation	*S. typhimurium* TM677	16-oxo-steviol methylester	NS	NS	Negative[a]	Terai et al. (2002)
Forward mutation	*S. typhimurium* TM677	13,16-*seco*-13-oxo-steviol methylester	NS	NS	Positive[f]	Terai et al. (2002)
Forward mutation	*S. typhimurium* TM677	13,16-*seco*-13α-hydroxy-steviol methylester	NS	NS	Negative[a]	Terai et al. (2002)
Forward mutation	*S. typhimurium* TM677	Steviol methylester 8,13-lactone	NS	NS	Positive[e]	Terai et al. (2002)
Gene mutation (*umu*)	*S. typhimurium* TA1535/pSK1002	Steviol	99	625–1250 μg/ plate[h] 1259–2500 μg/plate[f]	Positive Positive	Matsui et al. (1996)
Gene mutation	*B. subtilis* H17 *rec*$^+$, M45 *rec*$^-$	Steviol	99	10 mg/disk	Negative[a]	Matsui et al. (1996)
Gene mutation	Chinese hamster lung fibroblasts	Steviol	99	400 μg/ml[f]	Positive	Matsui et al. (1996)

Table 2. (contd)

End-point	Test system	Material	Purity (%)	Concentration/dose	Result	Reference
Gene mutation	Mouse lymphoma L5178Y cells, *Tk*$^{+/-}$ locus	Steviol	NS	340 μg/ml	Negative[a, b]	Oh et al. (1999)
Chromosomal aberration	Chinese hamster lung fibroblasts	Steviol	NS	0.5 g/ml[h] 1–1.5 mg/ml[f]	Negative Positive	Matsui et al. (1996)
Chromosomal aberration	Human lymphocytes	Steviol	NS	0.2 mg/ml	Negative	Suttajit et al. (1993)
DNA damage (comet assay)	TK6 and WTK1 cells	Steviol	NS	62.5–500 μg/ml	Negative[a]	Sekihashi et al. (2002)
In vivo						
DNA damage (comet assay)	Male BDF1 mouse stomach, colon, liver; male CRJ: CD1 mouse liver kidney, colon and testes	Steviol	>99	250–2000 mg/kg	Negative[c]	Sekihashi et al. (2002)
Micronucleus formation	MS/Ae mice	Steviol	99	1000 mg/kg bw	Negative	Matsui et al. (1996)
Micronucleus formation	Swiss mouse bone marrow	Steviol	About 90	8000 mg/kg	Negative[g]	Temcharoen et al. (2000)
Micronucleus formation	Wistar rat bone marrow	Steviol	About 90	8000 mg/kg	Negative[g]	Temcharoen et al. (2000)
Micronucleus formation	Syrian golden hamster bone marrow	Steviol	About 90	4000 mg/kg	Negative[g]	Temcharoen et al. (2000)
Micronucleus formation	ddY Mouse regenerating liver	Steviol	NS	50–200 mg/kg	Negative[b]	Oh et al. (1999)

NS, not specified.
[a] With and without metabolic activation (source not specified in original monograph).
[b] Inadequate detail available.
[c] Killed at 3 and 24 h.
[e] The presence of metabolic activation decreased the mutagenicity.
[f] With metabolic activation.
[g] Killed at 24, 30, 48 and 72 h. Ratio of polychromatic to normochromatic erythrocytes was decreased at later time-point(s) in females.
[h] Without metabolic activation.

In a study with limited reporting, available in Korean, groups of five partially hepatectomized ddY mice were given steviol (purity not stated) at an oral dose of 0, 50, 100 or 200 mg/kg bw. Steviol had no significant effect on the numbers of micronucleated hepatocytes. A group of mice treated with mitomycin C, the positive control, did show a positive response (Oh et al., 1999).

2.2.4 Reproductive toxicity

At its fifty-first meeting, the Committee reviewed a number of studies of reproductive and developmental toxicity with stevioside and *Stevia* extracts and noted that administration of stevioside (purity, 90–96%) at doses of up to 2500 mg/kg bw per day in hamsters and 3000 mg/kg bw per day in rats had no effect. The Committee also noted that, although an aqueous infusion of *S. rebaudiana* administered orally to female rats was reported to cause a severe, long-lasting reduction in fertility, the contraceptive effect of *Stevia* was probably not due to stevioside. Stevioside (purity, 95.6%) had neither teratogenic nor embryotoxic effects at doses of up to 1000 mg/kg bw per day in rats treated by gavage. At its present meeting, the Committee reviewed two additional studies.

Rats

Ten male Wistar rats (aged 25–30 days) were each given 2 ml of a crude aqueous extract of *S. rebaudiana* (corresponding to 0.67 g of dried leaves per ml), by gastric intubation, daily for 60 days. Ten control animals received saline only. There were no significant effects on food consumption or body-weight gain. Animals treated with *Stevia* extract showed decreased relative weights of the cauda epididymides, seminal vesicles and testes, accompanied by a reduction in plasma concentration of testosterone and in numbers of spermatazoa in the cauda epididymidis. The fructose content of the prostate and seminal vesicle was also decreased, which was considered by the author to be caused at least in part by a deficiency in testosterone stimulation (Melis, 1999).

Chickens

On day 7 of incubation, fertile broiler eggs were injected with 0.08–4.00 mg of stevioside (purity, >96%) or 0.025–1.25 mg of steviol (purity, >98%). The chicks were examined at hatching and 1 week later. There were no effects on embryonic mortality, body weight, malformations or body-weight gain during the first week after hatching. No stevioside or steviol was detected in the blood of the hatchlings sacrificed at age 1 day (Guens et al., 2003c).

2.2.5 Special study: effects on human microflora

Forty mg of stevioside (purity, 85%) and 40 mg of rebaudioside A (purity, 90%) were incubated for 72 h under anaerobic conditions with 40 ml of faecal bacterial suspensions from eleven healthy volunteers (six men and five women). Stevioside and rebaudioside A did not significantly influence the composition of faecal cultures. However, stevioside caused a weak inhibition of the growth of anaerobic

bacteria, while rebaudioside A caused a weak inhibition of the growth of aerobic bacteria, particularly coliforms (Gardana et al., 2003).

2.3 Observations in humans

In a multicentre randomized, double-blind, placebo-controlled trial of hypertensive Chinese men and women (aged 28–75 years), 60 patients were given capsules containing 250 mg of stevioside (purity not stated) three times per day, corresponding to a total intake of 750 mg of stevioside per day (equivalent to 12.5 mg/kg bw per day, assuming an average body weight of 60 kg) and followed up at monthly intervals for one year. Forty-six patients were given a placebo. After 3 months, systolic and diastolic blood pressure in men and women receiving stevioside decreased significantly and the effect persisted over the year. Blood biochemistry parameters, including lipids and glucose, showed no significant changes. Three patients receiving stevioside and one receiving the placebo withdrew from the study as a result of side-effects (nausea, abdominal fullness, dizziness). In addition, four patients receiving stevioside experienced abdominal fullness, muscle tenderness, nausea and asthenia within the first week of treatment. These effects subsequently resolved and the patients remained in the study (Chan et al., 2000).

A follow-up multicentre randomized, double-blind placebo-controlled trial was conducted in hypertensive Chinese men and women (aged 20–75 years). Eighty-five patients were given capsules containing 500 mg of stevioside (purity not stated) three times per day, corresponding to a total intake of 1500 mg of stevioside per day (equivalent to 25 mg/kg bw per day, assuming an average body-weight of 60 kg). Eighty-nine patients were given a placebo. Three patients in each group withdrew during the course of the study. There were no significant changes in body mass index or blood biochemistry parameters throughout the study. In the group receiving stevioside, mean systolic and diastolic blood pressure was significantly decreased compared with the baseline, commencing from about 1 week after the start of treatment. After 2 years, 6 out of 52 patients (11.5%) in the group receiving stevioside had left ventricular hypertrophy compared with 17 of 50 patients (34%) in the group receiving the placebo ($p < 0.001$). Eight patients in each group reported minor side-effects (nausea, dizziness and asthenia), which led two patients in each group to withdraw from the study. Four patients in the group receiving stevioside experienced abdominal fullness, muscle tenderness, nausea and asthenia within the first week of treatment. These effects subsequently resolved and the patients remained in the study (Hsieh et al., 2003).

In a paired cross-over study, 12 patients with type-2 diabetes were given either 1 g of stevioside (stevioside, 91%; other *Stevia* glycosides, 9%) or 1 g of maize starch (control group), which was taken with a standard carbohydrate-rich test meal. Blood samples were drawn at 30 min before and for 240 min after ingestion of the test meal. Stevioside reduced postprandial blood glucose concentrations by an average of 18% and increased the insulinogenic index by an average of 40%, indicating beneficial effects on glucose metabolism. Insulin secretion was not significantly increased. No hypoglycaemic or adverse effects were reported by the

patients or observed by the investigators. Systolic and diastolic blood pressure was not altered by stevioside administration (Gregersen et al., 2004).

Forty-eight hyperlipidaemic volunteers were recruited to a randomized, double-blind trial designed to investigate the hypolipidaemic and hepatotoxic potential of steviol glycoside extract. The extract used in this study was a product containing stevioside (73 ± 2%), rebaudioside A (24 ± 2%) and other plant polysaccharides (3%). The subjects were given two capsules, each containing 50 mg of steviol glycoside extract or placebo, twice daily (i.e. 200 mg/day, equivalent to 3.3 mg/kg bw per day assuming an average body weight of 60 kg), for 3 months. One volunteer receiving placebo, and three volunteers receiving steviol glycoside failed to complete the study for personal reasons, not related to adverse reactions. At the end of the study, both groups showed decreased serum concentrations of total cholesterol and of low density lipoproteins. Analyses of serum concentrations of triglycerides, liver-derived enzymes and glucose indicated no adverse effects. The authors questioned the subjects' compliance with the dosing regime, in view of the similarity of effect between treatment and placebo (Anonymous, 2004a). In a follow-up study, 12 patients were given steviol glycoside extract in incremental doses of 3.25, 7.5 and 15 mg/kg bw per day, for 30 days per dose. Preliminary results indicated no adverse responses in blood and urine biochemical parameters (Anonymous, 2004b).

3. INTAKE

3.1 Introduction

The Committee evaluated information on exposure to steviol glycosides submitted by Japan and China. Additional information was taken from a report on *S. rebaudiana* Bertoni plants and leaves that was prepared for the European Commission by the Scientific Committee on Food (European Commission, 1999). All of the intake results are presented in terms of equivalents of steviol, based on a conversion of 40% from steviol glycosides.

3.2 Use in foods

Steviol glycosides are used to sweeten a number of foods in China, Japan, and South America. Table 3 summarizes the information submitted to the Committee.

It is also known that *Stevia* leaves are used to prepare a sweetened tea in a number of countries throughout the world. The concentrations of steviol glycosides in these teas are likely to be lower than those reported in Table 3.

3.3 International estimates of intake

The WHO Global Environment Monitoring System — Food Contamination Monitoring and Assessment Programme (GEMS/Food) database was used by the Committee to prepare international estimates of intake of steviol glycosides

Table 3. Food use levels of steviol glycosides reported to the Committee

Food type	Maximum use level reported (mg/kg)
Beverages	500
Desserts	500
Yogurt	500
Cold confectionery	500
Sauces	1000
Pickles	1000
Delicacies	1000
Sweetcorn	200
Bread	160
Biscuits	300

(as steviol). It was assumed that steviol glycosides would replace all sweeteners used in or as food, reflecting the minimum reported relative sweetness of steviol glycosides and sucrose of 200 : 1. The estimates are shown in Table 4.

These estimates are conservative in that it is very unlikely that a user of steviol glycosides would replace all commodity sweeteners found in their diets (WHO, 2003).

3.4 National estimates of intake of steviol glycosides

Japan submitted an estimate of intake of steviol glycosides per capita based on the total demand for steviol glycosides in Japan, estimated at 200 tonnes per year. The estimate assumed a population of 120 million persons and an average body weight of 50 kg. The resulting estimate of intake of steviol glycosides (as steviol) was 0.04 mg/kg bw per day.

Additionally, the Japanese submission included two ‘maximum’ consumption estimates for steviol glycosides. These assumed that 10% of all added sugar in the diets of Japan or the USA would be replaced by steviol glycosides, at a ratio of 200 : 1, based on sweetness. The consumption of sugar in Japan was taken as 25 kg/person per year, while that in the USA was 125 pounds/person per year (57 kg/person per year). The average body weight for both Japan and the USA was assumed to be 50 kg. The resulting estimates of maximum consumption of steviol glycosides (as steviol) were 0.3 mg/kg bw per day for Japan and 0.6 mg/kg bw per day for the USA. The Committee concluded that there was no evidence to suggest that only 10% of sugar consumed would be replaced. Therefore, the Committee calculated ‘maximum’ intakes based on the replacement of all sugar in diets in Japan and the USA, resulting in estimates of 3 mg/kg bw per day for a 50 kg consumer in Japan and 5 mg/kg bw per day for a 60 kg consumer in the USA.

Table 4. International estimates of intakes of steviol glycosides as steviol

Food code	Food type (CM)	GEMS/Food diet									
		Middle Eastern		Far Eastern		African		Latin American		European	
		CM	SG	CM	SG	CM	SG	CM	SG	CM	SG
GS659	Refined sugar	73 (g/person per day)	2.4	43	1.4	25.5	0.8	97.3	3.3	96.8	3.3
GS659	Total sugar and honey	95.8 (g/person per day)	3.2	50.5	1.6	42.7	1.3	104.3	3.5	107.3	3.5

CM, commodity sweetener (refined sugar or total sugar and honey); SG, steviol glycosides.
CM intakes are given in grams per person per day, while SG intakes are given in mg/kg bw per day, using a factor of 200 for the relative sweetness and assuming a body weight of 60 kg.

Table 5. Summary of estimates of intakes of steviol glycosides (as steviol)

Estimate	Intake (mg/kg bw per day)
GEMS/Food (international)[a]	1.3–3.5 (60 kg person)
Japan, per capita	0.04
Japan, maximum consumption[b]	3
USA, maximum consumption[b]	5

GEMS/Food, WHO Global Environment Monitoring System — Food Contamination Monitoring and Assessment Programme.

[a] 'International' refers to the international estimates presented in Table 4.

[b] These estimates were prepared in parallel to those for the international estimates: it was assumed that all dietary sugars in diets in Japan and the USA would be replaced by steviol glycosides, at a ratio of 200 : 1.

The submission from China contained information on the annual production of steviol glycosides. It was reported that up to 1000 tonnes were produced each year, with 200 tonnes retained for domestic consumption. In view of the larger population in China than in Japan or the USA, the Committee noted that any estimates prepared using these data would result in lower exposures than those reported above.

3.5 Summary of intakes

Table 5 contains a summary of the intakes of steviol glycosides evaluated or derived by the Committee.

The Committee concluded that the replacement estimates were highly conservative and that intake of steviol glycosides (as steviol) would be likely to be 20–30% of these values.

4. COMMENTS

After oral administration, steviol glycosides are poorly absorbed in experimental animals and in humans.

Intestinal microflora metabolize steviol glycosides to the aglycone, steviol, by successive hydrolytic removal of glucose units. Data reviewed by the Committee at its current and fifty-first meetings (Annex 1, reference *149*) indicated that this process is similar in rats and humans. The hydrolysis of rebaudioside A to steviol was slower than that of stevioside. In humans treated orally with stevioside, small amounts of steviol were detected in the plasma, with considerable interindividual variability. The major route by which steviol is metabolized in humans in vivo appears to be via conjugation with glucuronide and/or sulfate. Studies with liver microsomal preparations indicated that steviol is also metabolized to a number of hydroxy and dihydroxy derivatives via CYP-dependent pathways.

Stevioside and/or steviol affected a variety of biochemical parameters in models in vitro, indicating possible mechanisms of antihypertensive and antiglycaemic effects that involve modulation of ion channels. High concentrations (e.g. 1 mmol/l) of stevioside were required to produce a maximal increase in insulin secretion, while steviol was effective at a concentration that was about three orders of magnitude lower. Stevioside also affected a variety of biochemical parameters in different animal species in vivo, mostly with parenteral administration; these studies were considered by the Committee to be of limited relevance to dietary exposure.

No new long-term studies of toxicity or carcinogenicity were available at the present meeting. At its fifth-first meeting, the Committee noted that oral administration of stevioside (purity, 95.6%) at a dietary concentration of 2.5%, equal to 970 and 1100 mg/kg bw per day in male and female rats, respectively, for 2 years was not associated with toxicity. Reduced body-weight gain and survival rate were observed with stevioside at a dietary concentration of 5%. In a new study, stevioside was found to inhibit the promotion of skin tumours by TPA in a model of skin carcinogenesis in mice.

The Committee reviewed new data on genotoxicity that, considered together with data reviewed by the Committee at its fifth-first meeting, allowed a number of conclusions to be drawn. Stevioside and rebaudioside A have not shown evidence of genotoxicity in vitro or in vivo. Steviol and some of its oxidative derivatives show clear evidence of genotoxicity in vitro, particularly in the presence of a metabolic activation system. However, studies of DNA damage and micronucleus formation in rats, mice and hamsters in vivo indicate that the genotoxicity of steviol is not expressed at doses of up to 8000 mg/kg bw.

One new study of developmental toxicity was available at the present meeting. Adverse effects on the reproductive apparatus, which could be associated with impaired fertility, were observed in male rats given a crude extract of *S. rebaudiana*, at a dose corresponding to 1.34 g of dried leaves. However, at its fifth-first meeting, the Committee reviewed a number of studies of reproductive and developmental toxicity with stevioside (purity, 90% or 96.5%). Doses of up to 2500 mg/kg bw per day in hamsters and 3000 mg/kg bw per day in rats had no effect in studies of reproductive toxicity. No teratogenic or embryotoxic effects were observed in rats given stevioside at a dose of up to 1000 mg/kg bw per day by gavage. The Committee considered that the adverse reproductive effects associated with administration of aqueous extracts of *S. rebaudiana*, noted at the present and fifty-first meeting, were unlikely to be caused by steviol glycosides.

Stevioside is being investigated as a potential treatment for hypertension and diabetes. Administration of stevioside at a dose of 750 or 1500 mg per day for 3–24 months resulted in decreased blood pressure in hypertensive patients, with no adverse effects. These studies, in a limited number of subjects, provided some reassurance that stevioside at a dose of up to 25 mg/kg bw per day (equivalent to 10 mg/kg bw per day expressed as steviol) for up to 2 years shows no evidence of significant adverse effects in these individuals. There is no information on the effects of repeated administration of stevioside on blood pressure in normotensive individuals. A small study in 12 patients with type-2 diabetes showed that a single

dose of 1 g of stevioside reduced postprandial glucose concentrations and had no effect on blood pressure.

The Committee evaluated information on intake of steviol glycosides, submitted by Japan and China. Additional information was available from a report on *S. rebaudiana* Bertoni plants and leaves that was prepared for the European Commission by the Scientific Committee on Food. All the intake results are presented in terms of equivalents of steviol, based on a conversion of 40% from the steviol glycoside, stevioside (relative molecular mass: steviol, 318, stevioside, 805).

The Committee used the GEMS/Food database to prepare international estimates of intake of steviol glycosides (as steviol). It was assumed that steviol glycosides would replace all dietary sugars, at the lowest reported relative sweetness ratio for steviol glycosides and sucrose, 200 : 1. The intakes ranged from 1.3 mg/kg bw per day (African diet) to 3.5 mg/kg bw per day (European diet).

The Committee evaluated estimates of per capita intake derived from disappearance (poundage) data supplied by Japan and China. The Committee also evaluated estimates of intake of steviol glycosides based on the replacement of all dietary sugars in the diets for Japan and the USA. These results are summarized in Table 5.

The Committee concluded that the replacement estimates were highly conservative and that intake of steviol glycosides (as steviol) would be likely to be 20–30% of these values.

5. EVALUATION

The Committee noted that most of the data requested at its fifty-first meeting, e.g. data on the metabolism of stevioside in humans, and on the activity of steviol in suitable studies of genotoxicity *in vivo*, had been made available.

The Committee concluded that stevioside and rebaudioside A are not genotoxic in vitro or in vivo and that the genotoxicity of steviol and some of its oxidative derivatives in vitro is not expressed in vivo. The no-observed-effect level (NOEL) for stevioside was 970 mg/kg bw per day in a long-term study evaluated by the Committee at its fifty-first meeting.

The Committee noted that stevioside has shown some evidence of pharmacological effects in patients with hypertension or with type-2 diabetes at doses corresponding to about 12.5–25 mg/kg bw per day (equivalent to 5–10 mg/kg bw per day expressed as steviol). The evidence available at present was inadequate to assess whether these pharmacological effects would also occur at lower levels of dietary exposure, which could lead to adverse effects in some individuals (e.g. those with hypotension or diabetes). The Committee therefore decided to allocate a temporary acceptable daily intake (ADI), pending submission of further data on the pharmacological effects of steviol glycosides in humans.

A temporary ADI of 0–2 mg/kg bw was established for steviol glycosides, expressed as steviol, on the basis of the NOEL for stevioside of 970 mg/kg bw per day (or 383 mg/kg bw per day expressed as steviol) in the 2-year study in rats and

a safety factor of 200. This safety factor incorporates a factor of 100 for inter- and intraspecies differences and an additional factor of 2 because of the need for further information. The Committee noted that this temporary ADI only applies to products complying with the specifications.

The Committee required additional information, to be provided by 2007, on the pharmacological effects of steviol glycosides in humans. These studies should involve repeated exposure to dietary and therapeutic doses, in normotensive and hypotensive individuals and in insulin-dependent and insulin-independent diabetics.

6. REFERENCES

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D-TAGATOSE (addendum)

First draft prepared by

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1. EXPLANATION

D-Tagatose is a ketohexose, an epimer of D-fructose isomerized at C4. It is obtained from D-galactose by isomerization under alkaline conditions in the presence of calcium. Its properties permit its use as a bulk sweetener, humectant, texturizer and stabilizer.

D-Tagatose was evaluated by the Committee at its fifty-fifth, fifty-seventh and sixty-first meetings (Annex 1, references *149*, *154* and *166*). At its fifty-fifth meeting, the Committee concluded that D-tagatose was not genotoxic, embryotoxic or teratogenic. It also concluded that an acceptable daily intake (ADI) could not be allocated for D-tagatose because of concern about its potential to induce glycogen deposition and hypertrophy in the liver and to increase the concentrations of uric acid in serum. At its fifty-seventh meeting, the Committee evaluated the results of four studies in experimental animals, the results of a study in volunteers and some publications concerning the increased concentration of uric acid in serum after intake of D-tagatose and other substances. The Committee decided to base its evaluation on the human data reviewed in the course of these two meetings. A no-observed-effect level (NOEL) of 0.75 g/kg bw per day was identified in a 28-day study in which no effects were observed in humans receiving three doses of 15 g of D-tagatose per day. An ADI of 0–80 mg/kg bw for D-tagatose was established on the basis of this NOEL and a safety factor of 10.

At its sixty-first meeting, the Committee reviewed the results of two new studies of toxicity in rats, and of two new studies of plasma concentrations of uric acid in human volunteers; these studies were submitted by the sponsor with a request for a re-evaluation of D-tagatose.

The Committee concluded that the 2-year study in rats demonstrated that the previously-reported liver glycogen deposition and hypertrophy did not result in

histopathological changes after long-term administration of D-tagatose, and thus addressed the concerns expressed at the fifty-fifth meeting. However, this study also identified new findings, namely increased adrenal, kidney and testes weights. The Committee considered that these changes might have been caused by high osmotic load resulting from the high dietary doses administered, but this could not be confirmed in the absence of histopathological examination of these tissues. Pending provision of the results of histopathological examination, the Committee confirmed that the human data provided the most relevant basis for assessing the acceptable intake of D-tagatose.

Results of a study in hyperuricaemic individuals indicated that the NOEL identified for normal individuals was also applicable to this vulnerable group. The Committee considered that a safety factor of 3 would be appropriate to allow for interindividual variation. In view of the additional uncertainty regarding the nature of the effects observed in the adrenals, kidneys and testes in the 2-year study in rats, the Committee concluded that the ADI should be temporary and applied an additional safety factor of 2. The previous ADI was removed, and the Committee allocated a temporary ADI for D-tagatose of 0–125 mg/kg bw on the basis of the NOEL of 0.75 g/kg bw per day and a safety factor of 6.

The Committee considered that the temporary ADI did not apply to individuals with hereditary fructose intolerance resulting from deficiency of 1-phosphofructoaldolase (aldolase B) or fructose 1,6-diphosphatase.

The Committee requested information on the histological examination of the adrenals, kidneys and testes of the rats from the 2-year study by 2006. This information was provided to the Committee for evaluation at its present meeting, together with additional data on the risk to individuals with hereditary fructose intolerance.

2. BIOLOGICAL DATA

2.1 Toxicological studies: long-term studies of toxicity and carcinogenicity

The Committee considered an addendum to a report that had been discussed at its sixty-first meeting, on a modified study of carcinogenicity conducted to investigate the effects of long-term administration of 2.5%, 5%, or 10% D-tagatose, 20% fructose or 10% D-tagatose plus 10% fructose on the liver of Wistar rats. In response to the Committee's request, the addendum included the results of histopathological examination of the testes, kidneys and adrenals of all rats. The incidence of nephrocalcinosis showed an apparent dose-related trend in both sexes. This was statistically significant in all groups of males treated with D-tagatose. There was a high (88%) incidence of nephrocalcinosis in the female control group, and a significant treatment-related effect was observed only in the groups given 10% D-tagatose. Mineralization occurred mainly in the pelvic and medullary regions of the kidneys. The incidence of adrenomedullary proliferative lesions was significantly increased in males at 5% and 10% D-tagatose, and also at 5% D-tagatose in females. This was predominantly associated with

pheochromocytomas in males and medullary hyperplasia in females. There were no other reported treatment-related effects. The incidence of Leydig cell hyperplasia and adenomas was similar and within the range for historical controls in all groups (Lina & Bär, 2003).

3. INTAKE

At its fifty-seventh meeting, the Committee estimated that the mean intake of D-tagatose was between 3 and 9 g/day and the 95th percentile of consumption was up to 18 g/day. These estimates, based on data on food consumption from Australia, Member States of the European Union and the USA, were considered to be still valid.

4. COMMENTS

Additional histopathological examinations were conducted on the adrenals, kidneys and testes of Wistar rats fed diets containing 2.5, 5 or 10% D-tagatose, or 10% D-tagatose plus 10% fructose for 2 years. The observed changes were similar to those reported in studies with other carbohydrates of low digestibility. The Committee has previously noted that gross dietary imbalance caused by high doses of polyols may result in metabolic and physiological disturbances in rats, and are associated with changes in calcium uptake and excretion accompanied by nephrocalcinosis and adrenal medullary hyperplasia (Annex 1, reference *62*). These changes were not considered to be of relevance to the safety evaluation of D-tagatose. Carbohydrates of low digestibility do not increase the intestinal absorption of calcium in humans to the same extent as in rats. Rats, especially females, are particularly prone to the development of nephrocalcinosis. The Committee has previously noted the unique features of the rat adrenal medulla and concluded that the occurrence of proliferative lesions of the adrenal medulla in rats fed with polyols and lactose is a species-specific phenomenon (Annex 1, reference *122*). An increased incidence of Leydig cell tumours has been reported in male Wistar rats fed diets containing 10% lactitol or 20% D-tagatose. This study demonstrated that there were no toxicologically significant findings in rats fed D-tagatose at dietary levels of up to 10% for 2 years (equal to approximately 4 and 5 g/kg bw per day for males and females, respectively).

The Committee further considered the risk to individuals with hereditary fructose intolerance, which if untreated leads to metabolic disturbances, liver damage, renal tubular disease and defective blood coagulation. Treatment requires almost complete exclusion of sucrose, fructose and sorbitol. There is no direct evidence establishing that individuals with hereditary fructose intolerance are also intolerant to D-tagatose, but in view of their common biochemical pathways it is probable that D-tagatose could produce the same adverse effects as fructose. At its fifty-fifth meeting (Annex 1, reference *149*), the Committee noted that the absorption of D-tagatose by humans is not expected to exceed 20% of the administered dose. However, the rate of gluconeogenesis from D-tagatose is slower than that from fructose. Thus the Committee could not discount the possibility that, in individuals

with hereditary fructose intolerance, tissue concentrations of D-tagatose could be elevated or prolonged compared with those of fructose, leading to adverse reactions.

The Committee has previously noted that gastrointestinal effects (nausea, flatulence, diarrhoea) have been reported in some individuals after the consumption of 30 g of D-tagatose in a single dose.

The Committee at its fifty-seventh meeting estimated that the mean daily intake of D-tagatose would range from 3 to 9 g/day and the 95th percentile of consumption would be up to 18 g/day. These estimates, based on data on food consumption from Australia, Member States of the European Union and the USA, were considered to be still valid.

5. EVALUATION

At its sixty-first meeting, the Committee concluded that, pending provision of the results of histopathological examinations from a 2-year study in rats, the human data provided the most relevant basis for assessing the acceptable intake of D-tagatose. The histopathological data had now been provided and demonstrated that there were no toxicologically significant findings in rats given D-tagatose at levels of up to 10% in the diet for 2 years (equal to approximately 4 and 5 g/kg bw per day for males and females, respectively). On the basis of the data reviewed by the Committee at its sixty-first meeting and at its present meeting, and taking into account the fact that D-tagatose has physiological and toxicological properties similar to those of other carbohydrates of low digestibility, the Committee removed the temporary ADI and allocated an ADI 'not specified' for D-tagatose.

The fact that ingestion of 30 g or more of D-tagatose on a single occasion may cause gastrointestinal effects in humans should be taken into account when considering appropriate levels of use.

The ADI 'not specified' does not apply to individuals with hereditary fructose intolerance arising from 1-phosphofructoaldolase (aldolase B) deficiency or fructose 1,6-diphosphatase deficiency.

6. REFERENCE

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XYLANASES FROM BACILLUS SUBTILIS *EXPRESSED IN* B. SUBTILIS

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1. EXPLANATION

Xylanases from *Bacillus subtilis* expressed in *B. subtilis* have not been evaluated previously by the Committee. Xylanase (β-1,4-D-xylan xylanohydrolase, β-1,4-D-xylanohydrolase, 1,4-xylanase, endo-1,4-xylanase) is an enzyme that catalyses the hydrolysis of xylans and arabinoxylans to mono- and oligosaccharides. The activity of the petitioned enzyme is measured relative to that of the standard enzyme and is expressed in total xylanase units (TXU)[1]. The Committee received information on three xylanases, designated BS1, BS2, and BS3. These xylanases are derived from nonpathogenic and nontoxigenic, genetically modified strains of *B. subtilis*. *B. subtilis* has been a source of enzymes used in

[1] Xylanase activity can also be expressed in Danisco xylanase units (DXU); 1 TXU = 24.15 DXU.

food for many years. Xylanases BS1 and BS2 are identical to the native xylanase of *B. subtilis*. Xylanase BS3 differs from the native enzyme by two amino acids and is resistant to the xylanase inhibitor present in flour. Xylanases BS2 and BS3 are used as processing aids in baking applications to increase tolerance towards variations in process parameters, improve the dough, and increase the volume of baked goods. Use levels range from 500 to 13 300 TXU/kg of flour for xylanase BS3, and from 3000 to 40 000 TXU/kg of flour for xylanase BS2. The xylanase BS2 preparation contains 0.3 mg of total organic solids (TOS) per 1000 TXU, and the xylanase BS3 preparation contains 1.5 mg of TOS per 1000 TXU.

1.1 Genetic modification

Three production strains for xylanases BS1, BS2 and BS3 were developed by transformation of the *B. subtilis* host strain with an appropriate transformation vector. The host strain is derived from the well-characterized, nonpathogenic and nontoxigenic *B. subtilis* wild-type strain 168. Three transformation vectors were constructed based on the commonly used plasmid pUB110. The vectors contain the xylanase gene derived from *B. subtilis* strain 168. Two vectors encode xylanases BS1 and BS2, both of which are identical to the native xylanase A from strain 168. The vector encoding xylanase BS1 also contains genes encoding proteins that inactivate the antibiotics kanamycin/neomycin and phleomycin. These proteins are intracellular and are not carried over into the xylanase preparation. The vector encoding xylanase BS2 was genetically modified to remove the genes conferring resistance to the antibiotics. The third transformation vector encodes xylanase BS3, which was genetically modified by two amino acid substitutions to be resistant to the xylanase inhibitor present in flour. This vector does not contain genes conferring resistance to the antibiotics. Each vector was introduced into the host strain to obtain the corresponding xylanase production strain. All the introduced DNA is well-characterized and would not result in the production of any toxic or undesirable substances. The production strain is stable with respect to the introduced DNA.

1.2 Product characterization

Each xylanase is produced by pure culture fermentation of the respective production strain. Xylanase is secreted into the fermentation medium, from which it is subsequently recovered, concentrated, and formulated using substances suitable for use in food, such as starch and salt. Two xylanase preparations, one containing the native xylanase BS2 and the other containing the modified xylanase BS3, which is resistant to the xylanase inhibitor in flour, have been marketed. These xylanases would be denatured at temperatures >50 °C and would not be enzymatically active in food as consumed. Both xylanase preparations[2] conform

[2] Two specification monographs were prepared for xylanase preparations containing xylanases BS2 and BS3, the respective titles being *Xylanase from* Bacillus subtilis *expressed in* B. subtilis, and *Xylanase (resistant to xylanase inhibitor) from* Bacillus subtilis *containing a modified xylanase gene from* B. subtilis.

to the *General specifications for enzyme preparations used in food processing* (Annex 1, reference *156*). The xylanase preparation containing xylanase BS1 is not intended for commercialization.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

No information was available.

2.2 Toxicological studies

Toxicological studies have been performed with different test batches of the three enzyme preparations, each being brown, water-soluble liquid concentrates from a fermentation with the recombinant strain. Although for the enzyme preparation designated as xylanase BS3 the enzyme content was originally expressed in DXU, this was converted to TXU in the study descriptions below, for comparison purposes.

2.2.1 Acute toxicity

Studies of acute toxicity have been performed with three enzyme test preparations, designated as xylanases BS1 (or Bacillus xylanase), BS2 and BS3, with enzyme contents of 100 000 TXU/ml, 106 000 TXU/g and 110 000 TXU/g, respectively. The TOS contents of these preparations were 20.1%[3], 3.25%, and 15.9%, respectively. The studies followed OECD test guideline 420 (fixed dose procedure, 1992/2001), and were certified for compliance with good laboratory practice (GLP) and quality assurance (QA). The results are summarized in Table 1.

2.2.2 Short-term studies of toxicity

Rats

Groups of five male and five female Wistar rats (aged 6–7 weeks) were given xylanase BS3 (batch TOX2; enzyme content, 41 125 TXU/g; TOS content, 6.25%) at a dose equivalent to 0, 20 000, 50 000, or 200 000 TXU/kg bw by gavage (in sterile water), daily for 4 weeks. The study was performed according to OECD test guideline 407 (1995), and was certified for compliance with GLP and QA. All visible signs of ill health or behavioural changes were recorded daily, as were morbidity and mortality. Once per week, body weight and food consumption were recorded, and detailed clinical observations were performed outside the cage. In week 4, all animals were examined for sensory reactivity to different types of stimuli, grip strength, and motor activity. At termination of treatment, blood and urine samples were collected from all animals for haematology, clinical chemistry, and urine

[3] This is a theoretical value, since dry matter and ash content were not known.

Table 1. Acute toxicity of xylanase administered orally

Enzyme preparation	Species	Sex	LD_{50} (mg/kg bw)	Reference
BS1, batch 150699-3	Rat	M, F	>2000[a]	Kaaber (1999); Harbak & Thygesen (2002)
BS2, batch TOX1	Rat	F	>2000[b]	Bollen (2003a)
BS3, batch TOX1	Rat	F	>2000[c]	Bollen (2003b)

F, female; M, male.
[a] Equivalent to 200 000 TXU/kg bw.
[b] Equivalent to 212 000 TXU/kg bw.
[c] Equivalent to 220 000 TXU/kg bw.

analysis determinations. At necropsy, a macroscopic examination was performed on all animals, and absolute and relative (to body weight) weights of 11 organs were determined. Microscopy was carried out on about 40 organs and tissues from all animals in the control group and in the group receiving the highest dose.

No treatment-related changes were observed in any of the parameters examined. The no-observed-effect level (NOEL) was 200 000 TXU/kg bw per day (equivalent to an intake of TOS of 304 mg/kg bw per day), the highest dose tested (Kaaber, 2003).

Groups of ten male and ten female Wistar rats (aged 5–6 weeks) were given xylanase BS1 (or Bacillus xylanase; batch 150699-1; enzyme content, 38 900 TXU/ml; and TOS content, 3.04%) at a dose equivalent to 0, 8000, 20 000, or 80 000 TXU/kg bw by gavage (in sterile water), daily for 13 weeks. The study was performed according to OECD test guideline 408 (1998), and was certified for compliance with GLP and QA. All visible signs of ill health or behavioural changes were recorded daily, as were morbidity and mortality. Once weekly, body weight and food consumption were recorded, and detailed clinical observations were performed outside the cage. Ophthalmoscopy was performed on all animals at the start of the experiment, and animals in the control group and at the highest dose were re-examined before termination. In week 12, all animals were examined for sensory reactivity to different types of stimuli, grip strength, and motor activity. At termination of treatment, blood samples were collected from all animals for haematology and clinical chemistry determinations. At necropsy, a macroscopic examination was performed on all animals, and absolute and relative (to body weight) weights of 11 organs were determined. Microscopy was carried out on about 40 organs and tissues from all animals in the control group and at the highest dose, on all organs and tissues from animals dying or sacrificed during the study, and on all gross lesions from all animals.

No treatment-related effects were seen on mortality, clinical signs, ophthalmoscopy, sensory reactivity, body weights, and (in females) food consumption. In males, statistically significant changes in food consumption were observed during some weeks of treatment. These changes showed no dose–response relationship, and there was no statistically significant change in food consumption over the whole period of treatment. The only effects seen on haematology were small, but statistically significantly decreased relative numbers of lymphocytes in males in all treatment groups (without a clear dose–response relationship) and somewhat greater, but not statistically significantly decreased absolute numbers of lymphocytes in males at the intermediate and highest doses. Females also showed decreases in relative (at the highest dose only) and absolute numbers of lymphocytes (at the intermediate and highest dose), but these decreases were not statistically significant. Examination of the individual data revealed a wide variation in relative and absolute numbers of lymphocytes in animals in the control group and animals in the treated groups. In a 4-week study (Kaaber, 2003; described above), higher doses than those used in the present study did not result in significant effects on lymphocytes. Taking the results as a whole, the findings on lymphocytes in this study were not considered to be toxicologically relevant. Clinical chemistry parameters were not affected by treatment, nor were organ weights, or findings on macroscopy and microscopy. The NOEL was 80 000 TXU/kg bw per day (equivalent to 63 mg of TOS/kg bw per day), the highest dose tested (Glerup, 1999; Harbak & Thygesen, 2002).

2.2.3 Long-term studies of toxicity and carcinogenicity

No information was available.

2.2.4 Genotoxicity

The results of four studies of genotoxicity with xylanase in vitro are summarized in Table 2. The first three studies followed OECD test guideline 471 (1997), and were certified for compliance with GLP and QA. In these studies, three enzyme preparations designated as xylanases BS1 (or Bacillus xylanase; TOS content[4], 7.8%; enzyme content, 38 900 TXU/ml), BS2 (TOS content, 6.1%; enzyme content, 156 000 TXU/g) and BS3 (TOS content, 8.2%; enzyme content, 39 875 TXU/g) were tested. Xylanase BS1 was also tested in the fourth study, which followed OECD test guideline 473 (1997), and was also certified for compliance with GLP and QA.

2.2.5 Reproductive toxicity

No information was available.

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[4] Values given for total organic solids contents are theoretical values. Since ash contents were not known, it was assumed that all dry matter was organic material.

Table 2. Studies of genotoxicity with xylanase in vitro

Enzyme preparation	End-point	Test system	Concentration	Result	Reference
In vitro					
BS1, batch 150699-2	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	50–5000 μg/plate, ±S9. Solvent: sterile distilled water.	Negative[a]	Edwards (1999a); Harbak & Thygesen (2002)
BS2, batch TOX2	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	50–5000 μg/plate, ±S9. Solvent: sterile distilled water.	Negative[b]	Edwards (2003a)
BS3, batch TOX3	Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	50–5000 μg/plate, ±S9. Solvent: sterile distilled water.	Negative[b]	Edwards (2003b)
BS1, batch 150699-2	Chromosomal aberration	Human lymphocytes	First experiment: 1250, 2500, or 5000 μg/ml, ±S9 Second experiment: 156, 313, or 625 μg/ml, −S9; Third experiment: 1250, 2500, or 4000 μg/ml, +S9. No solvent.	Negative[c]	Edwards (1999b); Harbak & Thygesen (2002)

S9, 9000 × *g* supernatant from rat liver.

[a] With and without metabolic activation from S9, using the 'treat-and-plate' method (to avoid any problems that might have been caused had the test substance contained significant levels of bioavailable histidine). No cytotoxicity was observed.

[b] With and without metabolic activation from S9, using the plate incorporation method and the preincubation method. No cytotoxicity was observed.

[c] With and without metabolic activation from S9. In the first experiment, the cell cultures were treated for 3 h without and with S9 and were harvested 17 h later. Dose-related reductions in mitotic index were observed without S9 (to 94, 90 and 21% that of the negative control at 1250, 2500 and 5000 μg/ml, respectively) and with S9 (to 99, 76 and 26% that of the negative control at 1250, 2500 and 5000 μg/ml, respectively). In the second experiment, the cells were exposed continuously for 20 h without S9 and then harvested (the mitotic index was reduced to 94, 55 and 48% that of the negative control at 156, 313 and 625 μg/ml, respectively). In the third experiment, the cells were treated for 3 h with S9 and harvested 17 h later (the mitotic index was reduced to 86, 62 and 37% that of the negative control at 1250, 2500 and 4000 μg/ml, respectively).

2.3 Observations in humans

No information was available.

3. INTAKE

The petitioned enzyme preparations would be used in the milling and baking industries, mainly to improve the dough. They may be used in yeast-raised or chemically-leavened wheat and rye-based bakery products (including bread, cakes, pastries, biscuits, crackers, pasta and noodles) at use levels ranging from 12 000 to 320 000 DXU/kg of flour for xylanase BS3 (equivalent to 500–13 300 TXU/kg of flour or 0.06–1.6 mg of xylanase/kg of flour, given the specific activity of 202 900 DXU (or 8400 TXU)/mg). The use levels for xylanase BS2 range from 3000 to 40 000 TXU/kg of flour (equivalent to 0.12–1.6 mg of xylanase/kg of flour, given the specific activity of 25 000 TXU/mg). A lower minimum dosage might be sufficient for xylanase BS3, despite the fact that it has a lower specific activity than that of xylanase BS2, because its amino-acid sequence had been modified such that the enzyme does not bind so easily to a xylanase inhibitor found naturally in flour.

As the enzyme is inactivated during baking (data provided suggest that it is completely inactivated after 5 min at 70 °C), it is not active in the final product as eaten. It can thus be regarded as a processing aid.

In order to estimate the daily intake resulting from consumption of bakery products in a 'worst-case' situation, the following assumptions were made:

—All baking products are produced using the xylanase enzyme preparations as a processing aid;

—The flour content in bakery products is 66%;

—The dosage is set at the maximum recommended level, that is: 1.6 mg of xylanase/kg of flour for all preparations (equivalent to 13 300 TXU/kg of flour for xylanase BS3, and to 40 000 TXU/kg of flour for xylanase BS2).

The upper physiological consumption of food is 50 g/kg bw per day according to the budget method (Hansen, 1979). A 'worst-case' situation is that of the ingestion of bakery products at 25 g/kg bw per day. The hypothetical intake of flour from bakery products would then be 16.5 g of flour/kg bw per day (25×0.66), resulting in a daily enzyme intake of 26.4 μg of xylanase/kg bw, equivalent to 219 TXU (or 0.3 mg of TOS)/kg bw per day for xylanase BS3 and 660 TXU (or 0.2 mg of TOS)/kg bw per day for xylanase BS2.

4. COMMENTS

Xylanases naturally present in food and xylanases used as enzymes in food processing have not been reported to cause allergic reactions. By analogy, it is not likely that the *B. subtilis* xylanases under evaluation will cause allergic reactions after ingestion of food containing the residues of these enzymes.

Toxicological studies were performed with test batches of the water-soluble liquid enzyme concentrates. These bacterial enzyme preparations were not acutely toxic when tested in rats, nor were they mutagenic in assays in bacteria in vitro or clastogenic in an assay for chromosomal aberrations in mammalian cells in vitro. No significant treatment-related effects were seen in a 4-week study in rats treated by gavage with xylanase BS3 at doses up to and including 200 000 TXU/kg bw per day (equivalent to 304 mg of TOS/kg bw per day), the highest dose tested, or in a 13-week study in rats treated by gavage with xylanase BS1 at doses up to and including 80 000 TXU/kg bw per day (equivalent to 63 mg of TOS/kg bw per day), the highest dose tested. These highest doses were therefore considered to be the NOELs in these studies.

Conservative estimates of daily intakes resulting from the use of xylanase in baking applications were 660 TXU/kg bw per day (or 0.2 mg of TOS/kg bw per day) for xylanase BS2, and 219 TXU/kg bw per day (or 0.3 mg of TOS/kg bw per day) for xylanase BS3. When these intakes were compared with the NOEL of 200 000 TXU/kg bw per day (equivalent to 304 mg of TOS/kg bw per day), the highest dose tested in the 4-week study of oral toxicity, the margins of safety were >1000 for both enzyme preparations. When these intakes were compared with the NOEL of 80 000 TXU/kg bw per day (equivalent to 63 mg of TOS/kg bw per day), the highest dose tested in the 13-week study of oral toxicity, the margins of safety were >200 for both enzyme preparations.

5. EVALUATION

The Committee allocated an acceptable daily intake (ADI) 'not specified' for xylanase from this recombinant strain of *B. subtilis*, used in the applications specified and in accordance with good manufacturing practice.

6. REFERENCES

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ZEAXANTHIN (synthetic)

First draft prepared by

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1. EXPLANATION

Zeaxanthin (3*R*,3′*R*-dihydroxy-β-carotene), a naturally occurring xanthophyll pigment, is an oxygenated carotenoid that has no provitamin A activity. It occurs together with the isomeric xanthophyll pigment, lutein (see monograph in this

Figure 1. Chemical structure of zeaxanthin

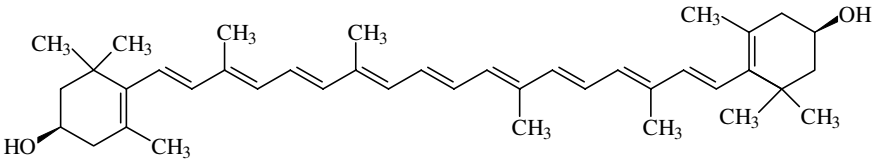
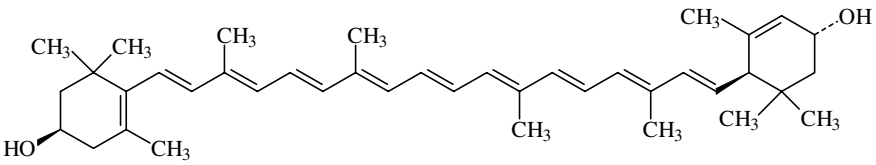


Figure 2. Chemical structure of lutein



volume, p 49), in many foods, particularly vegetables and fruit. It is intended to be used as a food colour and as a nutritional supplement in a wide range of applications at concentrations ranging from 0.5 to 70 mg/kg. An extract from *Tagetes erecta* L. containing primarily lutein with variable amounts of antheraxanthin and other xanthophylls was considered by the Committee at its thirty-first meeting (Annex 1, reference *77*). At that time, no toxicological data were available and no evaluation was made. For the present meeting, information was received for two different products: synthetic zeaxanthin and zeaxanthin-rich extract from *Tagetes erecta* L. However, the Committee did not receive any toxicological data supporting the safety evaluation of the extract. A number of toxicological studies have been carried out with respect to the safety of synthetic zeaxanthin for addition to food and these were evaluated by the Committee at its present meeting.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 Absorption, distribution, and excretion

(a) Absorption and availability

Xanthophylls may be ingested in either free or esterified forms, although unesterified zeaxanthin is the subjet of the present evaluation. Before absorption, the esters are hydrolysed by pancreatic esterases and lipases (Breithaupt et al., 2002) such that only the free forms are found in the circulation (Wingerath et al., 1995). Once released from their food matrix as a lipid emulsion, like other non-polar lipids, these compounds must be solubilized within micelles in the gastrointestinal tract to permit absorption by mucosal cells (Erdman et al., 1993). The transfer of carotenoids from lipid emulsion droplets to mixed micelles depends on

their hydrophobicity, as well as pH and concentration of bile acid (Tyssandier et al., 2001). Other carotenoids such as lycopene and xanthophylls can impair the transfer of β-carotene, but neither β-carotene nor other xanthophylls affect the transfer of lutein (Tyssandier et al., 2001). The more polar carotenoids such as the xanthophylls are preferentially solubilized in the surface of lipid emulsion droplets and micelles, while the less polar carotenoids are incorporated into the core area (Borel et al., 1996). This facilitates the transfer of compounds like zeaxanthin from the lipid droplets to the aqueous phase. Indeed, it has been demonstrated that the xanthophylls are more readily incorporated into micelles than other carotenoids (Garrett et al., 1999, Garrett et al., 2000).

The absorption of carotene is higher when fat is added to the diet (Roodenburg et al., 2000) and lower in disease-induced fat malabsorption (Erdman et al., 1993). The presence of fat in the small intestine stimulates the secretion of bile acids from the gall bladder and improves the absorption of carotenoids by increasing the size and stability of micelles, thus allowing more carotenoids to be solubilized. Absorption of carotenoids by mucosal cells is believed to occur by passive diffusion (Hollander & Ruble, 1978).

After uptake into mucosal cells, carotenoids are incorporated into chylomicrons and released into the lymphatics. When mucosal cells are sloughed off, carotenoids that have been taken up by the cells, but not yet incorporated into chylomicrons, are lost into the lumen of the intestine. The carotenoids within the chylomicrons are transported to the liver where they are distributed among the lipoprotein fractions. In contrast to the less polar carotenoids, a significant fraction of the xanthophylls is carried in the blood stream by high-density lipoprotein (HDL) (Romanchik et al., 1995).

The absorption of carotenoids including zeaxanthin is potentially affected by the food matrix in which the carotenoids are consumed, dietary fat, and the presence of other carotenoids in the diet (Castenmiller & West, 1998; Zaripheh & Erdman, 2002).

While no information on zeaxanthin itself was provided, the relative availability of lutein from a mixed vegetable diet has been shown to be 67% relative to that from a diet supplemented with pure lutein (van het Hof et al., 1999a). In another study, the relative bioavailability of lutein and β-carotene from various spinach products was compared with that from supplements (6.6 mg of lutein plus 9.8 mg of β-carotene). The values ranged from 45–54% for lutein to only 5.1–9.3% for β-carotene (Castenmiller et al., 1999). The presence of dietary fibre may explain, at least in part, the low availability of carotenoids from plant foods. It has been suggested that fibre interferes with the formation of micelles by partitioning bile salts and fat in the gel phase of the fibre. Processing, such as mechanical homogenization or heat treatment, has been shown to increase the availability of β-carotene in vegetables from 18% to 600% (van het Hof et al., 2000). There is evidence, however, that disruption of the matrix affects the availability of carotenoids differentially, possibly because of differences in their lipophilic character. For example, the plasma response of lutein was increased by about 14% when spinach was consumed chopped instead of whole, while β-carotene was not affected (van het Hof et al., 1999b). The matrices of formulated natural or synthetic carotenoids (e.g.

water-dispersible beadlets, crystalline powders, oil suspensions) and whether the compounds are esterified or non-esterifed may clearly affect availability (Swanson et al., 1996; Boileau et al., 1999).

Because the absorption of carotenoids occurs via incorporation into mixed micelles, ingestion of fat affects their availability. The amount of dietary fat required to ensure the absorption of carotenoids seems to be low (3–5 g/meal), although it depends on the physico-chemical characteristics of the carotenoids ingested. In one experiment, lutein, added as esters, gave plasma concentrations about 100% higher when consumed with 35 g of fat than with 3 g of fat (van het Hof et al., 2000). The small amount of fat may have limited the solubilization of lutein esters and/or the release of esterases and lipases (Roodenburg et al., 2000). Availability of carotenoids is also affected by the absorbability of the dietary fat (Borel et al., 1998).

Egg yolk is an example of a source of highly available zeaxanthin and lutein. The lipid matrix of the egg yolk containing cholesterol, triacylglycerols and phospholipids provides a vehicle for the efficient absorption of the xanthophylls (Handelman et al., 1999).

Interactions between carotenoids may decrease absorption. Competition for absorption may occur at the level of micellar incorporation, intestinal uptake, lymphatic transport or at more than one level. Alternatively, simultaneous ingestion of various carotenoids may induce an antioxidant-sparing effect in the intestinal tract resulting in increased levels of uptake of the protected carotenoids. It has been demonstrated that in the presence of high amounts of β-carotene, the uptake of xanthophylls from the intestinal lumen by chylomicrons is greater than that of β-carotene (Gärtner et al., 1996). A number of studies on the interaction of dietary β-carotene and lutein have reported varying effects of one carotenoid on the absorption or plasma concentrations of the other (Micozzi et al., 1992; Kostic et al., 1995; van den Berg, 1998; Tyssandier et al., 2002). Van den Berg (1999) has concluded that in general, long-term supplementation with β-carotene has limited or no effect on plasma concentrations of other carotenoids. However, in the α-Tocopherol, β-Carotene Cancer Prevention Study (ATBC Study), a total of 29 133 male Finnish smokers aged 50–69 years were given daily supplements of 20 mg of β-carotene (0.3 mg/kg bw per day) for an average of 6.7 years. Significantly decreased serum concentrations of lutein (no changes in zeaxanthin) were observed in comparison with groups that did not receive β-carotene supplements (Albanes et al., 1997).

A number of non-dietary factors also negatively affect the availability of carotenoids, including exposure to tobacco smoke, alcohol consumption, intestinal parasites, malabsorption diseases, liver and kidney diseases, hormone status, poor iron, zinc and protein intake, gastric pH and hyperthyroidism (Albanes et al., 1997; Williams et al., 1998., Patrick, 2000; Alberg, 2002).

(b) Pharmacokinetic studies

Pharmacokinetic studies with zeaxanthin have been performed in mice, rats and humans.

Mice

In a study designed to investigate the uptake of lutein/zeaxanthin, BALB/c mice received diets containing an extract of marigold petals for up to 28 days (Park et al., 1998). Based on data on food intake and body weight, daily intakes of lutein for each group of 36 mice corresponded to approximately 0, 75, 150, 300, and 600 mg/kg bw, while intakes of zeaxanthin were approximately 0, 1.0, 2.0, 4.0, and 8.0 mg/kg bw, respectively. Six mice per group were killed on days 0, 3, 7, 14, 21 and 23. Body, liver and spleen weights did not differ throughout the experiment. Plasma uptake of lutein and zeaxanthin (in all cases analysed together) was rapid and reached maximum concentrations (about 3 μmol/l) by day 3 of dosing (the first time-point examined after the start of dosing) and did not differ between groups thereafter until day 28. There was also a rapid increase to day 3 in concentrations of lutein and zeaxanthin in liver and spleen with continued, though small, increases to day 28. The liver was considered to be the major storage organ for lutein and zeaxanthin.

Rats

The absorption, excretion, tissue distribution and plasma kinetics of zeaxanthin were investigated in groups of male and female Han Wistar rats given a single oral (gavage) dose of [^{14}C]*R*, *R*-all-*E*-zeaxanthin (99%) at 2 or 20 mg/kg bw in a beadlet formulation containing gelatin and vegetable oil (three to five rats per measurement). Before administration of the radiolabeled dose, rats had been treated with nonradiolabelled synthetic zeaxanthin (99%) added to the diet as a beadlet formulation for 2 weeks at the same daily dose (2 or 20 mg/kg bw per day) to establish steady-state conditions. Radioactivity was determined in expired air, urine, bile, faeces, plasma, blood organs and tissues.

Peak plasma concentrations of zeaxanthin were observed at 6 and 3 h after administration of the lower dose, and at 8 and 6 h after the higher dose, in males and females respectively. Plasma concentrations were below the limit of detection by 36–48 h after dosing. Tissue concentrations of zeaxanthin were highest in the gastrointestinal tract (stomach, and small and large intestines), liver, spleen, kidney, pancreas, and lungs, with measurable amounts also in the thyroid and adrenals. Radioactivity was almost completely cleared from tissues by 96 h, the last time-point evaluated, and there was no evidence of accumulation. Saturation of the absorption pathway for zeaxanthin at the higher dose was suggested both by a non-linear increase in the area under the curve of concentration–time (AUC) (an approximately twofold increase for a tenfold increase in dose) and by data on urinary excretion. Chromatograms obtained for each of the pooled plasma samples of each sex at both doses indicated the presence of up to seven components, none of which was identified.

Most of the administered radiolabel was excreted in the faeces of both male and female rats (about 90% and about 100% at doses of 2 mg/kg and 20 mg/kg, respectively). Urinary excretion accounted for a mean of only 3–4% and about 1% at the lower and higher doses, respectively, in both sexes. Excretion was rapid, with most of the radioactivity being excreted in the first 48 h after dosing. Biliary

excretion was minimal after either dose (approximately 0.5% of the administered dose). No radiolabel was associated with the expired air in this study, indicating that the site of labelling was stable. From the data on urinary excretion, the oral absorption of total radioactivity was about 4% in both sexes at the lower dose, and only about 1% in both sexes at the higher dose. There were reported to be no relevant differences between males and females (Froescheis et al., 2001).

A distribution study with zeaxanthin of a higher specific activity than that used in the study reported above was conducted in groups of three male albino Ibm: RORO rats pre-treated with zeaxanthin-poor (basic rat diet containing a maximum of 0.0001% zeaxanthin, equivalent to about 0.08 mg/kg bw per day) or zeaxanthin-enriched diet (containing 0.001% zeaxanthin, equivalent to about 0.8 mg/kg bw per day) for 5 weeks. A single dose of (7,8,7′,8′[^{14}C])-zeaxanthin in a liposomal preparation was administered orally by gavage. As in the previous experiment, zeaxanthin was mainly excreted in the faeces (50–70% of administered radiolabel in 24 h), with 6–11% of the radioactivity in the urine after 24 h. Approximately 1% of the applied dose, or about 4–7% of the absorbed dose, was measured in the expired air during the first 24 h after the administration of radiolabeled zeaxanthin. Based on the urine and tissue measurements, and assuming that there was no enterohepatic circulation, absorption ranged between 9–15% for the rats given the zeaxanthin-poor diet to 13–19% for the rats given the zeaxanthin-enriched diet (biliary excretion was not considered). After 24 h, about one-third of the administered radiolabel was still present in the body and intestinal tract, while after 1 week, <0.5% was present. It was concluded that the radioactivity from [^{14}C]zeaxanthin is rapidly depleted from the body and the gastrointestinal tract in rats (Glatzle et al., 1999a, 1999b).

The distribution of zeaxanthin was investigated in groups of 20 male FU-albino (RORO) rats fed diets containing nonradiolabelled zeaxanthin at a concentration of 10 mg or 100 mg/kg of feed, added as a beadlet formulation (a dose of approximately 0.8 mg or 8 mg/kg bw per day) (Bausch et al., 1999). Five rats per group were killed after 35 days of accumulation (receiving a diet containing zeaxanthin), then after 7, 21 and 35 days of depletion (receiving a zeaxanthin-free diet). A dose-dependent accumulation of zeaxanthin was found in several tissues, with the exception of the thyroid gland and the eye, where concentrations were undetectable. After 35 days of accumulation, the highest concentrations of zeaxanthin were found in the small intestine (about 3.3 μg/g) and spleen (about 2.75 μg/g), followed by liver (about 0.4 μg/g), fat (about 0.14 μg/g) and adrenal glands (about 0.12 μg/g) (values are for the higher dose). There was a pronounced decrease in tissue concentrations of zeaxanthin during a subsequent 5-week period when rats received a zeaxanthin-free diet. For many tissues, >50% of zeaxanthin was lost in the first week, followed by a slower rate of loss.

Humans

Concentrations of lutein and zeaxanthin in serum and tissues have been shown to be quite variable (Boileau et al., 1999), but to increase, as expected, with increased intake either from dietary sources or from supplements (e.g. Hammond et al., 1997; Landrum et al., 1997a, 1997b; Carroll et al., 1999; Tucker et al., 1999; Berendschot et al., 2000; Johnson et al., 2000; Curran-Celantano et al., 2001;

Olmedilla et al., 2001; Schalch et al., 2001; Bone et al., 2003). In a population-based study, Brady et al. (1996) reported that lower serum concentrations of lutein and zeaxanthin are generally associated with males, smoking, younger age, lower concentrations of HDL cholesterol, greater consumption of ethanol and higher body mass index. Carotenoids are present in variable amounts in many tissues such as kidneys, buccal mucosal cells and adrenal glands, but the main sites of storage are adipose tissue and liver (Parker, 1996). As in serum, β-carotene, lutein and lycopene are the main carotenoids found in tissues, although β-cryptoxanthin and zeaxanthin are also present in significant amounts (Boileau et al., 1999). The eye in general, and the retina in particular, contain extraordinarily high concentrations of zeaxanthin and lutein (Bone et al., 1993). Other carotenoids are present in only trace amounts in the retina and lens (Khachik et al., 1997a, 1997b, Yeum et al., 1999, Bernstein et al., 2001). Zeaxanthin and lutein are the pigments responsible for the colouration of the macula lutea (yellow spot) (Landrum & Bone, 2001).

There have been two pharmacokinetic studies with zeaxanthin in humans. The plasma concentrations of lutein and zeaxanthin were measured in a small pilot study in groups of eight volunteers (four men and four women) receiving daily supplements comprising capsules containing either 4.1 mg of lutein (with 0.34 mg of zeaxanthin) or 20.5 mg of lutein (with 1.7 mg of zeaxanthin) for 42 days (Cohn et al., 2001). Subjects were monitored for a further 25 days after the dosing phase. Steady-state concentrations of xanthophyll were reached between days 38 to 43 (0.06 μmol/l and 0.13 μmol/l for the lower and higher doses, respectively). Dose-normalized incremental maximum and average steady state concentrations of lutein and zeaxanthin were found to be comparable, indicating that they have similar bioavailability. The elimination half-life was calculated to be approximately 5–7 days for either compound.

In a subsequent study, after a run-in period of 3 days to define base-line concentrations, capsules providing doses of either 1 mg or 10 mg of zeaxanthin were administered daily to groups of five men and five women for 42 days (Cohn et al., 2002, Hartmann et al., 2004). Accumulation of plasma zeaxanthin was monitored between days 1 and 42, and the kinetics of disappearance were followed from day 42 to day 76. Concentrations of zeaxanthin increased from 0.048 ± 0.026 μmol/l at baseline to 0.20 ± 0.07 and 0.92 ± 0.28 μmol/l at 1 and 10 mg of zeaxanthin respectively. The dose-normalized bioavailability of zeaxanthin after the 10 mg dose was 40% lower than after the 1 mg dose. After 17 days of dosing, >90% of the concentration at steady-state was reached, which was compatible with an effective half-life of accumulation of 5 days. The terminal elimination half-life was 12 ± 7 days. Multiple doses of 1 or 10 mg of zeaxanthin did not affect plasma concentrations of other carotenoids, retinol, α-tocopherol or lipids. 3′-Dehydrolutein was shown to be derived from zeaxanthin and had the same plasma concentration profile as zeaxanthin. It is reported that both doses of zeaxanthin were well tolerated by all subjects.

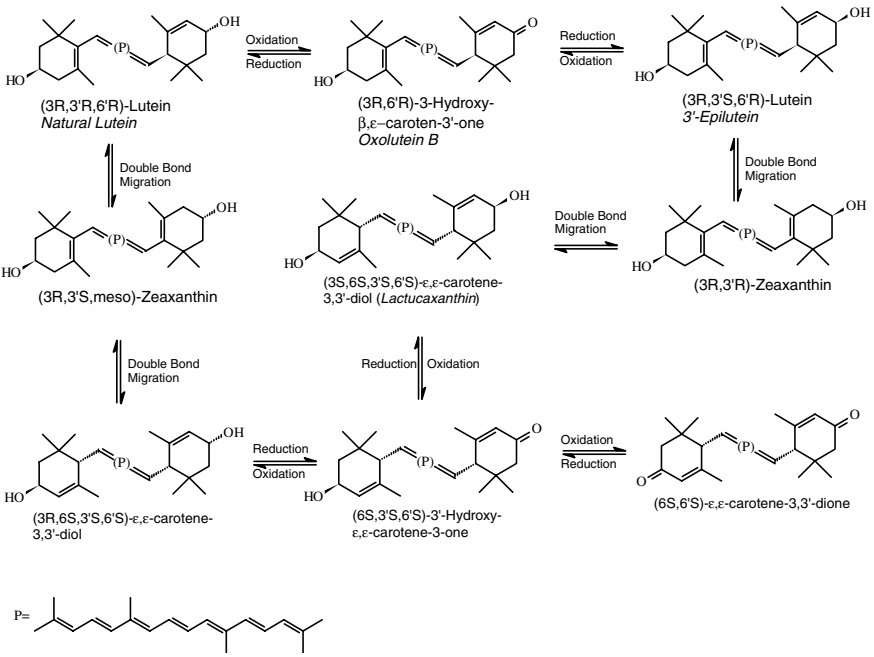
2.1.2 Biotransformation

A number of compounds derived from lutein and zeaxanthin have been identified in human serum (Figure 3) (reviewed by Khachik et al., 1995a). These are

called 'metabolites', but they undoubtedly are formed by chemical rather than enzymatic reactions. The metabolites result principally from three types of reactions involving the end groups of these carotenoids — oxidation, reduction and double-bond migration (Figure 3).

Lutein and zeaxanthin can exist in equilibrium involving the intermediate carotenoid 3′-epilutein. Allylic oxidation of lutein at C3 results in the formation of oxolutein B, which can exist in equilibrium with lutein and 3′-epilutein through reduction reactions. 3′-Epilutein and zeaxanthin can also exist in equilibrium through reversible double-bond migration. Thus, presence of 3′-epilutein in human serum may be due to conversion of lutein and/or zeaxanthin. Acid-catalysed dehydration is another reaction of carotenoids with 3-hydroxy-ε end groups. Lutein is believed to undergo dehydration in stomach acid to form 3-hydroxy-3′,4′-didehydro- β,γ-carotene and 3-hydroxy-2′,3′-didehydro-β,ε-carotene (anhydroluteins) that have been isolated from serum. In addition to their presence in human serum, these metabolites have also been detected in breast milk as well as retinal extracts (Khachik et al., 1995b; Khachik et al., 1997a, 1997b). The toxicological importance of these compounds is not known.

Figure 3. Proposed reactions of lutein and zeaxanthin in humans



Adapted from Khachik et al. (1995a, 1997b)

2.1.3 Effects on enzymes and other biochemical parameters

The xanthophylls are not considered to be precursors of retinol. Indeed, they have been shown to have little or no activity as substrates of β-carotene-15,15′-dioxygenase, although they are able to inhibit the conversion of β-carotene to retinol (Ershov et al., 1993; van Vliet et al., 1996; Grolier et al., 1997). However, in a model in rats, Weiser & Korman (1993) showed that the xanthophylls have small but significant provitamin A activity (4–5% of the activity of β-carotene), probably via a vitamin A-sparing effect. Furthermore, Weiser & Korman reported that dietary zeaxanthin is able to induce duodenal 15,15′-dioxygenase activity in chicks aged 1 day.

Although there are no studies on the effects of zeaxanthin on the enzymes of drug metabolism, studies have shown that lutein (extracted from marigold petals and likely to contain small amounts of zeaxanthin) has no effect on a number of phase I and phase II enzymes in rat liver, lung and kidney (Gradelet et al., 1996; Jewell & O'Brien, 1999).

2.2 Toxicological studies

2.2.1 Acute toxicity

Studies of acute toxicity with zeaxanthin were performed in rats and mice (Baechtold, 1977a, 1977b). All mice and rats survived for 10 days after a single oral dose of zeaxanthin of up to 4000 mg/kg bw in rats and 8000 mg/kg bw in mice. The LD_{50} values in rats and mice are therefore >4000 and 8000 mg/kg bw, respectively.

2.2.2 Short-term studies of toxicity

Mice

In a study of oral toxicity, which did not comply with good laboratory practice (GLP), albino-SPF mice were diets containing all-*trans*-3*R*,3′*R*-zeaxanthin incorporated into gelatin-coated beadlets containing a fine suspension of 9.3% of the pure compound (purity, 97.6%) for 13 weeks. Some batches of beadlets contained up to 0.15% chloroform, a residue from the synthetic procedure. Groups of 10 male and 10 female mice received zeaxanthin at nominal doses of 250, 500, or 1000 mg/kg bw per day. Using 'placebo' beadlets, adjustments were made such that all four groups received the same amount of beadlets (about 10% of feed). In addition, there was a control group that received beadlets that did not contain zeaxanthin. No treatment-related effects were observed throughout the study. Haematology, blood chemistry and urine analysis measurements showed no evidence of toxicity caused by. There were no treatment-related findings by ophthalmoscopic examination. In contrast with later studies in rats and dogs, no discolouration of adipose tissue was reported. Findings at necropsy and histopathological examination of tissues revealed no significant treatment-related changes. The no-observed-effect level (NOEL) was 1000 mg/kg bw per day, the highest dose tested (Ettlin et al., 1980a).

Rats

In a 13-week study of oral tolerance study, which did not comply with GLP, albino-SPF rats were given diets containing a beadlet formulation of zeaxanthin exactly as described above for the study in mice. Groups of 16 male and 16 female rats were given zeaxanthin at a dose of 250, 500, or 1000 mg/kg bw per day. The addition of 'placebo' beadlets ensured that all four groups received similar amounts of beadlets (about 20% of feed), and a 'beadlet-only' control group was included. Towards the end of the study, rats at the highest dose tended to avoid eating the beadlets, so the dose was reduced by about 40% in females and about 65% in males. One male rat at the lowest dose died at week 8; there had been no previous clinical signs of toxicity and there were no obvious histopathological findings. There was a slight reduction in body-weight gain in all groups consuming the beadlets, which was unrelated to intake of zeaxanthin. As for the mice, there were no treatment-related effects throughout the study. Haematology, blood chemistry and urine analysis measurements showed no evidence of toxicity caused by zeaxanthin. There were no treatment-related findings by ophthalmoscopic examination. In contrast with later 13-week studies of toxicity in rats and dogs, no discolouration of adipose tissue was reported in the current study. Findings at necropsy and histopathological examination of tissues revealed no significant treatment-related changes. The NOEL was 500 mg/kg bw per day (in view of the reduced intake at the highest dose during the latter part of the study, the data from this group could not be used) (Ettlin et al., 1980b).

In a second 13-week study of oral tolerance in rats, a different batch of beadlets was used in which methylene chloride was employed instead of chloroform in the synthesis of the zeaxanthin. The residue of methylene chloride after evaporation was estimated to be approximately 150–250 mg/kg. In this study, which complied with GLP, the rats were unable to exclude the beadlets from their diets. Groups of 12 male and 12 female rats were given zeaxanthin at the same doses as those used previously (0, 250, 500, and 1000 mg/kg bw per day). As before, there were no biologically significant changes resulting from exposure to zeaxanthin, but there were a number of statistically significant changes: decreased numbers of leukocytes (in males at the highest dose), decreased concentrations of bilirubin (in males and females at the highest dose), and increased concentrations of sodium, decreased concentrations of total serum protein and decreased concentrations of α-1-globulin (in females at the intermediate and highest doses). All these changes, however, were considered by the investigators to be within normal limits for the rat. There were no treatment-related changes in organ weights, and no macroscopic or microscopic findings that could be attributed to toxic effects of zeaxanthin. Overall, the results were similar to those of the earlier study described above, with the exception that there was a yellow-orange discolouration of the faeces and a slight orange discolouration of the adipose tissue, particularly at the higher doses. This was attributed to colour imparted by the zeaxanthin beadlet. The NOEL was 1000 mg/kg bw per day, the highest dose tested (the discolouration not being considered to be an adverse effect) (Buser, 1985).

Dogs

In a 13-week study of oral toxicity, which complied with GLP, groups of three male and three female beagle dogs aged 10 months were given diets containing a beadlet formulation of zeaxanthin, exactly as described above for mice and rats. Methylene chloride was used instead of chloroform in the formulation of the beadlets. Beadlets were incorporated into feed pellets at concentrations of 0, 4, 8, or 16%, to achieve doses of zeaxanthin of 0, 123, 204, or 422 mg/kg bw per day in males and 0, 104, 238, and 442 mg/kg bw per day in females, respectively. The percentage of beadlets present in the feed was kept constant (16% w/w) for all treatment groups. Actual concentrations of zeaxanthin measured throughout the study were within 90–106% of the nominal concentrations. There were no differences in body weights between the groups throughout the study. Furthermore, no treatment-related toxicity was observed in any of the dogs. Haematology, blood chemistry and urine analysis measurements showed no evidence of toxicity caused by zeaxanthin. The test compound was found to discolour strongly and to slightly soften the faeces, particularly at the highest dose. There was also a slight orange discolouration of the adipose tissue, particularly in males at the highest dose. There were no treatment-related findings by ophthalmoscopic examination. At necropsy, male dogs in the groups receiving the intermediate or highest doses showed slight to moderate discolouration (yellow to reddish) of the adipose tissue (attributed to colour imparted by the zeaxanthin beadlet). Histolopathological examination of the tissues showed splenic adhesions (subacute haemorrhagic perisplenitis) in one male at the intermediate dose, which was considered not to be treatment-related (possibly arising owing to trauma). Slight changes in organ weights (increased weights of the thyroid in females at the lowest dose and males at the highest dose compared with controls, decreased weights of the heart in females at the lowest dose, and decreased weights of the kidney in dogs at the intermediate dose) were not accompanied by histological changes and were not dose-dependent. Overall, it was concluded that there were no treatment-related microscopic findings. The NOEL was 420 mg/kg bw per day, the highest dose tested (the discolouration not being considered to be an adverse effect) (Ettlin, 1985).

Monkeys

In a study that complied with GLP and that was designed primarily to investigate the effects on the eye of long-term exposure to zeaxanthin, cynomologus monkeys aged 4–7 years were given zeaxanthin at a dose of 0, 0.2, or 20 mg/kg bw per day by gavage for 52 weeks. The solutions for gavage were prepared by dissolving beadlets in water. The control was prepared using beadlets that contained no zeaxanthin. There were two males and two females in each group, with an additional male and female included in the group receiving the higher dose, designated for examination at 6 months. All animals survived the treatment period. All animals at 20 mg/kg per day showed orange/yellow discolouration of the faeces from day 2 of the study onwards (attributed to treatment with zeaxanthin). There was no effect on overall mean body-weight gain and on overall group mean feed intake in either of the treatment groups. There were no treatment-related changes

in haematology, blood chemistry, or urine analysis measurements. There were no changes in electrocardiogram waveform or data on blood pressure that could be regarded as being related to the administration of zeaxanthin. There were no treatment-related organ weight changes. Most of the animals showed dark yellow-coloured mesenteric fat at interim sacrifice and gold-yellow mesenteric fat at terminal sacrifice (attributed to treatment with zeaxanthin). Histopathological examinations revealed no treatment-related findings (a single adenoma of the thyroid observed in a single female at the higher dose was considered to be an isolated incident and not of toxicological significance). Comprehensive ophthalmic examinations showed no evidence of treatment-related adverse changes. Animals showed a dose-related increase in plasma and liver concentrations of zeaxanthin. Thus, zeaxanthin was considered to be well tolerated. The NOEL in cynomologus monkeys was 20 mg/kg bw per day, the highest dose tested (Pfannkuch et al., 2000a, 2000b; Pfannkuch, 2001).

2.2.3 Long-term studies of toxicity and carcinogenicity

There have been no studies of toxicity with zeaxanthin with a duration of greater than 1 year.

2.2.4 Genotoxicity

There is some concern, in the first three studies listed in Table 1, that zeaxanthin precipitated out of solution, despite the use of dimethylsulfoxide (DMSO), at the highest concentrations tested (Strobel, 1986, 1987) and at all concentrations tested (Strobel & Bonhoff, 1987). It is clear, however, that zeaxanthin in a beadlet formulation gave negative results in an assay for micronucleus formation in bone marrow of mice. In these studies, there was no evidence for genotoxicity. The concentrations and doses used in some of the studies were considered to be low, but were the maximum feasible doses.

In addition to the studies reported in Table 1, a beadlet formulation of zeaxanthin was evaluated for mutagenic activity in an Ames assay, which complied with GLP, using both the plate incorporation and the pre-incubation methods (Gocke, 1987). Seven *Salmonella typhimurium* standard tester strains were employed (TA1535, TA1537, TA1538, TA97, TA98, TA100, and TA102), with and without an exogenous microsomal fraction (S9) derived from livers of male albino rats treated with phenobarbital/β-naphthoflavone. Owing to precipitation of the test compound in the aqueous medium, concentrations of 2.4 to 1500 μg/plate and 5 to 500 μg/plate were tested in the plate incorporation and pre-incubation methods, respectively. There was no increase of the numbers of mutants in any of the tester strains, while the positive controls verified the sensitivity of the strains and the activity of the S9 mix.

Table 1. Studies of genotoxicity with zeaxanthin

End-point	Test system	Concentration or dose	Result	Reference[a]
In vitro				
Gene mutation	Chinese hamster lung V79 cells	1–16 μg/ml (0.002–0.03 mmol/l)	Negative	Strobel, 1986
Unscheduled DNA synthesis	Hepatocytes from male FU-albino rats	1–16 μg/ml; 20 h exposure	Negative	Strobel, 1987
Chromosome aberration	Human peripheral blood lymphocytes	6, 30, 60, or 120 μg/ml, 1 h exposure; 60 and 120 μg/ml, 2 h exposure; both +S9 40, 50, 60, 70, or 80 μg/ml, 24 h exposure, −S9	Negative	Strobel & Bonhoff, 1987
In vivo				
Micronucleus formation	Mouse bone-marrow cells	500, 1000, or 2000 mg/kg bw, beadlet powder peroral (equivalent to 44.5, 89.0, and 178.0 mg/kg bw pure zeaxanthin, respectively); exposure for 6 or 30 h	Negative	Gallandre, 1980

S9, 9000 × *g* supernatant of rat liver.
[a] All studies complied with GLP.

2.2.5 Reproductive toxicity

(a) Multigeneration studies

No studies of this type were available.

(b) Developmental toxicity

Rats

In a segment II study of teratology, which complied with GLP, groups of 36 mated female FU-albino outbred rats (aged 2–3 months at the beginning of the experiment) were given diets containing zeaxanthin at a dose of 0, 250, 500, or 1000 mg/kg bw per day orally as a dietary admixture in a 10% beadlet formulation from days 7 to 16 of gestation (Kistler, 1984). Actual doses of zeaxanthin were close to the nominal levels, based on food intake. Dams were necropsied on day 21 and uteri were examined for numbers and locations of implantations and resorptions. Fetuses from 15 litters per group underwent skeletal or soft tissue examinations. Litters from each group were raised until weaning and examined for abnormalities. There were no treatment-related maternal deaths and no signs of maternal toxicity. There were no reproductive effects (resorption rates, average litter sizes, mean body weights of live fetuses) and no teratagenic or developmental effects (no skeletal, soft tissue, or external abnormalities related to treatment). One fetus in the group receiving the highest dose exhibited severe malformations (representing 1 out of 422 fetuses at this dose). The NOEL was 1000 mg/kg bw per day, the highest dose tested.

Rabbits

In a segment II study of teratology, which complied with GLP, groups of 20 mated female FU-albino rabbits (aged 2–3 months at the beginning of the experiment) were fed zeaxanthin at a dose of 0, 100, 200, or 400 mg/kg orally in rapeseed oil from days 7 to 19 of gestation (Kistler, 1983). Dams were necropsied on day 30 of gestation and uteri were examined for numbers and locations of implantations and resorptions. Fetal viability in incubators was tested and gross and skeletal examinations were conducted. There were no treatment-related maternal deaths and no signs of maternal toxicity. There were no reproductive effects (resorption rates, average litter sizes, mean body weights of live fetuses, survival rates of foetuses) and no teratogenic effects (no skeletal, soft tissue, or external abnormalities). Some malformed fetuses were observed, but there was no consistency in the pattern and no dose-dependence with distribution in all groups including controls. Thus, the incidence of malformed fetuses was considered to be unrelated to treatment. The NOEL was 400 mg/kg bw per day, the highest dose tested.

2.2.6 Special studies

(a) Immune responses

In order to assess the allergenic potential of zeaxanthin, a maximization test for skin sensitization, which complied with GLP, was performed in 15 (10 test and

5 control) female Himalayan spotted guinea-pigs, aged 4–6 weeks (Csato & Arcelin, 2000a). The intradermal induction of sensitization in animals in the test group was performed using a 3% solution of zeaxanthin (purity, 98.3%) in polyethylene glycol 300 (PEG 300) and in a 1:1 mixture of Freund complete adjuvant (FCA) and physiological saline, applied in the nuchal region. The epidermal induction of sensitization was conducted for 48 h under an occlusive dressing with 25% zeaxanthin in PEG 300, 1 week after the intradermal induction and after pretreatment of the test areas with 10% sodium dodecyl sulfate (SDS). Animals in the control group were treated identically except for the absence of zeaxanthin in the vehicle solutions. Two weeks after epidermal induction, the control and test animals were challenged by epidermal application of 25% zeaxanthin in PEG 300 and PEG 300 alone under the occlusive dressing. Cutaneous reactions were evaluated at 24 and 48 h after removal of the dressing. None of the control or test animals showed skin reactions after the challenge treatment and it was concluded that zeaxanthin is not a skin sensitizer, and that the risk, if any, to humans is low.

(b) Ocular toxicity

The long-term ingestion of canthaxanthin at high doses has been shown to lead to accumulation and crystallization in the retina of humans (Arden & Barker, 1991) and monkeys (Goralczyk et al., 1997), and the question has therefore arisen as to whether zeaxanthin behaves similarly. The effects of zeaxanthin on the eye were investigated in a GLP-compliant study in cynomolgus monkeys treated orally with zeaxanthin in a 10% beadlet formulation (described above) by gavage for 52 weeks. The cynomolgus monkey was chosen since it was shown to be an excellent model to investigate the induction and dose-dependency of carotenoid crystal formation in the retina (Goralczyk et al., 1997; Goralczyk, 2000; Goralczyk et al., 2002). Groups of two male and two female monkeys were given zeaxanthin at a dose of 0 (placebo beadlet), 0.2, or 20 mg/kg bw per day, with one additional male and female included in the group receiving the higher dose. One male and one female in the group receiving the higher dose were also killed at 6 months. Occasional retinal changes, such as inclusions in the macula, were observed in some groups of animals, including the controls, and were considered to be unrelated to treatment. Overall, comprehensive ophthalmic examinations (ophthalmoscopy and biomicroscopy examinations, fundus photography, electroretinography (considered to be a very sensitive procedure for the detection of early signs of generalized retinal degeneration), and post-mortem examinations of the right retina including macroscopic inspection, microscopic pathology under polarized and bright light for peripheral retina and macula, confocal microscopy of the macula and histopathological examination of the peripheral retinal) showed no evidence of treatment-related adverse changes, including no evidence for crystal formation in the eyes during or after 52 weeks of treatment with zeaxanthin. Dose-dependent increases in concentrations of zeaxanthin were reported in the peripheral retina. In the central retina and lens, zeaxanthin content was markedly increased in animals at the higher dose, but there was no evidence for crystalline deposits. It was concluded that administration of zeaxanthin for 52 weeks in cynomolgus monkeys at doses of 0.2 and 20 mg/kg bw per day resulted in no toxic effects to the eye (Pfannkuch et al., 2000a, 2000b; Pfannkuch, 2001).

(c) Ocular irritation

Zeaxanthin, as described above, was also tested in a study of primary eye irritation in three adult New Zealand white rabbits. The primary irritation score for zeaxanthin was 0.78 (maximum potential score is 13.0) and it was classified as 'not irritating' to the rabbit eye in this study, which complied with GLP (Csato & Arcelin, 2000b).

2.3 Observations in humans

2.3.1 Clinical studies

There have been a number of studies designed to investigate the pharmacokinetics of lutein and zeaxanthin that did not necessarily include safety end-points, but also did not report any adverse effects caused by the xanthophylls (see sections 2.1.1 and 2.1.2). A relatively large number of studies in humans has examined correlations between dietary intake of lutein and/or zeaxanthin, the effects of dietary supplements, or serum concentrations of lutein and/or zeaxanthin and the incidence of age-related macular degeneration (AMD), macular pigment density or cataractogenesis with varying results (Eye Disease Case–Control Study Group, 1993; Seddon et al., 1994; Mares-Perlman et al., 1995a, 1995b; Khachik et al., 1997c; Lyle et al., 1997b; Beatty et al., 1999; Chasan-Taber et al., 1999; Lyle et al., 1999a; Pratt, 1999; Richer, 1999; Bone et al., 2000; Johnson et al., 2000; Gale et al., 2001; Schalch et al., 2001; Bone et al., 2003; Gale et al., 2003). These studies will not be reviewed here, but in many cases, weak inverse associations have been found, although it is apparent that the protective effect of the xanthophylls against AMD or cataract formation remains unproven. Of importance here, however, is that none of these studies reported adverse effects, including ocular toxicity, caused by the xanthophylls, even though in some cases, high dietary levels or supplements were consumed.

In a pharmacokinetic study described earlier (section 2.1.1), which complied with GLP, and in which groups of five men and five women were given capsules containing zeaxanthin at either 1 mg or 10 mg of zeaxanthin per day for 42 days (corresponding to doses of approximately 0.014 and 0.14 mg/kg bw per day for a 70 kg adult), clinical chemistry measures (haematology, blood chemistry and urine analysis) and adverse events were recorded. Several clinical laboratory results at different assessment times fell outside of the normal ranges, but all were deemed to be without clinical relevance. There were no relevant changes in blood pressures, heart rate, or body temperature; one subject in the group receiving the higher dose showed an electrocardiogram (ECG) abnormality that was deemed not to be of clinical relevance. In the groups receiving the lowee dose and higher doses, there was one adverse event (an infection of the upper respiratory tract) deemed to have only a remote possibility of being related to dosing. In the group receiving the higher dose there were three adverse events (one case of bilirubinaemia, one case of abnormal vision, and one case of abnormal accommodation) that were deemed to be remotely or possibly related to treatment. The case of abnormal accommodation was accompanied in the same subject by reports of dyspnoea and sleep disorder, both of which were deemed to have only a remote

possibility of being related to treatment. There was also one report of syncope in the group receiving the lower dose, which was deemed to be unrelated to treatment. All the adverse events were rated as mild to moderate in severity (Cohn et al., 2002).

2.3.2 Epidemiological studies

Most epidemiological studies on the xanthophylls have addressed the hypothesis that intake of these compounds is inversely related to development of cancer. A number of such studies have suggested that dietary xanthophylls may protect against the development of a variety of cancers including those of the oesophagus, colon, breast, prostate and lung (e.g. Le Marchand et al., 1995; Freudenheim et al., 1996; Zhang et al., 1997; Franceschi et al., 2000; Levi et al., 2000; Lu et al., 2001; Nkondjock & Ghadirian, 2004), although recent studies on breast and lung cancer have indicated that these compounds are not protective (Terry et al., 2002; Mannisto et al., 2004).

A recent large prospective study in a region of China with epidemic rates of oesophageal and gastric cancer examined the relationship between serum concentrations of carotenoids and subsequent risk of developing cancers of the stomach and upper digestive tract (Abnet et al., 2003). There was an association between the incidence of gastric non-cardia cancer and serum concentrations of lutein and/or zeaxanthin derived from normal dietary sources. These observations, however, are only correlative and, indeed, in a Dutch cohort study, dietary intake of lutein/zeaxanthin was not associated with risk of gastric cancer (pathological type not specified), although intakes of retinal and β-carotene were positively associated with risk of this cancer (Botterweck et al., 2000).

In view of the structural similarities between xanthophylls and β-carotene, the Committee considered the outcome of two trials that showed that supplementation with β-carotene increases risk of lung cancer in heavy smokers; one study involved the administration of β-carotene at 30 mg/day plus 25 000 IU of retinyl palmitate in 18 314 smokers, former smokers and workers exposed to asbestos (Omenn et al., 1996), while in the second study, β-carotene at 20 mg/day with or without 50 mg of α-tocopherol was given to 29 133 male smokers (The α-Tocopherol and β-Carotene Cancer Prevention Study Group, 1994). However, in the light of the negative results in studies of genotoxicity and the absence of tumour-promoting activity of lutein, it was considered that these intervention studies on β-carotene were not appropriate for the risk assessment of zeaxanthin.

The results of a number of epidemiological studies, including descriptive, cohort and case–control studies, suggest that diets rich in carotenoids or β-carotene are associated with a reduced risk of cardiovascular disease (reviewed in Institute of Medicine, 2000). Furthermore, no adverse outcomes have been reported between increased serum concentrations of lutein and zeaxanthin and risk of subsequent myocardial infarction (Street et al., 1994). Recent epidemiological findings, as well as those from studies in vitro and in mouse models, support the hypothesis that increased dietary intake of zeaxanthin protects against the development of early atherosclerosis (Dwyer et al., 2001).

3. INTAKE

3.1 Concentrations in foods

A database of concentrations of carotenoids, including lutein and zeaxanthin, in 120 foods was assembled by Mangels et al. (1993), and was updated by Holden et al. (1999). It should be noted that the carotenoid content of food is highly variable and depends on a number of factors, including geographical area and growing conditions, cultivar or variety, processing techniques, preparation and length and conditions of storage (Holden et al., 1999 and references therein). Major sources of lutein/zeaxanthin are leafy green vegetables (e.g. raw spinach, 11.9 mg/100 g), corn (boiled, 1.8 mg/100 g) and green vegetables such as broccoli (raw, 2.4 mg/100 g), brussel sprouts (boiled, 1.3 mg/100 g), green beans (boiled, 0.7 mg/100 g), and peas (canned, 1.3 mg/100 g). Although it is not a major part of the diet in western Europe and North America, kale has the highest lutein/zeaxanthin content of all foods analysed (raw, 39.5 mg/100 g). A number of foods have been analysed specifically for zeaxanthin. Major sources are corn (canned, 0.5 mg/100 g), corn meal (0.5 mg/100 g), Japanese persimmons (0.5 mg/100 g) and leafy greens (e.g. raw spinach, 0.3 mg/100 g).

3.2 Dietary intake

Dietary recall data from 1102 adult women participating in the 1986 Continuing Survey of Food Intake by Individuals indicate mean intakes of lutein/zeaxanthin of 1.3 mg/day, with a total intake of carotenoids of 6 mg/day (Chug-Ahuja et al., 1993). Food frequency data from 8341 adults participating in the 1992 National Health Interview Survey indicate that mean intakes of xanthophylls for men were 2.2 mg/day and for women 1.9 mg/day (Nebeling et al., 1997). The Nutritional Factors in Eye Disease Study reported mean dietary intakes of lutein/zeaxanthin of 0.7–0.8 mg/day (VandenLangenberg et al., 1996). In a pooled analysis of seven cohort studies designed to assess the effect of dietary carotenoids on risk of lung cancer, intakes of lutein/zeaxanthin were energy-adjusted by using the predicted intake of 2100 kcal/day for men and 1600 kcal/day for women (Mannisto et al., 2004). Food consumption was assessed at baseline using a validated dietary questionnaire for each study population. For these seven populations, the mean intake of lutein/zeaxanthin for men and women combined was 3.7 mg/day (range, 1–6 mg/day).

The mean and 90th percentile consumption of zeaxanthin in the United States of America (USA) estimated by surveyed food samples was 1.42 and 2.68 mg/day respectively (Table 2). Simulations considering proposed food use levels in the total population of the USA resulted in estimated mean and 90th percentile intakes for all users of zeaxanthin of 1.46 and 2.68 mg/day respectively (DSM Nutritional Products, 2004) (Table 2). The same method was applied to the United Kingdom (UK) using data on food consumption from the UK. The estimated mean and 90th percentile consumption of total zeaxanthin in the sample foods surveyed was 1.02 and 1.81 mg/day respectively for males, and 0.95 and 1.63 mg/day for females (DSM Nutritional Products, 2004) (Table 2).

Lutein/zeaxanthin formuations are also available as dietary supplements, but there are no reliable estimates of intake from these sources.

Table 2. Estimated daily intake of zeaxanthin

Country	Estimated daily intake (mg/person per day)	Target year	Method/compound	Standards for use	Reference
USA	1.42 (mean), 2.68 (90th)	1994–1996, 1998	Food-uses and food consumption amount, all persons, zeaxanthin		DSM Nutritional Products and Nemin Foods, LC, for the Committee at its 63rd Meeting (from: Dietary Reference Intakes, Institute of Medicine, 2000)
	1.46(mean), 2.68(90th)	1994–1996, 1998	Food-uses and food consumption amount, all users, zeaxanthin		
UK (EU), male adults	1.02 (mean), 1.81 (90th)	1986–1987	Food-uses and food consumption amount, all persons, zeaxanthin		DSM Nutritional Products and Nemin Foods, LC, for the Committee at its 63rd meeting
	1.02 (mean), 1.81 (90th)		Food-uses and food consumption amount, all users, zeaxanthin		
UK (EU), female adults	0.95 (mean), 1.63 (90th)	1986–1987	Food-uses and food consumption amount, all persons, zeaxanthin		
	0.95 (mean), 1.63 (90th)		Food-uses and food consumption amount, all users, zeaxanthin		

EU: European Union.

4. COMMENTS

In rats given diets containing zeaxanthin for 5 weeks, the highest tissue concentrations were present in the small intestine, spleen, liver and adipose tissue. Seven days after cessation of administration, concentrations in plasma and tissues had decreased by between two- and fourfold, indicating that the elimination half-life was about 4–5 days.

In humans treated with daily administration of zeaxanthin at a dose of 1 or 10 mg for 42 days, the time to steady-state plasma concentrations was about 30 days. This is consistent with an elimination half-life of about 5 days. The plasma concentrations indicated that uptake and availability were not proportional to dose.

The food matrix, including its fibre and lipid contents, and the concentrations of other carotenoids in the diet may influence the extent of absorption of carotenoid compounds. Studies have shown that zeaxanthin/lutein does not influence the absorption of β-carotene.

Zeaxanthin has oral LD_{50} values of >4000 mg/kg bw in rats and >8000 mg/kg bw in mice. Ninety-day studies of toxicity with zeaxanthin in rats given doses of up to 1000 mg/kg bw per day, and in dogs given doses of up to 442 mg/kg bw per day, produced no treatment-related effects even at the highest doses. In a 52-week study in monkeys, which was designed primarily to investigate possible adverse effects on the eye, zeaxanthin was administered at a dose of 0.2 or 20 mg/kg bw per day by gavage. This study was performed because adverse ocular effects had been seen with canthaxanthin (Annex 1, references *78*, *89*, *117*). There were no treatment-related effects on a wide range of toxicological end-points. Furthermore, comprehensive ophthalmic examinations, including electroretinography, showed no evidence of treatment-related adverse changes.

No long-term studies of toxicity or carcinogenicity were available.

Zeaxanthin gave negative results in several studies of genotoxicity in vitro and in vivo. Although the Committee noted that the doses in these tests were low, it recognized that maximum feasible doses were used.

In a study of developmental toxicity with zeaxanthin in rats, there was no evidence for toxicity at doses of up to 1000 mg/kg bw per day, the highest dose tested.

In the pharmacokinetic study in humans described above, a variety of clinical chemistry measurements as well as any adverse events were recorded during the study. In the groups of five men and five women receiving zeaxanthin at a dose of 1 or 10 mg per day for 42 days, there were no reported treatment-related adverse effects. There has been a relatively large number of human studies that have examined correlations between macular degeneration and exposure to lutein/zeaxanthin via intake from traditional food or from dietary supplements, or via measurements of serum concentrations. Although these studies were designed to look for ocular effects, where clinical or biochemical parameters were also examined, no adverse effects of the xanthophylls were reported.

Dietary intake

Dietary intake data from a number of studies in North America and the UK indicate that intake of zeaxanthin from natural sources is in the range of 1–2 mg/day (about 0.01–0.03 mg/kg bw per day). Simulations considering proposed use levels as a food ingredient resulted in an estimated mean and 90th percentile intake of lutein plus zeaxanthin of approximately 7 and approximately 13 mg/day, respectively. Formulations containing lutein and zeaxanthin are also available as dietary supplements, but there were no reliable estimates of intakes from these sources.

5. EVALUATION

In several studies of toxicity, including developmental toxicity, no adverse effects were documented in animals, including monkeys, or humans. Taking into account data showing that zeaxanthin was not genotoxic, had no structural alert, that the isomeric xanthophyll lutein did not exhibit tumour promoting activity, and that zeaxanthin is a natural component of the body (the eye), the Committee concluded that there was no need for a study of carcinogenicity.

Zeaxanthin has some structural similarities to β-carotene, which has been reported to enhance the development of lung cancer when given in supplement form to heavy smokers. The available data indicated that zeaxanthin in food would not be expected to have this effect. The Committee was unable to assess whether zeaxanthin in the form of supplements would have the reported effect in heavy smokers.

In view of the toxicological data and structural and physiological similarities between the xanthophylls lutein and zeaxanthin, the Committee decided to include zeaxanthin in the acceptable daily intake (ADI), 0–2 mg/kg bw, for lutein, which had a stronger toxicological database, and to make this a group ADI for these two substances. This group ADI does not apply to other zeaxanthin preparations that do not comply with established specifications.

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SAFETY EVALUATIONS OF GROUPS OF RELATED FLAVOURING AGENTS

INTRODUCTION

Eight groups of flavouring agents were evaluated using the Procedure for the Safety Evaluation of Flavouring Agents as outlined in Figure 1 (Annex 1, references *116*, *122*, *131*, *137*, *143*, *149*, *154* and *160*). In applying the Procedure, the chemical is first assigned to a structural class as identified by the Committee at its forty-sixth meeting (Annex 1, reference *122*). The structural classes are as follows:

- Class I. Flavouring agents that have simple chemical structures and efficient modes of metabolism, which would suggest a low order of toxicity by the oral route.
- Class II. Flavouring agents that have structural features that are less innocuous than those of substances in Class I but are not suggestive of toxicity. Substances in this class may contain reactive functional groups.
- Class III. Flavouring agents that have structural features that permit no strong initial presumption of safety, or may even suggest significant toxicity.

A key element of the Procedure involves determining whether a flavouring agent and the product(s) of its metabolism are innocuous and/or endogenous substances. For the purpose of the evaluations, the Committee used the following definitions, adapted from the report of its forty-sixth meeting:

Innocuous metabolic products are defined as products that are known or readily predicted to be harmless to humans at the estimated intake of the flavouring agent.

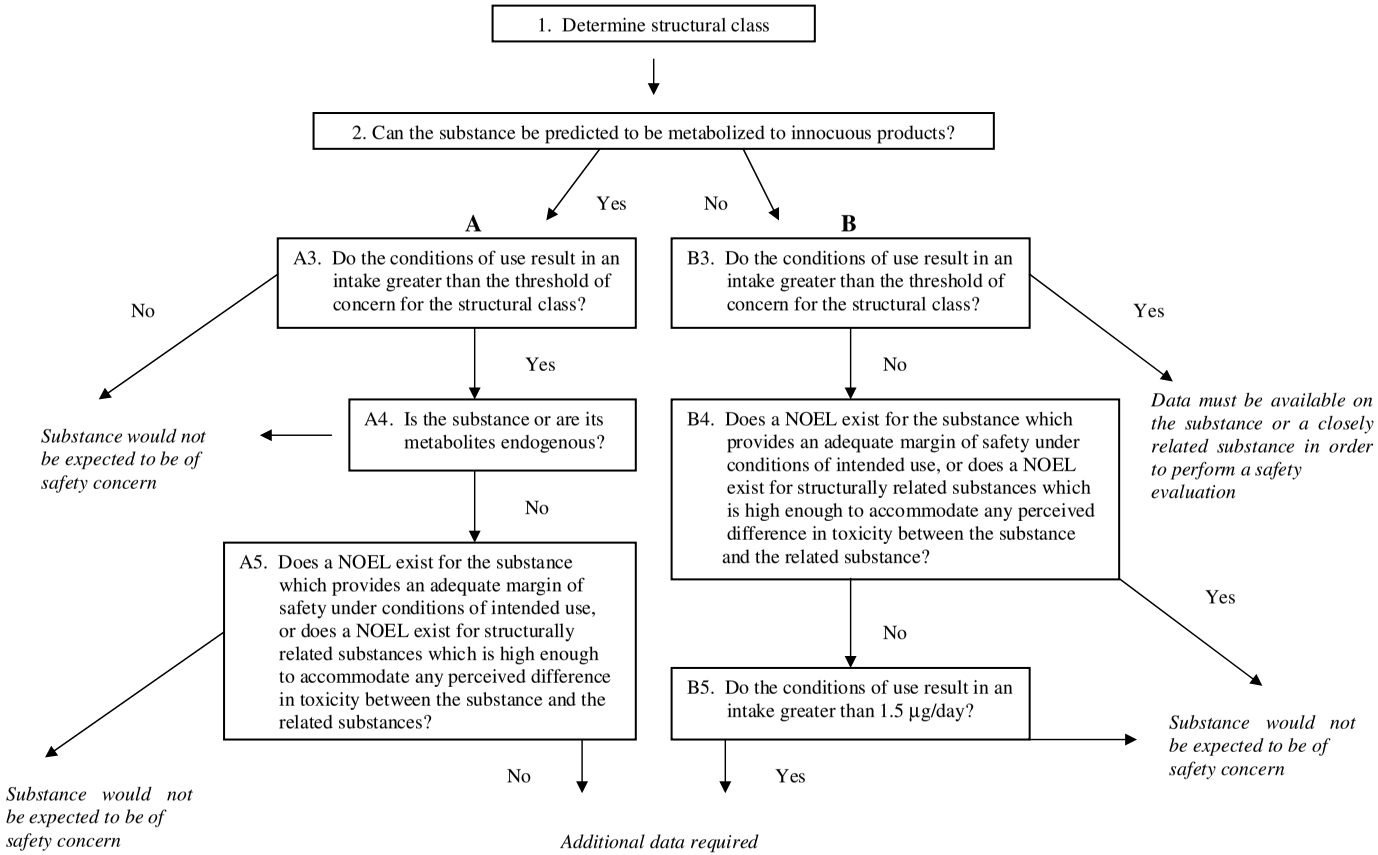
Endogenous substances are intermediary metabolites normally present in human tissues and fluids, whether free or conjugated; hormones and other substances with biochemical or physiological regulatory functions are not included. The estimated intake of a flavouring agent that is, or is metabolized to, an endogenous substance should be judged not to give rise to perturbations outside the physiological range.

Intake

Estimates of the intake of flavouring agents by populations typically involve the acquisition of data on the amounts used in food. These data were derived from surveys in Europe and the USA. In Europe, a survey was conducted in 1995 by the International Organization of the Flavour Industry, in which flavour manufacturers reported the total amount of each flavouring agent incorporated into food sold in the European Union during the previous year. Manufacturers were requested to exclude use of flavouring agents in pharmaceutical, tobacco or cosmetic products.

In the USA, a series of surveys was conducted between 1970 and 1987 by the National Academy of Sciences National Research Council (under contract to the Food and Drug Administration) in which information was obtained from ingredient manufacturers and food processors on the amount of each substance destined for

Figure 1. Procedure for the safety evaluation of flavouring agents



addition to the food supply and on the usual and maximal levels at which each substance was added in a number of broad food categories.

In using the data from these surveys to estimate intakes of flavouring agents, it was assumed that only 60% of the total amount used is reported in Europe and 80% of the amount used is reported in the USA and that the total amount used in food is consumed by only 10% of the population.

$$\text{Intake } (\mu\text{g per person per day}) = \frac{\text{annual volume of production (kg)} \times 10^9\ (\mu\text{g/kg})}{\text{population of consumers} \times 0.6 \text{ (or } 0.8) \times 365 \text{ days}}$$

The population of consumers was assumed to be 32×10^6 in Europe and 26×10^6 in the USA.

Several of the flavouring agents that were evaluated at the present meeting were not included in the above surveys or were placed on the market after the surveys were conducted. Intakes of these flavouring agents were estimated on the basis of anticipated use by the manufacturer in the USA, and the standard formula was applied.

PYRIDINE, PYRROLE AND QUINOLINE DERIVATIVES

First draft prepared by

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of 22 flavouring agents (Table 1) by the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, p 192). This group included:

—six pyrroles (Nos 1314, 1305–1307, 1310 and 1319);

—two indoles (Nos 1301 and 1304);

—12 pyridine derivatives (Nos 1308, 1309, 1311–1313, 1315–1318 and 1320–1322); and

—a quinoline derivative and an isoquinoline derivative (Nos 1302 and 1303).

The Committee has not previously evaluated any member of the group.

Nineteen of the 22 substances (Nos 1301–1307, 1309, 1310, 1312–1320 and 1322) have been reported to occur naturally in foods. They have been detected in fresh and cooked vegetables, uncured meats, a variety of whole grains, green and black teas, coffee, alcoholic beverages, whiskeys, shellfish, and a wide variety of fresh fruits (Nijssen et al., 2003).

1.2 Estimated daily intake

The total annual volume of production of the 22 flavouring agents in this group is approximately 1000 kg in Europe (International Organization of the Flavor Industry, 1995) and 650 kg in the USA (National Academy of Sciences, 1982; Lucas et al., 1999). More than 41% of the total annual volume of production in Europe and >79% in the USA is accounted for by a single substance in this group, namely 2-acetylpyridine (No. 1309). The estimated daily intakes of 2-acetylpyridine in Europe and the USA are 59 and 68 μg/person, respectively. The daily intakes of all other flavouring agents in the group ranged from 0.001 to 30 μg/person (National Academy of Sciences, 1982; International Organization of the Flavor Industry, 1995; Lucas et al., 1999), most values being at the lower end of this range. The estimated daily per capita intake of each agent is reported in Table 1.

1.3 Absorption, distribution, metabolism, and elimination

Pyridine, pyrrole and quinoline derivatives are expected to be rapidly absorbed from the gastrointestinal tract, oxidized to polar metabolites, and eliminated primarily in the urine and, to a minor extent, in the faeces.

Alkyl-substituted pyrroles and indoles may undergo cytochrome P450 (CYP)-mediated side-chain oxidation to yield the corresponding alcohol, which may be excreted as the glucuronic acid or sulfate conjugate (Ruangyuttikarn et al., 1992; Thornton-Manning et al., 1993; Gillam et al., 2000). To a lesser extent, the double bond of the indole ring may undergo epoxidation (Skiles et al., 1991; Smith et al., 1993).

Alkyl-substituted pyridines and quinolines are principally subject to side-chain oxidation, primarily at the C1 position. Minor pathways include ring hydroxylation and epoxidation for substituted quinolines. *N*-Oxide formation has also been reported (Cowan et al., 1978; Schwartz et al., 1978; Damani et al., 1980; Nguyen et al., 1988).

Methyl nicotinate (No. 1320), the only ester in the group, is rapidly hydrolysed by carboxyesterase to yield nicotinic acid and methanol (Heymann, 1980; White et al., 1990; Durrer et al., 1992).

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Substances

Step 1. In applying the Procedure, the Committee assigned three (Nos 1301, 1304 and 1314) of the 22 agents to structural class I. Thirteen agents (Nos 1305–1307, 1309, 1312, 1313, 1315–1320 and 1322) were assigned to structural class II and the remaining six (Nos 1302, 1303, 1308, 1310, 1311, and 1321) were assigned to structural class III (Cramer et al., 1978).

Step 2. Twenty flavouring agents in this group are predicted to be metabolized to innocuous products (Nos 1301–1307, 1309 and 1311–1322). The evaluation of these flavouring agents therefore proceeded via the A-side of the decision-tree. Two flavouring agents (Nos 1308 and 1310) cannot be predicted to be metabolized to innocuous products. The evaluation of these two flavouring agents therefore proceeded via the B-side of the decision-tree.

Step A3. The estimated daily intakes of all three of the flavouring agents in structural class I (Nos 1301, 1304 and 1314), all thirteen of the flavouring agents in structural class II (Nos 1305–1307, 1309, 1312, 1313, 1315–1320 and 1322), and of the four flavouring agents in structural class III (Nos 1302, 1303, 1311 and 1321) are below the respective thresholds of concern (i.e. 1800 μg/person for class I, 540 μg/person for class II, and 90 μg/person for class III). According to the Procedure, the use of these 20 flavouring agents raises no safety concern at estimated current intakes.

Step B3. The estimated daily intakes in Europe and the USA of the remaining two flavouring agents in this group (Nos 1308 and 1310), which cannot be predicted to be metabolized to innocuous products, are also below the threshold of concern for structural class III (i.e. 90 μg/person). Accordingly, the evaluation of both flavouring agents in the group proceeded to step B4.

Step B4. For *N*-furfurylpyrrole (No. 1310), the no-observed-effect level (NOEL) of 12 mg/kg bw per day from a 90-day feeding study in rats (Morgareidge, 1971) is >1 000 000 greater than the estimated current intake of this substance as a flavouring agent. For 2-pyridinemethanethiol (No. 1308), the NOEL of 3.4 mg/kg bw per day from a 90-day feeding study in rats (Posternak et al., 1969) is >20 000 000 times greater than the estimated current intake of this substance as a flavouring agent.

The intake considerations and other information used to evaluate the 22 flavouring agents in this group according to the Procedure are summarized in Table 3.

1.5 Consideration of secondary components

No flavouring agents in this group have minimum assay values of <95%.

1.6 Consideration of combined intakes from use as flavouring agents

In the event that all three agents in structural class I were consumed concurrently on a daily basis, the estimated combined intake would not exceed the human intake threshold for class I (1800 μg/person per day). In the unlikely event that all 13 agents in structural class II were consumed concurrently on a daily basis, the estimated combined intake would not exceed the human intake threshold for class II (540 μg/person per day). In the unlikely event that all six agents in structural class III were consumed concurrently on a daily basis, the estimated combined intake would not exceed the human intake threshold for class III (90 μg/person per day). Overall evaluation of the data indicated that combined intake would not raise safety concerns at estimated current intakes.

1.7 Conclusions

The Committee concluded that none of the flavouring agents in this group of pyridine, pyrrole and quinoline derivatives would present safety concerns at estimated current intakes. Other available data on the toxicity and metabolism of these pyridine, pyrrole and quinoline derivatives were consistent with the results of the safety evaluation.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

The background information summarizes key data relevant to the safety evaluation of the 22 pyridine, pyrrole and quinoline derivatives used as flavouring agents.

2.2 Additional considerations on intake

Quantitative data on natural occurrence and consumption ratios reported for indole (No. 1301), skatole (No. 1304), 1-methyl-2-acetylpyrrole (No. 1306), 2-acetylpyridine (No. 1309), *N*-furfurylpyrrole (No. 1310), and pyrrole (No. 1314) indicate that exposure occurs predominantly from consumption of traditional foods (i.e. consumption ratio >1) (Stofberg & Kirschman, 1985; Stofberg & Grundschober, 1987). Volumes of production and intake values for each flavouring agent in this group are shown in Table 2.

Table 1. Summary of the results of safety evaluations of pyridine, pyrrole and quinoline derivatives used as flavouring agents

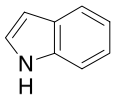
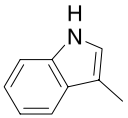
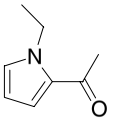
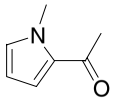
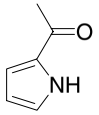
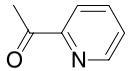
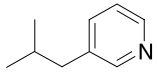
Flavouring agent	No.	CAS No. and structure	Step 2 Predicted to be metabolized to innocuous metabolites?	Step A3 Does intake exceed the threshold for human intake?[a]	Comments	Conclusion based on current intake
Structural class I						
Indole	1301	120-72-9 	Yes	No Europe: 30 USA: 10	See notes 2, 5	No safety concern
Skatole	1304	83-34-1 	Yes	No Europe: 3 USA: 0.07	See notes 2, 5	No safety concern
Pyrrole	1314	109-97-7 	Yes	No Europe: 0.1 USA: 0.01	See note 1	No safety concern
Structural class II						
1-Ethyl-2-acetylpyrrole	1305	39741-41-8 	Yes	No Europe: ND USA: 0.009	See notes 1, 4	No safety concern

Table 1. (contd)

Flavouring agent	No.	CAS No. and structure	Step 2 Predicted to be metabolized to innocuous metabolites?	Step A3 Does intake exceed the threshold for human intake?[a]	Comments	Conclusion based on current intake
1-Methyl-2-acetylpyrrole	1306	932-16-1 	Yes	No Europe: 1 USA: 0.02	See notes 1, 4	No safety concern
Methyl 2-pyrrolyl ketone	1307	1072-83-9 	Yes	No Europe: 4 USA: 0.2	See note 1	No safety concern
2-Acetylpyridine	1309	1122-62-9 	Yes	No Europe: 59 USA: 68	See notes 3, 4	No safety concern
3-(2-Methylpropyl) pyridine	1312	14159-61-6 	Yes	No Europe: ND USA: 0.07	See note 3	No safety concern

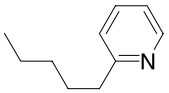
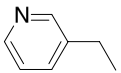
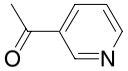
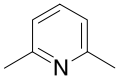
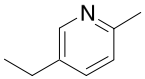
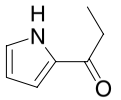
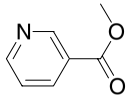
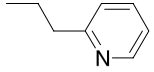
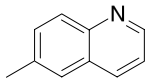
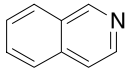
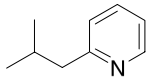
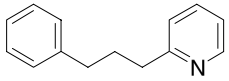
2-Pentylpyridine	1313	2294-76-0 	Yes	No Europe: 0.07 USA: 0.07	note 3	See concern	No safety
Pyrrole	1314	109-97-7 	Yes	No Europe: 0.1 USA: 0.01		See note 1	No safety concern
3-Ethylpyridine	1315	536-78-7 	Yes	No Europe: 11 USA: 3		See note 3	No safety concern
3-Acetylpyridine	1316	350-03-8 	Yes	No Europe: 27 USA: 0.8		See notes 3, 4	No safety concern
2,6-Dimethylpyridine	1317	108-48-5 	Yes	No Europe: 0.3 USA: 0.007		See note 3	No safety concern
5-Ethyl-2-methylpyridine	1318	104-90-5 	Yes	No Europe: 0.1 USA: 0.04		See note 3	No safety concern

Table 1. (contd)

Flavouring agent	No.	CAS No. and structure	Step 2 Predicted to be metabolized to innocuous metabolites?	Step A3 Does intake exceed the threshold for human intake?[a]	Comments	Conclusion based on current intake
2-Propionylpyrrole	1319	1073-26-3 	Yes	No Europe: 0.01 USA: 2	See notes 1, 4	No safety concern
Methyl nicotinate	1320	93-60-7 	Yes	No Europe: 0.6 USA: 0.2	See note 6	No safety concern
2-Propylpyridine	1322	622-39-9 	Yes	No Europe: ND USA: 0.9	See note 3	No safety concern
Structural class III						
6-Methylquinoline	1302	91-62-3 	Yes	No Europe: 4 USA: 0.01	See notes 2, 5	No safety concern

Isoquinoline	1303	119-65-3 	Yes	No Europe: 0.01 USA: 0.07		See note 2	No safety concern
2-(2-Methylpropyl) pyridine	1311	6304-24-1 	Yes	No Europe: ND USA: 0.9		See note 3	No safety concern
2-(3-Phenylpropyl) pyridine	1321	2110-18-1 	Yes	No Europe: 2 USA: 0.7	note 3	See concern	No safety

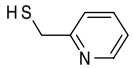
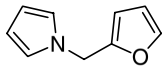
Flavouring agent	No.	CAS No. and structure	Step 2 Predicted to be metabolized to innocuous metabolites?	Step B3 Does intake exceed the threshold for human intake?[a]	Step B4 Adequate margin of safety for the flavouring agent or related chemical?	Comments	Conclusion based on current intake
Structural class III							
2-Pyridinemethanethiol	1308	2044-73-7 	No	No Europe: 0.001 USA: 0.007	Yes. The NOEL of 3.42 mg/kg bw per day in rats (Posternak et al., 1969) is >20 million times the estimated daily intake of 2-pyridinemethanethiol.	See note 3	No safety concern

Table 1. (contd)

Flavouring agent	No.	CAS No. and structure	Step 2 Predicted to be metabolized to innocuous metabolites?	Step B3 Does intake exceed the threshold for human intake?[a]	Step B4 Adequate margin of safety for the flavouring agent or related chemical?	Comments	Conclusion based on current intake
rfurylpyrrole	1310	1438-94-4 	No	No Europe: 0.1 USA: 0.07	Yes. The NOEL of 12.2 mg/kg bw per day in rats (Morgareidge, 1971) is >1 million times the estimated daily intake of *N*-furfurylpyrrole.	See notes 1, 4	No safety concern

CAS: Chemical Abstracts Service; ND: No intake data reported; NR: Not required for evaluation because consumption of the substance was determined to be of no safety concern at step A3 of the Procedure.

[a] The thresholds for human intake for structural classes I, II, and III are 1800, 540 and 90 μg/person per day, respectively. All intake values are expressed in μg/person per day. The combined intake of the flavouring agents in structural class I is 33 μg/person per day in Europe and 11 μg/person per day in the USA. The combined intake of the flavouring agents in structural class II is 103 μg/person per day in Europe and 76 μg/person per day in the USA. The combined intake of the flavouring agents in structural class III is 6 μg/person per day in Europe and 1 μg/person per day in the USA.

Notes:
1 The pyrrole ring undergoes hydroxylation at the C2 position and is excreted in the urine as the corresponding glucuronic acid conjugate.
2 The ring system undergoes hydroxylation at the C3 position and is excreted in the urine as the corresponding glucuronic acid conjugate.
3 Alkyl side-chain oxidation followed by glucuronic acid conjugation and excretion or oxidation to nicotinic acid.
4 The acetyl group is reduced and conjugated with glucuronic acid.
5 Forms a reactive epoxide metabolite that is detoxified through glutathione conjugation.
6 Ester readily undergoes hydrolysis and resulting nicotinic acid is either used in numerous metabolic processes or excreted as the mercapturic acid conjugate.

Table 2. Annual volumes of production of pyridine, pyrrole and quinoline derivatives used as flavouring agents in Europe and the USA

Flavouring agent (No.)	Annual volume of production (kg)[a]	Intake[b]		Annual intake from natural occurrence in foods (kg)[c]	Consumption ratio[d]
		µg/day	µg/kg bw per day		
Indole (1301)					
Europe	213	30	0.51		
USA	73	10	0.2	1593	22
6-Methylquinoline (1302)					
Europe	31	4	0.07		
USA	0.1	0.01	0.0002	+	NA
Isoquinoline (1303)					
Europe	0.1	0.01	0.0002		
USA	0.5	0.07	0.001	+	NA
Skatole (1304)					
Europe	20	3	0.05		
USA	0.5	0.07	0.001	1990	3980
1-Ethyl-2-acetylpyrrole (1305)					
Europe	N/D	N/D	N/D		
USA[f]	0.05	0.009	0.0001	+	NA
1-Methyl-2-acetylpyrrole (1306)					
Europe	10	1	0.02		
USA[f]	0.1	0.02	0.0003	5633	56330
Methyl 2-pyrrolyl ketone (1307)					
Europe	27	4	0.06		
USA	0.9	0.2	0.003	+	NA
2-Pyridinemethanethiol (1308)					
Europe	0.01	0.001	0.00002		
USA	0.05	0.007	0.0001	–	NA

Table 2. (contd)

Flavouring agent (No.)	Annual volume of production (kg)[a]	Intake[b]		Annual intake from natural occurrence in foods (kg)[c]	Consumption ratio[d]
		μg/day	μg/kg bw per day		
2-Acetylpyridine (1309)					
Europe	414	59	1		
USA	514	68	1	4462	9
N-Furfurylpyrrole (1310)					
Europe	1	0.1	0.002		
USA	0.5	0.07	0.001	5764	11528
2-(2-Methylpropyl)pyridine (1311)					
Europe	N/D	N/D	N/D		
USA[e]	5	0.9	0.01	–	NA
3-(2-Methylpropyl)pyridine (1312)					
Europe	N/D	N/D	N/D		
USA[f]	0.4	0.07	0.001	+	NA
2-Pentylpyridine (1313)					
Europe	0.5	0.07	0.001		
USA	0.5	0.07	0.001	+	NA
Pyrrole (1314)					
Europe	0.9	0.1	0.002		
USA	0.1	0.01	0.0002	2315	23150
3-Ethylpyridine (1315)					
Europe	76	11	0.2		
USA	24	3	0.05	+	NA
3-Acetylpyridine (1316)					
Europe	189	27	0.4		
USA	6.4	0.8	0.01	+	NA
2,6-Dimethylpyridine (1317)					
Europe	2.1	0.3	0.005		

USA	0.05	0.007	0.0001	+	NA
5-Ethyl-2-methylpyridine (1318)					
Europe	1	0.1	0.002		
USA[f]	0.2	0.04	0.0006	+	NA
2-Propionylpyrrole (1319)					
Europe	0.1	0.01	0.0002		
USA[e]	11	2	0.03	+	NA
Methyl nicotinate (1320)					
Europe	4	0.6	0.01		
USA	1.8	0.2	0.004	+	NA
2-(3-Phenylpropyl)pyridine (1321)					
Europe	15	2	0.04		
USA[e]	4	0.7	0.01	–	NA
2-Propylpyridine (1322)					
Europe	N/D	N/D	N/D		
USA[e]	5	0.9	0.01	+	NA
Total					
Europe	1005				
USA	648				

NA, not available; N/D, no intake data reported; +, reported to occur naturally in foods (Nijssen et al., 2003), but no quantitative data; –, not reported to occur naturally in foods.

[a] From International Organization of the Flavour Industry (1995) and Lucas et al. (1999) or National Academy of Sciences (1982).

[b] Intake expressed as μg/person per day was calculated as follows: [(annual volume, kg) × (1 × 10^9 μg/kg)]/[population × survey correction factor × 365 days], where population (10%, 'eaters only') = 32 × 10^6 for Europe and 26 × 10^6 for the USA. The correction factor = 0.6 for Europe and 0.8 for the USA, representing the assumption that only 60% and 80% of the annual volume of the flavour, respectively, was reported in the poundage surveys (International Organization of the Flavour Industry, 1995; Lucas et al., 1999; National Academy of Sciences, 1982) or in the anticipated annual volume.
Intake expressed as μg/kg bw per day was calculated as follows: [(μg/person per day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.

[c] Quantitative data for the USA reported by Stofberg & Grundschober (1987).

[d] The consumption ratio was calculated as follows: (annual consumption in food, kg)/(most recently reported volume as a flavouring agent, kg).

[e] The volume cited is the anticipated annual volume, which was the maximum amount of flavour estimated to be used annually by the manufacturer at the time the material was proposed for use as a flavouring agent. National surveys (National Academy of Sciences, 1970, 1982, 1987; Lucas et al., 1999), if applicable, revealed no reported use as a flavouring agent.

[f] Annual volume reported in previous USA survey (National Academy of Sciences, 1982).

2.3 Biological data

2.3.1 Biochemical data

(a) *Absorption, distribution, and excretion*

(i) *Pyrroles (Nos 1314, 1305–1307, 1310 and 1319) and indoles (Nos 1301 and 1304)*

Weak bases with pK_a values of >3, such as pyrroles and indoles, are readily absorbed from the intestine by passive diffusion because the base will not be ionized and pass through intestinal membranes with ease (Hogben et al., 1959). Pyrrole (No. 1314) has a pK_a of 3.8, while indole (No. 1301) has a pK_a of 3.2.

Female albino Wistar rats given [2^{14}C]indole as a single oral dose at 64 to 80 mg/kg bw eliminated >80% of the radiolabel in the urine within 48 h. Urine, faeces and expired air contained 80.6, 11.1, and 2.4% of the administered dose, respectively (King et al., 1966).

Groups of 24 male Holtzman rats were maintained on diets supplemented with indole at 0 (control), 0.25, 0.50, or 0.75% for 3 weeks. During week 3, all groups (including controls) received a single dose of ^{14}C-labelled indole via stomach tube. Urine collected for 48 h before and for an additional 24 h after administration of the single dose revealed that 32.1, 46.8, 49.4, 50.9, and 61.5% of indole and indole metabolites were recovered from the groups receiving indole at 0 (control), 0.25, 0.50, 0.75%, respectively, demonstrating a rapid elimination of indole (Martinez & Roe, 1972).

Mice and rats given a single intraperitoneal injections of [^{14}C-3]methylindole at 400 mg/kg bw excreted 69.4 and 66.2% respectively in the urine within 48 h (Skiles et al., 1991).

(ii) *Pyridines (Nos 1308, 1309, 1311–1313, 1315–1318, 1320–1322) and quinolines (Nos 1302 and 1303)*

Pyridines (pK_a = 5.2), quinolines (pK_a = 4.85), and isoquinolines (pK_a = 5.14) are weak tertiary bases and undergo rapid absorption in the gastrointestinal tract (Hogben et al., 1959).

2-Methylpyridine, at a dose of 500 mg/kg bw administered orally to rats, was distributed to the liver, heart spleen, lungs, and muscles within 10–20 min and an unidentified amount was excreted in the urine 48 h after dosing (Kupor, 1972).

In dogs treated orally with 3-acetylpyridine at a dose of 40 mg/kg bw per day for 2 days, metabolites were detected in the urine within 48 h (McKennis et al., 1964).

(b) Metabolism

(i) Pyrroles (Nos 1314, 1305–1307, 1310, and 1319) and indoles (Nos 1301 and 1304)

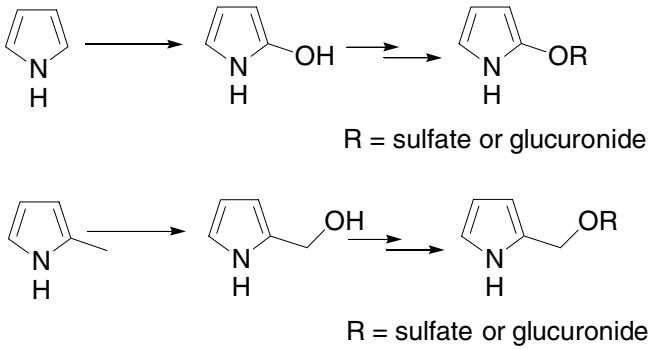
Unsubstituted pyrrole and indole are metabolized primarily by ring hydroxylation at the C2 position in pyrrole (Figure 1) (Town et al., 1992) and the C3 position in indole (Figure 2); Posner et al., 1961; King et al., 1966; Ruangyuttikarn et al., 1992; Thornton-Manning et al., 1993; Gillam et al., 2000). The resulting hydroxyl derivative is subsequently conjugated with glucuronic acid or sulfuric acid and excreted in the urine. A minor pathway involves epoxidation of the pyrrole ring double bond to yield an epoxide that is readily conjugated with glutathione (York et al., 1993).

Alkyl-substituted pyrroles and indoles may also undergo CYP-induced side-chain oxidation to yield the corresponding alcohol, which may be excreted as the glucuronic acid or sulfuric acid conjugate (Ruangyuttikarn et al., 1992; Thornton-Manning et al., 1993; Gillam et al., 2000). To some extent, epoxidation of the indole ring double bond has been considered as another metabolic pathway for metabolism of alkyl-substituted pyrroles and indole derivatives (Skiles & Yost, 1989; Smith et al., 1993).

Experiments in vitro have demonstrated that pyrroles and indoles also undergo ring hydroxylation. Hydroxylation at the C2 position of the pyrrole ring occurs when human liver microsomes are incubated with a pyrrole-substituted heterocyclic derivative (HIV *tat* inhibitor Ro 5-3335) (see Figure 1) (Town et al., 1992). The pyrrole ring or its metabolites also react with glutathione. A novel class of α-glutathione-*S*-transferase (GST) isozymes is expressed in rat liver fractions after treatment with pyrroles (York et al., 1993; Primiano & Novack, 1989).

Ring hydroxylation of indole (No. 1301) has been well documented (see Figure 2). Approximately 63% of total 3-hydroxyindole was present as 3-hydroxyindole sulfate (49.6%) and 3-hydroxyindole glucuronide (13.2%) in pooled samples of urine at 48 h after oral treatment of three albino Wistar rats with [^{14}C]2-indole as a

Figure 1. Metabolic options for pyrrole and alkyl-substituted pyrroles



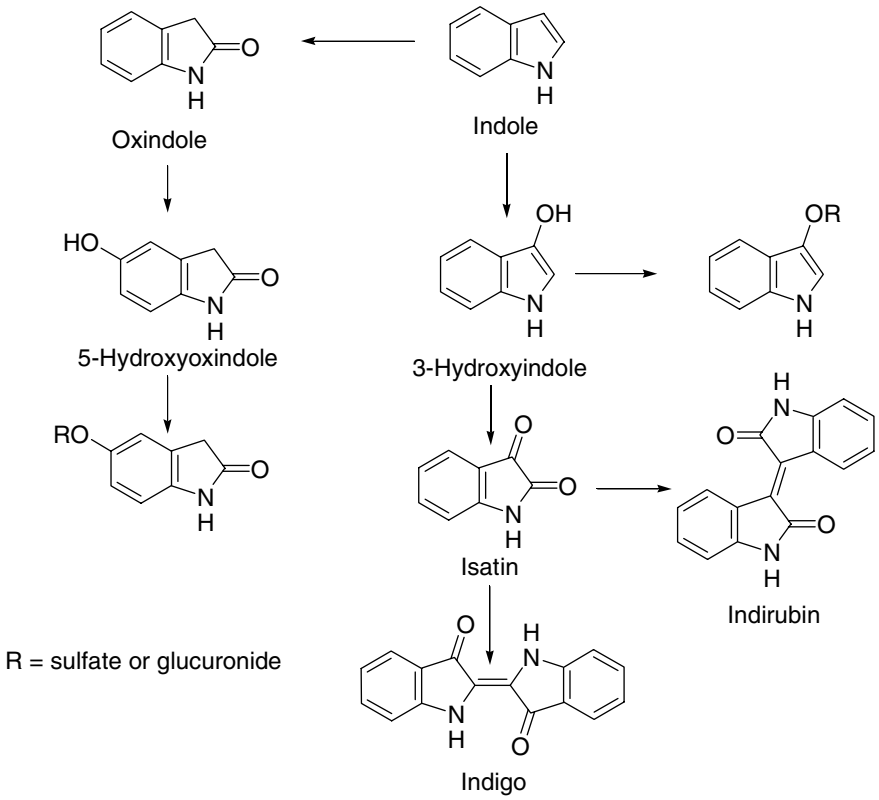
single dose at 64 to 74 mg/kg bw. In two of the rats, other metabolites identified in the urine included 5-hydroxyindole (3.5%), 2*H*-indole-2-one (1.4%), and indole-2,3-dione (5.8%). Analysis of the faecal excretions showed that 0.14, 0.40, and 0.64% of the radiolabel was present as indole, 3-hydroxyindole sulfate and total 3-hydroxyindole metabolites, respectively (King et al., 1966). In a parallel study, bile samples collected at 48 h via cannulation of the common bile duct of two female albino Wistar rats treated orally with ^{14}C-labelled 2-indole at a dose of 49 to 63 mg/kg bw showed that 0.56, 0.80, and 0.82% of the radiolabel was present as 5-hydroxyindole, 3-hydroxyindole sulfate, and total 3-hydroxyindole metabolites, respectively (King et al., 1966).

3-Hydroxyindole was the primary metabolite isolated when indole was incubated with freshly prepared rabbit liver microsomes (Posner et al., 1961). 3-Hydroxyindole may further oxidize to indigo (2-(1,3-dihydro-3-oxo-2*H*-indol-2-ylidene)-1,2-dihydro-3*H*-indol-3-one) (Posner et al., 1961). Aerobic incubation of indole with rat liver microsomes in the presence of glucose-6-phosphate, nicotinamide, and nicotinamide adenine dinucleotide phosphate, reduced (NADPH) for 1 h demonstrated the formation of ring-oxidized metabolites including indigo, indirubin (3-(1,3-dihydro-3-oxo-2*H*-indol-2-ylidene)-1,3-dihydro-2*H*-indol-2-one), and oxindole (1,3-dihydro-2*H*-indol-2-one). Under anaerobic conditions, oxindole was detected (King et al., 1966). The presence of metabolites of indole observed under aerobic conditions were also reported when indole was incubated with recombinant human CYP enzymes, 2A6, 2C19, and 2E1 coexpressed with CYP reductase in *Escherichia coli* (Gillam et al., 2000). These studies support the role of CYP enzymes in the oxidation of the indole ring.

Alkyl-substituted pyrroles and indoles undergo mainly side-chain oxidation, although there is some evidence that epoxidation of the indole ring alkene also occurs. Mice and rats given ^{14}C-labelled 3-methylindole (No. 1304) as a single intraperitoneal injection at 400 mg/kg bw excreted 69.4 and 66.2%, respectively, of the administered dose as indole-3-carbinol (i.e. 3-hydroxymethylindole) and 2.6 and 7.3%, respectively, as the mercapturic acid conjugate of 3-methylindole, 3-[(*N*-acetylcystein-*S*-yl)methyl]indole (see Figure 2) (Skiles et al., 1991). The mercapturic acid conjugate is likely to be formed via a reactive 3-methylene iminium ion (Figure 1) that may be generated either directly via CYP mediated oxidation of the methyl substituent or indirectly via ready dehydration of indole-3-carbinol (Skiles & Yost, 1992).

At least six metabolites were isolated from the urine of male Swiss-Webster mice at 36 h after administration of ring-labelled [^{14}C]3-methylindole at a dose of 400 mg/kg bw by intraperitoneal injection. Two primary pathways were characterized. In one, side-chain oxidation yields indole-3-carbinol that is dehydrated to 3-methyleneindolenine which subsequently is conjugated with glutathione to yield 3-[(*N*-acetylcystein-*S*-yl)methyl]indole. Indole-3-carbinol is then oxidized to the corresponding carboxylic acid. In the other pathway, the 2,3-alkene is epoxidized to yield 3-methyloxindole or 3-hydroxy-3-methylindolenine intermediates. These intermediary metabolites are conjugated with glucuronic acid or sulfuric aicd, followed by excretion in the urine, or are further oxidized to yield a series of dihydroxy-3-methyloxindole metabolites that are also conjugated and excreted (Smith

Figure 2. Metabolism of indole under aerobic conditions



et al., 1993). When (^{2}H-2-)-3-methylindole was incubated with microsomal CYP in the presence of $^{18}O_2$, 3-methyloxindole was formed with an ^{18}O label incorporated at position 2. Also, an intramolecular shift (NIH shift) of ^{2}H from position 2 to position 3 was observed. These experiments provide evidence for the formation of the epoxide followed by a well recognized NIH shift to yield 3-methyloxindole (Skordos et al., 1998).

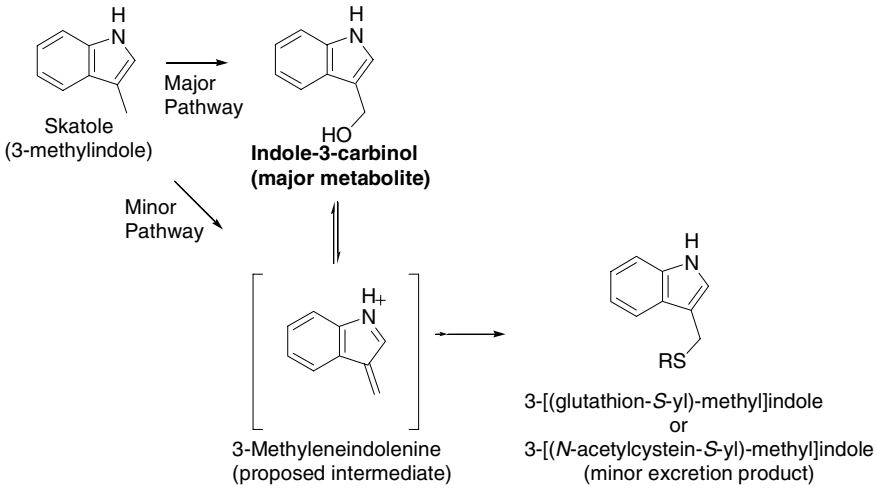
Evidence for the presence of the 3-methyleneindolenine intermediate has been demonstrated by numerous experiments in vitro. Incubation of 3-methylindole (0.5 mmol/l) with rabbit Clara cells and alveolar macrophages yielded four metabolites: the two metabolites derived from side-chain oxidation were indole-3-carbinol and the glutathione conjugate, 3-(*N*-acetylcysteine-*S*-yl)-3-methylindole. Two other metabolites, presumably derived from epoxidation, were 3-methyloxindole, and 2-(*N*-acetylcystein-*S*-yl)-3-hydroxy-3-methylindoline, a mercapturic acid (Thornton-Manning et al., 1993). Incubation with microsomal CYP, NADPH and excess glutathione (4 mmol/l) produced the corresponding glutathione conjugates in place

of the mercapturic acid conjugates (Thornton-Manning et al., 1993). Human liver microsomes were incubated with [^{14}C]3-methylindole in the presence of NADPH. Hydrolysis of the isolated protein fraction indicated the presence of a cysteine conjugate at position 3 of 3-methylindole. The authors suggested that a reactive 3-metyhyleneindolenine intermediate reacts with the cysteine thiol groups of target proteins (Ruangyuttikarn et al., 1992).

Experiments have been performed in vitro to better characterize the enzyme-catalysed formation of the reactive intermediate 3-methyleneindolenine and the intermediates produced in subsequent reaction with glutathione and cellular proteins. When liver homogenates isolated from rats treated with β-naphthoflavone or phenobarbitone, known inducers of CYP, were incubated with 3-methylindole (skatole, No. 1304) at 1 mmol/l, rates of glutathione depletion were significantly higher than rates of depletion for untreated liver homogenates (i.e. 32.1 ($p < 0.05$) and 48 ($p < 0.001$) nmol/mg protein per 30 min, respectively, versus 20 nmol/mg protein per 30 min for untreated controls) (Garle & Fry, 1989). Incubation of 3-methylindole with HepG2 cell lysates containing vaccinia-expressed CYP2A6 or CYP2F1 in vitro produced an intermediate that binds covalently to cellular proteins, presumably through a similar mechanism (Thornton-Manning et al., 1992).

Swiss-Webster mice given L-buthionine-(*S*,*R*)-sulfoximine, a specific inhibitor of GSH synthesis, at a dose of 0–6 mmol/kg bw, 3 h before treatment with [^{14}C-methyl]3-methylindole at a dose of 75 mg/kg bw, showed that the covalent binding of 3-methylindole-derived radiolabel to cellular proteins increased with increasing concentration of L-buthionine-(*S*,*R*)-sulfoximine. The binding was greater in the renal tissue (3.4-fold increase) than in pulmonary (2.1-fold increase) or hepatic (1.5-fold increase) tissues. Increased binding to cellular proteins correlated with lower concentrations of glutathione (Yost et al., 1990).

Figure 3. Metabolic pathway of skatole in rats and human microsomal preparations



(ii) Pyridines (Nos 1308, 1309, 1311–1313, 1315–1318, 1320–1322) and quinolines (Nos 1302 and 1303)

Alkyl-substituted pyridines and quinolines are subject to side-chain oxidation, usually at the C1 position (Hawksworth & Scheline, 1975). The polar hydroxy- or acyl-substituted metabolites are readily excreted in conjugated form (see Figure 4). Ring hydroxylation and epoxidation have also been observed for substituted quinolines. In addition, pyridine derivatives may form polar *N*-oxides (Cowan et al., 1978) (Figure 4). Pyridine itself has been found to undergo *N*-oxidation and *N*-methylation in vivo in most species (Damani & Crooks, 1982). Instances of combined side-chain oxidation and *N*-oxide formation have been observed (Cowan et al., 1978; Schwartz et al., 1978; Damani et al., 1980; Nguyen et al., 1988).

Methyl nicotinate is the only ester in the group and is rapidly hydrolysed to yield pyridinecarboxylic acid (nicotinic acid) and methyl alcohol by carboxylesterases (Heymann, 1980; White et al., 1990; Durrer et al., 1992).

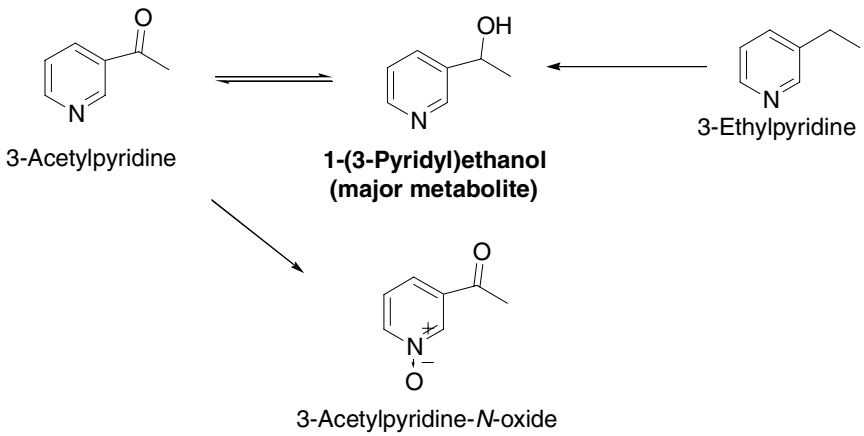
In a study of the effect of substrate on the rate of carboxylesterase-catalysed hydrolysis, the steady state kinetic constants for a series of nicotinate esters were determined. Purified hog liver carboxylesterase and human plasma containing carboxylesterase were incubated with methyl nicotinate. The maximal velocity (V_{max}) for ester hydrolysis in homogeneous hog liver carboxyesterase and human plasma is 24.4 and 46.4 mmol/min per mg protein, respectively, indicating rapid hydrolysis of ester (Durrer et al., 1992).

Nicotinic acid, or niacin, is ubiquitous in living cells and is essential for the production of nicotinamide adenine dinucleotide (NAD) and its derivatives. As a result of normal turnover of NAD or excess dietary intake, nicotinic acid is excreted as the glycine conjugate, nicotinuric acid (Miller et al., 1960). In humans, approximately 88% of an oral dose of 3000 mg of nicotinic acid was recovered in the urine within 1 h (Miller et al., 1960).

There is substantial experimental evidence that alkyl-substituted pyridines undergo extensive side-chain oxidation to yield the corresponding carboxylic acid derivatives. Ninety per cent of a dose of 2-methylpyridine of 100 mg/kg bw or 96% of a dose of 2,6-dimethylpyridine (No. 1317) of 100 mg/kg bw administered by gavage to male albino Wistar rats was excreted as the glycine conjugate of the corresponding pyridine-2-carboxylic acid derivative (Hawksworth & Scheline, 1975).

In groups of three male and three female Wistar rats given 4-methylpyridine at a dose of 300 mg/kg bw by gavage, >50% was excreted in the urine principally as pyridine-4-carboxylic acid (50%) or its glycine conjugate (5%) after 24 h. Minor metabolites included unchanged 4-methylpyridine (approximately 2.5%) and 4-methylpyridine-*N*-oxide (1.5%), demonstrating that *N*-oxidation is also a minor metabolic pathway (Nguyen et al., 1988). In fact, in mice, hamsters, rats, guinea-pigs, or rabbits given the related substance 3-methylpyridine at a dose of 40 mg/kg bw by intraperitoneal administration, 6.4, 0.3, 4.0, 0.7, and 0.1%, respectively, of the administered dose was excreted in the urine as the corresponding *N*-oxide within 24 h (Gorrod & Damani, 1980).

Figure 4. Metabolic pathway for 3-alkyl- and 3-acetylpyridine



When incubated with hepatic and pulmonary microsomal preparations isolated from male Wistar rats, albino Dunkin-Hartley guinea-pigs, albino New Zealand rabbits, and LACA albino mice, 3-methylpyridine, 3-ethylpyridine (No. 1315), and 3-acetylpyridine (No. 1316) were converted to *N*-oxides by the hepatic microsomal fractions and not the pulmonary microsomal fractions (Cowan et al., 1978).

Although no metabolic data are available on 2-acetylpyridine, data are available on the structurally related compound, 3-acetylpyridine. 3-Acetylpyridine is metabolized by both *N*-oxidation and side-chain reduction. In rats, 3-acetylpyridine is reported to be metabolized to 1-(3-pyridyl)ethanol and 1-(3-pyridyl-*N*-oxide)ethanol (Schwartz et al., 1978). Both 1-(3-pyridyl)ethanol and (3-pyridyl)-1,2-ethandiol were isolated as *N*-oxide derivatives from the urine of a dog given daily oral doses of 3-acetylpyridine for 8 consecutive days (McKennis et al., 1964). Similar results were obtained in vitro when 3-acetylpyridine was incubated successively with rat microsomal and cytoplasmic preparations. In both experiments, metabolites included 1-(3-pyridyl-*N*-oxide)ethanol and 1-(3-pyridyl)ethanol (Damani et al., 1980) (Figure 2).

2-Pyridinemethanethiol (No. 1308) is oxidized to the polar sulfonic acid metabolite and subsequently excreted in the urine. The oxidation of thiol is catalysed by two enzyme systems, CYP and the flavin-containing monooxygenases (Renwick, 1989). Aromatic and aliphatic sulfides are primarily oxidized by flavin-containing monooxygenases and, to a lesser extent, CYP to form sulfonic acids. Based on the numerous examples of successive oxidation of thiols to sulfonic acids by flavin-containing monooxygenases and CYP enzymes in a variety of test systems (Cashman & Williams, 1990; Cashman et al., 1990; Rettie et al., 1990; Yoshihara & Tatsumi, 1990; Sadeque et al., 1992, 1995; Cashman et al., 1995a, 1995b; Elfarra et al., 1995; Nnane & Damani, 1995), it is concluded that the *S*-oxidation pathway is the major route of detoxication of 2-pyridinemethanethiol in humans (Ziegler, 1980; Nickson & Mitchell, 1994).

Alkyl-substituted quinolines undergo side-chain oxidation, ring epoxidation and ring hydroxylation. When 6-methylquinoline (No. 1302) was incubated with rat liver microsomes, 6-hydroxylmethylquinoline, 5-hydroxyl-6-methylquinoline, and 6-methyl-7,8-oxoquinoline were the major metabolites. Other minor metabolites include 6-methyl-5,6-oxoquinoline, quinoline-6-carboxaldehyde, and quinoline-6-carboxylic acid (Scharping et al., 1993).

Isoquinoline (No. 1303), lacking ring substituents, is subject to *N*-oxide formation, epoxidation and ring hydroxylation. When groups of five male Wistar rats were given isoquinoline at a dose of 75 mg/kg bw via intragastric tube for three consecutive days, induction of UDP-glucuronosyltransferase, microsomal epoxide hydrolase, and GST activities were observed. Isoquinoline showed a 1.4 to 1.8-fold increase in UDP-glucuronosyltransferase activity and a 20% increase in GST activity ($p < 0.05$) (Le & Franklin, 1997).

On the basis of the evidence above, pyrrole and indole undergo ring hydroxylation. Alkyl-substituted pyrroles and indoles mainly undergo CYP-mediated oxidation of the side-chain to yield the corresponding side-chain alcohol that may be excreted as the glucuronic acid or sulfuric acid conjugate or further oxidized. To some extent, epoxidation of the pyrrole ring may occur leading to hydroxylated polar metabolites excreted mainly in the urine. In a similar manner, alkyl-substituted pyridines and quinolines are subject to side-chain oxidation and in the case of substituted quinolines, ring epoxidation and ring hydroxylation. The polar hydroxy- or acyl-substituted metabolites are readily excreted in conjugated form. In addition, pyridine and quinoline derivatives may form polar *N*-oxides. Instances of combined side-chain oxidation and *N*-oxide formation have been observed.

2.3.2 Toxicological studies

The available data on the toxicity of pyridine, pyrrole and quinoline derivatives in this group of 22 flavouring agents are presented below. Although the studies of acute toxicity and and short-term studies of toxicity were of limited use for evaluating the safety of these substances, because of their short duration, they are included for completeness.

(a) Acute toxicity

Oral median lethal dose (LD_{50}) values have been reported for 10 of the 22 agents in this group (see Table 3). In rats, LD_{50} values are in the range from 51 to 3450 mg/kg bw; however most LD_{50} values range from approximately 300 to 1500 mg/kg bw (Smyth et al., 1951, 1962; Spanjers & Til, 1968; McGee, 1974; Posternak et al., 1975; Moreno, 1976; Izamerov et al., 1982; Costello et al., 1992; Myers & Ballantyne, 1997), demonstrating that the acute oral toxicity of pyridine, pyrrole and quinoline derivatives is low. In mice, LD_{50} values are in the range of 282 to 2800 mg/kg bw (Shellenberger, 1971; Pellmont, 1977; Moran et al., 1980; Izamerov et al., 1982).

Table 3. Studies of the acute toxicity of pyridine, pyrrole and quinoline derivatives administered orally

No.	Flavouring agent	Species	Sex	LD_{50} (mg/kg bw)	Reference
1301	Indole	Rat	M	1000	Smyth et al. (1962)
1302	6-Methylquinoline	Rat	NR	1260	Moreno (1976)
1303	Isoquinoline	Rat	NR	360	Smyth et al. (1951)
1304	Skatole	Rat	NR	3450	McGee (1974)
1309	2-Acetylpyridine	Rat	NR	2280	Posternak et al. (1975)
1309	2-Acetylpyridine	Rat	M, F	2160[a]	Spanjers & Til (1968)
1310	*N*-Furfurylpyrrole	Mice	F	335	Shellenberger (1971)
1310	*N*-Furfurylpyrrole	Mice	M	580	Shellenberger (1971)
1310	*N*-Furfurylpyrrole	Mice	M, F	380	Moran & Easterday (1980)
1316	3-Acetylpyridine	Rat	M	57[b]	Costello et al. (1992)
1316	3-Acetylpyridine	Rat	F	51[b]	Costello et al. (1992)
1318	5-Ethyl-2-methylpyridine	Rat	NR	1540	Smyth et al. (1951)
1318	5-Ethyl-2-methylpyridine	Rat	NR	368	Izamerov et al. (1982)
1318	5-Ethyl-2-methylpyridine	Mice	NR	282	Izamerov et al. (1982)
1318	5-Ethyl-2-methylpyridine	Rat	M	1195[c]	Myers & Ballantyne (1997)
1319	2-Propionylpyrrole	Mice	M, F	1620	Moran & Easterday (1980)
1320	Methyl nicotinate	Mice	NR	2800	Pellmont (1977)

F, female; M, male; NR, not reported.

[a] Calculated using density = 1.08 g/ml (Sigma-Aldrich, 2003; available from http://www.sigmaaldrich.com).

[b] Calculated using density = 1.102 g/ml (Sigma-Aldrich, 2003; available from http://www.sigmaaldrich.com).

[c] Calculated using density = 0.919 g/ml (Sigma-Aldrich, 2003; available from http://www.sigmaaldrich.com).

(b) Short-term studies of toxicity

The results of short-term studies with representative pyridine, pyrrole and quinoline derivatives are summarized in Table 4 and are described below.

(i) Indole (No. 1301)

Rats

Groups of six male Holtzman rats were fed a diet containing indole at a concentration of 0 (control), 0.25, 0.50, or 0.75% for 3 weeks (equivalent to a dose of approximately 0, 125, 250, or 375 mg/kg bw per day. An additional group received a diet containing indole at 0.75%, supplemented with methionine at 0.25%. The animals were monitored for food consumption and food efficiency, uptake, body-weight gain, and haematological effects. At termination of the study, the animals were necropsied and the livers were weighed. A statistically significant reduction in food intake was reported at 0.50 and 0.75%. All groups fed indole, including the group receiving indole at 0.25% in the diet, exhibited a statistically significant reduction in body-weight gain. However, animals in the group given the diet sup-

Table 4. Short-term studies of toxicity and long-term studies of toxicity and carcinogenicitywith pyridine, pyrrole and quinoline derivatives used as flavouring agents

No.	Agent	Species; sex	No. of test groups[a]/ no. per group[b]	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
Short-term studies of toxicity							
1301	Indole	Rat; M	4/6	Diet	21	<125	Martinez & Roe (1972)
1302	6-Methylquinoline	Rat; M, F	1/20–32	Diet	90	2.2 (M)[c] 2.7 (F)[c]	Posternak et al. (1969)
1307	Methyl 2-pyrrolyl ketone	Rat; M, F	1/32	Diet	91	87.5 (M)[c] 86.3 (F)[c]	Posternak et al. (1975)
1308	2-Pyridinemethanethiol	Rat; M, F	1/20–32	Diet	90	3.4 (M)[c] 3.4 (F)[c]	Posternak et al. (1969)
1309	2-Acetylpyridine	Rat; M, F	1/32	Diet	91	3.1 (M)[c] 3.1 (F)[c]	Posternak et al. (1975)
1309	2-Acetylpyridine	Rat; M, F	4/20	Gavage	91	37	Til & van der Meulen (1971)
1310	*N*-Furfurylpyrrole	Rat; M, F	1/30	Diet	90	12.2	Morgareidge (1971)
Long-term study of toxicity and carcinogenicity							
1301	Indole	Rat; M, F	1/25	Diet	590	<100	Kaiser (1953)

[a] Total number of test groups does not include control animals.
[b] Total number per test group includes both male and female animals.
[c] Study performed with either a single dose or multiple doses that produced an adverse effect.

plemented with methionine at 0.25% had body-weight gains similar to those of the controls. There were no significant differences between test and control animals with respect to concentration of haemoglobin or erythrocyte volume fraction, and relative weights of the liver (Martinez & Roe, 1972).

(ii) Indole-3-carbinol, a metabolite of skatole (No. 1304)

Indole-3-carbinol is a major urinary metabolite formed by side-chain oxidation of skatole (3-methylindole, No. 1304) and is therefore available data on its toxicity is included in this evaluation

Rats

In a study designed to evaluate the ability of indole-3-carbinol to prevent cancer, groups of 20 female Sprague-Dawley rats were given a tricapylin vehicle with or without different tumour-inducing agents on days 1, 8, 15 to 26, and 29. Animals were maintained on a basal diet with access to food and water ad libitum. From weeks 5 to 30, control animals continued to be given a basal diet or a diet supplemented with indole-3-carbinol at a concentration of 2000 mg/kg of diet (approximately equivalent to an intake of 100 mg/kg bw per day). Animals were weighed weekly and the size and location of palpable mammary gland tumours were recorded. After 30 weeks, animals were killed and subjected to macroscopic and histopathological examination. Total foci and aberrant crypts per focus were evaluated by light microscopy. Mean body weights in the groups treated with indole-3-carbinol were significantly ($p < 0.05$) lower than those of the control group. There was no significant difference in survival in the control group and in the groups treated with indole-3-carbinol. A higher incidence of mammary gland tumours (10%) was detected in the control group that was treated with vehicle only, when compared with that (0%) in the group treated with vehicle and maintained on a diet supplemented with indole-3-carbinol at a concentration of 2000 mg/kg of diet from weeks 5 to 30. Treatment with indole-3-carbinol resulted in a 100-fold increase in liver GST-P (placental form of glutathione-*S*-transferase) foci, but a decreased number of aberrant crypts of the colon compared with the control group (Stoner et al., 2002).

In a 6-week study, groups of 10 female Sprague-Dawley rats were given indole-3-carbinol at a dose of 0, 5, 25, 50, 100, or 200 mg/kg bw per day by gavage 5 days per week. Animals underwent daily observation for clinical signs of toxicity and weekly measurements of body weight. In addition, at the conclusion of the study, gross necropsy, organ weight measurements, and histopathological examination were conducted. At the highest dose, a 10% reduction in body-weight gain and a 20% increase in absolute weight of the liver was reported. No other adverse effects were reported (Grubbs et al., 1995).

A group of male inbred ACI/N rats was maintained on a basal diet or a diet containing indole-3-carbinol at a concentration of 1000 mg/kg of diet (approximately equivalent to a dose of 50 mg/kg bw per day) for 37 weeks. Animals were observed daily for general health, and at 37 weeks, body weights were measured and animals were killed. At necropsy, liver weights were recorded and organs were evaluated for gross lesions. Histological examination of major organs was per-

formed and the oral cavity was evaluated for pre-neoplastic and neoplastic lesions. There were no treatment-related effects on body and liver weights, or on gross pathology. Histopathological examinations did not reveal any differences between treated and control animals, and there was no treatment-related increase in the incidence of neoplasms of the oral cavity (Tanaka et al., 1992)

(iii) 6-Methylquinoline (No. 1302), methyl- 2-pyrrolyl ketone (No. 1307), 2-pyridinemethanethiol (No. 1308), and 2-acetylpyridine (No. 1309)

Rats

In a series of 90-day single-dose studies, groups of 10–16 Charles River CD rats were fed diets containing either 6-methylquinoline, methyl 2-pyrrolyl ketone, 2-pyridinemethanethiol, or 2-acetylpyridine. The rats were housed in same-sex pairs and given access to water and food ad libitum. The concentration of the test material in the diet was adjusted during the study to maintain constant levels of dietary intake. The doses were calculated to be >100 times greater than the possible average daily intake, which was determined by multiplying usual levels of use of the flavouring agent in each of 33 food categories (e.g. baked goods and meat products) by the average amount of that food category consumed daily and summing the intake over all 33 food categories. Body weights of the individual rats and the food consumption of pairs of rats were measured weekly and the efficiency of food utilization was calculated. Haematological examinations were carried out on half the animals at 7 weeks and on all animals at 13 weeks. After 13 weeks, all animals were killed, liver and kidney weights were measured, and gross and histological examinations were carried out on a wide range of organs. Tissues samples from various organs from each animal were preserved for histopathological evaluation.

Based on measurements of growth, food intake, haematological and clinical chemistry parameters, organ weights, and gross and histopathological examination, no differences were observed between groups of animals receiving 2-pyridinemethanethiol, 6-methylquinoline, or 2-acetylpyridine and control animals. Similarly, with the exception of a significant decrease in body-weight gain of <10% accompanied by a decrease in food utilization in males only, no other variations were reported in groups of rats receiving methyl 2-pyrrolyl ketone, compared with controls. Therefore, the NOELs were: 2-acetylpyridine, 3.13 and 3.06 mg/kg bw per day (Posternak et al., 1975); 2-pyridinemethanethiol, 3.42 mg/kg bw per day (Posternak et al., 1969); 6-methylquinoline, 2.2 and 2.7 mg/kg per day (Posternak et al., 1969); and methyl-2-pyrrolyl ketone, 87.5 and 86.3 mg/kg bw per day, for males and females, respectively (Posternak et al., 1975).

(iv) 2-Acetylpyridine (No. 1309)

Rats

Groups of 10 male and 10 female albino rats were given 2-acetylpyridine at a dose of 0, 37, 110, 330, or 1000 mg/kg bw per day by gavage in a propylene glycol

vehicle, 6 days a week for 13 weeks. Clinical signs of toxicity, body-weight gain, and food intake were recorded and haematological parameters were assessed. Serum enzyme activities of serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, and serum alkaline phosphatase were monitored. In addition, urine analysis was performed at the end of the study. At week 14, all surviving animals were killed and examined macroscopically. Selected organs were weighed and microscopic examinations were performed. 2-Acetylpyridine had no effect on general appearance, behaviour, mortality, growth, water intake, serum enzyme activity, or urine composition. All early deaths were attributed to dosing errors. Slight anaemia was indicated by a decrease in erythrocytes in males at the two higher doses (330 and 1000 mg/kg bw per day) and in females at 110 and 330 mg/kg bw per day. While the decrease in erythrocyte count was less distinct at 1000 mg/kg bw per day, females at the highest dose (1000 mg/kg bw per day) exhibited decreased lymphocyte counts and increased neutrophil counts. Relative weights of the kidney, liver, and spleen were increased at the highest dose in both males and females. Slightly elevated absolute weights of the liver were also observed in both sexes at 330 mg/kg bw per day; however, the increase was not statistically significant. Microscopic examination revealed changes in centrolobular hepatocytes (slightly swollen cells with homogeneous, eosinophilic cytoplasm, individual cell necrosis, and increased number of mitotic figures), slight proliferation of bile duct epithelium, and increased haematopoietic activity in the spleen at the two higher doses (330 and 1000 mg/kg bw per day) in both sexes, with the exception of the hepatic changes, which were observed only at the highest dose (1000 mg/kg bw per day) in females. The NOEL for 2-acetylpyridine was 37 mg/kg bw per day in albino rats (Til & van der Meulen, 1971).

(v) N-Furfurylpyrrole (No. 1310)

Rats

Groups of 15 male and 15 female albino FDRL rats were fed a diet containing *N*-furfurylpyrrole, which was adjusted at 2-week intervals to maintain an intake of 11.2 mg/kg bw per day for 90 days. The average actual intake of *N*-furfurylpyrrole was calculated to be 12.21 mg/kg bw per day. Rats were given access to drinking-water ad libitum. Daily observation for survival, behaviour and physical appearance produced no indications of changes in behaviour or signs of toxicity. All animals survived to the end of the study. Weekly measurements of body weight, food consumption, and calculation of efficiency of food utilization, revealed no differences between test and control animals. Haematological examination, clinical chemistry (blood urea), and urine analysis conducted during weeks 6 and 12 on eight males and eight females from each group showed normal values. After 90 days, all animals were killed and subjected to a detailed gross examination, and liver and kidney weights were recorded. Histological examination of a wide range of tissues and organs from each animal revealed no treatment-related changes (Morgareidge, 1971).

(c) Long-term studies of toxicity and carcinogenicity

The results of a study of carcinogenicity with indole are summarized in Table 4 and are described below.

(i) Indole (No. 1301)

Rats

Groups of five male and twenty female strain W rats were maintained on a diet providing indole at a dose of 0 or 100 mg/kg bw per day for 460 days, followed by a 30-day period of no treatment to examine the possible reversibility of any treatment-related effects. Following the reversibility period, treatment with a diet providing indole at a dose of 200 mg/kg bw per day was continued for 100 additional days. A concurrent control group was maintained, but was not described. Animals were monitored every 2 weeks for food consumption, body weight, survival and haematological effects. At necropsy, liver, kidney, and spleen were examined microscopically. Rats maintained on a diet supplemented with indole showed a 20% reduction in body-weight gain compared with controls. The haematological profile obtained for the first 460 days revealed slightly reduced concentrations of haemoglobin and erythrocyte counts compared with thoseof the controls (i.e. haemoglobin, 15.2 versus 13.8 g Hb/100 ml blood; and erythrocyte count, 8 versus 6.4 million cells/ml blood, respectively), which were reversible upon cessation of treatment. By day 460, the leukocyte counts reached twice their original values, which the authors stated were still within the normal range of values for rats. No leukaemia or other tumours attributable to administration of indole were observed in the animals. The average life expectancy of the rats was not altered by the inclusion of indole in the diet. Except for indications of moderate reversible anaemia, no other adverse effects were reported (Kaiser, 1953).

(d) Genotoxicity

Studies of mutagenicity and genotoxicity were available for nine pyridine, pyrrole and quinoline derivatives. The results of these tests are summarized in Table 5 and described below.[1]

(i) In vitro

There was no evidence of mutagenicity in the assay for reverse mutation in bacteria when various strains of *Salmonella typhimurium* (TA97, TA98, TA100, TA102, TA104, TA1535, TA1537, TA1538, and TM677) were incubated with indole

[1] For conversions used, see Table 5

Table 5. Results of studies of genotoxicity with pyridine, pyrrole and quinoline derivatives used as flavouring agents

No.	Flavouring agent	End-point	Test system	Concentration or dose	Result	Reference
In vitro						
1301	Indole	Reverse mutation	*S. typhimurium* TA100	≤20 μg/plate	Negative[a]	Ochiai et al. (1986)
1301	Indole	Reverse mutation	*S. typhimurium* TM677	4 mmol/l (469 μg/ml)[b]	Negative[c]	Kaden et al. (1979)
1301	Indole	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1538	4–2500 μg/plate	Negative[d]	Anderson & Styles (1978)
1301	Indole	Reverse mutation	*S. typhimurium* TA98 and TA100	≤500 nmol/plate (59 μg/plate)[b]	Negative[a]	Vance et al. (1986)
1301	Indole	Reverse mutation	*S. typhimurium* TA100, TA1535 and TA1537	3 μmol/plate (351 μg/plate)[b]	Negative[d]	Florin et al. (1980)
1301	Indole	Reverse mutation	*S. typhimurium* TA98	0.03–30 μmol/plate (3.5–3515 μg/plate)[b,e]	Negative[d]	Florin et al. (1980)
1301	Indole	Reverse mutation	*S. typhimurium* TA 97 and TA102	10–1000 μg/plate	Negative[d]	Fujita et al. (1994)
1301	Indole	Reverse mutation	*S. typhimurium* TA98 and TA100	≤0.4 μmol/plate (47 μg/plate)[b]	Negative[d]	Sasagawa & Matsushima (1991)
1301	Indole	Mutation	*E. coli* WP2 *uvrA*/pKM101	≤0.4 μmol/plate (47 μg/plate)[b]	Negative[d]	Sasagawa & Matsushima (1991)
1302	6-Methylquinoline	Reverse mutation	*S. typhimurium* TA100	100–600 μg/plate	Positive[c]	Dong et al. (1978)
1302	6-Methylquinoline	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA1538	≤3 600 μg/plate	Negative[a] Positive[c,f]	Wild et al. (1983)

1302	6-Methylquinoline	Reverse mutation	*S. typhimurium* TA98 and TA100	≤6 μmol/plate (859 μg/plate)[g]	Negative[a] Positive[c]	Nagao et al. (1977)
1302	6-Methylquinoline	Reverse mutation	*S. typhimurium* TA98 and TA100	≤1000 μg/plate	Negative[a] Positive[c]	Zeiger et al. (1992)
1302	6-Methylquinoline	Reverse mutation	*S. typhimurium* TA98 and TA100	NR	Negative[a] Positive[c]	Sugimura et al. (1976)
1302	6-Methylquinoline	Reverse mutation	*S. typhimurium* TA100	5 μmol/plate (716 μg/plate)[g]	Positive[c]	Takahashi et al. (1988)
1302	6-Methylquinoline	Reverse mutation	*S. typhimurium* TA98	NR	Negative[d]	Debnath et al. (1992)
1302	6-Methylquinoline	Reverse mutation	*S. typhimurium* TA100	3.3–333 μg/plate	Negative[a] Positive[c]	Debnath et al. (1992)
1303	Isoquinoline	Reverse mutation	*S. typhimurium* TA98 and TA100	20–50 μg/ml	Negative[d]	Sideropoulos & Specht (1984)
1303	Isoquinoline	Reverse mutation	*S. typhimurium* TM677	≤8 mmol/l (1033 μg/ml)[h]	Negative[c]	Kaden et al. (1979)
1303	Isoquinoline	Reverse mutation	*S. typhimurium* TA98 and TA100	NR	Negative[d]	Sugimura et al. (1976)
1303	Isoquinoline	Reverse mutation	*S. typhimurium* TA98 and TA100	1–20 μmol/plate (129–2583 μg/plate)[h]	Negative[d]	Nagao et al. (1977)
1303	Isoquinoline	Reverse mutation	*S. typhimurium* TA98 and TA100	10000–20000 μg/ml	Negative[d]	Epler et al. (1979)
1303	Isoquinoline	Mutation	*E. coli* B/r HCR+	50 μg/ml	Negative[d]	Sideropoulos & Specht (1984)
1303	Isoquinoline	Unscheduled DNA synthesis	Rat hepatocytes	NR	Negative	Williams (1984)
1304	Skatole	Reverse mutation	*S. typhimurium* TA100, TA1535 and TA1537	3 μmol/plate (394 μg/plate)[i]	Negative[d]	Florin et al. (1980)
1304	Skatole	Reverse mutation	*S. typhimurium* TA98	0.03–30 μmol/plate (3.9–3935 μg/plate)[i]	Negative[d,j]	Florin et al. (1980)
1304	Skatole	Reverse mutation	*S. typhimurium* TA98 and TA100	NR	Negative[c]	Kim et al. (1989)

Table 5. (contd)

No.	Flavouring agent	End-point	Test system	Concentration or dose	Result	Reference
1304	Skatole	Reverse mutation	*S. typhimurium* TA98 and TA100	≤0.4 mol/plate (52 μg/plate)[i]	Negative[d]	Sasagawa & Matsushima (1991)
1304	Skatole	Reverse mutation	*S. typhimurium* TA100	≤100 μg/plate	Negative[a]	Ochiai et al. (1986)
1304	Skatole	Mutation	*E. coli* WP2 *uvrA*/pKM101	≤0.4 μmol/plate (52 μg/plate)[i]	Negative[d]	Sasagawa & Matsushima (1991)
1304	Skatole	Mutation	*E. coli* Sd-4-73	0.01–0.025 ml/disk	Negative	Szybalski (1958)
1304	Skatole	DNA single strand break	Bovine kidney cells	10 μmol–1 mmol/l (1.31–131.18 μg/ml)[i]	Positive	Kim et al. (1989)
1307	Methyl 2-pyrrolyl ketone	Reverse mutation	*S. typhimurium* TA98 and TA100	12.5–200 μg/plate	Negative[d]	Wang et al. (1994)
1307	Methyl 2-pyrrolyl ketone	Reverse mutation	*S. typhimurium* TA98	4–100 μmol/plate (437–10913 μg/plate)[k]	Negative[c] Positive[a]	Lee et al. (1994)
1307	Methyl 2-pyrrolyl ketone	Reverse mutation	*S. typhimurium* TA100	4–100 mol/plate (437–10913 μg/plate)[k]	Negative[d]	Lee et al. (1994)
1309	2-Acetylpyridine	Mitotic aneuploidy	*S. cerevisiae* D61.M	0.50–0.87% (54000–939600 μg/ml)[l]	Positive	Zimmermann et al. (1986)
1314	Pyrrole	Reverse mutation	*S. typhimurium* TA98, TA100 and TA102	14 nmol/plate–1.4 mmol/plate (0.94–93926 μg/plate)[m]	Negative[d]	Aeschbacher et al. (1989)
1314	Pyrrole	Reverse mutation	*S. typhimurium* TA100, TA1535 and TA1537	3 μmol/plate (201 μg/plate)[l]	Negative[d]	Florin et al. (1980)
1314	Pyrrole	Reverse mutation	*S. typhimurium* TA98	0.03–30 μmol/plate (2.01–2013 μg/plate)[m]	Negative[d]	Florin et al. (1980)

1314	Pyrrole	Reverse mutation	*S. typhimurium* TA98 and TA100	NR	Negative[d]	Lee et al. (1994)
1314	Pyrrole	Unscheduled DNA synthesis	Rat hepatocytes	NR	Negative	Williams (1984)
1315	3-Ethylpyridine	Reverse mutation	*S. typhimurium* TA98, TA100, TA 1535 and TA1537	3 μmol/plate (321 μg/plate)[n]	Negative[d]	Florin et al. (1980)
1316	3-Acetylpyridine	Mutation	*E. coli* WP2 *uvrA*	5000–10000 μg/plate	Negative	Pai et al. (1978)
1316	3-Acetylpyridine	Mitotic aneuploidy	*S. cerevisiae* D61.M	0.5–1.11% (55100–122322 μg/ml)[o]	Positive	Zimmermann et al. (1986)
In vivo						
1302	6-Methylquinoline	Sex-linked recessive mutation	*Drosophila melanogaster*	10 mmol/l (1432 μg/ml)[g]	Negative	Wild et al. (1983)
1302	6-Methylquinoline	Micronucleus formation	NMRI mice	0, 286, 429, or 572 mg/kg bw	Negative	Wild et al. (1983)

NR, not reported.
[a] Without metabolic activation.
[b] Calculated based on relative molecular mass = 117.15.
[c] With metabolic activation.
[d] With and without metabolic activation.
[e] Toxic at concentrations >3.0 μmol/plate (351 μg/plate).
[f] TA100 and TA1535.
[g] Calculated based on relative molecular mass = 143.19.
[h] Calculated based on relative molecular mass = 129.16.
[i] Calculated based on relative molecular mass = 131.18.
[j] Toxic at concentrations of >3.0 μmol/plate (394 μg/plate).
[k] Calculated based on relative molecular mass = 109.13.
[l] Calculated based on density = 1.08 g/ml (Sigma-Aldrich, 2003; available at http://www.sigmaaldrich.com).
[m] Calculated based on relative molecular mass = 67.09.
[n] Calculated based on relative molecular mass = 107.16.
[o] Calculated based on density = 1.102 g/ml (Sigma-Aldrich, 2003; available at http://www.sigmaaldrich.com).

(No. 1301) at a concentration of up to 30 μmol/plate (3515 μg/plate) (Anderson & Styles, 1978; Kaden et al., 1979; Florin et al., 1980; Ochiai et al., 1986; Vance et al., 1986; Sasagawa & Matsushima, 1991; Fujita et al., 1994), isoquinoline (No. 1303) at a concentration of up to 20 000 μg/ml (Sugimura et al., 1976; Nagao et al., 1977; Epler et al., 1979; Kaden et al., 1979; Sideropoulos & Specht, 1984;), skatole (No. 1304) at a concentration of up to 3 μmol/plate (394 μg/plate) (Florin et al., 1980; Ochiai et al., 1986; Kim et al., 1989; Sasagawa & Matsushima, 1991), pyrrole (No. 1314) at a concentration of up to 1.4 mmol/plate (93 926 μg/plate) (Florin et al., 1980; Aeschbacher et al., 1989; Lee et al., 1994), and 3-ethylpyridine (No. 1315) at a concentration of up to 3 μmol/plate (321 mg/plate) (Florin et al., 1980) with and without metabolic activation. Methyl 2-pyrrolyl ketone (No. 1307) at concentrations of 4 to 100 μmol/plate induced a > 2-fold increase in the number of revertants per plate compared with the control when tested in *S. typhimurium* TA98 in the absence of metabolic activation (Lee et al., 1994). However, negative results were obtained with metabolic activation as well as in *S. typhimurium* TA100 (both with and without metabolic activation). Furthermore, no mutagenic activity was reported in either strain when incubated with methyl 2-pyrrolyl ketone at a concentration of up to 200 μg/plate with and without metabolic activation (Wang et al., 1994). 6-Methylquinoline (No. 1302) at a concentration of 3.3 to 3600 μg/plate) gave uniformly positive results in the presence of metabolic activation (Sugimura et al., 1976; Nagao et al., 1977; Dong et al., 1978; Wild et al., 1983; Takahashi et al., 1988; Debnath et al., 1992; Zeiger et al., 1992). Methylquinolines, tested at a concentration of 400 μg/plate, showed a potent bactericidal or bacteriostatic effect, with only 6% survival of *S. typhimurium* TA100 treated with 6-methylquinoline (Dong et al., 1978).

There was no evidence of mutagenicity when *Escherichia coli* (strains WP2 *uvr*4A/pKM101, SD-4-73, or B/r HCR+) were incubated with indole (No. 1301) at a concentration of up to 0.4 μmol/plate (47 mg/plate) (Sasagawa & Matsushima, 1991), isoquinoline (No. 1303) at a concentration of up to 50 μg/ml, skatole (No. 1304) at a concentration of up to 0.4 μmol/plate (52 mg/plate) (Szybalski, 1958; Sasagawa & Matsushima, 1991), or 3-acetylpyridine (No. 1316) at a concentration of up to 10 000 mg/plate of (Pai et al., 1978).

In non-standardized assays, 2-acetylpyridine (No. 1309) at 0.50 to 0.87% (54 000 to 939 600 μg/ml) and 3-acetylpyridine (No. 1316) at 0.5 to 1.11% (55 100 to 122 322 μg/ml) caused a dose-dependent increase in mitotic aneuploidy in strain D61.M of *Saccharomyces ceverisiae* (Zimmermann et al., 1986). At the higher test concentrations, the growth of D61.M was strongly or completely inhibited. The authors noted that it is generally recognized that there is a threshold dose for induction of aneuploidy in yeast (Zimmermann et al., 1985a, 1985b, 1985c).

Assays in mammalian cell lines have been performed for isoquinoline (No. 1303) (Williams, 1984), skatole (No. 1304) (Kim et al., 1989), and pyrrole (No. 1314) (Williams, 1984). There was no evidence of increased unscheduled DNA synthesis when freshly isolated rat liver cells were incubated with pyrrole or isoquinoline (concentrations not specified) (Williams, 1984). Single-strand DNA breaks and inhibition of growth were reported when undeuterated or deuterated (at C2 or C3 positions) 3-methylindole (skatole) at 10 μmol/l to 1 mmol/l (1.31 to 131.18 mg/ml) was incubated with isolated cultured bovine kidney cells. However,

there was no evidence of DNA interstrand crosslinks (Kim et al., 1989). These observations are consistent with reports that, at high concentrations, indoles deplete glutathione, leading to increased formation of DNA adducts (Nichols et al., 2000; Regal et al., 2001).

(ii) In vivo

There was no evidence for mutation in a standard assay for sex-linked recessive lethal mutation when adult *Drosophila melanogaster* were fed 6-methylquinoline (No. 1302) at a concentration of 10 mmol/l (1432 µg/ml) in a 5% sucrose solution for 3 days (Wild et al., 1983). Furthermore, 6-methylquinoline did not induce micronucleus formation in bone marrow cells obtained from male and female NMRI mice 30 h after treatment with the test compound as a single intraperitoneal dose at 0, 286, 429, or 572 mg/kg bw (Wild et al., 1983).

(iii) Conclusions

Overall, negative results were reported in assays for reverse mutation in bacteria for six representative pyridine, pyrrole and quinoline derivatives (i.e. indole, No. 1301; isoquinoline, No. 1303; skatole, No. 1304; methyl 2-pyrrolyl ketone, No. 1307; pyrrole, No. 1314; and 3-ethylpyridine, No. 1315). Although 6-methylquinoline gave positive results with metabolic activation, it gave negative results in studies in vivo, indicating that there are adequate detoxication mechanisms for the rapid absorption, distribution, biotransformation, and elimination of the *N*-containing heteroaromatic derivatives. 2-Acetylpyridine and 3-acetylpyridine produced positive results in yeast, but this is unlikely to occur at low doses because yeast is generally believed to have a threshold for the induction of aneuploidy. The positive results reported in bacteria for skatole are consistent with observations that, at high concentrations, indoles depletes glutathione, leading to reduced detoxification.

On the basis of the available evidence, the 22 pyridine, pyrrole and quinoline derivatives in this group do not demonstrate genotoxic potential.

(e) Other relevant studies

(i) DNA adducts

Numerous studies have been undertaken to investigate the alkylating potential of 3-methylindole and its principal metabolite indole-3-carbinol. A recent study has investigated the formation of DNA adducts with metabolites of 3-methylindole (skatole) in vitro. When 3-methylindole at a concentration of 200 µmol/l is incubated with calf thymus DNA in the presence of CYP obtained from goat lung, rat liver, or human liver microsomes, DNA adducts are formed. In all three microsomal preparations, the 3-methylindole-deoxyguanosine adduct was the primary adduct. 3-Methylindole-deoxyadenosine and 3-methylindole-deoxycytosine adducts were formed in smaller amounts. No adducts were reported in untreated hepatocytes. Analysis of adducts formed when 3-[$^{2}H_{3}$]-methylindole was incubated with microsomes revealed that the 3-methyleneindolenine intermediate undergoes nucle-

ophilic attack with the nucleotide amine function. When mammalian cell lines were exposed to *3*-methylindole at 200 μmol/l, or more likely its activated metabolite(s), concentrations of GSH were depleted, leading to increased formation of protein and DNA adducts (Regal et al., 2001).

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ALIPHATIC AND ALICYCLIC HYDROCARBONS

First draft prepared by

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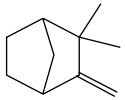
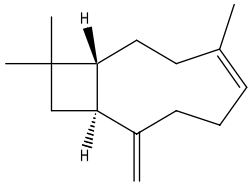
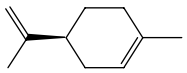
1. EVALUATION

1.1 Introduction

The Committee evaluated a group of 20 aliphatic and alicyclic hydrocarbons (Table 1) by the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, p 192). One member of this group, *d*-limonene (No. 1326), was evaluated by the Committee at its thirty-ninth meeting (Annex 1, reference *101*) and was assigned an acceptable daily intake (ADI) of 0–1.5 mg/kg bw. The Committee at that meeting recommended, however, that intake of this substance as a food additive be restricted to 0.075 mg/kg bw per day, or 5% of the ADI. At its forty-first meeting (Annex 1, reference *107*), the Committee re-evaluated the ADI for

Table 1. Summary of the results of safety evaluations of aliphatic and alicyclic hydrocarbons used as flavouring agents[a]

Flavouring agent	No.	CAS No. and structure	Step A3[b] Does intake exceed the threshold for human intake?	Step A4 Is the flavouring agent or are its metabolites endogenous?	Step A5 Adequate margin of safety for the flavouring agent or related substance?	Comments	Conclusion based on current intake
Structural class I							
Camphene	1323	79-92-5 	No Europe: 16 USA: 28	NR	NR	See note 1	No safety concern
β-Caryophyllene	1324	87-44-5 	No Europe: 389 USA: 508	NR	NR	See note 1	No safety concern
d-Limonene	1326	5989-27-5 	Yes Europe: 39307 USA: 12726	No	Yes. Given that there is an ADI 'not specified' for *d*-limonene (see footnote c), the daily intakes of 660 μg/kg bw	See note 1	See footnote c

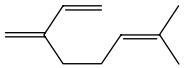
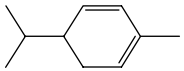
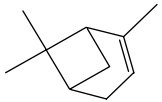
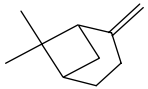
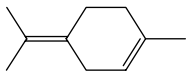
					in Europe and 210μg/kg bw in the USA were considered not to pose a safety concern.		
Myrcene	1327	123-35-3 	Yes Europe: 8287 USA: 156	No	Yes. The LOEL/NOEL of 250mg/kg bw per day for myrcene (National Toxicology Program, 2004a, 2004b) is approximately 1800 and 83000 times the daily intakes of 140μg/kg bw in Europe and 3μg/kg bw in the USA, respectively.	See notes 2, 3	No safety concern
α-Phellandrene	1328	99-83-2 / 4221-98-1 	No Europe: 92 USA: 410	NR	NR	See note 1	No safety concern

Table 1. (contd)

Flavouring agent	No.	CAS No. and structure	Step A3[b] Does intake exceed the threshold for human intake?	Step A4 Is the flavouring agent or are its metabolites endogenous?	Step A5 Adequate margin of safety for the flavouring agent or related substance?	Comments	Conclusion based on current intake
α-Pinene	1329	80-56-8 	Yes Europe: 2152 USA: 2444	No	Yes. The daily intakes of 36 μg/kg bw in Europe and 41 μg/kg bw in the USA are approximately 5% and 20%, respectively, of those of the structural analogue *d*-limonene, for which an ADI 'not specified' was established (see footnote c).	See note 1	No safety concern
β-Pinene	1330	127-91-3 	No Europe: 1550 USA: 759	NR	NR	See note 1	No safety concern
Terpinolene	1331	586-62-9 	No Europe: 772 USA: 70	NR	NR	See note 1	No safety concern

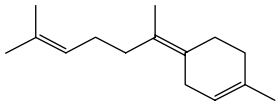
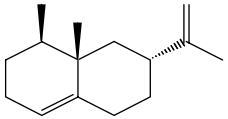
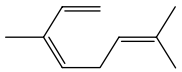
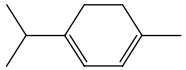
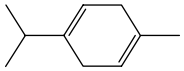
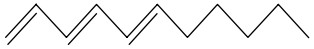
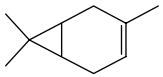
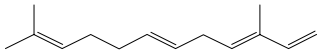
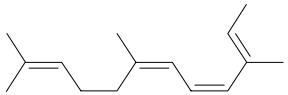
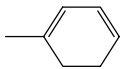
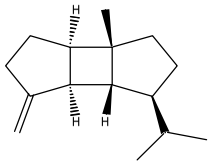
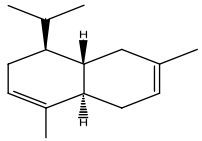
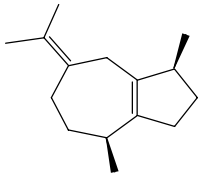
Bisabolene	1336	495-62-5 	No Europe: 15 USA: 10	NR	NR	See note 1	No safety concern
Valencene	1337	4630-07-3 	No Europe: 62 USA: 26	NR	NR	See note 1	No safety concern
3,7-Dimethyl-1, 3,6-octatriene	1338	13877-91-3 	No Europe: 65 USA: 11	NR	NR	See notes 2, 3	No safety concern
p-Mentha-1, 3-diene	1339	99-86-5 	No Europe: 32 USA: 93	NR	NR	See note 1	No safety concern
p-Mentha-1, 4-diene	1340	99-85-4 	No Europe: 1372 USA: 321	NR	NR	See note 1	No safety concern
1,3,5-Undecatriene	1341	16356-11-9 / 19883-29-5 	No Europe: 0.2 USA: 0.2	NR	NR	See note 3	No safety concern

Table 1. (contd)

Flavouring agent	No.	CAS No. and structure	Step A3[b] Does intake exceed the threshold for human intake?	Step A4 Is the flavouring agent or are its metabolites endogenous?	Step A5 Adequate margin of safety for the flavouring agent or related substance?	Comments	Conclusion based on current intake
δ-3-Carene	1342	13466-78-9 	No Europe: ND USA: 40	NR	NR	See note 1	No safety concern
Farnesene (α and β)	1343	502-61-4   farnesene	No Europe: ND USA: 40	NR	NR	See notes 2, 3	No safety concern
1-Methyl-1, 3-cyclohexadiene	1344		No Europe: ND USA: 313	NR	NR	See note 1	No safety concern
β-Bourbonene	1345	5208-59-3 	No Europe: ND USA: 0.2	NR	NR	See note 1	No safety concern

Cadinene (mixture of isomers)	1346	523-47-7  β-Cadinene, a principal isomer of cadinene	No Europe: ND USA: 0.05	NR	NR	See note 1	No safety concern
Guaiene	1347	88-84-6 	No Europe: ND USA: 3	NR	NR	See note 1	No safety concern

CAS: Chemical Abstracts Service; ND: No intake data reported; NR: Not required for evaluation because consumption of the agent was determined to be of no safety concern at step A3 of the Procedure.

[a] Step 2: All the agents in this group are expected to be metabolized to innocuous products.

[b] The threshold for human intake for structural class I is 1800 μg/per person per day. All intake values are expressed in μg/person per day. The combined intake of flavouring agents in structural class I is 54 111 μg/person per day in Europe and 17 959 μg/person per day in the USA.

[c] An ADI 'not specified' was established for *d*-limonene by the Committee at its forty-first meeting (Annex 1, reference *107*), which was maintained at the present meeting.

Notes:

1. Allylic oxidation, epoxidation and hydrolysis to yield diols or by ring cleavage followed by conjugation with glucuronic acid and excretion in the urine.
2. Side-chain oxidation followed by subsequent conjugation with glycine, glucuronic acid, or glutathione.
3. Epoxidation to yield the corresponding diol that is conjugated with glucuronic acid and excreted in the urine.

d-limonene and recommended that it be withdrawn and replaced with an ADI 'not specified'.

Nineteen of the 20 flavouring agents in this group (Nos 1323, 1324, 1326–1331, 1336–1343 and 1345–1347) have been reported to occur naturally in foods. They have been detected in, for example, coffee, alcoholic beverages, baked and fried potato, heated beans, tea, bread and cheese (Nijssen et al., 2003). The substance with the highest natural occurrence is *d*-limonene (No. 1326).

1.2 Estimated daily intake

The total annual volume of production of the 20 flavouring agents in this group is approximately 380 000 kg in Europe (International Organization of the Flavor Industry, 1995) and 140 000 kg in the USA (National Academy of Sciences, 1989; Lucas et al., 1999). *d*-Limonene (No. 1326) accounts for approximately 73% of the total annual volume of production in Europe and 71% in the USA. The estimated daily intakes of *d*-limonene in Europe and the USA are approximately 40 000 μg and 13 000 μg/person, respectively. Myrcene (No. 1327), α- and β-pinene (Nos 1329 and 1330, respectively), terpinolene (No. 1331), β-caryophyllene (No. 1324), α-phellandrene (No. 1328), and *p*-mentha-1,4-diene (No. 1340) account for most of the remaining (approximately 26–27%) total annual volume of production. The estimated daily intakes of these flavouring agents are in the range of 92–8300 μg/person in Europe and 70–2400 μg/person in the USA. The reported annual volumes of production of the remainder of the flavouring agents in this group are extremely low, accounting for <1 and 3% of the total annual volume of production in Europe and the USA, respectively. The estimated daily intakes of these agents range from <0.1 to 93 μg/person in Europe and the USA, except for 1-methyl-1,3-cyclohexadiene (No. 1344) which has an estimated daily intake of approximately 300 μg/person in the USA. The estimated daily per capita intake of each agent is reported in Table 2.

1.3 Absorption, distribution, metabolism and elimination

Being lipophilic, the aliphatic and alicyclic hydrocarbons in this group are likely to cross biological membranes by passive diffusion. After oral and inhalation exposure, they are rapidly absorbed and distributed to body tissues, elimination from blood being triphasic, with a slow terminal phase.

On the basis of the available data, it is anticipated that all the aliphatic and alicyclic hydrocarbons in this group will participate in similar pathways of metabolic detoxification in mammals, including humans. After absorption, these hydrocarbons are oxidized to polar oxygenated metabolites via cytochrome P450 (CYP) enzymes and alcohol and aldehyde dehydrogenases. The aliphatic and alicyclic substances are oxidized either by side-chain oxidation or by epoxidation of an exocyclic or endocyclic double bond. Alkyl oxidation initially yields hydroxylated metabolites that may be excreted in conjugated form or undergo further oxidation, yielding more polar metabolites that are also excreted in conjugated form in the urine. If a double bond is present, epoxide metabolites may form and these metabolites are detoxified either by hydrolysis to yield diols, or by conjugation with glutathione.

Table 2. Annual volumes of production of aliphatic and alicyclic hydrocarbons used as flavouring agents in Europe and the USA

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		µg/d	µg/kg bw per day		
Camphene (1323)					
Europe	110	16	0.3		
USA	213	28	0.5	27 350	128
β-Caryophyllene (1324)					
Europe	2728	389	6		
USA	3860	508	8	279 587	72
d-Limonene (1326)					
Europe[e]	275 461	39 307	655		
USA	96 615	12 726	212	1 300 995	13
Myrcene (1327)					
Europe	58 076	8 287	138		
USA	1 188	156	3	66 842	56
α-Phellandrene (1328)					
Europe	648	92	2		
USA	3 116	410	7	133 334	43
α-Pinene (1329)					
Europe	15 080	2 152	36		
USA	18 552	2 444	41	189 253	10
β-Pinene (1330)					
Europe	10 865	1 550	26		
USA	5761	759	13	275 128	48
Terpinolene (1331)					
Europe	5409	772	13		
USA	531	70	1	25 220	47

Bisabolene (1336)					
Europe	104	15	0.2		
USA	77	10	0.2	33 865	440
Valencene (1337)					
Europe	437	62	1		
USA	200	26	0.4	444	2
3,7-Dimethyl-1,3,6-octatriene (1338)					
Europe	454	65	1		
USA	82	11	0.2	102	1
p-Mentha-1,3-diene (1339)					
Europe	227	32	0.5		
USA	708	93	2	24 720	35
p-Mentha-1,4-diene (1340)					
Europe	9613	1 372	23		
USA	2436	321	5	98 317	40
1,3,5-Undecatriene (1341)					
Europe	2	0	0.004		
USA[g]	1	0	0.003	+	NA
δ-3-Carene (1342)					
Europe	NR	NA	NA		
USA[g]	225	40	0.7	+	NA
Farnesene (α and β) (1343)					
Europe	NR	NA	NA		
USA[g]	225	40	0.7	+	NA
1-Methyl-1,3-cyclohexadiene (1344)					
Europe	NR	NA	NA		
USA[g]	1783	313	5	–	NA
β-Bourbonene (1345)					
Europe	NR	NA	NA		
USA[f]	1	0.2	0.003	+	NA
Cadinene (mixture of isomers) (1346)					
Europe	NR	NA	NA		
USA	0.4	0.05	0.0009	12 154	30 385

Table 2. (Contd)

Flavouring ratio[d]	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption agent (No.)
		µg/d	µg/kg bw per day		
Guaiene (1347)					
Europe	NR	NA	NA		
USA[f]	18	3	0.05	+	NA
Total					
Europe	379 214				
USA	135 592				

NR, no data reported; NA, not applicable; +, reported to occur naturally in foods (Nijssen et al., 2003), but no quantitative data available; –, not reported to occur naturally in foods.

[a] From International Organization of the Flavor Industry (1995) and Lucas et al. (1999) or National Academy of Sciences (1989).

[b] Intake expressed as µg/person per day was calculated as follows: [(annual volume, kg) × (1×10^9 µg/kg)/(population × survey correction factor × 365 days)], where population (10%, "eaters only") = 32×10^6 for Europe and 26×10^6 for the USA. The correction factor = 0.6 for Europe, and 0.8 for the USA, representing the assumption that only 60% and 80% of the annual volume of the flavour, respectively, was reported in the poundage surveys (International Organization of the Flavor Industry, 1995; Lucas et al., 1999; National Academy of Sciences, 1989). Intake (µg/kg bw per day) was calculated as follows: [(µg/person per day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.

[c] Quantitative data for the USA reported by Stofberg & Grundschober (1987).

[d] Consumption ratio was calculated as follows: (annual consumption in food, kg)/(most recent reported volume as a flavouring agent, kg).

[e] Annual volume reported for limonene (CAS No. 138-86-3).

[f] Annual volume reported in previous USA surveys (National Academy of Sciences 1970 or 1982, as reported in National Academy of Sciences, 1989).

[g] The volume cited is the anticipated annual volume, which was the maximum amount of flavour estimated to be used annually by the manufacturer at the time the material was proposed for flavour use. National surveys (National Academy of Sciences 1970, 1975, 1976, 1982 or 1987, as reported in National Academy of Sciences, 1989; Lucas et al., 1999) revealed no reported use as a flavouring agent at that time.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure, the Committee assigned all the 20 flavouring agents in this group to structural class I (Cramer et al., 1978).

Step 2. All the flavouring agents in this group are expected to be metabolized to innocuous products. The evaluation of all agents in this group therefore proceeded via the A-side of the decision-tree.

Step A3. The estimated daily intakes of 17 of the 20 flavouring agents (Nos 1323, 1324, 1328, 1330, 1331 and 1336–1347) are below the threshold of concern (i.e. 1800 μg/person per day for class I). According to the Procedure, the use of these 17 flavouring agents raises no safety concern at estimated current intakes. The estimated daily per capita intakes of the remaining three agents in this group, *d*-limonene (No. 1326), myrcene (No. 1327) and α-pinene (No. 1329), exceed the threshold of concern for class I. Accordingly, the evaluation of these three agents proceeded to step A4.

Step A4. *d*-Limonene, myrcene and α-pinene are not endogenous in humans. Therefore, the evaluation of these agents proceeded to step A5.

Step A5. For myrcene (No. 1327) a lowest-observed-effect level (LOEL) of 250 mg/kg bw per day was reported for male mice and male and female rats treated by gavage for 13 weeks (National Toxicology Program, 2004a, 2004b), while the same dose was the no-observed-effect level (NOEL) in female mice. This dose is approximately 1800 times greater than the estimated intake of myrcene from its use as a flavouring agent in Europe (140 μg/kg bw per day) and 83 000 times greater than the estimated intake of myrcene in the USA (3 μg/kg bw per day). The Committee concluded that myrcene would not pose a safety concern at estimated current intake.

At its forty-first meeting, the Committee established an ADI 'not specified' for *d*-limonene (No. 1326) on the basis of short- and long-term studies of toxicity in female rats and male and female mice, and studies of developmental toxicity in mice, rats and rabbits. In these studies, *d*-limonene was tested at doses ranging from 250 to 2800 mg/kg bw per day. Based on the ADI 'not specified', the Committee concluded that *d*-limonene would not pose a safety concern at the estimated current intakes (660 μg/kg bw per day in Europe and 210 μg/kg bw per day in the USA).

No toxicological data on α-pinene (No. 1329) were available. *d*-Limonene shares structural characteristics with α-pinene in that both contain a methyl-substituted cyclohexene ring, which contains a second alkyl substituent. In *d*-limonene, this is an isopropenyl group, while in α-pinene the second substituent is a dimethyl-substituted methylene bridge. Based on these chemical structures, it would be predicted that the toxicity of α-pinene would be unlikely to exceed that of *d*-limonene. Both compounds are predicted to be metabolized to innocuous products. Metabolism of

both compounds is by hydroxylation of the cyclohexene ring and oxidation of its methyl substituent. *d*-Limonene undergoes epoxidation of the endocyclic and allylic double bonds, leading to dihydroxy products. α-Pinene is converted to several metabolites, including *d*-limonene, by rat liver microsomes in vitro. The Committee concluded that *d*-limonene shared sufficient chemical and metabolic similarities with α-pinene to be used as a structural analogue for α-pinene at this step of the Procedure. The estimated current per capita intakes of α-pinene in Europe (36 μg/kg bw per day) and in the USA (41 μg/kg bw per day) are approximately 5% and 20%, respectively, of those of *d*-limonene, and are almost four orders of magnitude lower than the lowest doses of *d*-limonene considered in the establishment of its ADI 'not specified'. On the basis of these considerations, the Committee concluded that α-pinene would not pose a safety concern at estimated current intakes.

The intake considerations and other information used to evaluate the 20 aliphatic and alicyclic hydrocarbons in this group according to the Procedure are summarized in Table 1.

1.5 Consideration of secondary components

Nine members (Nos 1323, 1324, 1327, 1337–1339 and 1341–1343) of this group of flavouring agents have assay values of <95%. The Committee evaluated the secondary components in No. 1339 (1,4- and 1,8-cineole) at a previous meeting and considered that they did not present a safety concern. The secondary components in Nos 1323, 1324, 1337 and 1343 ($C_{15}H_{24}$ terpene hydrocarbons) and in No. 1342 (β-pinene, *d*-limonene, myrcene and *p*-cymene) were all evaluated according to the Procedure by the Committee at its present meeting. The Committee did not consider any of these secondary components to present a safety concern. The secondary components in No. 1327 (dihydromyrcene), No. 1338 (*cis*-β-ocimene), No. 1341 (2,4,6-undecatriene), and the remaining secondary components in No. 1343 (other isomers of farnesene) are all structurally related to the primary flavouring agents and are expected to share the same metabolic fate. Therefore none of these secondary components was considered to present a safety concern.

1.6 Consideration of combined intakes from use as flavouring agents

In the unlikely event that all 20 agents in this group were consumed concurrently on a daily basis, the estimated combined intake would exceed the human intake threshold of 1800 μg/person per day for class I. However, these 20 agents are all expected to be efficiently metabolized and would not saturate metabolic pathways. Overall evaluation of the data indicated that combined intake of these agents would not raise a safety concern.

1.7 Conclusions

The Committee maintained the previously established ADI 'not specified' for *d*-limonene (Annex 1, reference *107*). The Committee concluded that use of the

flavouring agents in this group of aliphatic and alicyclic hydrocarbons would not present a safety concern at estimated current intakes. The Committee also noted that the available data on the toxicity and metabolism of these flavouring agents were consistent with the results of the safety evaluation.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

The relevant background information summarizes the key scientific data applicable to the safety evaluation of 20 aliphatic and alicyclic hydrocarbons used as flavouring agents (see Table 1). All substances in this group are unsaturated hydrocarbons that are acyclic, monocyclic, or bicyclic. Members of the group that exhibit the highest annual volumes of use as flavouring agents include a series of naturally occurring C10 terpene hydrocarbons (e.g. *d*-limonene, myrcene, and α- and β-pinene).

2.2 Additional considerations on intake

Volumes of production and intake values for each flavouring agent are reported in Table 2. The majority of flavouring agents in this group are products of plant biosynthesis.

Nineteen of the 20 flavouring agents in the group have been reported to occur naturally in traditional foods (Nijssen et al., 2003; Table 2). Quantitative data on natural occurrence data have been reported for 14 flavouring agents in the group (Stofberg & Grundschober, 1987). The consumption of all of these 14 agents is derived predominantly from their presence in traditional foods (i.e. they have a consumption ratio of ≥1; Table 2).

Owing to their volatility, several C_{10} terpene hydrocarbons in this group have also been reported to emit from vegetation to the atmosphere. In North America, the annual emissions for these C_{10} terpene hydrocarbons approach 18 million tonnes, with major contributions coming from α-pinene (No. 1329), β-pinene (No. 1330), and δ-3-carene (No. 1342) (Guenther et al., 2000).

2.3 Biological data

2.3.1 Biochemical data

(a) Absorption, distribution, and excretion

(i) Acyclic hydrocarbons

In male Japanese white rabbits given myrcene (No. 1327) at a dose of 670 mg/kg bw per day by gavage for 2 days, approximately 25% of the total administered amount (19 g to six rabbits) could be recovered from the urine excreted over a period of 3 days after administration. More than 80% of the metabolites in urine were neutral metabolites, the rest were acidic metabolites (Ishida et al., 1981).

(ii) Monocyclic hydrocarbons

Male Wistar rats were given [$^{14}C_9$]*d*-limonene (No. 1326) by stomach tube at a dose of 800 mg/kg bw, and the concentration of radiolabel was determined in blood, tissues (fat not included), excreta, bile, and expired air. The animals were sacrificed 48 h after dosing. Radiolabel reached a peak plasma concentration at 2 h after dosing, and after maintaining high levels for 10 h, declined to negligible levels at 48 h. In most tissues, peak concentrations of radiolabel were reached within 2 h after dosing, indicating rapid distribution. Liver, kidney and adrenals contained the highest concentrations of radiolabel (higher than blood or serum); other tissues (including brain) contained <0.2% of the administered radiolabel. Hardly any radiolabel could be detected at 48 h after dosing. Whole body autoradiography confirmed these findings. At 48 h after dosing, about 60% of the administered radiolabel was recovered from the urine, 5% from faeces and 2% from exhaled air as carbon dioxide (CO_2). Approximately 25% of the administered radiolabel was excreted in the bile during the first 24 h after administration. Total recovery of radiolabel was <100% and as there was hardly any radiolabel present in the tissues at 48 h, this could point to loss of volatile ^{14}C from the excreta or to the elimination of volatile ^{14}C-labelled compounds other than CO_2 (Igimi et al., 1974). When a similar dose of [$^{14}C_9$]*d*-limonene was given to male rabbits, 72% and 7% of the radiolabel was excreted in the urine and faeces during the first 72 h, respectively (Kodama et al., 1974).

In an additional study with several species (rats, hamsters, guinea-pigs, rabbits, dogs, and humans) dosed orally with [$^{14}C_9$]-*d*-limonene, urinary excretion of radiolabel in rodents and rabbits comprised 82–96% of the dose within 72 h and faecal excretion was 2–9%. The total excretion rate in dogs was somewhat lower (77% via urine and 9% via faeces within 72 h), while two human volunteers excreted 55–83% of the administered dose in the urine. Faecal excretion in humans was not measured, but may have been considerable in the person with lower urinary excretion as this person developed diarrhoea shortly after administration. In all species, most excretion occurred within the first 24 h (Kodama et al., 1976).

In vitro, the solubility of *d*-limonene in blood and olive oil was high, but low in water (partition coefficients were 42, 5700, 1.8, and 140 for blood/air, oil/air, water/air, and oil/blood, respectively). This suggests a high respiratory uptake and accumulation in adipose tissues (Falk et al., 1990a). Indeed, uptake was rapid and high (68%) in an experiment in which human volunteers were exposed to *d*-limonene in air at 0.225 and 0.450 mg/l for 2 h while doing light physical exercise. The absorbed *d*-limonene was metabolized rapidly. Elimination followed a triphasic pattern, with a short half-life in blood immediately after exposure (2.6 min) but a long half-life during the late elimination phase (12.5 h), which indicates slow elimination from adipose tissues. Approximately 1% of the total uptake was eliminated unchanged in expired air, while approximately 0.003% was eliminated unchanged in urine (Falk-Filipsson et al., 1993).

(iii) Bicyclic hydrocarbons

When male Japanese white rabbits were given (+)-α-pinene (No. 1329) in a single dose at 560 mg/kg bw by gavage, only 3% of the total administered dose

(10 g to six rabbits) could be recovered from the urine collected for 3 days after administration. All the metabolites in urine were neutral metabolites (Ishida et al., 1981). The same authors also investigated the urinary excretion of δ-3-carene (No. 1342) and (–)-β-pinene (isomer of (+)-β-pinene, No. 1330) in rabbits, and found that approximately 18% of the total administered amount (26 g to six rabbits) of δ-3-carene could be recovered from the 3-day urine, with approximately 55% of the metabolites in urine being acidic metabolites, and the remaining 45% being neutral metabolites. For (–)-β-pinene, approximately 15% of the total administered dose (12 g to six rabbits) could be recovered from the urine, of which 43% were neutral and 57% were acidic metabolites (Ishida et al., 1981).

In vitro data on the solubility of α-pinene, β-pinene (No. 1330) and δ-3-carene in blood, olive oil and water suggest a high respiratory uptake and accumulation in adipose tissues (partition coefficients were 15–32, 2900–5000, 0.12–0.41, and 160–190 for blood/air, oil/air, water/air, and oil/blood, respectively). For α-pinene this is supported by a high estimated brain/blood partition coefficient of 18 (Falk et al., 1990a). Experiments in which human volunteers were exposed to (+)- and (–)-α-pinene or δ-3-carene at 0.225 and 0.450 mg/l in air for 2 h while doing light physical exercise confirmed that uptake was rapid and high for these agents (58–60% for (+)- and (–)-α-pinene and 70% for δ-3-carene), and that they were metabolized rapidly. Elimination followed a triphasic pattern, with (+)- and (–)-α-pinene exhibiting a rapid initial (distribution) phase (4.8 and 5.6 min, respectively), a rapid second distribution phase (38 and 40 min, respectively), and a slow elimination phase (695 and 555 min, respectively). Triphasic elimination was also observed for δ-3-carene with half-lives of 4.5, 35, and 1800 min for the initial, rapid, and slow phases, respectively. It was estimated that it would require more than 2 or 6 days to eliminate α-pinene or δ-3-carene, respectively, from the body. The long half-lives indicate slow elimination from adipose tissues. Less than 0.001% of the total uptake of α-pinene or δ-3-carene was eliminated unchanged in the urine, while 7.5–7.8% and 3% of the inhaled amount of the α-pinenes and δ-3-carene was exhaled (Falk et al., 1990b, 1991).

In another study, humans were exposed for 4 or 6 h to atmospheres containing a mixture of volatile organic substances, which included α-pinene, at total concentrations of 0.012 or 0.024 mg/l. At 0.024 mg/l, the air concentration of α-pinene was 0.775 μg/l. The mean pre-exposure blood concentration of α-pinene of 0.035 μg/l increased to an average concentration of 1.9 μg/l during the 4 h of exposure (50–240 min). Thereafter (330–450 min), the mean blood concentration decreased to 0.15 μg/l. Changes proportional to those observed at 0.024 mg/l were recorded at 0.012 mg/l. Similar results were recorded for 6 h of exposure. Plasma elimination for α-pinene was best described with a three-exponential curve, with half-lives ranging from 0.22–7.8 min, 19–58 min and >150 min for the initial, intermediate and terminal phases, respectively (Ashley & Prah, 1997).

In summary

Being lipophilic, the aliphatic and alicyclic hydrocarbons in this group are likely to cross biological membranes by passive diffusion. After oral and inhalation exposure, they are rapidly absorbed and distributed to body tissues, with elimination from blood being triphasic with a slow elimination phase.

(b) Metabolism

(i) Acyclic hydrocarbons

In the urine of rabbits given myrcene (No. 1327) via oral gavage, the main metabolites identified were myrcene-3,10-glycol, myrcene-1,2-glycol, and uroterpenol (as acetate) (40.7, 20.8 and 11.8%, respectively, of the neutral metabolites). Additionally, the glycols underwent further oxidation to yield 2-hydroxymyrcene-1-carboxylic acid and 3-hydroxymyrcene-10-carboxylic acid (no quantitative data were given for these acidic metabolites). The authors suggested that uroterpenol (or limonene-8,9-diol) may have been formed from limonene, which is derived from cyclization of myrcene in the acidic conditions of the rabbit stomach (Ishida et al., 1981).

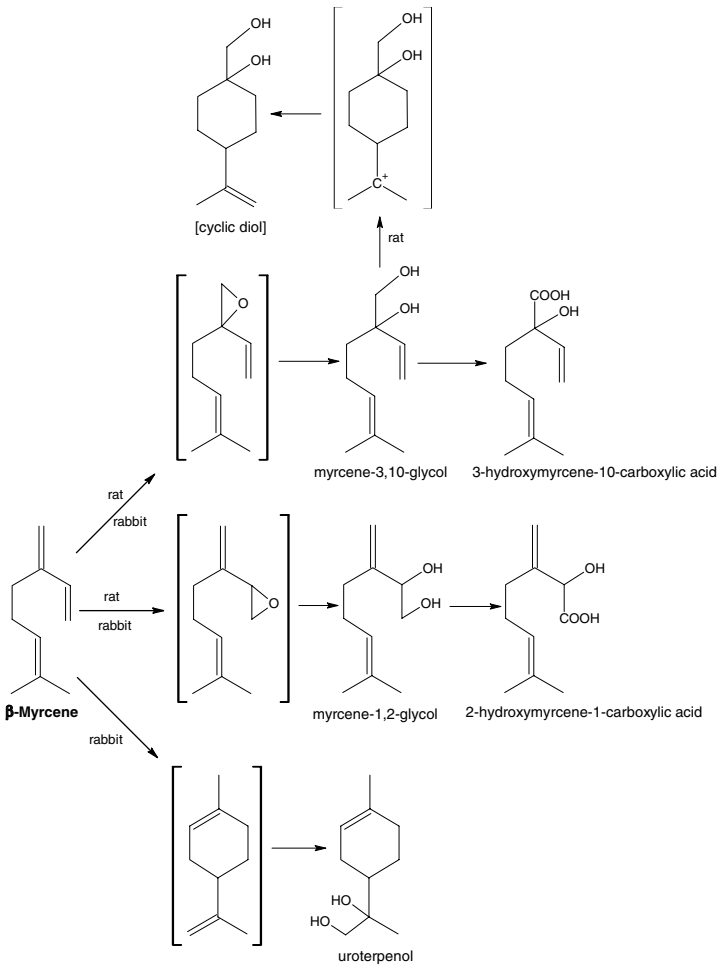
When rats were given myrcene at a dose of 800 mg/kg bw per day orally by gavage for 20 days, the principal metabolites isolated from the urine were 10-hydroxylinalool (or myrcene-3,10-glycol) and, to a lesser extent, 7-methyl-3-methylene-oct-6-ene-1,2-diol (or myrcene-1,2-glycol). Other minor metabolites included the hydroxy acids of both the 3,10- and 1,2-glycols (10-carboxylinalool (or 3-hydroxymyrcene-10-carboxylic acid) and 2-hydroxy-7-methyl-3-methylene-oct-6-enoic acid (or 2-hydroxymyrcene-1-carboxylic acid), respectively) and a cyclic diol, 1-hydroxymethyl-4-isopropenylcyclohexanol (or *p*-menth-8-ene-1,7-diol), formed by intramolecular cyclization of an open chain metabolite (Madyastha & Srivatsan, 1987). It was demonstrated that the biotransformation of myrcene was CYP-mediated and that it could be enhanced by pre-treatment of animals with phenobarbital (Madyastha & Srivatsan, 1987). Aside from being a substrate for CYP enzymes, myrcene has also been shown to induce these enzymes, especially those from the CYP2B (phenobarbital-inducible) subfamily (De-Oliveira et al., 1997).

These results indicate that the principal urinary metabolites in rats and rabbits after administration of myrcene by gavage are myrcene-3,10-glycol and myrcene-1,2-glycol formed from the hydration of the respective epoxide intermediates. In both species, epoxidation of the 3,10-double bond was favoured over epoxidation of the 1,2-double bond, while epoxidation of the 6,7-double bond did not seem to occur. Further oxidized biotransformation products, notably carboxylic acids, and cyclization products were observed in both rats and rabbits. The proposed metabolism scheme is given in Figure 1.

(ii) Monocyclic hydrocarbons

More than 10 metabolites were found in the urine of rats given *d*-limonene (or *p*-mentha-1,8-diene; No. 1326) at a dose of 800 mg/kg bw by oral gavage. Four of the metabolites were identified as perillic acid, *p*-menth-1-ene-8,9-diol (or uroterpenol, or limonene-8,9-diol), perillic acid-8,9-diol, and 8-hydroxy-*p*-menth-1-en-9-yl-β-D-glucopyranosiduronic acid (the glucuronic acid conjugate of uroterpenol). The bile of these rats contained three metabolites, of which the most important was 8-hydroxy-*p*-menth-1-en-9-yl-β-D-glucopyranosiduronic acid (Igimi et al., 1974). Six metabolites were identified in the urine of rabbits given the same oral dose. In addition to the four metabolites identified in rat urine, the rabbit urine contained *p*-mentha-1,8-dien-10-ol (or limonene-10-ol) and *p*-mentha-1,8-dien-10-

Figure 1. Metabolism of myrcene in rats and rabbits



yl-β-D-glucopyranosiduronic acid (the glucuronic acid conjugate of *p*-mentha-1,8-dien-10-ol). Although not determined quantitatively, perillic acid, perillic acid-8,9-diol and both glucuronic acid conjugates were the major metabolites in rabbit urine, and no unchanged *d*-limonene was detected (Kodama et al., 1974). The same authors identified an additional five metabolites in the urine of rats and dogs treated orally with *d*-limonene. These were characterized as 2-hydroxy-*p*-menth-8-en-7-oic acid, perillylglycine, perillyl-β-D-glucopyranosiduronic acid (the glucuronic acid of perillic acid), *p*-mentha-1,8-dien-6-ol (or limonene-6-ol), and *p*-menth-1-ene-6,8,9-triol. They also found some species differences in the nature of the major metabolites in urine. Perillic acid-8,9-diol was the main metabolite in rats and rabbits, perillyl-β-D-glucopyranosiduronic acid in hamsters, uroterpenol in dogs, and the

glucuronic acid conjugate of uroterpenol in guinea-pigs and humans. It should be noted that the fate of only 40–65% of the *d*-limonene dose administered orally to these animals and humans was accounted for (Kodama et al., 1976).

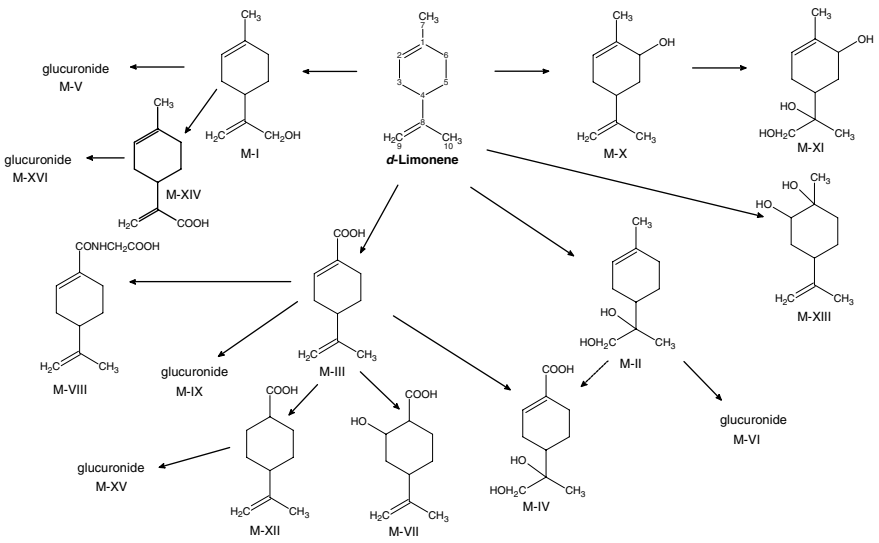
Perillic acid, dihydroperillic acid, and limonene-1,2-diol were the major metabolites identified in the plasma of humans given an oral dose of *d*-limonene. Minor metabolites were the methyl esters of perillic acid and dihydroperillic acid, and *d*-limonene itself (Crowell et al., 1994). Apart from the parent compound, Poon et al. (1996) and Vigushin et al. (1998) also identified perillic acid, dihydroperillic acid, and limonene-1,2-diol as major metabolites in human plasma. However, they also found two other metabolites, i.e. *p*-mentha-1,8-diene-10-carboxylic acid and limonene-8,9-diol (or uroterpenol), while they did not detect the methyl esters of perillic acid and dihydroperillic acid. Peak plasma concentrations of all metabolites were achieved 4–6 h after administration, with the exception of limonene-8,9-diol which reached its peak 1 h after administration (Poon et al., 1996). Metabolites in human urine comprised the glucuronic acid conjugates of perillic acid, dihydroperillic acid, *p*-mentha-1,8-diene-10-carboxylic acid, limonene-8,9-diol, and limonene-10-ol (Poon et al., 1996).

These results indicate that metabolism of *d*-limonene (see Figure 2) proceeds either by allylic oxidation of the exocyclic methyl group to yield perillic acid derivatives (e.g. perillic acid and dihydroperillic acid), by epoxidation and hydrolysis to yield diols (e.g. limonene-1,2-diol and limonene-8,9-diol), or by hydroxylation to yield monohydroxy compounds (e.g. limonene-6-ol, limonene-10-ol). Experiments in vitro have shown that epoxidation of the C8 double bond is favoured over epoxidation of the C1 double bond, due to steric hindrance of the 1-methyl group: upon incubation with rat liver microsomes, the majority of *d*-limonene was converted to the 8,9-epoxide and the 8,9-diol, and to a much lesser extent, to the 1,2-epoxide and the 1,2-diol (Watabe et al., 1981). Other experiments in vitro have shown that male rats can convert *d*-limonene into perillyl alcohol (by hydroxylation of the methyl group at C7) and carveol (or limonene-6-ol; by ring C6-hydroxylation). These reactions are catalysed by CYP2C11 and, when pre-treated with phenobarbital, CYP2B1. In female rats, the activity for conversion to either alcohol is much lower. Apparently, the female-specific CYP2C12 has no activity with respect to *d*-limonene hydroxylation. In males, the hydroxylation activities were not detectable with fetal liver microsomes, but they increased after birth, closely related to the developmental increase in CYP2C11 (Miyazawa et al., 2002). Limonene has also been shown to induce CYP enzymes of the CYP2B and CYP2C subfamilies (Austin et al., 1988; Maltzman et al., 1991).

The metabolic products constitute the major plasma metabolites, but unchanged *d*-limonene was also present, as were perillic acid artefacts (methyl esters) in one study. Perillic acid can be excreted unchanged, or as the glycine or glucuronic acid conjugate in the urine, or it can be further oxidized to perillic acid-8,9-diol or 2-hydroxy-*p*-menth-8-en-7-oic acid. Glucuronic acid conjugates in urine have also been identified for dihydroperillic acid, for *p*-mentha-1,8-diene-10-carboxylic acid, for limonene-8,9-diol, and for limonene-10-ol. Although most metabolites have been identified in several species, there are species differences as to the importance of the different pathways. In the rat, the major pathway is via perillic acid

and metabolites thereof (especially perillic acid-8,9-diol). Minor pathways in the rat include epoxidation of the 8,9-double bond, with subsequent hydrolysis to the corresponding diol and glucuronidation, or oxidation at C10 or C6 positions, with subsequent glucuronidation. In the hamster, the perillic acid derivatives were more important (especially the conjugate of perillic acid) than the 8,9-epoxide-derivatives, while in guinea-pigs and in rabbits they were equally important. In dogs and humans, however, the 8,9-epoxide derivatives were more important than the perillic acid derivatives in the study by Kodama et al. (1976), while other investigators (Crowell et al., 1994; Poon et al., 1996; Vigushin et al., 1998) identified perillic acid and its derivatives as major metabolites in humans, next to 8,9- and 1,2-epoxide derivatives.

Figure 2. Metabolism of d-limonene



M-I	limonene-10-ol	M-IX	glucuronic acid of M-III
M-II	limonene-8,9-diol	M-X	limonene-6-ol
M-III	perillic acid	M-XI	*p*-menth-1-ene-6,8,9-triol
M-IV	perillic acid-8,9-diol	M-XII	dihydroperillic acid
M-V	glucuronic acid of M-I	M-XIII	limonene-1,2-diol
M-VI	glucuronic acid of M-II	M-XIV	*p*-mentha-1,8-diene-10-carboxylic acid
M-VII	2-hydroxy-*p*-menth-8-en-7-oic acid	M-XV	glucuronic acid of M-XII
M-VIII	perillylglycine	M-XVI	glucuronic acid of M-XIV

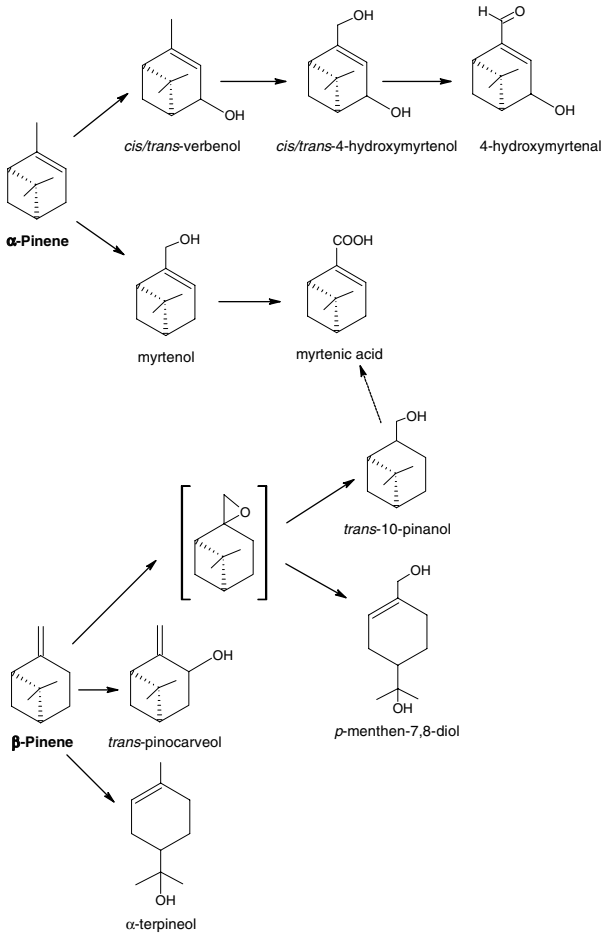
(iii) Bicyclic hydrocarbons

In the urine of sawmill workers exposed to an atmosphere containing α-pinene (No. 1329) at 0.031–0.210 mg/l, β-pinene (No. 1330) at 0.002–0.017 mg/l, and δ-3-carene (No. 1342) at 0.006–0.090 mg/l for 3 days, *cis* and *trans*-verbenol were identified as metabolites. They were excreted as conjugates, probably with glucuronic acid. The authors suggested that these metabolites were formed by hydroxylation of α-pinene (Eriksson & Levin, 1990). Analysis of urinary metabolites eliminated by human volunteers within 4 h after exposure to α-pinene at 0.010–0.450 mg/l for 2 h revealed that α-pinene is indeed eliminated as *cis*- and *trans*-verbenol, at a ratio of 1 : 10, within 20 h after exposure (Levin et al., 1992). In a more extensive metabolic study, urine was collected from sawmill workers at the end of an 8–9 h work shift or from chamber-exposed individuals. After hydrolysis of glucuronic acid conjugates, *cis*- and *trans*-verbenol were identified in the urine, together with two diols, *cis*- and *trans*-4-hydroxymyrtenol, formed by methyl group hydroxylation of *cis*- and *trans*-verbenol. *Trans*-4-hydroxymyrtenal was also detected (Eriksson & Levin, 1996).

In the urine of rabbits given (+)-α-pinene, (–)-α-pinene, (±)-α-pinene, (–)-β-pinene, or δ-3-carene orally via gavage, bicyclic terpene hydrocarbon metabolites were recovered as (glucuronic acid) conjugates or as further oxidized metabolites, notably carboxylic acids. The principal neutral metabolite formed by oxidation at the C4 position in the alicyclic ring of each of the three stereochemical forms of α-pinene was *trans*-verbenol. As a minor pathway, allylic oxidation of the exocyclic methyl group to yield myrtenol was observed for all three α-pinene stereoisomers, with also myrtenic acid as minor metabolite (Ishida et al., 1981). The presence of an exocyclic alkene function in (–)-β-pinene provided additional metabolic options, and four neutral and one acidic metabolites were identified. Allylic oxidation of the C2 position yields (+)-*trans*-pinocarveol, while epoxidation of the exocyclic alkene followed by hydration or rearrangement yields (–)-*trans*-10-pinanol and (–)-1-*p*-menthene-7,8-diol, respectively. Ring cleavage yields (–)-α-terpineol. These metabolites comprised 11, 39, 30 or 5% of the total urinary neutral metabolite fraction, respectively. The acidic metabolite identified was identical to the one identified for the α-pinenes (Ishida et al., 1981). The metabolism of α- and β-pinene is depicted in Figure 3. A study with rat liver microsomes in vitro demonstrated the involvement of CYP enzymes in the metabolism of α-pinene. Metabolites present were β-pinene and limonene together with smaller amounts of *trans*-verbenol, myrtenol, verbenone, and pinene oxide (White & Agosin, 1980). α-Pinene, as well as another bicyclic hydrocarbon, i.e. cadinene, have been shown to induce CYP enzymes, especially those from the CYP2B subfamily, and to a lesser extent also CYP3A2 (cadinene) and CYP4A2 (α-pinene) (Austin et al., 1988; Hiroi et al., 1995).

δ-3-Carene undergoes stereoselective hydroxylation at the *gem*-methyl group (yielding 3-caren-9-ol), followed by carboxylation, allylic oxidation of the C10 methyl group followed by carboxylation or, as the main route, allylic ring opening and hydroxylation at a secondary carbon atom, yielding (–)-*m*-mentha-4,6-dien-8-ol (72% of the total urinary neutral metabolite fraction) and *m*-cymen-8-ol (Ishida et al., 1981).

Figure 3. Metabolism of α-pinene and β-pinene



In rabbits, β-caryophyllene (No. 1324) undergoes epoxidation of the endocylic 5,6-double bond to yield a stable epoxide metabolite and hydroxylation at the *gem*-dimethyl group. Epoxidation of the exocyclic 2,12-double bond, ultimately resulting in 2,12-diol formation, was also reported (Asakawa et al., 1981, 1986).

In summary

On the basis of the available data, it is anticipated that all the aliphatic and alicyclic hydrocarbons in this group will participate in similar pathways of metabolic detoxification in mammals, including humans. After absorption, these hydrocarbons are oxidized to polar oxygenated metabolites via CYP enzymes and alcohol and aldehyde dehydrogenases. The aliphatic and alicyclic substances are oxidized

either by side-chain oxidation or by epoxidation of an exocyclic or endocyclic double bond. Alkyl oxidation initially yields hydroxylated metabolites that may be excreted in conjugated form or undergo further oxidation, yielding more polar metabolites that are also excreted in conjugated form in the urine. If a double bond is present, epoxide metabolites may form and these metabolites are detoxified either by hydrolysis to yield diols, or by conjugation with glutathione.

2.3.2 Toxicological studies

All toxicological studies with *d*-limonene (No. 1326) described in the following sections (a) to (e) have been evaluated previously by the Committee and formed the basis for setting the ADI 'not specified' for *d*-limonene (Annex 1, references *101* and *107*). Since *d*-limonene is now being evaluated as part of the group of aliphatic and alicyclic hydrocarbons, the studies are reported again in this monograph, in somewhat more detail, in order to have present all toxicological information on this group of flavouring agents.

(a) Acute toxicity

Oral median lethal dose (LD_{50}) values have been reported for 16 of the 20 substances in this group, three of them being tested in both mice and rats, and the other 13 only in rats (see Table 3). In mice, oral LD_{50} values were all >2000 mg/kg bw (Hoffmann-LaRoche, 1967; Pellmont, 1973; Tsuji et al., 1975a). In rats, oral LD_{50} values ranged from 1590 to >8000 mg/kg bw (Brownlee, 1940; Wong & Hart, 1971; Keating, 1972; Moreno, 1972a, 1972b, 1972c, 1972d, 1973a, 1973b; Pellmont, 1973; Moreno, 1974a, 1974b; Levenstein, 1975; Moreno, 1975; Tsuji et al., 1975a; Moreno, 1976a, 1976b, 1980). These LD_{50} values indicate that the acute oral toxicity of aliphatic and alicyclic hydrocarbons is low.

(b) Short-term studies of toxicity

Short-term studies of toxicity were available for four of the 20 substances in this group (Tsuji et al., 1975a, 1975b; Kanerva et al., 1987; Shapiro, 1988; Webb et al., 1989; National Toxicology Program, 1990, Webb et al., 1990; National Toxicology Program, 2004a, 2004b). The results of these studies are summarized in Table 4 (except for the substance *d*-limonene, for which already an ADI 'not specified' was established; see 1.1) and described below.

(i) d-Limonene (No. 1326)

Mice

Groups of five male and five female $B6C3F_1$ mice were given *d*-limonene at a dose of 0, 413, 825, 1650, 3300, or 6600 mg/kg bw per day in corn oil by gavage for 12 days, over a 16-day period. The animals were observed twice per day and weighed once per week. Necropsies were performed on all animals. Histopathology was performed on the survivors in the groups receiving the highest dose. The study complied with good laboratory practice (GLP).

Table 3. Studies of the acute toxicity of aliphatic and alicyclic hydrocarbons administered orally

No.	Flavouring agent	Species	Sex	LD_{50} (mg/kg bw)	Reference
1323	Camphene	Rat	NR	>5000	Moreno (1974a)
1324	β-Caryophyllene	Rat	M, F	>5000	Wong & Hart (1971)
1326	*d*-Limonene	Mouse	M, F	5600 (M) 6600 (F)	Tsuji et al. (1975a)
1326	*d*-Limonene	Rat	M	>5000	Moreno (1972a)
1326	*d*-Limonene	Rat	M, F	4400 (M) 5200 (F)	Tsuji et al. (1975a)
1327	Myrcene	Rat	M	>5000	Moreno (1972b)
1328	α-Phellandrene	Rat	M	5700	Moreno (1972c)
1328	α-Phellandrene	Rat	M, F	1.87 ml/kg (1590[a])	Brownlee (1940)
1330	β-Pinene	Rat	NR	>5000	Moreno (1975)
1331	Terpinolene	Rat	NR	4.39 ml/kg (3753[b])	Levenstein (1975)
1336	Bisabolene	Rat	NR	>5000	Moreno (1974b)
1336	Bisabolene	Mouse	M, F	>13360	Hoffmann-LaRoche (1967)
1337	Valencene	Rat	M	>5000	Moreno (1980)
1338	3,7-Dimethyl-1,3, 6-octatriene	Rat	M, F	5000	Moreno (1976a)
1339	*p*-Mentha-1,3-diene	Rat	NR	1680	Moreno (1973a)
1340	*p*-Mentha-1,4-diene	Rat	NR	3650	Moreno (1973b)
1341	1,3,5-Undecatriene	Mouse	M, F	>2000	Pellmont (1973)
1341	1,3,5-Undecatriene	Rat	M, F	>8000	Pellmont (1973)
1342	δ-3-Carene	Rat	M	4800	Moreno (1972d)
1346	Cadinene	Rat	NR	>5000	Keating (1972)
1347	Guaiene	Rat	NR	>5000	Moreno (1976b)

F, female; M, male; NR, not reported.
[a] Calculated using a density of α-phellandrene of 0.85 (0.835–0.865) g/ml (Lewis, 1999).
[b] Calculated using a density of terpinolene of 0.855 g/ml (Lewis, 1999).

All except one animal at 3300 or 6600 mg/kg bw per day died within 3 days of study initiation. At 1650 mg/kg bw per day, two animals died (one owing to gavage error). No treatment-related clinical signs were observed in mice that survived doses of 1650 mg/kg bw per day or lower, nor were treatment-related histopathological lesions observed (National Toxicology Program, 1990).

Groups of 10 male and 10 female $B6C3F_1$ mice were given *d*-limonene at a dose of 0, 125, 250, 500, 1000 or 2000 mg/kg bw per day in corn oil by gavage, 5 days per week for 13 weeks. The animals were observed twice per day and weighed once per week. Necropsies were performed on all animals. Histological examinations were performed on all control animals and on animals at the highest dose. Tissues examined included a whole range of organs and tissues. The study complied with GLP.

Table 4. Results of short-term studies of toxicity with aliphatic and alicyclic hydrocarbons used as flavouring agents

No.	Flavouring agent	Species, sex	No. of test groups[a]/no. per group[b]	Route	Duration (weeks)	NOEL (mg/kg bw per day)	Reference
1327	Myrcene	Mouse, M, F	5/20	Gavage	13	<250 (M) 250 (F)	National Toxicology Program (2004)[a]
1327	Myrcene	Rat, M, F	5/20	Gavage	13	<250	National Toxicology Program (2004)[b]
1330	β-Pinene[c]	Rat, M, F	1/10	Diet	2	8[d]	Shapiro (1988)
1341	1,3,5-Undecatriene[c]	Rat, M, F	1/10	Diet	2	2[d]	Shapiro (1988)

F, female; M, male.

[a] Long-term studies of toxicity and carcinogenicity with *d′*-limonene have not been summarized because an ADI 'not specified' was established for *d′*-limonene by the Committee at its forty-first meeting (Annex 1, reference *107*)[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] Administered as Galbelica, a formulation composed of 80% β-pinene and 20% 1,3,5-undecatriene. The dose of Galbelica given was 10 mg/kg bw per day.

[d] Only one dose was tested. As this dose produced no adverse effects, it is not a true NOEL, but the highest dose tested that had no adverse effects. The actual NOEL may be higher.

One male and two females at 2000 mg/kg bw per day, as well as one female at 500 mg/kg bw per day died before the end of the study. Several other animals died as a result of gavage error. At 1000 and 2000 mg/kg bw per day, male mice gained less weight than the control animals, with final body weights being 89–90% of those of the controls. Clinical signs of rough hair coats and decreased activity were observed for animals at the two highest doses. An alveolar cell adenoma was reported in the lung of one female at 2000 mg/kg bw per day (National Toxicology Program, 1990).

Rats

Groups of five male and five female F344/N rats were given *d*-limonene at a dose of 0, 413, 825, 1650, 3300, or 6600 mg/kg bw per day in corn oil by gavage for 12 days, over a 16-day period. The animals were observed twice per day and weighed once per week. Necropsies were performed on all animals. Histopathology was performed on the survivors in the group receiving the highest dose. The study complied with GLP.

All except two animals at 3300 or 6600 mg/kg bw per day died within 2 days of study initiation. At 1650 mg/kg bw per day, the animals gained less weight than the control animals, with final body weights being 90–92% of those of the controls. No treatment-related clinical signs or histopathological lesions were observed in rats receiving doses of 1650 mg/kg bw per day or lower (National Toxicology Program, 1990).

Groups of 9–10 male and 9–10 female Sprague Dawley JCL rats were given *d*-limonene (in 1% Tween 80) at oral doses of 0, 277, 554, 1385, or 2770 mg/kg bw per day for 1 month.

Final body weights and body-weight gains were reduced in all treated males (in a dose-dependent manner), and in females at 1385 mg/kg bw per day. Food consumption was also reduced in treated males. Blood and urine analysis revealed increases in haemoglobin, erythrocyte volume fraction, erythrocyte counts and total protein, and decreases in leukocyte count, alanine aminotransferase, total cholesterol, bilirubin and blood urea nitrogen. Changes were observed in both sexes, but to a greater extent in males than in females, and some changes were already observed at the lowest dose, 277 mg/kg bw per day. Absolute weights of the thymus, lungs, heart (males only), spleen, and ovaries were decreased, while absolute weights of the kidneys (males only), liver (females only), and adrenals were increased. Relative weights of these organs were sometimes also affected. Some organ weight changes were already observed at the lowest dose tested. No significant treatment-related changes compared with controls were observed upon histological examination, with the exception of granular cast formation in kidneys in males, starting at 277 mg/kg bw per day (Tsuji et al., 1975a).

Groups of 10 male and 10 female F344/N rats were given *d*-limonene at a dose of 0, 150, 300, 600, 1200, or 2400 mg/kg bw per day in corn oil by gavage, 5 days per week for 13 weeks. The animals were observed twice per day and weighed once per week. Necropsies were performed on all animals. Histological examinations were performed on all animals in the vehicle control group and at the highest dose, and all female rats at 1200 mg/kg bw per day. A range of organs and tissues

were examined. Kidneys were examined for all male rats. The study complied with GLP.

Nine female and five male rats at 2400 mg/kg bw per day died within the first week of the study. Male rats gained less weight than the control animals at the three highest doses, and final mean body weights at 600, 1200, or 2400 mg/kg bw per day were 94%, 88%, and 77% of those of the controls, respectively. The one surviving female at 2400 mg/kg bw per day also gained less weight, with a final body weight that was 11% lower than that of the controls. Rough hair coats, lethargy, and excessive lacrimation were observed for animals at the two highest doses. Nephropathy with a dose-dependent increase in severity was noted in all treated male rats. The nephropathy was characterized by degeneration of epithelium in the convoluted tubules, granular casts within tubular lumens, and regeneration of the tubular epithelium. Hyaline droplets were observed in the epithelium of the proximal convoluted tubules in all groups of male rats, including controls. Re-evaluation by two pathologists confirmed that there was no increase in the number of these droplets in treated males compared with controls (National Toxicology Program, 1990).

Special studies on kidney toxicity of d-limonene

In a short-term study to investigate renal effects, groups of five male F344 rats (aged 10 weeks) were given *d*-limonene at a dose of 0, 75, 150, or 300 mg/kg bw per day in corn oil via gavage, 5 days per week, for a total of 5 or 20 doses. Observations included daily and final body weights, weekly food intake, relative and absolute liver and kidney weights, and light microscopy of the liver and kidneys. The kidneys were examined for hyaline droplet formation, granular cast formation, and nephrosis. Examinations were conducted on animals that were killed on day 6 (after five doses) and day 27 (after 20 doses). In addition, two-dimensional gel electrophoresis evaluation of protein profiles was conducted on samples from the kidneys of animals in the control group and in the group receiving the intermediate dose killed on day 6.

No indications of toxicity were seen upon daily 'in-life' observations and gross necropsy, and weight gain and food consumption were comparable to those of controls. On days 6 and 27 of the study, increases in relative weights of the liver (not dose-dependent) and the kidney (dose-dependent) were observed in all treated rats, reaching statistical significance only at the highest dose. Light microscopy revealed no effects of *d*-limonene on the liver, but the kidney showed a dose-dependent exacerbation of the minimal hyaline droplet formation observed in controls, of approximately similar severity on days 6 and 27. On day 27, hyaline droplet formation was accompanied by granular cast formation (dose-dependent) and nephrosis. Compared with controls, rats at the intermediate dose had significantly greater concentrations of α_{2u}-globulin in renal cortical tissues (Kanerva et al., 1987).

A further study investigating the renal effects of *d*-limonene was carried out by Webb et al. (1989). In the first experiment, groups of three male and three female Fischer 344 rats (aged 12 weeks) received radiolabelled *d*-limonene in a single

dose at 0 or 200 mg/kg bw in corn oil by gavage. The animals were sacrificed 24 h after dosing and kidneys were removed and examined histopathologically. In the second experiment, groups of male Fischer 344 rats (aged 6 weeks) received *d*-limonene at a dose of 0, 2, 5, 10, 30, or 75 mg/kg bw per day in corn oil by gavage, 5 days per week for 13 weeks. Interim necropsies were conducted on five rats per group on days 8, 15, 22, and 29 in the control group and the groups receiving a dose of 10 (days 8 and 15 only) and 75 mg/kg bw per day. At the end of the 90-day study, 10 rats at each dose were sacrificed. At necropsy, liver and kidney weights were recorded, and a range of tissues were processed for histopathology. On a daily basis, rats were observed for signs of toxicity and body weights were recorded. Food consumption was recorded weekly.

In the first experiment, treatment resulted in an increase in the incidence and severity of hyaline droplets in the kidneys of males only. This was associated with an increase in α_{2u}-globulin and a greater accumulation of radiolabel in the renal cortex. Females had no hyaline droplets or α_{2u}-globulin accumulation.

In the second experiment, relative weights of the liver and kidney were increased in a dose-dependent manner at 30 and 75 mg/kg bw per day, with statistical significance reached only at the highest dose. No histopathological changes were noted in the livers of treated rats, while examination of the kidneys revealed changes characterized as hyaline droplet formation, granular casts and multiple cortical changes, all of which were classified as chronic nephrosis. Exacerbation of hyaline droplet formation and chronic nephrosis were time- and dose-dependent, and already observed at the earliest necropsy on day 8 of treatment (Webb et al., 1989).

After evaluation of a study of carcinogenicity with *d*-limonene in rats (see section (c) below), a supplemental short-term study was initiated to investigate effects on the rat kidney. Groups of 12 male and 12 female F344/N rats (aged 18 weeks) were given *d*-limonene at a dose of 0, 75, 150, 300, 600, or 1200 mg/kg bw per day in corn oil by gavage for 14 days, over a 21-day period. The animals were observed twice per day and weighed once per week. Microscopic examination of kidney sections from these rats indicated a treatment-related increase in intracytoplasmic granules in the proximal convoluted tubules of dosed male rats, but not of female rats. The granules were shown to contain α_{2u}-globulin by immunohistochemical staining for protein. α_{2u}-Globulin was shown to be increased in kidney homogenates from dosed male rats by enzyme-linked immunosorbent assay (ELISA) (National Toxicology Program, 1990).

Dogs

Groups of three male and three female beagle dogs were given *d*-limonene at a dose of 0, 0.4, 1.2, or 3.6 ml/kg bw per day (equivalent to approximately 0, 340, 1000, or 3000 mg/kg bw per day, respectively) by oral administration for 6 months.

Frequent vomiting and nausea were reported at the two higher doses. At the end of treatment, females at the two higher doses and males at the highest dose had lost weight. Food consumption was not affected. Several changes were

observed upon analysis of blood (after 1, 3 and 6 months of treatment) and urine (after 1 and 6 months), and determination of absolute and relative organ weights, with some of them already present at the lowest dose tested. However, as most changes were without a dose–response relationship, some were more pronounced in one sex than the other, statistics were not performed, and in some cases starting values already differed considerably between groups, it is difficult to interpret the findings. The authors only reported decreased total cholesterol and blood sugar concentrations in both males and females at the highest dose. Histological examinations revealed an increase in protein cast formation in the renal tubules of females and males at doses of 340 and 1000 mg/kg bw per day, respectively, but no other remarkable findings in other organs examined (Tsuji et al., 1975b).

The findings in the kidney reported in the previous study were not confirmed in a later study, in which groups of five male and five female beagle dogs (aged 10–15 months) were given *d*-limonene at a dose of 0 (tap water only), 0.12, or 1.2 ml/kg bw per day (equivalent to approximately 0, 100, or 1000 mg/kg bw per day) by gavage for 6 months. The highest dose was determined in a pilot study to be close to the maximum tolerated dose for emesis. To minimize emesis, the total daily doses were divided into two equal amounts and administered in the morning and the afternoon.

Treatment with *d*-limonene was associated with excretion of soft faeces. Diarrhoea and emesis occurred periodically, with the same frequency in animals at the lowest and highest doses. Food consumption and final body weights were unaffected by treatment. Absolute and relative weights of the kidney were increased with a dose-dependent trend, reaching statistical significance at the highest dose only in females (absolute and relative weight) and males (relative weight only). Haematology and clinical chemistry determinations at 1, 3 and 6 months, and urine analysis at 6 months revealed no treatment-related differences other than a 35% increase in serum cholesterol and a twofold increase in serum alkaline phosphatase activities in animals at the highest dose. Neither the gross post-mortem examinations nor the histological examinations revealed any evidence of treatment-related alterations in a whole range of tissues and organs. Specifically, no hyaline droplet nephropathy was observed (Webb et al., 1990).

(ii) Myrcene (No. 1327)

Mice

In a 13-week study of toxicity, groups of 20 male and 20 female B6C3F_1 mice (10 of each sex for the core study, 10 of each sex for the clinical chemistry part of the study) were given myrcene at a dose of 0, 250, 500, 1000, 2000, or 4000 mg/kg bw per day by gavage, on weekdays only. Animals were observed twice per day for moribundity and death, and clinical observations were recorded weekly. Weight measurements were taken initially and then weekly until termination of the study. On day 23, blood was drawn from the clinical chemistry study mice for clinical chemistry analysis, and at week 13 from all surviving animals in the core study for haematology and clinical chemistry analysis. At termination, all mice in the core study were necropsied and weights of heart, right kidney, liver, lung, right testis,

and thymus were recorded. A full histopathological examination was conducted on animals in the control group and in the groups receiving a dose of 1000, 2000, or 4000 mg/kg bw per day. In addition, the liver, forestomach, and kidneys were examined histopathologically to a no-effect level.

All animals at 4000 mg/kg bw per day died or were killed in a moribund condition within the first 3 days of treatment, while nine out of ten male and eight out of ten female mice at 2000 mg/kg bw per day died or were killed in a moribund condition before week 5 of treatment. Clinical signs of toxicity in these animals included lethargy, abnormal breathing, or thin appearance. All other animals appeared normal and survived until study termination. Compared with the controls, the surviving animals in the group receiving a dose of 2000 mg/kg bw per day had decreased body weights. In all other treatment groups, body weights were not affected. Effects on organ weights were mainly observed in the surviving animals at 2000 mg/kg bw per day: in the one male, the relative weights of liver, lung and kidney were increased, as were the absolute and relative weights of the thymus; the two females had increased absolute and relative weights of the liver and kidney. In the other treatment groups, organ weight changes did not exceed 20% in males, while in females the relative kidney weight and the absolute and relative liver weights were increased by more than 20% at 1000 mg/kg bw per day only. Haematology analysis revealed very small decreases (1.5–6.2%) in erythrocytes, haemoglobin, and erythrocyte volume fraction and very small increases (<3%) in mean corpuscular volume and mean corpuscular haemoglobin at 1000 mg/kg bw per day in both sexes. In the few surviving animals at 2000 mg/kg bw per day, these changes were somewhat more pronounced (5.1–21%). Treatment also affected the number of reticulocytes, platelets and leukocytes, but the changes were not consistent between doses and/or between sexes. Clinical chemistry analysis revealed decreased blood urea nitrogen concentrations at 1000 mg/kg bw per day and decreased alanine aminotransferase and sorbitol dehydrogenase activity at 500 and 1000 mg/kg bw per day in both sexes. In the animals that died before termination of the study, minimal centrilobular hypertrophy and minimal to marked necrosis of the liver, irritation of the nose and forestomach, atrophy and/or necrosis of the bone marrow, spleen and thymus, and necrosis of the renal tubules were observed. Minimal centrilobular hypertrophy of the liver was also observed in females at 1000 and 2000 mg/kg bw per day (8 out of 10, and 2 survivors, respectively), and in males at 250, 500, 1000 and 2000 mg/kg bw per day (1 out of 10, 4 out of 10, 10 out of 10 and 1 survivor, respectively). Minimal to moderate forestomach epithelial hyperplasia was found in females at 500 and 1000 mg/kg bw per day (2 out of 10 in both cases) and in 2 out of 10 males at 1000 mg/kg bw per day. Some females also showed irritation of the nose at 1000 and 2000 mg/kg bw per day (2 out of 10 and 1 out of 2 survivors, respectively). Thymus atrophy was observed in surviving animals at 2000 mg/kg bw per day. Minimal nephropathy and adrenal cortex hyperplasia were observed in animals of all treatment groups, including controls. In males, minimal renal cytoplasmic vacuolation was also observed, with the incidence decreasing upon treatment (10 out of 10, 8 out of 10, and 1 out of 10 at 0, 250, and 500 mg/kg bw per day, respectively). The NOEL was <250 mg/kg bw per day for male mice, on the basis of liver hypertrophy. The

NOEL for female mice was 250 mg/kg bw per day, on the basis of forestomach lesions (National Toxicology Program, 2004a; draft results).

Rats

In a 13-week study of toxicity, groups of 20 male and 20 female F344 rats (10 of each sex for the core study, 10 of each sex for the clinical pathology part of the study) were given myrcene at a dose of 0, 250, 500, 1000, 2000, or 4000 mg/kg bw per day by gavage, on weekdays only. Animals were observed twice daily for moribundity and death, and clinical observations were recorded weekly. Weight measurements were taken initially and then weekly until termination of the study. On day 23, blood was drawn from the rats in the clinical pathology part of the study, and at week 13 from all surviving animals in the core study for haematology and clinical chemistry analysis. At termination, all rats in the core study were necropsied and weights of heart, right kidney, liver, lung, right testis, and thymus were recorded. A full histopathological examination was conducted on animals in the control group and in the groups receiving a dose of 2000, and 4000 mg/kg bw per day. In addition, the nose, lymph nodes, liver, Harderian gland, and kidneys were histopathologically examined to a no-effect level.

All animals at 4000 mg/kg bw per day died or were killed in a moribund condition within the first 12 days of treatment. Treatment-related mortality was also observed at 2000 mg/kg bw per day, with two male and four female rats dying or killed a moribund condition. Clinical signs of toxicity in these animals included lethargy, ruffled fur, abnormal breathing, or thin appearance. Three accidental deaths occurred at lower doses (one male and one female rat at 1000 mg/kg bw per day, and one male rat at 500 mg/kg bw per day). A decrease in mean bodyweight gain of more than 10% was observed in males (at 500 — only during the last week of treatment — 1000, and 2000 mg/kg bw per day), but not in females. Absolute and relative kidney weights were increased in a dose-dependent manner by more than 20% in male and female rats at all doses. In males, dose-dependent changes of more than 20% were also observed in relative weight of the liver (increased at 1000 and 2000 mg/kg bw per day), relative weight of the testis (increased at 2000 mg/kg bw per day), and absolute and relative weights of the thymus (decreased at 1000 — absolute weight only — and 2000 mg/kg bw per day). In females, absolute and relative weights of the liver were increased in a dose-dependent manner by more than 20% at 500 (relative weight only), 1000, and 2000 mg/kg bw per day. Haematology analysis revealed decreases of 25–35% in leukocytes and lymphocytes at day 23, but not at day 93, in males and females at 2000 mg/kg bw per day. Increases of more than 30% (not dose-dependent) in reticulocytes were reported in males and females at 500, 1000, and 2000 mg/kg bw per day. Treatment affected clinical chemistry parameters, but most changes were not consistent between day 23 and week 13, between doses, and/or between sexes. At week 13, both males and females at 2000 mg/kg bw per day had decreased concentrations of creatinine, as well as slightly decreased blood urea nitrogen concentrations. Males, but not females, also had decreased alanine aminotransferase and sorbitol dehydrogenase activities at 500, 1000 and 2000 mg/kg bw per day. In the animals that died before termination of the study, mild to moderate irritation of the nose and forestomach, minimal to marked atrophy of the spleen,

necrosis of the thymus, degeneration of the renal tubules (except in males at 4000 mg/kg bw per day), and porphyrin pigmentation of the Harderian gland were observed. In the surviving animals, minimal forestomach epithelial hyperplasia was also found in one female at 2000 mg/kg bw per day, and minimal to marked nose irritation in males at 500, 1000 and 2000 mg/kg bw per day (one out of nine, five out of nine and eight out of eight, respectively) and in females at 1000 and 2000 mg/kg bw per day (nine out of nine and six out of six, respectively). At 2000 mg/kg bw per day, minimal to mild atrophy of the spleen was observed in all surviving animals, and in one out of two females minimal necrosis of the thymus was also observed. Nephropathy was observed in animals in all treatment groups, including controls, but with a somewhat higher incidence and severity in males than in females. All surviving males in all treatment groups, including controls, showed minimal to moderate accumulation of hyaline droplets in the renal tubules. All surviving animals in all treatment groups, but not in controls, showed degeneration of the renal tubules of increasing severity, which was accompanied by minimal mineralization in some females. Minimal porphyrin pigmentation of the Harderian gland was observed in animals of all treatment groups, including controls, with a dose-dependent increase in incidence in males but not in females. On the basis of the kidney findings, which were observed at all doses, the NOEL was <250 mg/kg bw per day (National Toxicology Program, 2004b; draft results).

(iii) β-Pinene (No. 1330) and 1,3,5-undecatriene (No. 1341)

Rats

In a 14-day feeding study, groups of five male and five female young adult Sprague-Dawley rats were fed diets calculated to provide Galbelica at a dose of 10 mg/kg bw per day. Galbelica is a formulation composed of 80% β-pinene and 20% 1,3,5-undecatriene. It was administered in a vehicle called Pinene Beta Supra, in a ratio of 1(Galbelica):4 (vehicle). Two additional groups served as controls and were maintained on a basal diet, one group with and the other without the Pinene Beta Supra vehicle. Daily observations for gross signs of toxicity and mortality were made. Body weight and food consumption were recorded weekly. On day 14, all animals were sacrificed and subjected to gross necropsy. Liver and kidney weights were recorded. Histopathology was performed only when findings were remarkable upon gross necropsy. The study complied with GLP, and was certified for quality assurance.

All rats survived and no clinical signs of toxicity were observed. Food consumption, body weights, and liver and kidney weights were not significantly different between groups. At gross necropsy, all findings were reported to be unremarkable. The NOEL for Galbelica was 10 mg/kg bw per day (equivalent to 8 and 2 mg/kg bw per day for β-pinene and 1,3,5-undecatriene respectively), the highest dose tested (Shapiro, 1988).

(c) Long-term studies of toxicity and carcinogenicity

Long-term studies of toxicity and carcinogenicity were only available for *d*-limonene (No. 1326) (National Toxicology Program, 1990). The results of these

studies are described below (they were not summarized in tabular form because an ADI 'not specified' for *d*-limonene was established by the Committee at its forty-first meeting (Annex 1, reference *107*)).

Mice

In a study of carcinogenicity, which complied with GLP, groups of 50 male and 50 female B6C3F_1 mice were given *d*-limonene at a dose of 0, 250, or 500 mg/kg bw per day for males, or 0, 500, or 1000 mg/kg bw per day for females, in corn oil by gavage, 5 days per week for 103 weeks. The animals were observed twice per day and weighed once per week for 12 weeks and once per month thereafter. Necropsies were performed on all animals. Histopathological examinations were performed on all animals in the control group and at the higher dose, and on animals at the lower dose that died before the end of the study. In addition, histopathological examinations were performed on all grossly visible lesions and target organs or tissues at all doses. Tissues examined included a range of organs and tissues, including the liver in females at the lower dose.

No treatment-related clinical signs were observed. Treatment did not affect survival in males and females at the higher dose, or in females at the lower dose. In males at the lower dose, survival was significantly lower than that of controls (24 out of 50 compared with 33 out of 50, respectively). No effects on body weight were observed in male mice in both treatment groups or in female mice at the lower dose. Females at the higher dose had mean body weights that were 5–15% lower than those of controls from week 28 to study termination, resulting in a 25% lower mean body-weight gain over the whole treatment period. The only findings in male mice were significantly increased incidences of multinucleated hepatocytes and centrilobular liver cytomegaly at the higher dose. However, the incidences of hepatocellular adenomas or carcinomas (combined) in this group were not different from those in controls. In fact, no treatment-related increases in neoplasms were observed in this study. A significant decrease was observed in incidence of neoplasms of the anterior pituitary gland in females at the higher dose. Overall, the National Toxicology Program concluded that 'under the conditions of this 2-year gavage study, there was no evidence of carcinogenic activity of d-limonene for male B6C3F_1 mice that received 250 or 500 mg/kg or for female mice that received 500 or 1000 mg/kg' (National Toxicology Program, 1990).

Rats

In a study of carcinogenicity, which complied with GLP, groups of 50 male and 50 female F344/N rats were given *d*-limonene at a dose of 0, 75, or 150 mg/kg bw per day for males, and 0, 300, or 600 mg/kg bw per day for females, in corn oil by gavage, 5 days per week for 103 weeks. The animals were observed twice per day and weighed once per week for 12 weeks and once per month thereafter. Necropsies were performed on all animals. Histopathological examinations were performed on all animals in the control group and at the higher dose, on animals at the lower dose animals that died before the end of the study, and on all females at the lower dose. In addition, histopathological examinations were performed on all grossly visible lesions and target organs or tissues at all doses. Tissues exam-

ined included a range of organs and tissues, including the adrenal glands, kidney, liver, spleen, and testis in male rats at the lower dose.

No treatment-related clinical signs were observed. Treatment did not affect survival in males and females at the lower dose. At the higher dose, the survival of males was significantly higher than that of controls (40 out of 50 compared with 29 out of 50, respectively), while at the higher dose, the survival of females was significantly lower than that of controls (24 out of 50 compared with 42 out of 50, respectively). Animals at the higher dose had mean body weights that were 4–7% lower than those of controls (in males from week 2 to study termination, in females from week 28 to study termination), but this did not result in lower mean body-weight gains over the whole treatment period. Histopathology showed the kidney to be the primary target organ in male rats. Spontaneous nephropathy occurred in all males, including controls, but in treated males the incidence and severity were increased in a dose-dependent manner. In treated males, the incidences of mineralization of the renal papilla and focal hyperplasia of the transitional epithelium overlying the papilla were also increased in a dose-dependent manner, as was the incidence of tubular cell hyperplasia. Tubular cell adenomas and tubular cell adenomas or adenocarcinomas (combined) occurred with significant positive trends in treated males. These neoplasms were not observed in controls or in treated females. Some statistically significant effects were found in the uterus of female rats and in the testis, haematopoietic system, and skin of male rats. However, these findings were not considered to be treatment-related, owing to the absence of a dose–response relationship, low incidence in controls compared with historical controls, low survival of controls, and/or because incidences were within the range for historical controls. The finding of increased incidences of cataracts in males and females at the higher dose was attributed to the position of the cages, and therefore not considered to be treatment-related. Overall, the National Toxicology Program concluded that 'under the conditions of this 2-year gavage study, there was clear evidence of carcinogenic activity of d-limonene for male F344/N rats, as shown by increased incidences of tubular cell hyperplasia, adenomas, and adenocarcinomas of the kidney. There was no evidence of carcinogenic activity of d-limonene for female F344/N rats that received 300 or 600 mg/kg' (National Toxicology Program, 1990).

Relevance of d-limonene-induced kidney tumours to humans

During the first evaluation of *d*-limonene by the Committtee at its thirty-ninth meeting, there was an extensive discussion of the mechanism involved in the exacerbation of the spontaneously occurring nephropathy in male rats, with the subsequent occurrence of renal tumours. A number of mechanistic studies were evaluated (Annex 1, reference *102*), also taking into account an extensive review on this topic by the United States Environmental Protection Agency (Environmental Protection Agency, 1991). Based on these data, the Committee concluded that the postulated mechanism behind the male rat-specific renal tubule cell carcinogenesis, being α_{2u}-globulin-associated nephropathy following a specific, time-dependent, sequence of pathological changes, was probably not relevant to

humans, and that toxic end-points associated with this effect were not an appropriate basis for the derivation of an ADI for *d*-limonene (Annex 1, reference *101*).

A few years after this evaluation, IARC examined the scientific basis for possible species differences in mechanisms by which, among others, renal tubule cell tumours in male rats may be produced, and addressed also the predictive value of this type of tumour for the identification of carcinogenic hazards to humans (IARC, 1999a). In the resulting concensus document, a set of criteria was formulated which must be met before it can be concluded that an agent causes kidney tumours through an α_{2u}-globulin-associated response. The criteria are:

—Lack of genotoxic activity (agent and/or metabolite) based on an overall evaluation of in vitro and in vivo data;

—Male rat specificity for nephropathy and renal tumorigenicity;

—Induction of the characteristic sequence of histopathological changes in shorter-term studies, of which protein droplet accumulation is obligatory;

—Identification of the protein accumulating in tubule cells as α_{2u}-globulin;

—Reversible binding of the chemical or metabolite to α_{2u}-globulin;

—Induction of sustained increased cell proliferation in the renal cortex;

—Similarities in dose–response relationship of the tumour outcome with the histopathological end-points (protein droplets, α_{2u}-globulin accumulation, cell proliferation).

If a particular agent meets all these criteria, according to the consensus document it can subsequently be concluded that the production of renal cell tumours in male rats by an α_{2u}-globulin-associated response is not predictive of carcinogenic hazard to humans. α_{2u}-Globulin is not present in humans, and although closely related proteins may be present, differences in ligand-binding of these proteins compared with the unique binding properties of α_{2u}-globulin preclude their involvement in the characteristic protein droplet nephropathy (IARC, 1999a).

When IARC evaluated the carcinogenic hazard by *d*-limonene (IARC, 1999b), *d*-limonene fulfilled all the criteria, hence it was concluded that '. . . *d*-limonene produces renal tubular tumours in male rats by a non-DNA-reactive mechanism, through an α_{2u}-globulin-associated response. Therefore, the mechanism by which *d*-limonene increases the incidence of renal tubular tumours in male rats is not relevant to humans.'

The Committee at its present meeting thus confirmed its earlier position that the mechanism behind the induction of renal tubule cell carcinogenesis by *d*-limonene in male rats is α_{2u}-globulin-associated nephropathy, and that neither these tumours, nor the associated non-tumorigenic lesions are relevant to humans.

(d) Genotoxicity

Testing for genotoxicity has been performed on 10 of the 20 flavouring agents in this group (Nos 1323, 1324, 1326–1330, 1340, 1342, 1346). The results of these tests are summarized in Table 5 and described below.

Table 5. Results of studies of genotoxicity with aliphatic and alicyclic hydrocarbons used as flavouring agents

No.	Flavouring agent	End-point	Test object	Dose or concentration	Result	Reference
In vitro						
1323	Camphene	Reverse mutation	*S. typhimurium* TA98, TA100	0.05–100 µl/plate (42.1–84 200 µg/plate)[1]	Negative[b]	Rockwell & Raw (1979)
1323	Camphene	Reverse mutation	*S. typhimurium* TA98, TA100, UTH8413, UTH8414	10–1000 µg/plate	Negative[c]	Connor et al. (1985)
1323	Camphene	Sister chromatid exchange	Chinese hamster ovary K-1 cells	10–1000 µmol/l (1.4–136.2 µg/ml)[d,e]	Negative[f]	Sasaki et al. (1989)
1324	β-Caryophyllene	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0.1–150 µl/plate (90.4–135 525 µg/plate)[g]	Negative[c]	Jagannath (1984a)
1324	β-Caryophyllene	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Up to 150 000 µg/plate	Negative[c]	Heck et al. (1989)
1324	β-Caryophyllene	Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA1535	3.3–333 µg/plate −S9[h] 1–10 000 µg/plate +S9[h]	Negative[c]	National Toxicology Program (2004c)
1324	β-Caryophyllene	Sister chromatid exchange	Chinese hamster ovary K-1 cells	10–1000 µmol/l (2.0–204.4 µg/ml)[i,e]	Negative[f]	Sasaki et al. (1989)
1324	β-Caryophyllene	Unscheduled DNA synthesis	Rat hepatocytes	Up to 10 000 µg/ml	Negative	Heck et al. (1989)

1326	*d*-Limonene[j]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	0.03–30 μmol/plate (4.1–4087 μg/plate)[k,l]	Negative[c]	Florin et al. (1980)
1326	*d*-Limonene	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	0.3–33 μg/plate –S9; 10–3333 μg/plate +S9	Negative[c]	Haworth et al. (1983); National Toxicology Program (1990)
1326	*d*-Limonene	Reverse mutation	*S. typhimurium* TA98, TA100, UTH8413, UTH8414	10–500 μg/plate	Negative[c]	Connor et al. (1985)
1326	*d*-Limonene	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Up to 150000 μg/plate	Negative[c]	Heck et al. (1989)
1326	*d*-Limonene[j]	Reverse mutation	*S. typhimurium* TA102	Up to 5000 μg/plate	Negative[b]	Müller et al. (1993)
1326	*d*-Limonene	Forward mutation, (non-)reciprocal recombination	*Saccharomyces cerevisiae* MP1	Up to 230 mmol/l (31335 μg/ml)[k]	Negative[f]	Fahrig (1984)
1326	*d*-Limonene	Forward mutation	Mouse lymphoma L5178Y $Tk^{+/-}$ cells	Up to 100 μg/ml	Negative[c]	Heck et al. (1989)
1326	*d*-Limonene	Forward mutation	Mouse lymphoma L5178Y $Tk^{+/-}$ cells	Up to 100 μg/ml[m]	Negative[c]	Myhr et al. (1990); National Toxicology Program (1990)

Table 5. (contd)

No.	Flavouring agent	End-point	Test object	Dose or concentration	Result	Reference
1326	*d*-Limonene	Sister chromatid exchange	Chinese hamster ovary K-1 cells	10–333 μmol/l (1.4–45.4 μg/ml)[k,e]	Negative[f]	Sasaki et al. (1989)
1326	*d*-Limonene	Sister chromatid exchange	Chinese hamster ovary cells	15–162 μg/ml –S9; 16.2–162 μg/ml +S9	Negative[c]	Anderson et al. (1990); National Toxicology Program (1990)
1326	*d*-Limonene	Chromosomal aberration	Chinese hamster ovary cells	10–100 μg/ml –S9; 50–500 μg/ml +S9	Negative[c]	Anderson et al. (1990); National Toxicology Program (1990)
1326	*d*-Limonene[j]	Cell transformation	Syrian hamster embryo cells	0.1–100 μg/ml	Negative	Pienta (1980)
1326	*d*-Limonene[j]	Cell transformation	Syrian hamster embryo cells	0.1–3 mmol/l (13.6–408.7 μg/ml)[k]	Positive[n]	Rivedal et al. (2000)
1327	Myrcene	Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA1535	33–3 333 μg/plate –S9°; 33–10 000 μg/plate +S9°	Negative[c]	National Toxicology Program (2004d)
1327	Myrcene	Gene mutation	Chinese hamster V79 *Hprt* cells	100–1 000 μg/ml	Negative[c]	Kauderer et al. (1991)
1327	Myrcene	Sister chromatid exchange	Human lymphocytes	100–1 000 μg/ml	Negative[c]	Kauderer et al. (1991)
1327	Myrcene	Sister chromatid exchange	Chinese hamster V79 cells	100–500 μg/ml –S9; 500 μg/ml +S9	Negative[c]	Röscheisen et al. (1991)
1327	Myrcene	Sister chromatid exchange	Hepatic tumour cells	100–500 μg/ml	Negative[p]	Röscheisen et al. (1991)

1327	Myrcene	Chromosomal aberration	Human lymphocytes	100–1 000 μg/ml	Negative[c]	Kauderer et al. (1991)
1328	α-Phellandrene	Sister chromatid exchange	Chinese hamster ovary K-1 cells	33.3–1 000 μmol/l (4.5–136.2 μg/ml)[q]	Negative[f]	Sasaki et al. (1989)
1329	α-Pinene	Reverse mutation	*S. typhimurium* TA98, TA100	0.05–100 μl/plate (43–85 920 μg/plate)[r]	Negative[b]	Rockwell & Raw (1979)
1329	α-Pinene	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	0.03–30 μmol/plate (4.1–4 087 μg/plate)[s,l]	Negative[c]	Florin et al. (1980)
1329	α-Pinene	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0.1–25 μl/plate (85.9–21 480 μg/plate)[r,t]	Negative[c]	Jagannath (1984b)
1329	α-Pinene	Reverse mutation	*S. typhimurium* TA98, TA100, UTH8413, UTH8414	10–500 μg/plate	Negative[c]	Connor et al. (1985)
1329	α-Pinene	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Up to 25 000 μg/plate	Negative[c]	Heck et al. (1989)
1329	α-Pinene	Unscheduled DNA synthesis	Rat hepatocytes	Up to 10 000 μg/ml	Negative	Heck et al. (1989)
1330	β-Pinene	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	0.03–30 μmol/plate (4.1–4 087 μg/plate)[u,v]	Negative[c]	Florin et al. (1980)
1330	β-Pinene	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	0.01–5 μl/plate (8.6–4 320 μg/plate)[w,x]	Negative[c]	DeGraff (1983)

Table 5. (contd)

No.	Flavouring agent	End-point	Test object	Dose or concentration	Result	Reference
1330	β-Pinene	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Up to 5 000 μg/plate	Negative[c]	Heck et al. (1989)
1330	β-Pinene	Sister chromatid exchange	Chinese hamster ovary K-1 cells	33.3–1 000 μmol/l (4.5–136.2 μg/ml)[u]	Negative[f]	Sasaki et al. (1989)
1340	*p*-Mentha-1,4-diene	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	Up to 50 000 μg/plate	Negative[c]	Heck et al. (1989)
1340	*p*-Mentha-1,4-diene	Unscheduled DNA synthesis	Rat hepatocytes	Up to 30 μg/ml	Negative	Heck et al. (1989)
1342	δ-3-Carene	Reverse mutation	*S. typhimurium* TA98, TA100, TA102	1.25–5 μl/plate (1 078–4 314 μg/plate)[v]	Positive[z]	Kurttio et al. (1990)
1346	Cadinene[j]	Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA1535, TA1537	1–100 μg/plate –S9[1]; 100–10 000 μg/plate +S9[1]	Negative; Positive[2]	National Toxicology Program (2004e)
1346	Cadinene[3]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	10–3 333 μg/plate –S9; 100–10 000 μg/plate +S9[o]	Negative[c]	Haworth et al. (1983); National Toxicology Program (2004f)

1346	Cadinene[3]	Forward mutation	Mouse lymphoma L5178Y $Tk^{+/-}$ cells	0.005–0.05 μg/ml −S9 (4.6–46.2 μg/ml)[4,5]; 0.01–0.08 μl/ml +S9 (9.2–73.9 μg/ml)[4,6]	Negative[c]	National Toxicology Program (2004g)
1346	Cadinene[3]	Sister chromatid exchange	Chinese hamster ovary cells	8.9–26.6 μg/ml −S9; 22.2–31.1 μg/ml +S9	Equivocal; Negative	National Toxicology Program (2004h)
1346	Cadinene[3]	Chromosomal aberration	Chinese hamster ovary cells	24.9–35.5 μg/ml −S9; 30.2–40 μg/ml +S9	Negative[c]	National Toxicology Program (2004h)
In vivo						
1326	*d*-Limonene	Mammalian spot test	Mouse (C57BLxT) embryos	215 mg/kg bw[7]	Negative	Fahrig (1984)
1327	Myrcene	Chromosomal aberration	Rat bone marrow cells	100–1 000 mg/kg bw[8]	Negative[9]	Zamith et al. (1993)
1327	Myrcene	Micronucleus formation	Mouse peripheral blood	250–2 000 mg/kg bw[10]	Negative	NTP (2004i)

[a] Calculated using a density of camphene of 0.842 g/ml (Lewis, 1999).
[b] With metabolic activation.
[c] With and without metabolic activation.
[d] Calculated using relative molecular mass of camphene of 136.24.
[e] Cytotoxicity observed at the highest dose/concentration tested.
[f] Without metabolic activation.
[g] Calculated using a density of β-caryophyllene of 0.9035 (0.897–0.910) g/ml (Lewis, 1999).
[h] Precipitation or slight toxicity was occasionally observed at the higher concentrations tested.
[i] Calculated using relative molecular mass of β-caryophyllene of 204.36.
[j] Isomer not specified.
[k] Calculated using relative molecular mass of *d*-limonene of 136.24.
[l] Cytotoxicity and precipitation observed at doses >3 μmol/plate.
[m] In some trials concentrations ≥50 μg/ml were lethal.

Table 5. (contd)

[n] Although not statistically significant ($p = 0.089$), a fourfold increase in transformation frequency was observed.
[o] Slight toxicity was occasionally observed at the highest concentration tested.
[p] Slight increase in sister chromatid exchanges, which was reproducible but not dose-dependent.
[q] Calculated using relative molecular mass of α-phellandrene of 136.24.
[r] Calculated using a density of α-pinene of 0.8592 g/ml (Lewis, 1999).
[s] Calculated using relative molecular mass of α-pinene of 136.24.
[t] Cytotoxicity observed at doses of 2.5 to 25 μg/plate, depending on the different tester strains.
[u] Calculated using relative molecular mass of β-pinene of 136.24.
[v] Cytotoxicity observed at doses >3 μmol/plate.
[w] Calculated using a density of β-pinene of 0.864 g/ml (Lewis, 1999).
[x] Cytotoxicity observed at doses of 2.5 to 5 μl/plate, depending on the different tester strains.
[y] Calculated using a density of δ-3-carene of 0.8627 (0.8586–0.8668) g/ml (Merck, 1996).
[z] Positive without metabolic activation in TA100 and TA102 at doses ≥2.5 μl/plate; negative with metabolic activation in all strains.

1. Slight toxicity was observed at various doses.
2. Equivocal/weak positive only in TA97 and TA100 with metabolic activation.
3. β-Cadinene was tested.
4. Calculated using a density of β-cadinene of 0.9239 g/ml (Merck, 1996).
5. The highest concentration of 0.05 μl/ml was lethal.
6. In some trials, concentrations ≥ 0.04 μl/ml were lethal.
7. Administered via injection into the peritoneal cavity of the dam.
8. Administered via gavage.
9. A dose-related increase in mitotic index was observed, but no clastogenicity.
10. Administered via gavage for 90 days.

(i) In vitro

No evidence of mutagenicity was observed in Ames assays when camphene (No. 1323; up to 84 200 μg/plate), β-caryophyllene (No. 1324; up to 150 000 μg/plate), *d*-limonene (No. 1326; up to 150 000 μg/plate), myrcene (No. 1327; up to 10 000 μg/plate), α-pinene (No. 1329; up to 25 000 μg/plate), β-pinene (No. 1330; up to 5000 μg/plate), or *p*-mentha-1,4-diene (No. 1340; up to 50 000 μg/plate) were incubated with *Salmonella typhimurium* strains TA97, TA98, TA100, TA1535, TA1537, TA1538, and/or UTH8413, UTH8414, or TA102 with and without metabolic activation (Rockwell & Raw, 1979; Florin et al., 1980; DeGraff, 1983; Haworth et al., 1983; Jagannath, 1984a, 1984b; Connor et al., 1985; Heck et al., 1989; National Toxicology Program, 1990; Müller et al., 1993; National Toxicology Program, 2004c, 2004d). Without metabolic activation, δ-3-carene (No. 1342) at doses of between 2157 and 4314 μg/plate gave positive results in the Ames assay in *S. typhimurium* strains TA100 and TA102, but gave negative results in both strains with metabolic activation (Kurttio et al., 1990). δ-3-Carene at doses of up to 4314 μg/plate also gave negative results in *S. typhimurium* strain TA98 with and without metabolic activation (Kurttio et al., 1990). In one Ames assay with *S. typhimurium* strains TA98, TA100, TA1535 and TA1537, the β isomer of cadinene (No. 1346) gave negative results at doses of up to 10 000 and 3333 μg/plate, respectively, with and without metabolic activation (Haworth et al., 1983; National Toxicology Program, 2004f). In another Ames assay, cadinene (isomer not specified) gave equivocal/weak positive results at doses of up to 10 000 μg/plate in *S. typhimurium* strains TA97 and TA100 with metabolic activation, but gave negative results at doses of up to 100 μg/plate in both strains without metabolic activation, as well as in strains TA98, TA1535 and TA1537 with and without metabolic activation (National Toxicology Program, 2004e).

Camphene (No. 1323; 1.4–136.2 μg/ml), β-caryophyllene (No. 1324; 2.0–204.4 μg/ml), α-phellandrene (No. 1328; 4.5–136.2 μg/ml), and β-pinene (No. 1330; 4.5–136.2 μg/ml) did not induce sister chromatid exchanges (SCE) in Chinese hamster ovary cells without metabolic activation (Sasaki et al., 1989).

β-Caryophyllene (No. 1324; up to 10 000 μg/ml), α-pinene (No. 1329; up to 10 000 μg/ml), and *p*-mentha-1,4-diene (No. 1340; up to 30 μg/ml) did not induce unscheduled DNA synthesis in rat hepatocytes (Heck et al., 1989).

d-Limonene (No. 1326) did not induce genetic effects when tested in *Saccharomyces cerevisiae* strain MP1, without metabolic activation, at concentrations of up to 230 mmol/l (Fahrig, 1984). In Chinese hamster ovary cells, *d*-limonene did not induce chromosomal aberrations at concentrations of 10–500 μg/ml, or SCE at concentrations of 1.4–162 μg/ml, with and without metabolic activation (Sasaki et al., 1989; Anderson et al., 1990; National Toxicology Program, 1990). In an assay for forward mutation in mouse lymphoma cells, *d*-limonene produced negative results in L5178Y cells with and without metabolic activation, at a concentration of up to 100 μg/ml (Heck et al., 1989; Myhr et al., 1990; National Toxicology Program, 1990). When incubated with Syrian hamster embryo cells, limonene (isomer not specified) did not induce cell transformation in one assay at a concentration of up to 100 μg/ml (Pienta, 1980), while in another assay concentrations up

to 408.7 μg/ml increased the transformation frequency, albeit not in a statistically significant manner (Rivedal et al., 2000). The latter study also tested the effects of limonene (isomer not specified) on gap junction intercellular communication in Syrian hamster embryo cells. Limonene at concentrations of 1.4–136.2 μg/ml did not show effects (Rivedal et al., 2000).

With and without metabolic activation, myrcene (No. 1327) did not induce gene mutations at the hypoxanthine guanine phosphoribosyl transferase (*Hprt*) locus in Chinese hamster V79 cells, at concentrations of up to 1000 μg/ml (Kauderer et al., 1991), nor did it induce SCE in these cells at concentrations up to 500 μg/ml (Röscheisen et al., 1991). Myrcene also did not induce SCE or chromosomal aberrations in human lymphocytes, with and without metabolic activation, at concentrations of up to 1000 μg/ml (Kauderer et al., 1991), nor did it induce SCE in hepatic tumour cells, at concentrations of up to 500 μg/ml, although a slight, reproducible but not dose-dependent increase was noted (Röscheisen et al., 1991).

The β isomer of cadinene (No. 1346) gave negative results in an assay for forward mutation in mouse lymphoma cells , at concentrations of up to 46.2 μg/ml without metabolic activation, and at up to 73.9 μg/ml with metabolic activation (National Toxicology Program, 2004g). In Chinese hamster ovary cells, this β isomer did not induce chromosomal aberration with or without metabolic activation at concentrations of 24.9–40 μg/ml, or SCE with metabolic activation at concentrations of up to 31.1 μg/ml (National Toxicology Program, 2004h). Without metabolic activation, an equivocal result was obtained for induction of SCE, at concentrations up to 26.6 μg/ml (National Toxicology Program, 2004g).

In a study conducted in vivo-in vitro, designed to investigate the mutagenicity of urinary metabolites of a number of food additives, Sprague-Dawley rats were given a single dose of 0.5 ml of camphene (No. 1323; approximately 1684 mg/kg bw) and α-pinene (No. 1329; approximately 1718 mg/kg bw) via gavage and the urine was collected for 24 h. Three types of urine sample were tested in the Ames assay with *S. typhimurium* strains TA98 and TA100 with metabolic activation: a direct urine sample, a urine-ether extract, and the aqueous fraction of the urine-ether extract. The urine samples of rats treated with α-pinene did not show any evidence of mutagenicity, either in the presence or absence of β-glucuronidase. Of the urine samples of camphene-treated rats only the urine-ether extract showed a weak mutagenic response, and only in TA100, not in TA98 (Rockwell & Raw, 1979).

(ii) In vivo

In a mammalian spot test, no evidence of mutagenicity was observed in mouse C57BLxT embryos in utero after intraperitoneal injection of the dam with *d*-limonene (No. 1326) at a dose of 215 mg/kg bw per day on days 9 and 10 of gestation (Fahrig, 1984).

In an assay for cytogenetic changes in bone marrow, groups of Wistar rats (two or four of each sex per group) were given myrcene (No. 1327) at a dose of 100, 500, or 1000 mg/kg bw via gavage. A negative control group (two rats of each sex) received only the vehicle (corn oil) via gavage, while a positive control group (two

rats of each sex) received cyclophosphamide at a dose of 30 mg/kg bw via intraperitoneal injection. A mitotic inhibitor (colchicine, administered at a dose of 5 mg/kg bw via intraperioneal injection) was administered 1 h before sacrifice at 24 or 48 h after treatment, at which time the bone-marrow cells were harvested. Compared with the negative control group, treatment with myrcene did not result in an increase of metaphase cells with chromosomal aberrations upon examination at 24 or 48 h. In contrast, in the positive control group chromosomal aberrations were found in 19% of the bone-marrow metaphase cells examined. Although not clastogenic, myrcene caused a dose-dependent increase in the mitotic index in bone-marrow cells, indicating that it was present at a sufficient dose in the target tissue (Zamith et al., 1993).

An assay for micronucleus formation in mouse peripheral blood erythrocytes was performed, with samples of peripheral blood obtained within 24 h of the final exposure in a 13-week study of toxicity in which male and female B6C3F_1 mice were given myrcene (No. 1327) at a dose of up to 2000 mg/kg bw per day via gavage. Scoring of 1000 normochromatic erythrocytes (NCEs) for micronuclei revealed no increase in micronucleated NCEs at any dose (National Toxicology Program, 2004i).

(iii) Conclusion

Seven substances in this group of flavouring agents have been tested in the Ames assay and found not to be mutagenic in bacteria in vitro. One flavouring agent, δ-3-carene, produced a positive result in this assay, only without metabolic activation, in *S. typhimurium* strains TA100 and TA102 but not TA98. Another flavouring agent, cadinene (isomer not specified), gave weakly positive results in the Ames assay, only with metabolic activation, in *S. typhimurium* strains TA97 and TA100 but not TA98, TA1535, and TA1537.

In mammalian cell systems, predominantly negative results were obtained for representative members of this group with respect to induction of SCE, chromosomal aberrations, unscheduled DNA synthesis, and gene mutations. In assays for cell transformation in Syrian hamster embryo cells, limonene (isomer not specified) gave negative results in one assay, but weak positive results in another, the increase in transformation frequency being not statistically significant.

Myrcene and *d*-limonene showed no signs of genetic toxicity in cytogenetic assays for micronucleus formation in bone marrow and peripheral erythrocytes (myrcene) and a mammalian spot test (*d*-limonene) performed in vivo.

On the basis of the results of available studies of genotoxicity, the Committee concluded that the flavouring agents in this group of aliphatic and alicyclic hydrocarbons are not genotoxic.

(e) Reproductive toxicity

(i) d-Limonene (No. 1326)

Three groups of 20 female Wistar rats were given *d*-limonene (in 1% gum arabic solution) at a dose of 0, 591, or 2869 mg/kg bw per day by oral administra-

tion on days 9–15 of gestation. Fifteen animals per group were sacrificed at day 20 of gestation. The remaining five animals per group were allowed to give birth to their offspring, and the offspring were followed until postnatal week 7.

Maternal toxicity was noted at the higher dose only, evidenced by mortality (8 out of 20 animals died compared with none of the 20 animals in the control group and in the group receiving the lower dose) and decreased body-weight gain. The fetuses of dams at the higher dose exhibited delayed ossification of the metacarpal bone and the proximal phalanx. This was reported to be restored to normal within several weeks after birth. At the higher dose, offspring showed decreases in postnatal weight gain in males, in absolute and relative weights of the thymus and spleen in males and females, and in absolute and relative weights of the ovary in females. No statistically significant differences were observed in physical signs of postnatal development (opening of the ear-shell, eyelid and vaginal orifice, odontiasis, descending of the testis, and coating with hair) at the doses tested (Tsuji et al., 1975c).

In a similar experiment, groups of 20 pregnant SLC-ICR mice were given *d*-limonene at a dose of 0, 591, or 2363 mg/kg bw per day by oral administration on days 7–12 of gestation. Fifteen animals per group were sacrificed at day 18 of gestation. The remaining five animals per group were allowed to give birth to their offspring, and the offspring was followed until postnatal week 7.

Compared with controls, dams at the higher dose gained less weight during administration, but weight gain was normal after treatment stopped. A similar effect was seen in dams at the lower dose, but to a smaller degree. At the higher dose, also the numbers of implantations and live fetuses were slightly decreased, and the fetal weights were slightly decreased at both doses. External and visceral examination of the fetuses did not reveal treatment-related changes. However, increased incidences of lumbar and fused ribs, as well as delayed ossification of some bones were observed in fetuses of the group receiving the higher dose. A delay in ossification of the middle phalanx was also observed in fetuses of the group receiving the lower dose. All retarded ossifications were restored to normal during postnatal development. Males born to dams in the group receiving the higher dose showed a significant decrease in postnatal body-weight gain. Several organ weights were affected by treatment (e.g. testes, ovaries, liver), mostly at the higher dose, but also incidently at the lower dose. Histology of the testes and ovaries did not reveal any abnormalities. No statistically significant differences were observed in physical signs of postnatal development at the doses tested (Kodama et al., 1977a).

The same research group also studied the developmental effects of *d*-limonene in rabbit fetuses and offspring. Groups of 13–21 pregnant Japanese White rabbits were given *d*-limonene at a dose of 0, 250, 500, or 1000 mg/kg bw per day by oral administration from days 6–18 of gestation. Ten to 18 animals per group were sacrificed on day 28 of gestation. The remaining three animals per group were allowed to give birth to their offspring, and the offspring were followed until postnatal week 7.

Signs of maternal toxicity included increased mortality at the highest dose (6 out of 21 animals died as compared with none of the 13 animals in the control

group and in other treatment groups) and a temporary decrease in body-weight gain and food consumption at the intermediate and highest dose. No abnormalities were noted in any of the fetuses upon external examination. Visceral and skeletal examinations revealed some anomalies (such as incomplete lobulation of the lungs, lumbar rib variations, and retarded ossification of the middle phalanx and fifth sternebrae) in all treatment groups, including controls, but without a dose–response relationship. Moreover, these anomalies were restored to normal during postnatal development. Postnatal weight gain was decreased in female offspring at the intermediate dose but not at the lowest or highest doses. Treatment did not affect opening of the ear-shell and eyelids, odontiasis or coating with hair (Kodama et al., 1977b).

(ii) Myrcene (No. 1327)

Groups of 16–29 female Wistar rats were given myrcene (dissolved in corn oil) at a dose of 250, 500, or 1200 mg/kg bw per day by gavage on days 6–15 of gestation. Control rats (16 in total) received either the vehicle only, or no treatment at all. All animals were sacrificed on day 20 of gestation. The gravid uterus was weighed, and the number of implantation sites, living and dead fetuses, resorptions, and corpora lutea were recorded. The fetuses were weighed, and examined for external, visceral, and skeletal abnormalities.

Signs of maternal toxicity were observed only at the highest dose and consisted of decreased maternal body-weight gain (especially during the first days of treatment) and gravid uterus weight, and mortality in one dam. Embryo- and fetotoxicity were also only observed at the highest dose, evidenced by reduced numbers of visible implantation sites and live fetuses, and increased incidences of fetuses with delayed ossification and other skeletal malformations. The NOEL for both maternal and embryo-fetotoxicity was 500 mg/kg bw per day (Delgado et al., 1993a).

In a follow-up study designed to test for peri- and postnatal developmental toxicity in rats, groups of 12–18 pregnant Wistar rats were given myrcene (in corn oil) at a dose of 250, 500, 1000, or 1500 mg/kg bw per day by gavage from day 15 of gestation until weaning of the offspring on postnatal day 21. A control group of 20 animals received the vehicle only, by gavage. The progeny were examined for mortality, weight gain, physical signs of postnatal development (ear unfolding, incisor eruption, fur development and eye opening), and when they reached maturity, for reproductive capacity.

Maternal toxicity was mainly evident at the highest dose; 5 of the 15 dams died within 4 days of treatment, and all dams showed a weight deficit at term which persisted after delivery (postnatal day 1). At 1000 mg/kg bw there was also a weight deficit observed at term, but this was no longer detectable on postnatal day 1. Necropsy of the dams revealed hyperkeratosis in the forestomach in most rats at the two higher doses. While the duration of gestation, litter size, and post-weaning mortality did not differ significantly between groups, the duration of labour was increased at 500 and 1000 mg/kg bw per day, as was the number of stillbirths at these doses. There was a dose-dependent decrease in birth weight at doses of 500 mg/kg bw and greater, which gradually returned to control values at weaning. Postnatal mortality was increased, especially during the first week of lactation, and

developmental landmarks were delayed at 500, 1000 and 1500 mg/kg bw per day. In addition, female offspring at the two higher doses displayed impaired fertility. The male sex organs were not affected by treatment, nor was the sperm count. The NOEL for peri- and postnatal toxicity was 250 mg/kg bw per day (Delgado et al., 1993b).

In a one-generation study, groups of 60 Wistar rats (15 male, 45 female) were given myrcene (in peanut oil) at a dose of 0, 100, 300, or 500 mg/kg bw per day by gavage. Males were treated for 91 days before mating and during the mating period, while females were treated continuously from 21 days before mating until the offspring were weaned at 21 days after birth. Males were sacrificed after the mating period, and one-third of the females in each group were sacrificed on day 21 of gestation. The weight of the gravid uterus was recorded, resorptions, living and dead fetuses, and implantation sites were counted, and all living fetuses were weighed and examined for external and skeletal abnormalities. The remaining pregnant females were allowed to give birth to their offspring, and were sacrificed after weaning (postnatal day 21). The numbers of viable and dead newborns were counted, and the pups were sexed, weighed on days 1, 6, 11, 16 and 21 and examined for signs of physical development.

The only effects observed in male rats were slight but statistically significant increases in the relative and absolute weights of the liver and kidney in the group receiving the highest dose. Body weight and body-weight gain were reduced at this dose, although not statistically significantly. Treatment did not affect the mating or pregnancy index, and maternal toxicity was not observed, except for slightly increased liver and kidney weights. However, at the highest dose a significant increase in the number of resorptions and a parallel decrease in the number of live fetuses per implantation site were observed, as well as a significant increase in the number of fetuses with skeletal malformations. The authors noted that most of the malformations that were observed (e.g. fused os zygomatic, dislocated sternum, and extra lumbar ribs) also occurred with high spontaneous frequency in the controls and historical controls of the strain of rats used. No effects were seen on maternal weight changes during gestation and lactation, and on offspring weight changes during lactation. During postnatal days 2–21, pup mortality was slightly increased at 500 mg/kg bw per day, but the increase was not statistically significant. Slight delays were noted in eye opening, incisor eruption, and primary coat appearance of offspring of dams treated with myrcene, but without a dose–response relationship. The NOEL was 300 mg/kg bw per day (Paumgartten et al., 1998).

(iii) Rowachol®

Groups of 12–17 JCL-Sprague Dawley rats were given Rowachol® at a dose of 0.16, 0.8, or 1.6 ml/kg bw per day by oral administration on days 9–14 of gestation. Rowachol is a liquid terpene mixture containing *l*-menthol (32%), α,β-pinene (17%), menthone (6%), borneol (5%), *d*-camphene (5%), cineol (2%), rheochrysin (0.1%), and olive oil (32.9%). A control group of 21 rats received olive oil at a oral dose of 0.80 ml/kg bw per day. Autopsies were performed on day 20 of gestation.

No adverse effects were observed at 0.16 and 0.8 ml/kg bw per day. In the group receiving a dose of 1.6 ml/kg bw per day, the dams lost weight during treatment, but gained weight again after treatment stopped. Placental weight and the numbers of implantations and live fetuses were reduced, as were fetal weight and body weight at birth. Within 1 week after birth, however, weight gain of offspring was comparable to that of control offspring. The fetuses of the group receiving the highest dose did not show any retarded ossification or gross or visceral anomalies, nor were there significant increases in the incidences of skeletal malformations. The NOEL for Rowachol® for maternal and embryo-fetotoxicity was 0.8 ml/kg bw per day (Hasegawa & Toda, 1978).

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AROMATIC HYDROCARBONS

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of five aromatic hydrocarbons (Table 1) by the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, p 192). One member of this group, biphenyl (No. 1332), was evaluated by the Committee at its eighth meeting (Annex 1, reference *8*) and was assigned an acceptable daily intake (ADI) for its use as a fungistatic agent. The fungistatic use of biphenyl was also evaluated by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) in 1966 and 1967 (WHO, 1967; WHO, 1968), when an ADI of 0–0.125 mg/kg bw was established.

Four (Nos 1325, 1332, 1333 and 1335) of the five flavouring agents in this group have been reported to occur naturally in foods. They have been detected in, for example, coffee, alcoholic beverages, baked and fried potato, heated beans, tea, bread and cheese (Nijssen et al., 2003). The substance with the highest natural occurrence is *p*-cymene (No. 1325).

1.2 Estimated daily intake

The total annual volume of production of the five flavouring agents in this group is approximately 7800 kg in Europe (International Organization of the Flavor Industry, 1995) and 3600 kg in the United States of America (USA) (National Academy of Sciences, 1989; Lucas et al., 1999) (see Table 2). More than 98% of the total annual volume of production in Europe and the USA is accounted for by the monoaromatic terpene hydrocarbon *p*-cymene. The estimated daily intakes of *p*-cymene in Europe and the USA are approximately 1100 μg/person and 470 μg/person, respectively. The reported annual volumes of production of the remainder of the flavouring agents in this group are low to very low. The estimated daily intakes of these agents range from 0.001 to 21 μg/person in Europe and the USA. The estimated daily per capita intake of each agent is reported in Table 1.

1.3 Absorption, distribution, metabolism and elimination

Being lipophilic, the aromatic hydrocarbons in this group are likely to cross biological membranes by passive diffusion. Available data on *p*-cymene and biphenyl indicated that these materials are readily absorbed from the gastrointestinal tract, widely distributed in the body, metabolized and excreted mainly in the urine.

On the basis of the available data, it is anticipated that the aromatic hydrocarbons in this group will participate in similar pathways of metabolic detoxification in mammals, including humans. After absorption, these hydrocarbons are oxidized to polar oxygenated metabolites via cytochrome P450 (CYP) enzymes and alcohol and aldehyde dehydrogenases. The major metabolic pathway of aromatic terpene hydrocarbons involves hepatic microsomal CYP-mediated oxidation of ring sidechains, yielding alcohols, aldehydes, and acids. The metabolites are then conjugated with glycine, glucuronic acid, or glutathione, and excreted in the urine and/or bile. The biotransformation of biphenyl proceeds via ring hydroxylation, preferentially at the C4 position, yielding phenolic derivatives that are subsequently metabolized to glucuronide and sulfate conjugates, which are excreted in the urine.

1.4 Application of the procedure for the safety evaluation of flavouring agents

Step 1. In applying the Procedure, the Committee assigned two (Nos 1325 and 1333) of the five flavouring agents in this group to structural class I. The remaining three flavouring agents (Nos 1332, 1334 and 1335) were assigned to structural class III (Cramer et al., 1978).

Table 1. Summary of the results of safety evaluations of aromatic hydrocarbons[a] used as flavouring agents

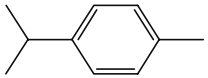
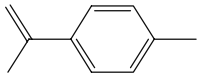
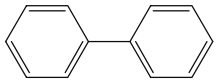
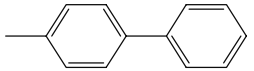
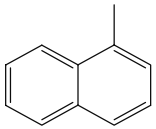
Flavouring agent	No.	CAS No. and structure	Step A3[b] Does intake exceed the threshold for human intake?	Comments	Conclusion based on current intake
Structural class I					
p-Cymene	1325	99-87-6 	No Europe: 1085 USA: 472	See note 1	No safety concern
p-α-Dimethylstyrene	1333	1195-32-0 	No Europe: 21 USA: 0.3	See note 1	No safety concern
Structural class III					
Biphenyl	1332	92-52-4 	No Europe: 0.001 USA: 0.7	See note 2	No safety concern
4-Methylbiphenyl	1334	644-08-6 	No Europe: 0.01 USA: 0.08	See notes 1, 2	No safety concern

Table 1. (Contd)

Flavouring agent	No.	CAS No. and structure	Step A3[b] Does intake exceed the threshold for human intake?	Comments	Conclusion based on current intake
1-Methylnaphthalene	1335	90-12-0 	No Europe: 0.9 USA: 0.06	See notes 1, 2	No safety concern

CAS: Chemical Abstracts Service.

[a] Step 2: All the agents in this group are expected to be metabolized to innocuous products.

[b] The thresholds for human intake for structural classes I and III are 1800 μg/person per day and 90 μg/person per day, respectively. All intake values are expressed in μg/person per day. The combined intake of flavouring agents in structural class I is 1106 μg/person per day in Europe and 472 μg/person per day in the USA. The combined intake of flavouring agents in structural class III is 0.9 μg/person per day in Europe and 0.8 μg/person per day in the USA.

Notes:

1. Side-chain oxidation followed by subsequent conjugation with glycine, glucuronic acid, or glutathione.
2. Ring hydroxylation yielding phenolic derivatives that are subsequently metabolized to glucuronide and sulfate conjugates, which are excreted in the urine.

Table 2. Annual volumes of use of aromatic hydrocarbons used as flavouring agents in Europe and the USA

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		μg/day	μg/kg bw per day		
***p*-Cymene (1325)**					
Europe	7602	1085	18		
USA	3583	472	8	29302	8
Biphenyl (1332)					
Europe	0.01	0.001	0.00002		
USA[e]	4	0.7	0.01	401	100
***p*,α-Dimethylstyrene (1333)**					
Europe	144	21	0.3		
USA	2	0.3	0.004	8	4
4-Methylbiphenyl (1334)					
Europe	0.1	0.01	0.0002		
USA[e]	0.45	0.08	0.001	—	NA
1-Methylnaphthalene (1335)					
Europe	6	0.9	0.01		
USA	0.45	0.06	0.001	218	484
Total					
Europe	7752				
USA	3590				

NA, not applicable; +, reported to occur naturally in foods (Nijssen et al., 2003), but no quantitative data available; –, not reported to occur naturally in foods.

[a] From International Organization of the Flavor Industry (1995) and Lucas et al. (1999) or National Academy of Sciences (1989).

[b] Intake expressed as μg/person per day was calculated as follows: [(annual volume, kg) × (1×10^9 μg/kg)/(population × survey correction factor × 365 days)], where population (10%, "eaters only") = 32×10^6 for Europe and 26×10^6 for the USA. The correction factor = 0.6 for Europe, and 0.8 for the USA, representing the assumption that only 60% and 80% of the annual volume of the flavour, respectively, was reported in the poundage surveys (International Organization of the Flavor Industry, 1995; Lucas et al., 1999; National Academy of Sciences, 1989).
Intake expressed as μg/kg bw per day was calculated as follows: [(μg/person per day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.

[c] Quantitative data for the USA reported by Stofberg & Grundschober (1987).

[d] Consumption ratio was calculated as follows: (annual consumption in food, kg)/(most recent reported volume as a flavouring agent, kg).

[e] The volume cited is the anticipated annual volume, which was the maximum amount of flavour estimated to be used annually by the manufacturer at the time the material was proposed for flavour use. National surveys (National Academy of Sciences 1970, 1975, 1976, 1982 or 1987, as reported in National Academy of Sciences, 1989; Lucas et al., 1999) revealed no reported use as a flavouring agent at that time.

Step 2. All the flavouring agents in this group are expected to be metabolized to innocuous products. The evaluation of all agents in this group therefore proceeded via the A-side of the decision-tree.

Step A3. The estimated daily per capita intakes of the two flavouring agents in structural class I and the three flavouring agents in structural class III are all below the thresholds of concern (i.e. 1800 μg for class I and 90 μg for class III). According to the Procedure, the use of these five flavouring agents raises no safety concern at estimated current intakes.

The intake considerations and other information used to evaluate the five aromatic hydrocarbons in this group according to the Procedure are summarized in Table 1.

1.5 Consideration of secondary components

All five flavouring agents in this group have minimum assay values of >95%. Hence, it is not necessary to consider secondary components.

1.6 Consideration of combined intakes from use as flavouring agents

In the event that the two agents in structural class I were consumed concurrently on a daily basis, the estimated combined intake would not exceed the human intake threshold of 1800 μg/person per day for class I. In the event that all three agents in structural class III were consumed concurrently on a daily basis, the estimated combined intake would not exceed the human intake threshold of 90 μg/person per day for class III. Overall evaluation of the data indicated that combined intake would not raise a safety concern.

1.7 Conclusions

The Committee concluded that none of the flavouring agents in this group of aromatic hydrocarbons would present safety concerns at current estimated intakes. The Committee noted that all the available data on toxicity and metabolism of the flavouring agents in the group were consistent with the results of the safety evaluation.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

The relevant background information summarizes the key scientific data applicable to the safety evaluation of five aromatic hydrocarbons used as flavouring agents (see Table 1).

2.2 Additional considerations on intake

Production Volumes of production and intake values for each flavouring agent are reported in Table 2.

Four of the five flavouring agents in the group have been reported to occur naturally in traditional foods (Nijssen et al., 2003; Table 2). Quantitative data on natural occurrence have been reported for all four agents (Stofberg & Grundschober, 1987). The consumption of all these agents is derived predominantly from their presence in traditional foods (i.e. they have a consumption ratio of ≥1; Table 2).

2.3 Biological data

2.3.1 Biochemical data

(a) Absorption, distribution, and excretion

In male Wistar rats or Dunkin Hartley guinea-pigs given *p*-cymene (No. 1325) orally at a dose of 100 mg/kg bw, 80% or 71%, respectively, of the administered dose was excreted in the form of extractable metabolites in the urine within the following 48 h. It was speculated that the rest of the dose was either excreted via the faeces or as unextractable metabolites in the urine (Walde et al., 1983). In a study in a single male Japanese white rabbit given *p*-cymene as a single oral dose at 670 mg/kg bw, Ishida et al. (1981) observed a total urinary excretion of 20% of the administered dose as neutral or acidic metabolites within 72 h.

In male albino rats given biphenyl (No. 1332) as a single oral dose at 100 mg/kg bw, nearly 30% of the administered dose was excreted in the urine within 4 days. Approximately equal amounts (5% of the administered dose) were recovered from the faeces and bile within 24 h. The metabolites found in excreta and bile mainly consisted of several mono-, di- and trihydroxy-metabolites of biphenyl. Biphenyl itself was present only in very small amounts in the urine, and was not detectable in faeces or bile. Most of the biotransformation products were excreted within 24–48 h after dosing (Meyer & Scheline, 1976). In a subsequent experiment following the same procedures, male guinea-pigs and rabbits were given biphenyl at the same oral dose. After 4 days, approximately 33% and 49% of the administered dose was excreted in the urine of guinea-pigs and rabbits, respectively, with most being excreted within the first 24–48 h, and only as biphenyl metabolites, since no unchanged biphenyl was detected. In the guinea-pig, approximately 20% of the dose was recovered in the faeces, nearly 75% of which was biphenyl itself. The rabbit excreted only 1.6% of the administered dose in the faeces, almost 90% of which was unmetabolized biphenyl. Small amounts of the administered dose were excreted via bile (3.3% and 0.3% in guinea-pigs and rabbits, respectively). No biphenyl was detected in bile of either species (Meyer, 1977). The guinea-pig thus exhibited a much higher level of faecal excretion of unchanged biphenyl than either the rat or the rabbit, suggesting decreased absorption compared with the other species.

Rats fed biphenyl at 1% in the diet (equivalent to a dose of 500 mg/kg bw per day), until a total of 15 g of diphenyl had been consumed, excreted nearly 60% of the total dose in the urine during the feeding period and the 48 h thereafter (West et al., 1956).

Six male rabbits given biphenyl as a single oral dose of 1000 mg in corn oil by gavage excreted nearly 65% of the administered dose in the urine as free phenolic

metabolites or glucuronic acid conjugates within 4 days after dosing (Block & Cornish, 1959).

In summary

Being lipophilic, the aromatic hydrocarbons in this group are likely to cross biological membranes by passive diffusion. Available data on *p*-cymene and biphenyl indicate that these materials are readily absorbed from the gastrointestinal tract, widely distributed in the body, metabolized and excreted mainly in the urine.

(b) Metabolism

The main metabolites in the urine of rabbits given *p*-cymene (No. 1325) orally were *p*-cymen-9-ol and *p*-cymen-8-ol (50% and 28%, respectively, of the neutral metabolites). Acidic metabolites identified were α-*p*-tolylpropionic acid, α-tolyl-α-hydroxylpropionic acid, *p*-isopropylbenzoic acid and *p*-1-hydroxyisopropylbenzoic acid. Ring hydroxylation did not occur (Ishida et al., 1981).

In male rats given *p*-cymene orally at a dose of 100 mg/kg bw, the principal urinary metabolites were *p*-isopropylbenzoic acid (19% of the administered dose) and 2-*p*-carboxyphenylpropionic acid (16%). Other less important urinary metabolites included 2-*p*-tolylpropan-1-ol (8%), 2-*p*-olylpropan-2-ol (9%), 2-*p*-carboxyphenylpropan-2-ol (9%), 2-*p*-(hydroxymethyl)phenylpropionic acid (4%), 2-*p*-carboxyphenylpropan-1-ol (11%), *p*-isopropylbenzoylglycine (2%), *p*-isopropylbenzyl alcohol (1%), and 2-*p*-tolylpropionic acid (1%) (Walde et al., 1983). When the same dose was given to male guinea-pigs, similar urinary metabolites were identified, however in different quantities. The primary urinary metabolite in guinea-pigs was *p*-isopropylbenzoylglycine (31%), indicating that conjugation with glycine was more prevalent in guinea-pigs than in rats. Another major metabolite in guinea-pigs was 2-*p*-tolylpropan-2-ol (14%). In addition, while ring hydroxylation of *p*-cymene was not reported in rats (Bakke & Scheline, 1970; Walde et al., 1983) and rabbits (Ishida et al., 1981), trace amounts of the ring hydroxylation metabolites carvacrol and hydroxycarvacrol were detected in the urine in guinea-pigs. Ring hydroxylation in guinea-pigs only occurred *ortho* to the methyl group (Walde et al., 1983).

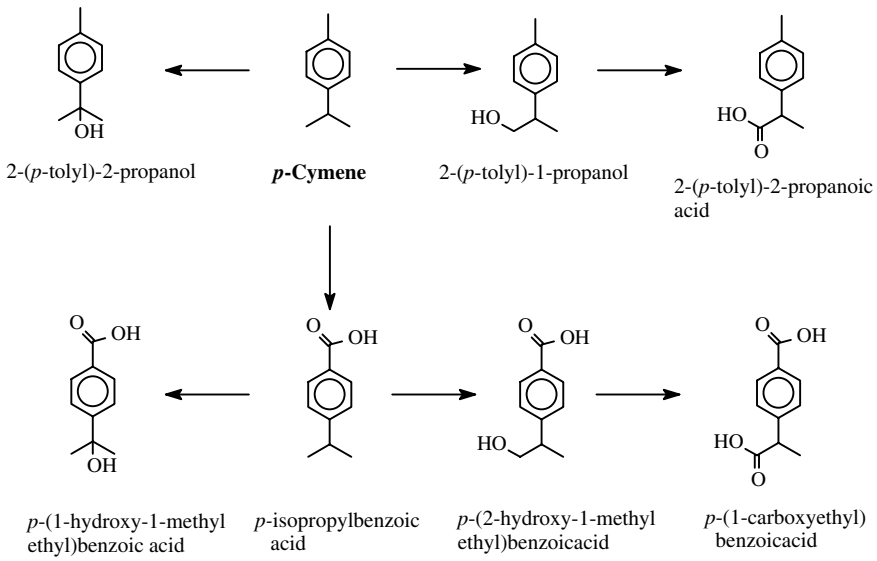
Boyle et al. (1999) studied the metabolite pattern of *p*-cymene in rats after oral administration of doses equivalent to 50 and 200 mg/kg bw. The major metabolites in the urine 0–48 h after administration of a dose of 50 mg/kg bw were 2-*p*-tolypropan-2-ol (39% of the recovered dose) and 2-*p*-carboxyphenylpropan-2-ol (19% of the recovered dose). The former metabolite is the product of allylic hydroxylation of the isopropyl substituent, while the latter metabolite is the product of allylic hydroxylation of both the isopropyl substituent and the methyl substituent. Minor metabolites in rat urine were 2-*p*-carboxyphenylpropan-1-ol (10%), 2-*p*-carboxyphenylpropionic acid (14%), and *p*-isopropylbenzoic acid (17%). A large percentage of the urinary metabolites at this dose was conjugated (66% conjugated versus 34% free). The same metabolites were observed after the highest dose, but conjugation was considerably reduced (18% conjugated versus 82% free), suggesting saturation of the conjugation pathway (Boyle et al., 1999).

In a study designed to identify the stereochemistry of *p*-cymene metabolites, four rabbits were given 2.5 g of *p*-cymene, after which urine was collected for 72 h. Seven different hydroxylated and carboxylated metabolites were recovered in the urine. Four were optically active and identified as 2-(*p*-tolyl)-1-propanol, 2-(*p*-tolyl)-propanoic acid, *p*-(2-hydroxy-1-methylethyl)benzoic acid, and *p*-(1-carboxyethyl) benzoic acid. Three were optically inactive and identified as 2-(*p*-tolyl)-2-propanol, *p*-isopropylbenzoic acid, and *p*-(1-hydroxy-1-methylethyl)benzoic acid. Oxidation of the methyl group of the isopropyl substituent yielded 2-(*p*-tolyl)-1-propanol in an *R/S* ratio of 65:35. The (*R*)-alcohol is then further oxidized to the corresponding acid (*R*)-2-(*p*-tolyl)propanoic acid, which undergoes complete stereochemical inversion to (*S*)-2-(*p*-tolyl)propanoic acid. Subsequently, the alcohol or acid metabolite may undergo oxidation of the tolyl methyl group to yield the corresponding hydroxy acid and diacid, respectively. If the tolyl methyl is oxidized before the isopropyl group, no stereochemical inversion is observed upon oxidation of the isopropyl side-chain. On the basis of the observed stereochemical changes, it was concluded that *omega*-hydroxylation of *p*-cymene or *p*-isopropylbenzoic acid occurs preferentially at the *pro-S*-methyl group of the isopropyl substituent (Matsumoto et al., 1992).

According to these studies, *p*-cymene undergoes extensive oxidation of the methyl substituent and isopropyl side-chain to yield polar oxygenated metabolites (Figure 1). These metabolites are either excreted unchanged in the urine, or undergo conjugation with glucuronic acid and/or glycine, followed by excretion in the urine.

Studies in vitro with supernatants of homogenized rat and mouse liver (Creaven & Parke, 1966) and rat hepatocytes (Wiebkin et al., 1976) have shown that

Figure 1. Metabolism of p-cymene



biphenyl (No. 1332) is largely metabolized by hydroxylation at C4, followed by conjugation and/or further hydroxylation. Hydroxylation at C2 in biphenyl occurred to a much smaller extent. Studies in rats in vivo (oral and intraperitoneal administration of biphenyl) and mice (intraperitoneal administration only) confirmed these findings, i.e. more 4-hydroxybiphenyl than 2-hydroxybiphenyl (both as conjugates) was present in the urine (Creaven & Parke, 1966).

In another study in rats, several hydroxylated metabolites of biphenyl were present in the urine after oral administration of biphenyl. The major metabolites were 4,4′-dihydroxybiphenyl and 4-hydroxybiphenyl, but several other mono-, di-, and trihydroxy compounds were reported, including 2- and 3-hydroxybiphenyl, 3,4- and 3,4′-dihydroxybiphenyl, and 3,4,4′-trihydroxybiphenyl. Trace amounts of the 3- and 4-methyl ethers of 3,4-dihydroxybiphenyl and 3,4,4′-trihydroxybiphenyl were also detected. Only small amounts were present as free phenolic compounds; nearly all the metabolites in urine were detected after enzymatic hydrolysis. The most prominent metabolites present in the bile of these rats were the conjugates of 4-hydroxybiphenyl, 4,4′-dihydroxybiphenyl and 3,4,4′-trihydroxybiphenyl (Meyer & Scheline, 1976).

West et al. (1956) identified diphenylmercapturic acid as an additional (minor) metabolite in the urine of biphenyl-treated rats, as well as 4-hydroxybiphenyl (plus conjugate), 4,4′-dihydroxybiphenyl and 3,4-dihydroxybiphenyl, demonstrating that formation of arene oxide is the initial step in the metabolism of biphenyl.

Studies in rabbits and guinea-pigs showed that most of an oral dose of biphenyl is excreted in the urine as 4-hydroxybiphenyl conjugated with either sulfate or glucuronic acid. Di- and/or trihydroxy metabolites were also detected in the urine in rabbits, but in much smaller amounts than previously reported for the rat. In guinea-pigs, only small amounts of dihydroxy metabolites and no trihydroxy metabolites were seen, (Meyer, 1977).

It can be concluded that the metabolism of biphenyl proceeds via ring hydroxylation, followed by conjugation and/or further hydroxylation and subsequent conjugation.

In summary

On the basis of the available data, it is anticipated that the aromatic hydrocarbons in this group will participate in similar pathways of metabolic detoxication in mammals, including humans. After absorption, these hydrocarbons are oxidized to polar oxygenated metabolites via CYP enzymes and alcohol and aldehyde dehydrogenases.

The major metabolic pathway of aromatic terpene hydrocarbons involves hepatic microsomal CYP-mediated oxidation of ring side-chains, yielding alcohols, aldehydes, and acids. The metabolites are then conjugated with glycine, glucuronic acid, or glutathione, and excreted in the urine and/or bile. The biotransformation of biphenyl proceeds via ring hydroxylation, preferentially at the C4 position, yielding phenolic derivatives that are subsequently metabolized to glucuronide and sulfate conjugates, which are excreted in the urine.

2.3.2 Toxicological studies

(a) Acute toxicity

Oral median lethal dose (LD_{50}) values have been reported for three of the five substances in this group, one being tested in both rats and rabbits, and the other two only in rats (see Table 3). The only reported oral LD_{50} value in rabbits was 2410 mg/kg bw (Deichmann et al., 1947). In rats, oral LD_{50} values ranged from 2570 to 5040 mg/kg bw (Deichmann et al., 1947; Pecchiai & Saffiotti, 1957; Jenner et al., 1964; Posternak et al., 1975; Clark et al., 1979; Hasegawa et al., 1989). These LD_{50} values indicate that the acute oral toxicity of aromatic hydrocarbons is low.

(b) Short-term studies of toxicity

Short-term studies of toxicity were available for three of the five substances in this group (Booth et al., 1961; Posternak et al., 1969, 1975). The results of these studies are summarized in Table 4 and described below.

(i) Biphenyl (No. 1332)

In order to further investigate the nephrotoxic effects of biphenyl as observed in a long-term study of toxicity in rats (see section 2.3.2(c)), a study was carried out to determine the extent of potential kidney damage in relation to dose and exposure time and to assess the reversibility of the injuries. Groups of 42 male and 42 female rats (strain not specified) were given diets containing 0, 0.1, 0.25, or 0.5% biphenyl, calculated (Food and Drug Administration, 1993) to provide average daily intakes of 0, 50, 125, or 250 mg/kg bw. Urine was collected periodically from five rats of each sex per group. After 30, 60, and 120 days of treatment, five rats of each sex per group were sacrificed and sections of the kidney were taken and prepared for histopathological examination. After 165 days of treatment, 10 rats of each sex at the highest dose were returned to the control diet; five rats were subsequently killed 30 days later, while the other five were killed 60 days later.

Table 3. Studies of the acute toxicity of aromatic hydrocarbons administered orally

No.	Flavouring agent	Species	Sex	LD_{50} (mg/kg bw)	Reference
1325	*p*-Cymene	Rat	M, F	4750	Jenner et al. (1964)
1332	Biphenyl	Rat	M, F	3150 (M) 3550 (F)	Hasegawa et al. (1989)
1332	Biphenyl	Rat	NR	3280	Deichmann et al. (1947)
1332	Biphenyl[a]	Rat	F	4100	Clark et al. (1979)
1332	Biphenyl	Rat	F	5040	Pecchiai & Saffiotti (1957)
1332	Biphenyl	Rabbit	NR	2410	Deichmann et al. (1947)
1334	4-Methylbiphenyl	Rat	M, F	2570	Posternak et al. (1975)

F, female; M, male; NR, not reported.
[a] Dowtherm A, a mixture of diphenyl (26%) and diphenyl oxide (72%), was tested.

Table 4. Results of short-term studies of toxicity and long-term studies of toxicity and carcinogenicity with aromatic hydrocarbons used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/No. per group[b]	Route	Duration	NOEL (mg/kg bw per day)	Reference
Short-term studies of toxicity							
1332	Biphenyl[c]	Rat; M, F	3/84	Diet	165 days	50	Booth et al. (1961)
1333	*p*,α-Dimethylstyrene	Rat; M, F	1/20–32	Diet	90 days	0.625[d]	Posternak et al. (1969)
1334	4-Methylbiphenyl	Rat; M, F	1/32	Diet	90 days	8.73[d]	Posternak et al. (1975)
Long-term studies of toxicity and carcinogenicity							
1332	Biphenyl	Mouse; M, F	1/72[e]	Diet	18 months	2.5[d]	Innes et al. (1969)
1332	Biphenyl	Rat; M, F	7/30	Diet	750 days	50	Ambrose et al. (1960)
1332	Biphenyl	Rat; M, F	3/100	Diet	105 weeks	25	Umeda et al. (2002)

F, female; M, male.

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both males and females.

[c] Special study on kidney toxicity.

[d] Only one dose was tested. As this dose produced no adverse effects, it is not a true NOEL, but is the highest dose tested that had no adverse effects. The actual NOEL may be higher.

[e] Two strains of mice were tested, 18 males and 18 females of each strain.

Compared with the controls, rats at the highest dose displayed an increase in urinary volume as well as urine turbidity and precipitate beginning on days 27 and 28, and continuing progressively until day 165. The same effect, but to a lesser degree, was seen in the group receiving the intermediate dose, but not in the group receiving the lowest dose. Within 10 days of returning to the control diet, the volumes of urine, turbidity and precipitate in rats at the highest dose decreased markedly and returned to normal within 30 days. Histopathological examinations of the kidney preparations revealed abnormalities that were only observed at the highest dose, with the exception of a single prominent lesion found in one female rat at the intermediate dose. After 30 days of treatment, the kidney lesions found at the highest dose included several small cysts and dilated tubules in the medulla and inner cortex of one male, and mild local tubular dilation with some epithelial flattening in two females. The frequency and severity of histopathological effects on the kidney increased as the study continued. After 60 days of treatment, the kidneys of three males and all females showed distinct lesions. After 120 days of treatment, all rats exhibited distinct focal tubular dilation of the kidneys, involving only a few tubules in females but several in males. Some small atrophic tubules and some cellular fibrous tissue were observed next to the dilated tubules. Upon returning to the control diet after 165 days of exposure to biphenyl, rats showed a regression of tubular dilation with scar formation, but the regression was more pronounced in males than in females. The no-observed-effect level (NOEL) was 50 mg/kg bw per day, since at this dose there was no evidence of either polyuria or histopathological damage to the kidney (Booth et al., 1961).

(ii) p-α-dimethylstyrene (No. 1333)

In a study designed to test the toxicity of 42 flavouring agents at a level equivalent to 100 times the maximum estimated daily dietary intake of a human with a body weight of 50 kg, groups of 10–16 male and 10–16 female Charles River CD rats were given diets containing *p*-α-dimethylstyrene for 90 days, resulting in a mean intake of 0.625 mg/kg bw per day. Body weight and food consumption were measured weekly and the efficiency of food utilization was calculated. Haematology examinations (on haemoglobin, erythrocyte volume fraction, erythrocyte count, and total and differential leukocyte counts) and blood urea determinations were carried out on half the animals at week 7 and on all animals at week 13. At autopsy, liver and kidney weights were measured and gross and histological examinations were carried out on a wide range of organs (not specified).

No effects of *p*-α-dimethylstyrene on any of the tested parameters were reported. The NOEL was 0.625 mg/kg bw per day, the highest dose tested (Posternak et al., 1969).

(iii) 4-methylbiphenyl (No. 1334)

In a study that was similar to that described above, in which 12 flavouring agents were tested, groups of 16 male and 16 female Charles River Sprague Dawley CD rats were given diets containing 4-methylbiphenyl for 90 days, resulting in a mean intake of 8.73 mg/kg bw per day.

Statistically significant, but very slight (<10%) decreases were observed in the mean terminal body weight of male rats and in food efficiency for female rats. No effects of 4-methylbiphenyl were reported on haematology parameters, on blood urea level, or on weights of the liver and kidney. Gross and histological examinations on a range of specified organs/tissues did not reveal any effects. The NOEL was 8.73 mg/kg bw per day, the highest dose tested (Posternak et al., 1975).

(c) Long-term studies of toxicity and carcinogenicity

Long-term studies of toxicity and carcinogenicity were only available for biphenyl (Ambrose et al., 1960; Innes et al., 1969; Umeda et al., 2002). The results of these studies are summarized in Table 4 and described below.

(i) Biphenyl (No. 1332)

Mice

In a study designed to evaluate the tumorigenicity of a series of substances used in industrial and agricultural applications, mice were given biphenyl for 18 months. Two strains of mice were used, obtained by mating C57BL/6 females with either C3H/Anf males or AKR males. Mice were given the maximal tolerated dose, which was defined as the highest dose that caused zero mortality in a series of preliminary studies in which biphenyl was administered for 1, 6, and then 19 consecutive days. The maximal tolerated dose of biphenyl was 2.5 mg/kg bw per day. This dose was given to 18 mice per sex per strain by gavage until the animals were aged 4 weeks, and then in the diet for the remainder of the study. The concentration of biphenyl in the diet was 517 mg/kg, which was calculated according to the weight and food consumption of the mice aged 4 weeks to provide the maximal tolerated dose. Groups of untreated negative controls and treated positive controls were maintained under the same conditions. Postmortem examinations at the end of the study included an external examination and a thorough examination of the thoracic and abdominal cavities, with histological examinations of all grossly visible lesions and all major organs, except the cranium. Blood smears were taken from all animals in the event that either splenomegaly or lymphadenopathy was observed. The authors reported no significant increase in tumours in mice of either strain treated with biphenyl when compared with negative controls. The NOEL was 2.5 mg/kg bw per day, the highest dose tested (Innes et al., 1969).

Rats

Groups of 15 male and 15 female weanling albino rats were given diets containing 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, or 1.0% biphenyl for 750 days, calculated (Food and Drug Administration, 1993) to provide average daily intakes of biphenyl of 0, 0.5, 2.5, 5, 25, 50, 250, or 500 mg/kg bw. Measurements of body weight and food consumption were recorded periodically for all rats throughout the study. Haemoglobin determinations were made at regular intervals for male and female rats assigned to the control group and groups receiving the three higher doses. At study termination, weights of liver, kidneys, heart and testes were deter-

mined and major organs/tissues were sectioned and stained for microscopic examinations. Bone-marrow smears were prepared from representative animals only.

Effects were mainly observed in animals in the groups receiving the two higher doses (0.5 and 1%). They included dose-dependent reductions in body-weight gain, food consumption and concentrations of haemoglobin. Mortality was increased at these doses in males (13 out of 15 died at both 0.5% and 1%, compared with 6 out of 15 controls) and females (10 out of 15 at 0.5% and 13 out of 15 at 1% died, compared with 6 out of 15 controls). Additional paired feeding experiments conducted at these doses for 98 days showed that the effects on body-weight gain were attributable to a decrease in food intake, possibly because of reduced palatability. Organs weights were not affected at doses of 0.1% and below. In the two surviving males at 0.5%, absolute and relative weights of kidneys were increased and of testes were decreased. Females at 0.5% also had increased absolute and relative kidney weights and, owing to reduced body weights, increased relative liver weights. The only histopathological change that could be attributed to administration of biphenyl occurred in the kidneys of both sexes of rats at the two higher doses. Prominent scarring, lymphocytic infiltration, tubular atrophy and dilation, and haemorrhage in the dilated tubules and renal pelvis were some of the effects observed. Hydronephrosis was common, as was squamous cell metaplasia of the renal pelvis epithelium, although this did not appear to be neoplastic. The frequency of scars and dilated tubules, as well as the severity of hydronephrosis, was greater in males at all doses (including controls) than in females. At lower doses (≤0.1%), histopathological findings in the kidneys were not different from those in the controls. Treatment with biphenyl did not result in increased incidences of tumours. The NOEL was 50 mg/kg bw per day on the basis of mortality and kidney findings (Ambrose et al., 1960).

In a long-term study of toxicity and carcinogenicity (reported to comply with OECD test guideline No. 453 and GLP), groups of 50 male and 50 female F344/DuCrj rats (aged 6 weeks) were given diets containing biphenyl at a dietary concentration of 0, 500, 1500, or 4500 mg/kg for 105 weeks. These dietary levels have been calculated (Food and Drug Administration, 1993) to provide average daily intakes of biphenyl of 0, 25, 75, or 225 mg/kg bw. The animals were observed daily for clinical signs, behavioural changes and mortality. Body weight and food consumption were recorded once a week for the first 14 weeks, and every 4 weeks thereafter. Urinary parameters, including pH and occult blood, were measured in all surviving rats in the final week of the study. Necropsies, organ weight determinations and histological examinations of a whole range of organs/tissues were performed on all animals.

Compared with those of the controls, the mean body weights of the animals at the highest dose were significantly decreased. Survival rates in all groups treated with biphenyl, with the exception of males at the highest dose, were comparable to those of the control animals. Nineteen out of 50 males at the highest dose died before the end of the study, and their death was attributed primarily to bladder tumours and haematuria. Although three females at the highest dose died from marked mineralization of the kidneys and the heart after 13–26 weeks of treatment with biphenyl, the survival rate did not differ from controls thereafter. Clinical

haematuria was observed in 32 males at the highest dose from weeks 40–105 of exposure; 14 of these animals had a skin or eye colour that suggested anaemia. No clinical haematuria was observed in the other groups of treated males or in any of the groups of treated females. Urine analysis revealed increased pH of the urine in males at the highest dose, while the incidence of positive occult blood was significantly increased in males and, to a lesser degree, in females at the highest dose. Relative weights of the kidney were significantly increased in both sexes at the intermediate and highest doses, while absolute weight of the kidney was significantly increased only in males at the highest dose. Gross findings included bladder calculi in both sexes at 4500 mg/kg (in 43 out of 50 males and 8 out of 50 females). No bladder calculi were found in animals at 500 or 1500 mg/kg. The bladder calculi first appeared at around week 40 of the study, together with the occurrence of haematuria. In addition, thickening of the bladder wall was reported in four of the eight calculi-bearing females. Forty-one of the 50 males at the highest dose had polyp-like or papillary nodules protruding from the bladder wall into the lumen; 38 of these 41 were bearing calculi. No tumour or tumour-related lesions were observed in organs other than the urinary tract. In the bladder, tumour or tumour-related lesions were almost exclusively found in animals at the highest dose, and included increased incidences of transitional cell hyperplasia in the epithelium of both males (45 out of 50) and females (10 out of 50), squamous metaplasia (in 19 out of 50 males and 4 out of 50 females), squamous cell hyperplasia (in 13 out of 50 males and 1 out of 50 females), inflammatory polyps (in 10 out of 50 males, not in females), and calculi (in 43 out of 50 males and 8 out of 50 females). The transitional cell hyperplasia were characterized as simple, nodular and papillary and developed focally on the bladder epithelium. Bladder tumours were only found in males at the highest dose, and included transitional cell papillomas (10 out of 50) and carcinomas (24 out of 50), and squamous cell papilloma and carcinoma (1 out of 50). The authors noted that all 24 of the male rats with transitional cell carcinomas and 8 of the 10 male rats with transitional cell papillomas were found to have bladder calculi. In other parts of the urinary tract, only non-neoplastic lesions were observed, no tumours. In the ureter, findings were limited to the highest dose and included increased incidences (statistically significantly only in males) of simple transitional cell hyperplasia (8 out of 50 males and 2 out of 50 females) and dilation (14 out of 50 males and 6 out of 50 females). In the kidneys, incidences of simple and nodular transitional cell hyperplasia of the renal pelvis were significantly increased in males at the highest dose and in females at the intermediate and highest doses. Mineralization of the renal pelvis was increased in males at the highest dose and in females at the intermediate and highest doses, but statistical significance was only reached for the latter. The incidences of calculi and desquamation of the renal pelvis were increased in animals at the highest dose (statistically significantly only in males). Other significant findings in the kidney included increased incidences of papillary mineralization, papillary necrosis and infarct in females at the highest dose, and of haemosiderin deposits in females at the intermediate and highest doses. In males, increased incidences of mineralization of the cortico-medullary junction and papillary necrosis (not statistically significantly) were observed at the highest dose, and increased incidences of papillary mineralization at the intermediate (not statistically significantly) and highest doses. Chronic nephropathy was reported for both sexes of

the control animals and the treated animals at all doses (in 45, 45, 43, and 34 out of 50 males, and in 33, 35, 30, and 26 out of 50 females, at 0, 500, 1500, and 4500 mg/kg, respectively). The NOEL in this study was 25 mg/kg bw per day (Umeda et al., 2002).

Mechanism of induction of bladder tumours by biphenyl in rats and relevance to humans

Studies have been conducted on the mechanism underlying the predominant formation of bladder calculi in male rats compared with female rats after administration of biphenyl. Analysis of the urinary calculi formed in the 105-week study described above revealed that the calculi consisted principally of potassium 4-hydroxybiphenyl *O*-sulfate (4-HBPOSK) in males and of 4-hydroxybiphenyl (4-HBP) and $KHSO_4$ in females. The shape and colour of the calculi were also different between sexes, as were the structure and distribution of component elements. The calculi in males had a multilayer structure with alternating layers of calcium phosphate and 4-HBPOSK, with the latter being the centre component and the inside area of each layer. In females, the calculi were described as being single layered with open holes where needle shaped crystals were present. The authors attributed the differences in the principal constituents and the structural formation of the calculi to the increased hydrolysis of 4-HBPOSK to 4-HBP and $KHSO_4$ in the female rat as compared with the male rat. They also noted that this was consistent with the observed lower pH of the female urine compared with the male urine, as the lower the pH, the greater the extent of hydrolysis (Ohnishi et al., 2000). Ohnishi et al. (2001) observed that co-adminstration of biphenyl and potassium bicarbonate ($KHCO_3$) in the diet of male rats for 13 weeks resulted in the formation of urine crystals consisting of 4-HBPOSK. This was induced by the higher concentration of potassium in the urine and the higher urinary pH as a consequence of feeding with $KHCO_3$. These urine crystals produced hyperplasia of the transitional epithelium of the ureter, uretal obstruction and hydronephrosis in the urinary tract (Ohnishi et al., 2001).

The mode of action by which long-term administration of biphenyl at high doses induces male-specific bladder tumours in rats is not fully understood. However, in all probability, the induction of tumours is secondary to the formation of bladder calculi, which in males results from the precipitation of the potassium salt of 4-hydroxybiphenyl-*O*-sulfate. These calculi then induce sustained mechanical damage, thereby evoking haematuria and a regenerative response. This is supported by the findings that bladder tumours occurred in close association with calculus formation and haematuria. It is also consistent with the observed sex differences in structure and composition of calculi and in occurrence of haematuria, which was absent in females. The postulated mechanism appears to be dose-dependent, given the very steep dose–response relationships found for the neoplastic and associated preneoplastic lesions, i.e. complete lack of these findings at 500 and 1500 mg/kg, but presence at the next higher dose of 4500 mg/kg.

When addressing the predictive value of certain types of tumours for the identification of carcinogenic hazards to humans, the International Agency for Research on Cancer (IARC) (IARC, 1999) concluded the following with respect to the

relevance of calculi- and microcrystalluria-associated urinary bladder neoplasms in rodents:

For chemicals producing bladder neoplasms in rats and mice as a result of calculus formation in the urinary bladder, the response cannot be considered to be species-specific; thus, the tumour response is relevant to an evaluation of carcinogenicity to humans. There are quantitative differences in response between species and sexes. Calculus formation is dependent on the attainment in the urine of critical concentrations of constituent chemicals which form the calculus; therefore, the biological effects are dependent on reaching threshold concentrations for calculus formation. Microcrystalluria is often associated with calculus formation, but its relevance to species-specific mechanisms cannot be assessed.

The Committee, however, concluded that the bladder tumours induced by long-term administration of biphenyl at high doses are not relevant for human risk assessment. Owing to the difference in anatomical position of the bladder (vertical in humans versus horizontal in rodents), humans will more easily lose calculi formed, if any. It is also very unlikely that humans will be exposed to biphenyl at the high doses needed to induce the formation of calculi, owing to precipitation of 4-hydroxybiphenyl *O*-sulfate.

(d) Genotoxicity

Testing for genotoxicity has been performed on four of the five flavouring agents in this group (Nos 1325, 1332, 1334, 1335). The results of these tests are summarized in Table 5 and described below.

(i) In vitro

No evidence of mutagenicity was observed in standard or modified Ames assays when *p*-cymene (No. 1325; up to 85 300 μg/plate), biphenyl (No. 1332; up to 10 000 μg/plate), 4-methylbiphenyl (No. 1334; up to 1000 μg/plate), or 1-methylnaphthalene (No. 1335; up to 4266 μg/plate) were incubated with *Salmonella typhimurium* strains TA97, TA98, TA100, TA1535, TA1537, TA1538, and/or TA1532, TA2636, TA2637, G46, C3076, or D3052 with and without metabolic activation (Clark et al., 1977, 1979; Anderson & Styles, 1978; Rockwell & Raw, 1979; Florin et al., 1980; Hirayama et al., 1981; Probst et al., 1981; Haworth et al., 1983; Pagano et al., 1983; Nohmi et al., 1985; Brams et al., 1987; Houk et al., 1989; National Toxicology Program, 2004a, 2004b, 2004c; Zeiger et al., 1992). Biphenyl also gave negative results when incubated with *Escherichia coli* strains WP2 and WP2 *uvrA*$^-$ in the modified Ames test (Probst et al., 1981), strain PQ37 in the SOS chromotest (up to 154 μg/ml) (Brams et al., 1987), and with strains WP2, WP2 *uvrA*, WP100, and CM571 in a test for DNA repair (up to 4000 μg/disk) (Hirayama et al., 1981).

In contrast to the negative results obtained for biphenyl in *S. typhimurium* and *E. coli* systems, biphenyl (No. 1332) produced genetic effects in an assay with *Saccharomyces cerevisiae* strain D7, with and without metabolic activation, at concentrations of up to 1 mmol/l (Pagano et al., 1983). In an assay for forward mutation in mouse lymphoma cells, biphenyl produced significant increases in mutation frequency in L5178Y cells at concentrations of 45.6–60.9 μg/ml without

Table 5. Results of studies of genotoxicity with aromatic hydrocarbons used as flavouring agents

No.	Flavouring agent	End-point	Test system	Dose or concentration	Result	Reference
In vitro						
1325	*p*-Cymene	Reverse mutation	*S. typhimurium* TA98, TA100	0.05–100 µl/plate (42.7–85 300 µg/plate)[a]	Negative[b]	Rockwell & Raw (1979)
1332	Biphenyl[c]	Reverse mutation	*S. typhimurium* TA98, TA1535, TA1537, TA1538	10–10 000 µg/plate[d]	Negative[e]	Clark et al. (1977)
1332	Biphenyl	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1538	4–2500 µg/plate	Negative[b]	Anderson & Styles (1978)
1332	Biphenyl[c]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	1–10 000 µg/plate	Negative[e]	Clark et al. (1979)
1332	Biphenyl	Reverse mutation	*S. typhimurium* TA98, TA100	1–100 µg/plate	Negative[e]	Hirayama et al. (1981)
1332	Biphenyl	Reverse mutation[f]	*S. typhimurium* G46, TA98, TA100, TA1535, TA1537, TA1538, C3076, D3052; *Escherichia coli* WP2 and WP2*uvrA*$^-$	NR[g]	Negative[e]	Probst et al. (1981)
1332	Biphenyl	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	1–100 µg/plate[h]	Negative[e]	Haworth et al. (1983); NTP (2004a/b)
1332	Biphenyl	Reverse mutation	*S. typhimurium* TA98, TA100, TA1532, TA1535, TA1537, TA1538, TA2636	0.1–500 µg/plate[i]	Negative[e]	Pagano et al. (1983)

Table 5. (contd)

No.	Flavouring agent	End-point	Test system	Dose or concentration	Result	Reference
1332	Biphenyl	Reverse mutation	*S. typhimurium* TA98, TA100, TA2637	10–5000 μg/plate[j]	Negative[e]	Nohmi et al. (1985)
1332	Biphenyl	Reverse mutation	*S. typhimurium* TA97, TA98, TA100	1–100 μg/plate	Negative[e]	Brams et al. (1987)
1332	Biphenyl	Reverse mutation	*S. typhimurium* TA98, TA100	≤2000 μg/plate	Negative[e]	Houk et al. (1989)
1332	Biphenyl	SOS induction	*Escherichia coli PQ37*	2.4–154 μg/ml	Negative[e]	Brams et al. (1987)
1332	Biphenyl	DNA repair	*E. coli* WP2, WP2*uvrA*, CM571, WP100	4000 μg/disc	Negative	Hirayama et al. (1981)
1332	Biphenyl	Mitotic recombination, gene conversion, reversion	*Saccharomyces cerevisiae D7*	≤1 mmol/l plate)[a] (154 μg/ml)[k]	Positive[e]	Pagano et al. (1983)
1332	Biphenyl	Forward mutation	Mouse lymphoma L5178Y $Tk^{+/-}$ cells	98.7–395 μmol/l –S9 (15.2–60.9 μg/ml)[k,l]; 5.01–60 μmol/l +S9 (0.8–9.3 μg/ml)[k,m]	Positive[n]; Positive[o]	Wangenheim & Bolcsfoldi (1988)
1332	Biphenyl	Sister chromatid exchange	Chinese hamster Don cells	0.1–1 mmol/l (15.4–154 μg/ml)[k]	Negative[p,q]	Abe & Sasaki (1977)
1332	Biphenyl	Chromosomal aberration	Chinese hamster Don cells	0.1–1 mmol/l (15.4–154 μg/ml)[k]	Negative[p]	Abe & Sasaki (1977)
1332	Biphenyl	Unscheduled DNA synthesis	Rat hepatocytes	0.01–1000 μmol/l (0.002–154 μg/ml)[k]	Negative	Brouns et al. (1979)
1332	Biphenyl	Unscheduled DNA synthesis	Rat hepatocytes	0.5–1000 nmol/ml (0.08–154 μg/ml)[k,r]	Negative	Probst et al. (1981)
1332	Biphenyl	Unscheduled DNA synthesis	Rat hepatocytes	0.1–100 μmol/l (0.02–15.4 μg/ml)[k]	Negative	Hsia et al. (1983)

1334	4-Methylbiphenyl[s]	Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA1535	0.1–10 μg/plate −S9[h]; 10–1000 μg/plate +S9[h]	Negative[e]	Zeiger et al. (1992)
1335	1-Methylnaphthalene	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	0.03–30 μmol/plate (4.3–4266 μg/plate)[t,u]	Negative[e]	Florin et al. (1980)
1335	1-Methylnaphthalene	Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA1535	0.3–33 μg/plate −S9[h]; 1–100 μg/plate +S9	Negative[e]	National Toxicology Program (2004c)

[a] Calculated using a density of *p*-cymene of 0.853 g/ml (Lewis, 1999).
[b] With metabolic activation.
[c] Dowtherm A, a mixture of diphenyl (26%) and diphenyl oxide (72%), was tested.
[d] Cytotoxicity observed at the highest dose/concentration tested.
[e] With and without metabolic activation.
[f] Modified Ames test.
[g] NR, not reported.
[h] Slight toxicity was occasionally observed at the highest concentration tested.
[i] Cytotoxicity observed at doses of 50 to 100 μg/plate, depending on the different tester strains.
[j] Lethality observed at doses of 2000 to 5000 μg/plate.
[k] Calculated using the relative molecular mass of biphenyl of 154.21.
[l] Cytotoxicity observed at 395 μmol/l (6% cell viability), while 345 μmol/l is near-leathal concentration (14% cell viability).
[m] 40 and 60 μmol/l are near-lethal concentrations (12–15% cell viability).
[n] A significant increase in mutation frequency was noted at 296–395 μmol/l (with a twofold increase only at 395 μmol/l), but not at 98.7–197 μmol/l.
[o] Significant increase in mutation frequency was noted at 20–60 μmol/l (with an increase of more than twofold only at 40 and 60 μmol/l), but not at 5.01–10 μmol/l.
[p] Without metabolic activation.
[q] Significant increase in induction of sister chromatid exchange was noted, but the increase was not dose-dependent and was less than twice the control value. At the highest concentration (1 mmol/l), the mitotic index was decreased to >50% of that for controls.
[r] Cytotoxicity observed at concentrations >100 nmol/ml.
[s] Mixed isomers of methylbiphenyl tested.
[t] Calculated using the relative molecular mass of 1-methylnaphthalene of 142.20.
[u] Cytotoxicity observed at doses >3 μmol/plate.

metabolic activation, and 3.1–9.3 μg/ml with activation (Wangenheim & Bolcsfoldi, 1988). However, the increases were ≥2-fold only at 60.9 μg/ml without activation, and 6.2–9.3 μg/ml with activation. At these concentrations, cell viability was ≤15%. At lower concentrations of 15.2–30.4 μg/ml without metabolic activation, and 0.8–1.5 μg/ml with activation, biphenyl gave negative results (Wangenheim & Bolcsfoldi, 1988). Cell viability was much higher at these lower concentrations (at least 49%). Biphenyl did not induce sister chromatid exchanges (SCE) or chromosomal aberrations in Chinese hamster Don cells at concentrations of up to 154 μg/ml without metabolic activation (Abe & Sasaki, 1977), nor did it induce unscheduled DNA synthesis in rat hepatocytes at concentrations of 0.002–154 μg/ml (Brouns et al., 1979; Probst et al., 1981; Hsia et al., 1983).

In a study designed to investigate the mutagenicity in vivo-in vitro of urinary metabolites of a number of food additives, Sprague-Dawley rats were given 0.5 ml of *p*-cymene (No. 1325; approximately 1706 mg/kg bw) by gavage and urine was collected for 24 h. Three types of urine samples were tested in the Ames assay with *S. typhimurium* strains TA98 and TA100 with metabolic activation: a direct urine sample, a urine-ether extract, and the aqueous fraction of the urine–ether extract. The urine samples of rats treated with *p*-cymene did not show any evidence of mutagenicity, either in the presence or absence of β-glucuronidase (Rockwell & Raw, 1979).

(ii) Conclusion

Four substances in this group of flavouring agents have been tested in the Ames assay and found not to be mutagenic in vitro in bacteria. In addition to showing no mutagenic potential in the Ames assay, biphenyl produced negative results in *E. coli* in the SOS chromotest and DNA repair test. On the other hand, biphenyl produced genetic effects in yeast (*S. cerevisiae*).

In mammalian cell systems, negative results were obtained for biphenyl with respect to induction of SCE, chromosomal aberration, and unscheduled DNA synthesis. The positive finding for biphenyl in an assay for forward mutation in mouse lymphoma cells was obtained at near lethal concentrations.

On the basis of the results of available studies of genotoxicity, the Committee concluded that the flavouring agents in this group of aromatic hydrocarbons are not genotoxic.

(e) Reproductive toxicity

(i) Biphenyl (No. 1332)

The potential effects of biphenyl on reproduction and survival of pups were examined in ten female and five male weanling albino rats (strain not specified) that were mated (one male to two female rats) after 60 days of being fed a diet containing biphenyl at 0 or 0.1%. Another nine females and three males were mated (one male to three females) after 60 days of dietary exposure to biphenyl at 0.5%. All the rats were kept on their respective diets until all the pups were

weaned. In a second experiment with rats aged 90 days, eight to nine females and three to four males were placed on diets containing biphenyl at 0, 0.1, or 0.5% for 11 days before being mated.

Biphenyl was reported to have no significant effect on reproduction, although the number of litters and pups born was slightly lower in the second experiment at the highest dose of 0.5% (Ambrose et al., 1960).

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ALIPHATIC, LINEAR α,β-UNSATURATED ALDEHYDES, ACIDS AND RELATED ALCOHOLS, ACETALS AND ESTERS

First draft prepared by

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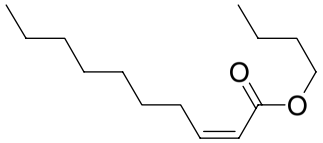
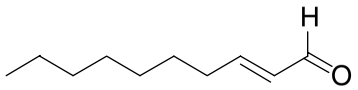
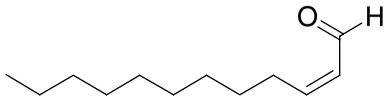
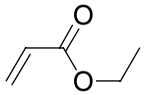
1. EVALUATION

1.1 Introduction

The Committee evaluated a group of 37 aliphatic, linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters flavouring agents (Table 1) by the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, p 192). The Committee has not previously evaluated any member of the group. The group included nine 2-alkenals (Nos 1349, 1350, 1353, 1359, 1360, 1362–1364 and 1366), six 2-alken-1-ols (Nos 1354, 1365, 1369, 1370, 1374 and 1384), five 2-alkenoic acids (Nos 1361, 1371–1373 and 1380), 16 related alkenoic and alkynoic acid esters (Nos 1348, 1351, 1352, 1355–1358, 1367, 1368, 1375–1379, 1381, 1382), and one acetal (No. 1383).

Table 1. Summary of the results of safety evaluations of aliphatic, linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters[a] used as flavouring agents

Flavouring agent	No.	CAS No. and structure	Step A3[b] Does intake exceed the threshold for human intake?	Comments	Conclusion based on current intake
Structural class I					
Butyl 2-decenoate	1348	7492-45-7 	No Europe: 0.01 USA: 0.3	See note 2	No safety concern
2-Decenal	1349	3913-71-1 	No Europe: 3 USA: 6	See note 4	No safety concern
2-Dodecenal	1350	4826-62-4 	No Europe: 16 USA: 2	See note 4	No safety concern
Ethyl acrylate	1351	140-88-5 	No Europe: 2 USA: 0.7	See note 2	No safety concern

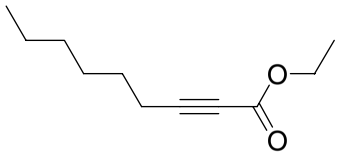
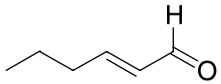
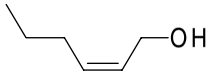
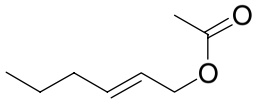
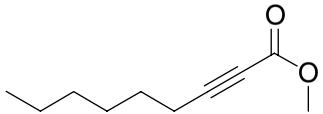
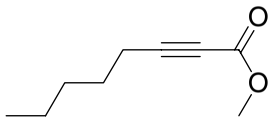
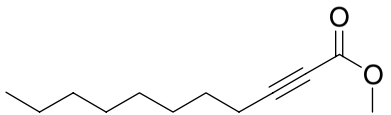
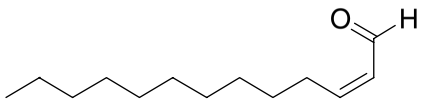
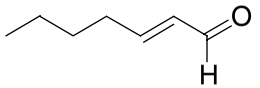
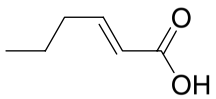
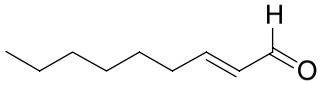
Ethyl 2-nonynoate	1352	10031-92-2 	No Europe: ND USA: 0.9	See note 2	No safety concern
2-Hexenal	1353	6728-26-3 	No Europe: 791 USA: 409	See note 4	No safety concern
2-Hexen-1-ol	1354	2305-21-7 	No Europe: 395 USA: 291	See note 5	No safety concern
(E)-2-Hexen-yl acetate	1355	10094-40-3 	No Europe: 199 USA: 56	See note 2	No safety concern
Methyl 2-nonynoate	1356	111-80-8 	No Europe: 2 USA: 21	See note 2	No safety concern
Methyl 2-octynoate	1357	111-12-6 	No Europe: 21 USA: 38	See note 2	No safety concern

Table 1. (contd)

Flavouring agent	No.	CAS No. and structure	Step A3[b] Does intake exceed the threshold for human intake?	Comments	Conclusion based on current intake
Methyl 2-undecynoate	1358	10522-18-6 	No Europe: ND USA: 0.04	See note 2	No safety concern
2-Tridecenal	1359	7774-82-5 	No Europe: 0.6 USA: 0.7	See note 4	No safety concern
trans-2-Heptenal	1360	8829-55-5 	No Europe: 6 USA: 30	See note 4	No safety concern
trans-2-Hexenoic acid	1361	13419-69-7 	No Europe: 18 USA: 36	See note 1	No safety concern
2-Nonenal	1362	2463-53-8 	No Europe: 2 USA: 0.4	See note 4	No safety concern

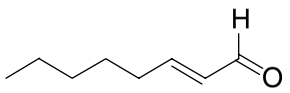
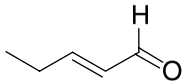
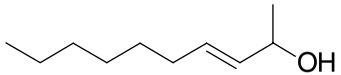
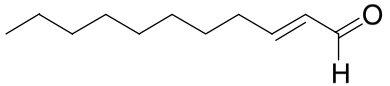
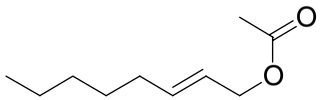
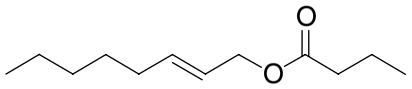
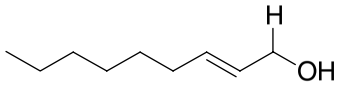
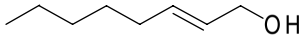
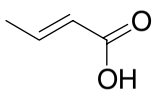
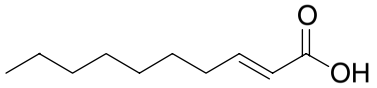
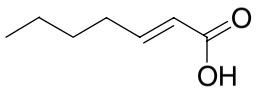
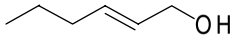
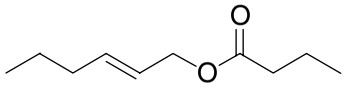
2-Octenal	1363	2363-89-5 	No Europe: 4 USA: 0.9	See note 4	No safety concern
2-Pentenal	1364	764-39-6 	No Europe: 0.9 USA: 0.1	See note 4	No safety concern
trans-2-Nonen-1-ol	1365	31502-14-4 	No Europe: 0.1 USA: 0.03	See note 5	No safety concern
2-Undecenal	1366	2463-77-6 	No Europe: 0.4 USA: 0.4	See note 4	No safety concern
trans-2-Octen-1-yl acetate	1367	3913-80-2 	No Europe: 0.3 USA: 0.7	See note 2	No safety concern
trans-2-Octen-1-yl butanoate	1368	84642-60-4 	No Europe: 0.3 USA: 0.7	See note 2	No safety concern
cis-2-Nonen-1-ol	1369	41453-56-9 	No Europe: 0.07 USA: 2	See note 5	No safety concern

Table 1. (contd)

Flavouring agent	No.	CAS No. and structure	Step A3[b] Does intake exceed the threshold for human intake?	Comments	Conclusion based on current intake
(E)-2-Octen-1-ol	1370	18409-17-1 	No Europe: ND USA: 0.2	See note 5	No safety concern
(E)-2-Butenoic acid	1371	107-93-7 	No Europe: ND USA: 7	See note 1	No safety concern
(E)-2-Decenoic acid	1372	334-49-6 	No Europe: ND USA: 4	See note 1	No safety concern
(E)-2-Heptenoic acid	1373	10352-88-2 	No Europe: ND USA: 4	See note 1	No safety concern
(Z)-2-Hexen-1-ol	1374	928-94-9 	No Europe: ND USA: 10	See note 5	No safety concern
trans-2-Hexenyl butyrate	1375	53398-83-7 	No Europe: ND USA: 4	See note 2	No safety concern

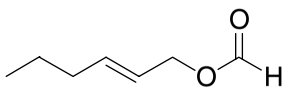
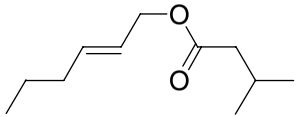
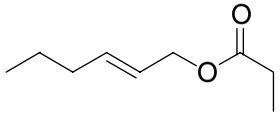
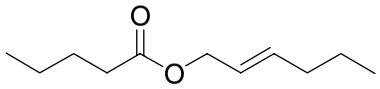
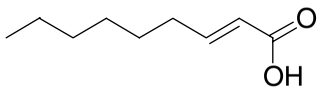
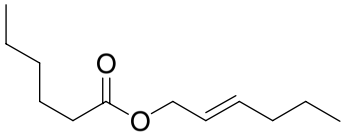
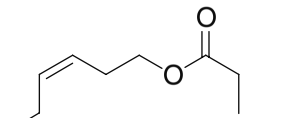
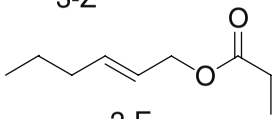
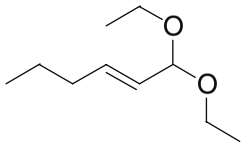
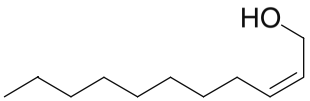
(E)-2-Hexenyl formate	1376	53398-78-0 	No Europe: ND USA: 7	See note 2	No safety concern
trans-2-Hexenyl isovalerate	1377	68698-59-9 	No Europe: ND USA: 4	See note 2	No safety concern
trans-2-Hexenyl propionate	1378	53398-80-4 	No Europe: ND USA: 4	See note 2	No safety concern
trans-2-Hexenyl pentanoate	1379	56922-74-8 	No Europe: ND USA: 4	See note 2	No safety concern
(E)-2-Nonenoic acid	1380	14812-03-4 	No Europe: ND USA: 3	See note 1	No safety concern

Table 1. (contd)

Flavouring agent	No.	CAS No. and structure	Step A3[b] Does intake exceed the threshold for human intake?	Comments	Conclusion based on current intake
(E)-2-Hexenyl hexanoate	1381	53398-86-0 	No Europe: ND USA: 0.09	See note 2	No safety concern
(Z)-3 & *(E)*-2-Hexenyl propionate	1382	33467-74-2 53398-80-4  3-Z  2-E	No Europe: ND USA: 0.7	See note 2	No safety concern
(E)-2-Hexenal diethyl acetal	1383	67746-30-9 	No Europe: 0.3 USA: 0.09	See notes 3, 4, and 5	No safety concern

2-Undecen-1-ol	1384	37617-03-1		No Europe: ND USA: 1.0	See note 5	No safety concern

CAS: Chemical Abstracts Service; ND: No intake data reported.

[a] Step 1: All the agents in this group are in structural class I.

[b] All 37 flavouring agents (Nos 1348–1384) in this group are expected to be metabolized to innocuous products. The evaluation of these flavouring agents therefore proceeded via the A-side of the decision-tree.

[c] The threshold for human intake for structural class I is 1800μg/person per day. All intake values are expressed in μg/person per day. The combined intake of the flavouring agents in structural class I is 1461μg/person per day in Europe and 949μg/person per day in the USA.

1. Undergoes β-oxidative cleavage and complete metabolism via the tricarboxylic acid cycle.
2. Hydrolysed to corresponding alcohols and acids, followed by complete metabolism in the fatty acid pathway or the tricarboxylic acid cycle.
3. Hydrolysed to corresponding aldehydes and alcohols.
4. Oxidized to acids, which may undergo β-oxidative cleavage and complete metabolism via the tricarboxylic acid cycle. Alternately, may undergo glutathione conjugation and excretion as mercapturic acid derivatives.
5. Oxidized to aldehydes and acids, which metabolize completely in the fatty acid β-oxidation pathway.

Twenty-eight of the 37 flavouring agents (Nos 1349–1351, 1353–1355, 1359–1366, 1369–1378, 1380–1382 and 1384) in this group have been reported to occur naturally in foods and have been detected in beef, chicken, fish, fresh fruit, cheese, tea, coffee and beer (Nijssen et al., 2003). Exposure to an important flavouring agent in the group, 2-hexenal (No. 1353), occurs primarily through consumption of traditional foods (Stofberg & Grundschober, 1987).

1.2 Estimated daily intake

The total annual volume of production of the 37 flavouring agents in this group is approximately 10 000 kg in Europe (International Organization of the Flavour Industry, 1995) and 7100 kg in the USA (National Academy of Sciences, 1970, 1982, 1987; Lucas et al., 1999) (see Table 2). Approximately 95% of the total annual volume of production in Europe and 81% in the USA is accounted for by 2-hexenal (No. 1353), the corresponding alcohol 2-hexen-1-ol (No. 1354), and the corresponding acetate ester (*E*)-2-hexen-yl acetate (No. 1355). Of these, 2-hexenal accounts for approximately 54% of the total annual volume of production in Europe and 44% in the USA. The estimated daily intakes of 2-hexenal in Europe and the USA were 791 and 409 μg/person, respectively. The daily intakes of all the other flavouring agents in the group were in the range of 0.01–395 μg/person (National Academy of Sciences, 1970, 1982, 1987; International Organization of the Flavour Industry, 1995; Lucas et al., 1999), with most values being at the lower end of this range. The estimated daily per capita intake of each agent is reported in Table 1.

1.3 Absorption, distribution, metabolism and elimination

In general, aliphatic esters formed from 2-alkenols and carboxylic acids are more rapidly hydrolysed than their saturated alcohol counterparts (Heymann, 1980). Hydrolysis of esters or acetals has been shown to occur in simulated stomach juice, simulated intestinal fluid, plasma, and liver microsomes (Knoefel, 1934; Morgareidge, 1962, Longland et al., 1977). If hydrolysed before absorption, the resulting aliphatic alcohols and carboxylic acids are rapidly absorbed in the gastrointestinal tract. The unsaturated alcohols are successively oxidized to the corresponding aldehydes and carboxylic acids, which participate in fundamental biochemical pathways, including the fatty acid pathway and tricarboxylic acid cycle (Nelson & Cox, 2000).

α,β-Unsaturated aldehydes are formed endogenously by lipid peroxidation of polyu0nsaturated fatty acids (Frankel et al., 1987), or they can be ingested as naturally occurring constituents of food (Stofberg & Grundschober, 1987; Nijssen et al., 2003) and, to a minor extent, as added flavouring agents. Under conditions of glutathione depletion and oxidative stress, high intracellular concentrations of α,β-unsaturated aldehydes have been shown to form adducts with proteins (Ichihashi et al., 2001) and DNA (Frankel et al., 1987; Eder et al., 1993; Eisenbrand et al., 1995; Golzer et al., 1996; Cadet et al., 1999; National Toxicology Program, 2001a), resulting in cellular toxicity and DNA fragmentation during apoptosis. At low intakes, α,β-unsaturated aldehydes undergo metabolic detoxication by

Table 2. Annual volumes of production of aliphatic, linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters used as flavouring agents in Europe and the USA

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Intake of aldehyde equivalents (μg/kg bw per day)[d]	Annual volume in naturally occurring foods (kg)[e]	Consumption ratio[f]
		μg/day	μg/kg bw per day				
Butyl 2-decenoate (1348)							
Europe	0.1	0.01	0.0002	0.00007			
USA	2	0.3	0.004	0.001		–	NA
2-Decenal (1349)							
Europe	19	3	0.045				
USA	45	6	0.099			34111	758
2-Dodecenal (1350)							
Europe	109	16	0.259				
USA	13	2	0.029			64	5
Ethyl acrylate (1351)							
Europe	11	2	0.026	0.01			
USA	5	0.7	0.011	0.005		386	77
Ethyl 2-nonynoate (1352)							
Europe	ND	ND	ND				
USA	7	0.9	0.015	0.005		–	NA
2-Hexenal (1353)							
Europe	5542	791	13				
USA	3103	409	7			155335	50
2-Hexen-1-ol (1354)							
Europe	2765	395	7				
USA	2209	291	5			7989	4
(E)-2-Hexen-yl acetate (1355)							
Europe	1397	199	3	2			
USA	426	56	1	0.6		424	1

Table 2. (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Intake of aldehyde equivalents (μg/kg bw per day)[d]	Annual volume in naturally occurring foods (kg)[e]	Consumption ratio[f]
		μg/day	μg/kg bw per day				
Methyl 2-nonynoate (1356)							
Europe	16	2	0.038	0.008			
USA	159	21	0.349	0.06		–	NA
Methyl 2-octynoate (1357)							
Europe	149	21	0.354	0.08			
USA	286	38	0.628	0.1		–	NA
Methyl 2-undecynoate (1358)							
Europe	ND	ND	ND				
USA[g]	0.2	0.04	0.001	0.0001		–	NA
2-Tridecenal (1359)							
Europe	4	0.6	0.010				
USA	5	0.7	0.011			+	NA
trans-2-Heptenal (1360)							
Europe	44	6	0.105				
USA	231	30	0.507			7614	33
trans-2-Hexenoic acid (1361)							
Europe	128	18	0.304				
USA	277	36	0.608			+	NA
2-Nonenal (1362)							
Europe	12	2	0.029				
USA	3	0.4	0.007			5413	1804
2-Octenal (1363)							
Europe	27	4	0.064				
USA	7	0.9	0.015			2046	292
2-Pentenal (1364)							
Europe	6	0.9	0.014				
USA	1	0.1	0.002			60	60

trans-2-Nonen-1-ol (1365)						
Europe	1	0.1	0.002			
USA	0.2	0.03	0.0004		1.4	7
2-Undecenal (1366)						
Europe	3	0.4	0.007			
USA	3	0.4	0.007		14 168	4723
trans-2-Octen-1-yl acetate (1367)						
Europe	2	0.3	0.005	0.004		
USA[h]	4	0.7	0.012	0.008	–	NA
trans-2-Octen-1-yl butanoate (1368)						
Europe	2	0.3	0.005	0.003		
USA[h]	4	0.7	0.012	0.006	–	NA
cis-2-Nonen-1-ol (1369)						
Europe	0.5	0.07	0.001			
USA[h]	11	2	0.032		+	NA
(E)-2-Octen-1-ol (1370)						
Europe	ND	ND	ND			
USA[h]	1	0.2	0.003		+	NA
(E)-2-Butenoic acid (1371)						
Europe	ND	ND	ND			
USA[h]	40	7	0.117		+	NA
(E)-2-Decenoic acid (1372)						
Europe	ND	ND	ND			
USA[h]	25	4	0.073		+	NA
(E)-2-Heptenoic acid (1373)						
Europe	ND	ND	ND			
USA[h]	20	4	0.059		+	NA
(Z)-2-Hexen-1-ol (1374)						
Europe	ND	ND	ND			
USA[h]	55	10	0.161		+	NA
trans-2-Hexenyl butyrate (1375)						
Europe	ND	ND	ND			
USA[h]	21	4	0.061	0.04	+	NA

Table 2. (contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Intake of alcohol equivalents (μg/kg bw per day)[c]	Intake of aldehyde equivalents (μg/kg bw per day)[d]	Annual volume in naturally occurring foods (kg)[e]	Consumption ratio[f]
		μg/day	μg/kg bw per day				
(E)-2-Hexenyl formate (1376)							
Europe	ND	ND	ND				
USA[h]	40	7	0.117	0.08		+	NA
trans-2-Hexenyl isovalerate (1377)							
Europe	ND	ND	ND				
USA[h]	20	4	0.059	0.03		+	NA
trans-2-Hexenyl propionate (1378)							
Europe	ND	ND	ND				
USA[h]	22	4	0.077	0.04		+	NA
trans-2-Hexenyl pentanoate (1379)							
Europe	ND	ND	ND				
USA[h]	25	4	0.070	0.04		–	NA
(E)-2-Nonenoic acid (1380)							
Europe	ND	ND	ND				
USA[h]	15	3	0.044			+	NA
(E)-2-Hexenyl hexanoate (1381)							
Europe	ND	ND	ND				
USA[h]	0.5	0.09	0.001	0.0005		+	NA
(Z)-3 & *(E)*-2-Hexenyl propionate (1382)							
Europe	ND	ND	ND				
USA	5	0.7	0.011	0.006		+[i]	NA
(E)-2-Hexenal diethyl acetal (1383)							
Europe	2	0.3	0.005	0.003	0.003		
USA[h]	0.5	0.09	0.001	0.0005	0.0005	–	NA

2-Undecen-1-ol (1384)					
Europe	ND	ND	ND		
USA[g]	3	0.5	0.008	+	NA
Total					
Europe	10240				
USA	7094				

NA, not available; ND, no intake data reported; +, reported to occur naturally in foods (Nijssen et al., 2003), but no quantitative data; –, not reported to occur naturally in foods.

[a] From International Organization of the Flavour Industry (1995) and Lucas et al. (1999) or National Academy of Sciences (1975, 1982).

[b] Intake expressed as μg/person/day was calculated as follows: [(annual volume, kg) × (1×10^9 μg/kg)/(population × survey correction factor × 365 days)], where population (10%, 'eaters only') = 32×10^6 for Europe and 26×10^6 for the USA; the correction factor = 0.6 for Europe, and 0.8 for the USA, representing the assumption that only 60 and 80% of the annual volume of the flavour, respectively, was reported in the poundage surveys (National Academy of Sciences, 1975, 1982; International Organization of the Flavour Industry, 1995; Lucas et al., 1999). Intake expressed as μg/kg bw per day was calculated as follows: [(μg/person per day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.

[c] Calculated as follows: (relative molecular mass of alcohol/relative molecular mass of acetal or ester) × daily per capita intake ('eaters only') of acetal or ester.

[d] Calculated as follows: (relative molecular mass of aldehyde/relative molecular mass of acetal) × daily per capita intake ('eaters only') of acetal.

[e] Quantitative data for the USA reported by Stofberg & Grundschober (1987).

[f] The consumption ratio is calculated as follows: (annual consumption in food, kg)/(most recent reported volume as a flavouring agent, kg).

[g] Annual volume reported in previous USA surveys (National Academy of Sciences, 1975, 1982).

[h] The volume cited is the anticipated annual volume, which was the maximum amount of flavouring agent estimated to be used annually by the manufacturer at the time the material was proposed for flavour use. Subsequent national surveys (National Academy of Sciences, 1975, 1982, 1987; Lucas et al., 1999) revealed no reported use of the substance as a flavour ingredient.

[i] Natural occurrence data applies to *(Z)*-3-hexenyl propionate (Nijssen et al., 2003).

enzymes of the high-capacity β-oxidation pathway or, to a lesser extent, by glutathione conjugation.

It is anticipated that humans will biotransform small quantities of 2-alkenols and 2-alkenals by oxidation to the corresponding acids, which may undergo β-oxidative cleavage and complete metabolism via the tricarboxylic acid cycle. An alternate minor pathway may involve conjugation of the unsaturated aldehyde with glutathione, followed by excretion as the mercapturic acid derivative.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure, the Committee assigned all 37 of the flavouring agents in this group to structural class I (Cramer et al., 1978).

Step 2. All 37 flavouring agents (Nos 1348–1384) in this group are expected to be metabolized to innocuous products. The evaluation of these flavouring agents therefore proceeded via the A-side of the decision-tree.

Step A3. The estimated daily intakes of all 37 flavouring agents in this group in Europe and the USA are below the threshold for concern for class I (i.e. 1800 μg/person). According to the Procedure, the safety of these 37 flavouring agents raises no concern when they are used at their estimated current intakes.

The intake considerations and other information used to evaluate the 37 aliphatic, linear, α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters in this group according to the Procedure are summarized in Table 1.

1.5 Consideration of secondary components

As many of the flavouring agents in this group are subject to conjugation with reduced glutathione, simultaneous consumption of the α,β-unsaturated aldehydes, at sufficiently high concentrations, could theoretically deplete glutathione, resulting in lipid peroxidation. However, under normal conditions and at the estimated current intakes resulting from use as flavouring agents, replenishable intracellular concentrations of glutathione (approximately 1–10 mmol/l) would be sufficient to detoxify the agents in this group. Additionally, since the α,β-unsaturated aldehydes provide similar flavouring characteristics, it is unlikely that all foods containing these flavouring agents will be consumed concurrently on a daily basis. On the basis of estimated current intakes of α,β-unsaturated aldehydes used as flavouring agents, and the constant replenishment of glutathione by biosynthesis, the Committee therefore concluded that the combined intake of these flavouring agents would not present a safety concern.

1.6 Consideration of combined intakes from use as flavouring agents

As many of the flavouring agents in this group are subject to conjugation with reduced glutathione, simultaneous consumption of the α,β-unsaturated aldehydes, at sufficiently high concentrations, could theoretically deplete glutathione, resulting

in lipid peroxidation. However, under normal conditions and at the estimated current intakes resulting from use as flavouring agents, replenishable intracellular concentrations of glutathione (approximately 1–10 mmol/l) would be sufficient to detoxify the agents in this group (Armstrong, 1987, 1991). Additionally, since the α,β-unsaturated aldehydes provide similar flavouring characteristics, it is unlikely that all foods containing these flavouring agents will be consumed concurrently on a daily basis. On the basis of estimated current intakes of α,β-unsaturated aldehydes used as flavouring agents, and the constant replenishment of glutathione by biosynthesis, the Committee therefore concluded that the combined intake of these flavouring agents would not present a safety concern.

1.7 Conclusions

The Committee concluded that none of the flavouring agents in this group of aliphatic, linear, α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters would present safety concerns at estimated current intakes. The Committee noted that the available data on the toxicity and metabolism of these aliphatic, linear, α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters were consistent with the results of the safety evaluation conducted according to the Procedure.

2. RELEVANT BACKGROUND INFORMATION

2.1 Additional considerations on intake

Quantitative data on natural occurrence and consumption ratios have been reported for 2-decenal (No. 1349), 2-dodecenal (No. 1350), ethyl acrylate (No. 1351), 2-hexenal (No. 1353), 2-hexen-1-ol (No. 1354), (*E*)-2-hexen-yl acetate (No. 1355), *trans*-2-heptenal (No. 1360), 2-nonenal (No. 1362), 2-octenal (No. 1363), 2-pentenal (No. 1364), *trans*-2-nonen-1-ol (No. 1365), and 2-undecenal (No. 1366) and demonstrate that consumption occurs predominantly from traditional foods (i.e. consumption ratio of >1). 2-Hexenal, the substance with the highest reported annual volume of production in Europe and the USA, is a common component of many foods. Intake of 2-hexenal from consumption of traditional foods exceeds intake as an added flavouring agent by a factor of >100 000 (Stofberg & Grundschober, 1987) (see Table 2). The highest dietary exposure to 2-hexenal occurs from fruits and vegetables, with an estimated daily intake of between 31 and 165 μg/kg bw (Eder et al., 1999).

2.2 Biological data

2.2.1 Biochemical data

(a) Hydrolysis

(i) Acetals

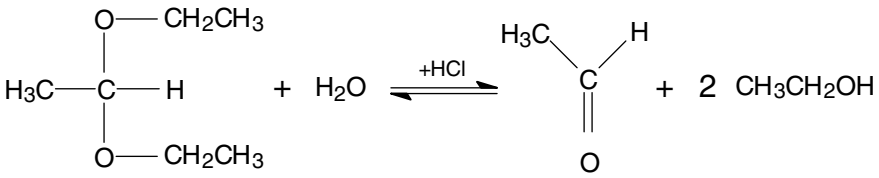
In general, aliphatic acetals undergo hydrolysis to their component aldehydes and alcohols (Knoefel, 1934; Morgareidge, 1962). Studies in vitro have shown that

1,1-dimethoxyethane[1], acetal[2], and related acetals are hydrolysed within 1–5 h in simulated gastric fluid, and to a lesser extent in simulated intestinal fluid (Morgareidge, 1962). Indirect evidence reported in a study in which rabbits were given 1,1-dimethoxyethane, acetal, and other aliphatic acetals in aqueous suspension by stomach tube indicate that rapid hydrolysis occurs in the stomach (see Figure 1) (Knoefel, 1934). A correlation was reported between narcotic effects, which are observed at high doses of acetals, and resistance to acid hydrolysis (Knoefel, 1934). It is anticipated that aliphatic acetals would undergo similar hydrolysis in humans.

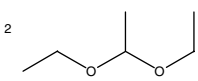
As part of a study to investigate the feasibility of using acetals as prodrugs, 2-propylpentanal acetals were synthesized and their metabolic conversion to valproic acid (2-propylpentanoic acid), an anticonvulsant, was investigated. The acid and alcohol of 2-propylpentanal were identified in the supernatant and microsomal fractions of rat liver incubated with the dimethyl, diethyl, and di-isopropyl acetals of 2-propylpentanal. These findings indicate that dimethoxy-, diethoxy-, and diisopropyl-2-propylpentane acetals hydrolyse to yield the corresponding alcohols and parent aldehyde 2-propylpentanal (Vicchio & Callery, 1989).

On the basis of this information, it can be concluded that acetals are readily hydrolysed in the acidic environment of the stomach, intestinal fluid, or in the liver to yield the component alcohol and aldehyde. Therefore, *trans*-2-hexenal diethyl acetal (No. 1383) is expected to hydrolyse to 2-hexenal (No. 1353) and ethanol. Certain aspects of the absorption, distribution, and excretion of these acetal metabolites have been studied in rodents and humans, respectively (Hald & Jacobsen, 1948; Wallgren & Barry, 1970; Halsted et al., 1973; Lame & Segall, 1986; Mitchell & Petersen, 1987).

Figure 1. Hydrolysis of acetal



[1]  1,1-dimethoxyethane

[2]  acetal

(ii) Esters

In general, aliphatic esters formed from 2-alkenols or 2-alkenoic acids (Nos 1348, 1351, 1352, 1355–1358, 1367, 1368, 1375–1379, 1381, 1382) are rapidly hydrolysed to their component alcohols and carboxylic acids by carboxylesterases (see Figure 2) (Heymann, 1980; Graffner-Nordberg et al., 1998; Anders, 1989). The substrate specificity of B-carboxylesterase isoenzymes has been correlated with the structure of the alcohol and the carboxylic acid components (e.g. *R* and *R′*, see Figure 1). Esters formed from 2-alkenols or 2-alkenoic acids are more rapidly hydrolysed than their saturated analogues (Heymann, 1980).

2-Propenyl esters are rapidly hydrolysed in vivo to yield 2-propenol (allyl alcohol) and the corresponding carboxylic acid (Silver & Murphy, 1978). Hydrolysis of these esters in vitro has been demonstrated repeatedly (Butterworth et al., 1975; Grundschober, 1977; Longland et al., 1977; Silver & Murphy, 1978). 2-Propenyl hexanoate[3] was readily hydrolysed in artificial pancreatic juice ($t_{1/2}$ = 1.98 min), rat liver ($t_{1/2}$ = 3.96 s), and rat small intestinal mucosa ($t_{1/2}$ = 0.096 s), but more slowly in artificial gastric juice ($t_{1/2}$ = 1120 min) (Longland et al., 1977). The tiglate ester, at a concentration of 400 µl/l, was completely hydrolysed in pig jejunum in <2 h at pH 7.5 (Leegwater & van Straten, 1974; Grundschober, 1977). Data on the acetate, propionate, hexanoate, isobutyrate, isovalerate, and 2-ethyl hexanoate esters of 2-propenol indicate that the rate of hydrolysis of straight-chain 2-propenyl esters is approximately 100 times greater than that of branched-chain 2-propenyl esters (Butterworth et al., 1975). The almost complete suppression of the hydrolysis of a series of 2-propenyl esters in rat liver homogenate by carboxylesterase inhibitors (triorthotolyl phosphate and *S*,*S*,*S*-tributylphosphotrithioate) provides evidence that hepatic carboxylesterases catalyse hydrolysis of 2-propenyl esters (Silver & Murphy, 1978).

The hydrolysis of esters formed from aliphatic alcohols and α,β-unsaturated carboxylic acids is similar to that of the esters discussed above. In a study of hydrolysis in vitro, a series of acrylate (2-propenoate) esters (1 µmol/l per ml) were incubated with rat liver, kidney and lung homogenates (see Table 3) (Miller et al., 1981). The rates of hydrolysis for methyl acrylate[4], ethyl acrylate (No. 1351), and butyl acrylate[5] in tissue homogenates were similar, although the rate of hydrolysis in the liver homogenate was approximately 20 times faster than in the kidney and lung homogenates (Miller et al., 1981). The three esters also disappeared rapidly when added to rat blood in vitro; the half-life for the alpha and beta phases for

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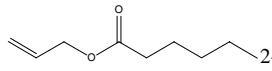
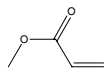
3  2-Propenyl hexanoate

4  methyl acrylate

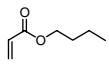
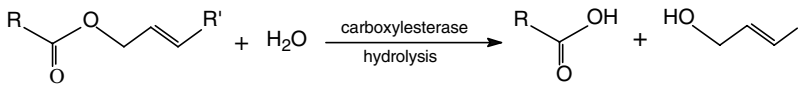
5  butyl acrylate

Figure 2. Hydrolysis of esters



R, R'=alkyl

Table 3. Hydrolysis of acrylate esters

	Methyl acrylate (nmol/min)	Ethyl acrylate (nmol/min)	Butyl acrylate (nmol/min)
Liver homogenate	17.2	26.8	23.6
Kidney homogenate	0.6	0.9	Negative
Lung homogenate	1.2	1.3	Negative
Whole blood	11.0	12.0	9.4

From Miller et al. (1981)

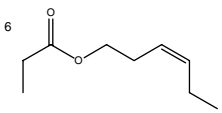
ethyl acrylate were 2.7 and 20.9 min, respectively (Miller et al., 1981). The short-chain acrylate esters (methyl acrylate, V_{max} = 0.241 mmol/l per min; ethyl acrylate, V_{max} = 0.568 mmol/l per min) are hydrolysed more rapidly than butyl acrylate (V_{max} = 0.141 mol/l per min) at enzyme-saturating levels of rat nasal mucosal carboxylesterase in vitro (Stott & McKenna, 1985). The specific activity of rat nasal mucosal carboxylesterase is reported to be equivalent to that of rat liver and greater than that of rat kidney, lung or blood (Stott & McKenna, 1985).

Hydrolysis of 2- and 3-hexenyl esters has also been reported. Studies conducted in vitro in simulated pancreatic fluid (100 ml) incubated at 37 °C indicate 100% hydrolysis of 0.015 mmol/l *trans*-2-hexenyl propionate (No. 1378) to the corresponding alcohol, 2-hexen-1-ol (No. 1354), and propionic acid within 2 h (Bennett, 1998). Similarly, varying concentrations (0.015, 0.020, or 0.025 mmol/l) of a related unsaturated ester, *cis*-3-hexenyl propionate[6], were shown to be hydrolysed completely to *cis*-3-hexenol within 2 h under the same experimental conditions (Bennett, 1998).

Once hydrolysed, the resulting alcohols, aldehydes, and carboxylic acids are readily metabolized in well-recognized biochemical pathways.

(b) Absorption, distribution, and excretion

Data on α,β-unsaturated aldehydes, carboxylic acids and their corresponding alcohols demonstrate that they are rapidly absorbed, distributed, metabolized and excreted in the urine, and to a lesser extent in the faeces. Groups of 10 male

6  *cis*-3-hexenyl propionate

Wistar albino rats were given the structurally related substances *trans*-2-nonenal or *trans*-2-pentenal (in olive oil) as single oral dose at 100 mg/kg bw. The control group received the vehicle only. Analyses by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) conducted on samples of urine collected before and after administration of the test materials confirmed that *trans*-2-nonenal is absorbed from the gastrointestinal tract into the systemic circulation and excreted in the urine mainly as C3 mercapturate conjugates within 24 h. Trace amounts of *trans*-2-nonenal were detected in the faeces. Similar results were obtained from the rats given *trans*-2-pentenal (Grootveld et al., 1998).

In rodents, the α,β-unsaturated aldehyde 3,7-dimethyl-2,6-octadienal is rapidly absorbed from the gastrointestinal tract and distributed throughout the body (Phillips et al., 1976; Diliberto et al., 1988). In Fisher F344 rats given [$^{14}C_1$ and $^{14}C_2$]3,7-dimethyl-2,6-octadienal at a dose of 5, 50, or 500 mg/kg bw orally or 5 mg/kg bw intravenously, most of the radiolabel was excreted in the urine, faeces, and expired air as radiolabelled carbon dioxide ($^{14}CO_2$) or [^{14}C]3,7-dimethyl-2,6-octadienal within 24 h. At 5 mg/kg bw administered orally, >45% and >6% of the administered dose was excreted in the urine and faeces, respectively, and approximately 16% and 1% was excreted as expired $^{14}CO_2$ and ^{14}C-labelled 3,7-dimethyl-2,6-octadienal, respectively, within 24 h. Production of $^{14}CO_2$ essentially ceased 12 h after dosing. The excretion profiles did not change at the other doses, indicating that distribution is independent of dose (Diliberto et al., 1988). At 5 mg/kg bw, >75% of the dose administered by intravenous injection was removed from the blood in the first 2 min. Elimination was essentially complete within 24 h (Diliberto et al., 1988). In a study on the effects of induction on metabolism, disposition, and excretion, rats were orally pre-treated with 3,7-dimethyl-2,6-octadienal at a dose of 5 mg/kg bw per day for 10 days. They were then given single doses of radiolabelled 3,7-dimethyl-2,6-octadienal at a dose of 5 mg/kg bw orally for the disposition study or 5 mg/kg bw intravenously for the biliary excretion study. After intravenous dosing, >20% of total radiolabel was excreted in the bile within the first hour. Five minutes after administration, unmetabolized 3,7-dimethyl-2,6-octadienal was not detected in the bile. Biliary excretion increased 34% in pre-treated animals compared with those receiving no pre-treatment, possibly indicating that some metabolism had been induced; however, pre-treatment had no effect on the disposition of 3,7-dimethyl-2,6-octadienal in rats (Diliberto et al., 1988). The authors concluded that 3,7-dimethyl-2,6-octadienal is rapidly absorbed, metabolized and excreted in the urine, faeces, and expired air. Tissue distribution is widespread, but there is no evidence to suggest that there is significant bioaccumulation (Diliberto et al., 1988).

The ethyl ester of 2-propenoic acid is also readily absorbed, metabolized and excreted (National Toxicology Program, 1986; DeBethizy et al., 1987; Ghanayem et al., 1987; Frederick et al., 1992). Ethyl acrylate (No. 1351) given orally to rats is rapidly absorbed and distributed to all major tissues (Ghanayem et al., 1987). Rats given [2,3-^{14}C]ethyl acrylate at doses of 100 to 400 mg/kg bw by intragastric instillation absorbed >90% of the radiolabel within 4 h of administration. The tissues with the highest concentration of radiolabel at 4 h after administration were the forestomach, glandular stomach, intestine, liver and kidney. Generally, the concentration of radiolabel in the blood and tissues was proportional to the dose, except in

the forestomach where rats given [2,3-^{14}C]ethyl acrylate at a dose of 400 mg/kg bw had lower concentrations of radiolabel at 4 h than rats given a dose of 200 mg/kg bw at the same time interval (Ghanayem et al., 1987). The major route of excretion of labelled ethyl acrylate is by exhalation of CO_2.

In a metabolic study, groups of three male Sprague-Dawley rats were given [2,3-^{14}C]ethyl acrylate as a single oral dose at 2, 20, or 200 mg/kg (DeBethizy et al., 1987). Total radiolabel recovered at the end of the study at 72 h ranged from 73 to 108% (see Table 4). Approximately 10–15% of the residual radiolabel was found in four major tissues — liver, stomach, gastrocnemius muscle, and epididymal fat. The dose did not affect the rate of expiration of $^{14}CO_2$, which was the primary mode of elimination and accounted for 52–61% of the original dose excreted. Approximately 45–60% of the total $^{14}CO_2$ recovered was excreted within the first 10 h after dose administration. A dose-dependent decrease in urinary and faecal excretion of radiolabel was reported (DeBethizy et al., 1987).

No ethyl acrylate was detected (limit of detection was 1 μg/ml) in peripheral blood at up to 60 min in male and female F344 rats given ethyl acrylate as a single dose at 200 mg/kg by gavage, indicating efficient absorption and rapid metabolic clearance (>95%) of ethyl acrylate after oral administration (National Toxicology Program, 1986; Frederick et al., 1992). Plasma half-lives reported in male and female F344 rats given ethyl acrylate were 14 and 11 min in blood, 74 and 94 min in forestomach tissue, 64 and 62 min in glandular stomach tissue, and 49 and 68 min in stomach contents, respectively (National Toxicology Program, 1986).

These data support the conclusion that 2-alkenals, 2-alkenoic acids and related 2-alken-1-ols and esters are rapidly absorbed, metabolized and excreted.

(c) Metabolism

The Committee has previously evaluated a group of 26 flavouring agents that included aliphatic, alicyclic, linear, α,β-unsaturated, di- and trienals and related alcohols, acids and esters (Annex 1, reference *166*) with similar metabolic profiles.

Table 4. Distribution of radiolabel (%) in male rats at 72 h after a single oral dose of [2,3-^{14}C]ethyl acrylate

Dose (mg/kg bw)	Radiolabel (%)				
	Expired CO_2	Urine	Major tissues	Faeces	Total recovered
2	61.1	28.4	13.0	5.9	108.4
20	56.8	13.5	14.9	3.7	88.8
200	52.3	8.4	10.4	1.8	72.8

From DeBethizy et al. (1987).

(i) Enzymatic conversion of aliphatic 2-alkenols and α,β-unsaturated aldehydes to carboxylic acids

Isoenzyme mixtures of NAD^+/NADH-dependent alcohol dehydrogenase (ADH) obtained from human liver microsomes catalyse the oxidation of aliphatic unsaturated alcohols (Pietruszko et al., 1973). In a comparison of saturated and unsaturated alcohols as substrates for human or horse ADH, the 2-alkenols exhibited increased binding (lower K_m) than their corresponding saturated analogues. There is also a correlation between increasing chain length (C1 to C6) of the alcohol substrate and increasing binding affinity (lower K_m) of ADH. However, maximum rates of reaction (higher V_{max}) for oxidation were essentially constant regardless of the alcohol structure (Klesov et al., 1977), indicating that the binding or release of the alcohol substrate is not the rate-limiting step for the reaction. The metabolism of 2-hexen-1-ol (No. 1354) and the corresponding aldehyde has been studied in mammals. Compared with six homologous saturated linear aliphatic alcohols, 2-hexen-1-ol exhibited the lowest K_m (i.e. greater enzyme-binding affinity) and highest V_m (maximum reaction rate) during oxidation catalysed by isoenzyme mixtures of NAD^+/NADH-dependent ADH obtained from human liver (Pietruszko et al., 1973).

Similarly, aldehyde dehydrogenase (ALDH) present predominantly in hepatic cytosol (Lame & Segall, 1986) exhibits broad specificity for the oxidation of aliphatic and aromatic aldehydes to yield the corresponding carboxylic acids (Feldman & Weiner, 1972). *trans*-2-Hexenal (No. 1353) is readily oxidized to *trans*-2-hexenoic acid (No. 1361) in mouse hepatic cytosol fractions (Lame & Segall, 1986) and in isoenzymes of rat ALDH present in mitochondrial, cytosolic, and microsomal fractions (Mitchell & Petersen, 1987). ALDH demonstrates higher catalytic activity in vitro for higher relative molecular mass, and more lipophilic aldehydes (Nakayasu et al., 1978). The molybdenum-containing enzymes xanthine oxidase and aldehyde oxidase also catalyse the oxidation of a wide range of aldehydes (Beedham, 1988). Examination of the stomach contents of rats 16 h after receiving *trans*-2-nonenal at a dose of 100 mg/kg showed that approximately 15% of the administered dose had been oxidized to *trans*-2-nonenoic acid (Grootveld et al., 1998). This assortment of oxidative enzymes provide the numerous metabolic options for the rapid conversion of 2-alkenols to α,β-unsaturated aldehydes and then to their corresponding α,β-unsaturated carboxylic acids.

(ii) Metabolism of aliphatic linear α,β-unsaturated carboxylic acids

The resulting linear α,β-unsaturated acids, such as *trans*-2-hexenoic acid (No. 1361), participate directly in fatty acid metabolism. In the fatty acid pathway, the α,β-unsaturated carboxylic acid is first condensed with coenzyme A (CoA) (Nelson & Cox, 2000). The resulting *trans*-2,3-unsaturated CoA ester (*trans*-Δ^2-enoyl CoA) is converted to the 3-ketothioester, which undergoes β-cleavage to yield an acetyl CoA fragment and a new thioester reduced by two carbons.

Cleavage of acetyl CoA units will continue along the carbon chain until the position of unsaturation is reached. If the unsaturation begins at an even-numbered carbon as in an α,β-unsaturated acid, acetyl CoA fragmentation will eventually

yield a Δ^2-enoyl CoA that is a substrate for further fatty acid oxidation. If the regiochemistry of the double bond is '*cis*', it is isomerized to the '*trans*' double bond by the action of 3-hydroxyacyl CoA epimerase prior to entering the fatty acid oxidation pathway. Even-numbered carbon acids continue to be cleaved to acetyl CoA. Acetyl CoA is completely metabolized in the citric acid cycle to yield CO_2 and water (Nelson & Cox, 2000).

Studies with radiolabelled α,β-unsaturated acids support the conclusion that α,β-unsaturated acids are completely metabolized to CO_2 and water. Between 77 and 85% of a single oral dose of [1-^{14}C](*E*,*E*)-2,4-hexadienoic acid of either 40 or 8000 mg/kg bw given to female mice is excreted as expired $^{14}CO_2$ within 4 days. Approximately 88% of the $^{14}CO_2$ was recovered within the first 24 h. Between 4 and 5% of the original dose was excreted in the urine as (*E*,*E*)-muconic acid[7] (i.e. (*E*,*E*)-2,4-hexadienedioic acid) and unchanged (*E*,*E*)-2,4-hexadienoic acid, respectively, accounting for 0.4 and 0.7% of the total radiolabel present in the urine collected over the first 24 h. Only about 1% of the 40 mg/kg bw dose was recovered in the faeces (Westoo, 1964). Regardless of dose, rats given [1-^{14}C](*E*,*E*)-2,4-hexadienoic acid at doses between 61 and 1213 mg/kg bw excreted >85% as exhaled $^{14}CO_2$ within 10 h. In the same period of time, approximately 2% of the radiolabel was detected in the urine. (*E*,*E*)-Muconic acid and unchanged (*E*,*E*)-2,4-hexadienoic acid were not detected (Fingerhut et al., 1962).

Similar results are available for esters formed from α,β-unsaturated carboxylic acids. Rats given [2,3-^{14}C]ethyl acrylate (ethyl 2-propenoate) at a dose of 200 mg/kg bw excreted approximately 27 and 70% as exhaled $^{14}CO_2$ in 4 and 24 h, respectively. A small amount of unchanged ethyl acrylate (1%) was also eliminated in the expired air within 24 h. Alternately, approximately 9 and 4% of the dose was excreted in the urine and faeces within 24 h, respectively (Ghanayem et al., 1987). Approximately 4% of the original dose (200 mg/kg bw) was excreted in the bile within 6 h of administration by gavage (Ghanayem et al., 1987). In the dose-dependent study discussed above, most of a single oral dose of [2,3-^{14}C]ethyl acrylate at 2, 20, or 200 mg/kg given to rats was exhaled as $^{14}CO_2$ with approximately half (45–60%) of the total $^{14}CO_2$ being recovered within the first 10 h (DeBethizy et al., 1987). On the basis of these data, it can be concluded that the predominant pathway for metabolism of 2,3-alkenols and α,β-unsaturated aldehydes involves oxidation to yield the corresponding α,β-unsaturated carboxylic acid followed by complete metabolism in the fatty acid pathway and tricarboxylic acid cycle.

(iii) Glutathione conjugation of α,β-unsaturated aldehydes

α,β-Unsaturated aldehydes conjugate with glutathione (GSH) directly or undergo allylic hydroxylation via lipid peroxidase to yield 4-hydroxyalkenals (Esterbauer et al., 1982) that also conjugate with GSH (Esterbauer et al., 1975; Winter et al., 1987). The GSH redox cycle maintains adequate levels of GSH in

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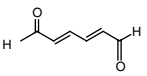
[7] 
(*E*,*E*)-muconic acid

animal cells (Nelson & Cox, 2000; Schulz et al., 2000) and is a major intracellular mechanism involved in the detoxication of α,β-unsaturated aldehydes (Reed et al., 1986). The addition of GSH across the electrophilic carbon–carbon double bond is catalysed by the enzyme glutathione *S*-transferase (GST), but can also occur at a lower rate in a non-enzymatic reaction (Eisenbrand et al., 1995; Grootveld et al., 1998).

Cultured primary rat hepatocytes, which are rich in GSH and GST, have been shown to metabolize greater amounts of 2-alkenals than human lymphoblastoid cells (Namalva cells) (Eisenbrand et al., 1995). The low levels of GSH, GST, and deactivating enzymes make the human lymphoblastoid cells more susceptible to the cytotoxic effects of 2-alkenals like *trans*-2-hexenal. In both cell types, the consumption of 2-alkenals was directly related to the depletion of intracellular GSH (Eisenbrand et al., 1995). A 75% decrease in the levels of liver GSH occurred when male rats were given the structurally related *trans*,*trans*-muconaldehyde[8] at a dose of 36 μmol/kg bw by intraperitoneal injection (Witz, 1989). Additionally, the report that the presence of GSH reduces the cytotoxicity of α,β-unsaturated aldehydes in *S. typimurium* TA104 in vitro provides additional evidence that GSH conjugation plays an important role in the detoxication process (Marnett et al., 1985).

The highly reactive α,β-unsaturated aldehyde acrolein (2-propenal) is metabolized via conjugation with GSH (Penttila et al., 1987) or other free thiol functions (Ohno et al., 1985). Conjugation of acrolein with GSH occurs with or without enzyme catalysis. The GSH adduct is subsequently reduced to the corresponding 3-hydroxypropyl GSH conjugate, which is then excreted principally as the mercapturic acid or cysteine derivative. Metabolic precursors of 2-propenal produce the same urinary metabolites as 2-propenal. 3-Hydroxypropylmercapturic acid (6–11%) was isolated from the urine of male albino CFE strain of rats given allyl alcohol (613 mg), acrolein (606 mg), allyl formate (758 mg), allyl propionate (1500 mg), or allyl benzoate (dose not stated) by subcutaneous injection (Kaye, 1973). The mercapturic acid derivative was also the primary urinary metabolite when allyl propionate was administered by intraperitoneal injection or by gavage. Similar mercapturic acid conjugates have been reported for 2-butenol and 2-butenal (Gray & Barnsley, 1971) and the higher homologues discussed below.

The major urinary metabolite isolated from the urine of male Wistar albino rats given a 100 mg/kg bw dose of *trans*-2-pentenal or *trans*-2-nonenal was the mercapturic acid conjugate of the corresponding alcohol — 3-*S*-(*N*-acetylcysteinyl)pentan-1-ol or 3-*S*-(*N*-acetylcysteinyl)nonan-1-ol, respectively (see Figure 3). Analysis of the faeces of animals dosed with 2-nonenal showed slight amounts of the unchanged aldehyde, while analysis of the stomach contents obtained 16 h after dosing showed that approximately 15% of the administered dose was present as *trans*-2-nonenoic acid. Low concentrations of glucuronic acid conjugates were also detected in the urine. The authors suggested that these

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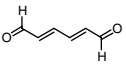
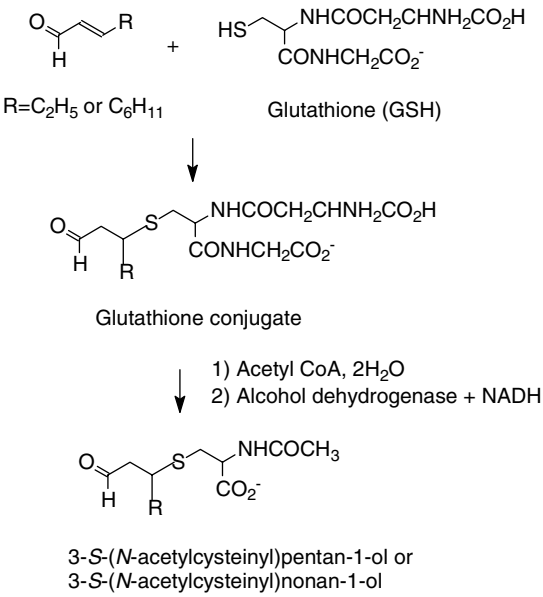
[8] 

trans,*trans*-muconaldehyde

Figure 3. Glutathione conjugation of α,β-unsaturated aldehydes



conjugates arose from a sequential pathway involving thiol conjugation, oxidation or reduction of the aldehyde functional group, followed by glucuronic acid conjugation of the resulting carboxylic acid or alcohol, respectively (Grootveld et al., 1998).

Increased conjugation with GSH is observed in α,β-unsaturated aldehydes for which β-oxidation is inhibited. The mercapturic acid conjugate was the major urinary excretion product isolated from rats given (*E*)-2-propyl 2,4-pentadienoic acid[9] as a single intraperitoneal dose at 100 mg/kg bw (Kassahun et al., 1991).

Alternatively, under conditions of oxidative stress (see section below), α,β-unsaturated aldehydes undergo lipid peroxidation before reaction with GSH. α,β-Unsaturated aldehydes have been reported to undergo C4 allylic hydroxylation catalysed by lipid peroxidase (Esterbauer et al., 1982) followed by conjugation with GSH (Esterbauer et al., 1975). Within 24 h of receiving 5-(H^3)-4-hydroxy-2-hexenal at a dose of 15 mg/kg bw by injection into the hepatic vein, Sprague-Dawley rats eliminated most (79.35%) of the radiolabel in the urine as a mercapturic acid metabolite (see Figure 4). The major excretion product resulted from Michael addition of GSH to the β-position of 4-hydroxy-2-hexenal followed by hemiacetal formation (Winter et al., 1987).

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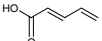
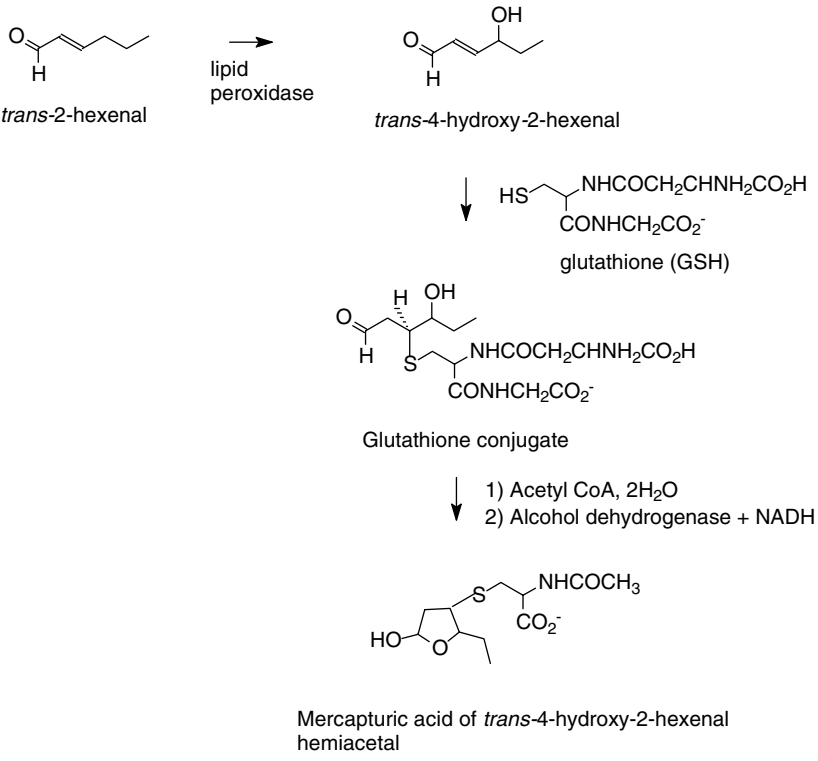
9  *(E)*-2-propyl 2,4-pentadienoic acid

Figure 4. Glutathione conjugation of α,β-unsaturated aldehydes under conditions of oxidative stress



(iv) Endogenous formation of α,β-unsaturated aldehydes

Glutathione conjugation, oxidative stress, lipid peroxidation, and apoptosis

α,β-Unsaturated aldehydes are formed endogenously by lipid peroxidation of polyunsaturated fatty acids (Frankel et al., 1987). The levels of α,β-unsaturated aldehydes obtained exogenously as naturally occurring constituents of food or as added flavouring agents are expected not to have a significant effect on the endogenous levels of α,β-unsaturated aldehydes. It may be expected that, under normal conditions, intracellular concentrations of GSH, which range from 1 to 10 mmol/l in mammalian cells (Armstrong, 1987, 1991), are sufficient to detoxify both endogenous and exogenous α,β-unsaturated aldehydes.

It should be noted that, at sufficiently high concentrations of α,β-unsaturated aldehydes, levels of cellular GSH may be depleted, resulting in a state of oxidative stress, which is characterized by the formation of reactive oxygen species or free radicals that react with various cellular components, particularly polyunsaturated fatty acids, leading to the formation of more aldehydes. In the aldehyde fragmenta-

tion pathway, abstraction of a diallylic hydrogen atom from a polyunsaturated fatty acid (e.g. the C11 hydrogen of 9,12-octadecadienoic acid, linoleic acid) and subsequent rearrangement yields a hydroperoxide intermediate. The unstable hydroperoxide readily degrades to an alkoxy radical that is prone to undergo either β-scission or hydrogen abstraction. β-Scission yields shortened, conjugated, α,β-unsaturated aldehydes such as 2-butenal, *trans*-2-hexenal, 4-hydroxy-2-nonenal, and 2,4-decadienal. Available data suggest that the formation of α,β-unsaturated aldehydes during lipid peroxidation may be involved in the pathophysiological effects associated with oxidative stress (Ichihashi et al., 2001). In addition to forming reactive unsaturated aldehydes, lipid peroxidation disturbs the structural integrity of the lipid bilayer, compromising cell permeability. This leads to membrane leakage, Na^+ influx, K^+ efflux, and influx of water leading to cytotoxic oedema. Additionally, aldehyde fragmentation products induce apoptotic cell death during oxidative stress (Esterbauer et al., 1991; Eckl et al., 1993; Dianzani, 1998).

The effectiveness of the GSH detoxication pathway for α,β-unsaturated aldehydes hinges on the ability of the cell to maintain the equilibrium between its pro-oxidant and antioxidant systems (Nelson & Cox, 2000). A decrease in the concentrations of antioxidants or an increase in the production of reactive oxygen species (e.g. oxygen, O_2; hydrogen peroxide, H_2O_2; hydroxyl radical, OH) can lead to oxidative stress, a condition in which the cell is unable to maintain the level of reactive oxygen species below a toxic threshold (Schulz et al., 2000). During periods of oxidative stress, the ratio of GSH to glutathione disulfide decreases owing to loss of GSH and accumulation of glutathione disulfide. The low levels of cellular GSH render the detoxication pathway inefficient and allow for increased interaction between the α,β-unsaturated aldehydes and cellular components (proteins and DNA), eventually leading to cytotoxicity and apoptosis (Eder et al., 1993; Ichihashi et al., 2001).

(v) Potential for macromolecular reactivity

In a series of experiments, the formation of protein adducts with endogenous and exogenous sources of α,β-unsaturated aldehydes was investigated (Ichihashi et al., 2001). When bovine serum albumin (1 mg/ml) was incubated with 2-butenal (crotonaldehyde) at a low concentration (2.5 mmol/l) for 24 h, the sum of the histidine and lysine residues lost on bovine serum albumin roughly corresponded to the protein carbonyls formed. These data suggest that at low concentrations, 2-butenal forms carbonyl adducts (Schiff bases) with histidine and lysine residues on bovine serum albumin. At higher concentrations, non-carbonyl and carbonyl adducts were detected. In adducts in which free carbonyls were present, the imidazole nitrogen on histidine and free amine nitrogen on lysine residues present in bovine serum albumin formed a covalent bond in a Michael-type addition to the *beta* position of 2-butenal.

Administration of [2,3-^{14}C]ethyl acrylate at a dose of 100, 200, or 400 mg/kg bw by intragastric instillation resulted in a high level of protein binding in the forestomach at 4 h (Ghanayem et al., 1987). Binding was also found in the liver.

In an effort to study the endogenous formation of α,β-unsaturated aldehydes and subsequent reaction with protein, a monoclonal antibody directed to protein-

bound 2-butenal was developed (Ichihashi et al., 2001). The antibody showed immunoreactivity for the 2-butenal-lysine modified protein adduct as well as those formed from 2-pentenal and 2-hexenal. Subsequently, the production of immunoreactive material was assessed in a model of renal carcinogenicity in which rats were given Fe^{3+}-nitrilotriacetate mixture by intraperitoneal injection to induce acute oxidative tissue damage in the renal proximal tubules. Animals were sacrificed at 0, 4, 8, 24, 48, and 72 h and their kidneys were excised and prepared for immunohistochemical study. Although incubation of stained kidney sections with the antibody showed little immunoreactivity at times up to and including 24 h, intense immunoreactivities were observed at 48 h in the cytoplasm and nuclei. The authors noted that the pattern of distribution and delayed onset of adduct formation in the rat kidney is consistent with the formation of membrane lipid peroxidation products (aldehydes) with cytosolic proteins (Ichihashi et al., 2001).

In two experiments conducted to link the formation of 2-alkenal-protein adducts with lipid peroxidation, low-density lipoprotein (LDL) was incubated with 5 μmol/l Cu^{2+}, and polyunsaturated fatty acids were incubated with an iron-acsorbate free radical generating system to induce the formation of lipid peroxidation products. Incubation of the antibody for the 2-butenal-lysine modified protein adduct with either the Cu-oxidized LDL product or the iron/ascorbate (oxidant), linolenic acid, bovine serum albumin mixture indicated the presence of 2-alkenal-lysine modified protein. These data strongly suggest that 2-alkenals formed endogenously by lipid peroxidation react with proteins in Schiff base and Michael addition-type reactions.

Studies in vitro indicate that 2-alkenal-DNA adducts form under conditions of oxidative stress. In cultured rat hepatocytes and human lymphoblastoid cells (Namalva cells) treated with various 2-alkenals, DNA single-strand breaks were detectable after intracellular concentrations of GSH were reduced to approximately 20% of those before treatment. Before incubation of Namalva cells and rat hepatocytes with *trans*-2-hexenal, concentrations of GSH were measured to be approximately 1.6 and 80 nmol/2 $\times$ 10^6 cells in each respective cell line. After the 1 h incubation, concentrations of GSH were reduced to approximately 10% of the control values. 2-Hexenal produced DNA damage in the Namalva cells, which are poor in GSH, at lower concentrations than in hepatocytes, which are rich in GSH. The authors concluded that metabolically proficient cells containing GSH and GST efficiently protect against the effects of 2-hexenal (Eisenbrand et al., 1995).

Studies using fluorescence spectroscopy revealed that α,β-unsaturated aldehydes bind DNA to form adducts in vitro and in vivo (Frankel et al., 1987; Eder et al., 1993; Cadet et al., 1999; National Toxicology Program, 2001a). *Trans*-2-Hexenal, a product of lipid peroxidation, has been shown to react with deoxyguanosine to produce low levels of exocyclic 1,N^2-propano adducts in calf thymus DNA, human lymphoblastoid Namalva cells, and in primary rat colon mucosa cells at concentrations of 0.2 and 0.4 mmol/l, respectively (Golzer et al., 1996).

In a study to evaluate the effect of GSH depletion on the oxidative DNA breakage, different alkenals (2-hexenal, 100 μmol/l; cinnamaldehyde, 300 μmol/l; 2,4-hexadienal, 300 μmol/l, and 2-cyclohexenone, 300 μmol/l) were incubated with V79 cells for 1 h. Under conditions in which levels of GSH were depleted to <20% of those of controls, DNA damage was reported for all four alkenals. However, at 3 h

after treatment, DNA damage decreased and concentrations of GST were increased. Using treatment with formamidopyrimidine DNA glycosylase (FPG) to monitor oxidative DNA damage, FPG-sensitive sites were detected with hexenal and cinnamaldehyde but not with 2,4-hexadienal or 2-cyclohexenone. The authors concluded that dose-dependent alkenal oxidative stress related to GSH depletion contributes to cytotoxic and genotoxic cell damage (Janowski et al., 2003).

The formation in vivo of *trans*- and *cis*-isomers of 1-N^2-propanodeoxyguanosine adducts of 2-hexenal was evaluated in various organs of groups of four male Fischer 344 rats sacrificed at different intervals (8, 24, 48, and 96 h) after administration of 2-hexenal as a single oral dose at 50, 200, or 500 mg/kg bw by gavage (Schuler & Eder, 1999). Using a ^{32}P-post-labelling technique with a limit of quantification of 0.03 adducts/10^6 nucleotides, no adducts were detected either in the organs of untreated rats or in the organs of treated rats sacrificed 8 h after treatment. The highest levels of DNA adducts were detected 48 h after treatment and occurred in organs that came into direct contact with the test substance (forestomach, oesophagus) or had high exposure (liver). In the groups receiving the two higher doses, levels of adducts determined 2 days after treatment at 500 mg/kg bw (forestomach, 3.1 adducts/10^6; liver, 1.7 adducts/10^6; oesophagus, 1.1 adducts/10^6) were disproportionately greater than those at 200 mg/kg bw (forestomach, 0.42 adducts/10^6; liver, 0.15 adducts/10^6; oesophagus, 0.1 adducts/10^6). The authors anticipated that higher doses of 2-hexenal deplete GSH and therefore, a higher fraction of the dose is available for DNA binding. The authors noted that adduct levels in animals sacrificed at 4 days were significantly lower than those sacrificed at 2 days, suggesting that some DNA repair was occurring. Except for the oesophagus (0.08 adducts/10^6), no quantifiable levels of DNA adducts were detected at 50 mg/kg bw, and only trace amounts of adducts (0.01–0.02 adducts/10^6) could be detected in other organs. This experiment did not account for the formation and reaction of 2-hexenal formed endogenously under conditions of GSH depletion and oxidative stress.

In a similar experiment, groups of male Fischer rats were given 2-butenal as single oral doses at 0, 200, or 300 mg/kg bw by gavage and DNA adducts were analysed using the same ^{32}P-post-labelling technique (Schuler & Eder, 1999). Delayed (20 h) formation of DNA adducts (2.9 adducts/10^8 nucleotides at 200 mg/kg bw and 3.4 adducts/10^8 nucleotides at 300 mg/kg bw) were detected in the liver. Significantly lower levels were observed at 12 h after the 200 mg/kg dose. DNA adducts (6.2 adducts/10^8 nucleotides or 2.0 adducts/10^8 nucleotides, respectively) were also detected in rats given 2-butenal at a dose of 1 or 10 mg/kg bw by gavage five times per week for 6 weeks. One week after male rats were given 2-butenal five times per week for 4 weeks, levels of DNA adducts were 69% of the peak level measured 24 h after the last dose. Two weeks after the last dose, adduct levels were reduced to 18% of the peak level, suggesting that continued repair was occurring.

In a study to assess the relationship between cytotoxicity, DNA damage, and GSH depletion, a series of α,β-unsaturated aldehydes (2-hexenal to 2-nonenal) were incubated with Chinese hamster fibroblast V79 cells or Caco-2 human colon adenocarcinoma cells for 1 h. In both cell lines, similar levels of cytotoxicity were reported, with 2-nonenal being the most cytotoxic. The degree of DNA damage in

V79 and Caco-2 cell lines overlapped to a high extent with cytotoxic concentrations, suggesting that cytotoxicity is directly correlated with interaction with DNA. After 1 h, concentrations of GSH were approximately 20% of those of controls. GSH depletion occurred at concentrations at least tenfold lower than those required for DNA damage, also suggesting that depletion of cellular GSH is a prerequisite for DNA damage (Glaab et al., 2001).

Studies using the ^{32}P-post-labelling procedure have shown that *trans*-2-hexenal forms cyclic 1,N^2-propano adducts with deoxyguanosine in primary colon mucosa cells from rats and humans at concentrations as low as 0.4 mol/l. Hexenal has been detected in flavoured foods at concentrations of up to 14 mg/kg (0.14 mmol/l), which is comparable to its natural occurrence in some fruits and vegetables (up to 30 mg/kg). This clearly indicates that hexenal induces genotoxic effects in cells from the rat and human gastrointestinal tracts at concentrations similar to those found in food (Golzer et al., 1996).

In addition to direct reaction with DNA, α,β-unsaturated aldehydes act to induce DNA fragmentation through apoptosis. Recent experiments (Ji et al., 2001) indicate that depletion of GSH by α,β-unsaturated aldehydes (4-hydroxy-2-nonenal), progressing in a dose- and time-dependent manner, induces poly-ADP-ribose polymerase (PARP) cleavage and DNA fragmentation. In one of the pathways of apoptosis, depletion of GSH results in the release of mitochondrial cytochrome c to the cytosol where a cascade of cytosolic cysteine proteases (i.e. caspases) is activated. Activation of caspase-3 causes cleavage of cellular proteins and PARP, leading to DNA fragmentation and subsequent cell death (Liu et al., 1996; Li et al., 1997; Zou et al., 1997; Green & Reed, 1998; Cain et al., 1999).

In summary, it is concluded that high cellular concentrations of 2-alkenals may deplete GSH, leading to oxidative stress and the formation of protein and DNA adducts. Under these conditions, alkenals may also form endogenously from the increased lipid peroxidation of membrane polyunsaturated fatty acids. To some extent, repair is observed after cessation of exposure. At the concentrations of alkenals that are present in the diet, there is no significant potential for oxidative stress or the formation of DNA adducts.

2.2.2 Toxicological studies

Typically, toxicological studies would be organized in this monograph according to duration (i.e. short-term, long-term, and carcinogenicity), flavouring agent and then species. However, in the interest of preserving the continuity of the studies performed by the National Toxicology Program, the short-term studies of toxicity and carcinogenicity will be discussed in the section on long-term studies (see 2.2.2 (c)) in the sequence in which they were conducted.

(a) Acute toxicity

Oral median lethal dose (LD_{50}) values have been reported for 19 of the 37 substances in this group. Seventeen of the α,β-unsaturated aldehydes and related alcohols, acids, and esters used as flavouring agents (Nos 1349–1353, 1356,

1357, 1359, 1360, 1362, 1369, 1371, 1375, 1377, 1378, 1381, and 1383) have rat oral LD_{50}s in the range of 767 to >5000 mg/kg bw (Pozzani et al., 1949; Bär & Griepentrog, 1967; Smyth et al., 1970; Gaunt et al., 1971; Moreno, 1972, 1973a, 1973b, 1973c, 1976, 1977a, 1977b, 1977c, 1978a, 1978b, 1978c, 1979; Freeman, 1980; Moreno, 1980a, 1980c; Mondino, 1981; Moreno, 1982).

In mice, oral LD_{50} values (Nos 1353, 1359, 1367, and 1368) are in the range of 1550 to >8000 mg/kg bw (Gaunt et al., 1971; Pellmont, 1974a, 1974b; Moreno, 1980b), demonstrating that the oral acute toxicity of α,β-unsaturated aldehydes and related alcohols, acids and esters is low (see Table 5).

(b) Short-term studies of toxicity

The results of short-term studies of toxicity on ethyl acrylate (No. 1351) and 2-hexenal (No. 1353) are described below and summarized in Table 6.

(i) Ethyl acrylate (No. 1351)

Rats

In a 2-week study, groups of 10 male F344/N rats were given ethyl acrylate at a dose of 2, 10, 20, 50, 100 or 200 mg/kg bw per day by intragastric instillation in corn oil for 5 days per week, or were given drinking-water containing ethyl acrylate at a concentration corresponding to a dose of 0, 23, 99, 197 or 369 mg/kg bw per day for 7 days per week. Concurrent control groups were maintained. At study termination, primary compound-related histopathological changes (i.e. hyperplasia, hyperkeratosis, inflammation, oedema, and ulcers/erosions) were observed in the forestomach of the rats treated by gavage. The incidence and severity of the epithelial hyperplasia increased in a dose-related manner at doses of ≥20 mg/kg bw per day administered by gavage. An increase in forestomach weight reaching 281% of the values for controls was reported in the group receiving a dose of 200 mg/kg bw per day by gavage. No compound-related effects were observed in the group receiving a dose of 10 mg/kg bw per day by gavage. Rats given drinking-water containing ethyl acrylate exhibited a lower incidence and severity of forestomach irritation than did rats dosed via gavage. Dose-dependent diffuse epithelial forestomach hyperplasia was reported at doses of ≥99 mg/kg bw per day, while oedema, erosions and ulcers were not observed in any of the animals given drinking-water containing ethyl acrylate. A slight increase in forestomach weight was reported only at the highest dose (369 mg/kg bw per day). No compound-related effects were observed in the groups of rats given drinking-water containing ethyl acrylate at a dose of 23 mg/kg bw per day. No lesions were observed in the glandular stomach of any treated animals. In comparison with controls, animals treated by gavage exhibited minimal depletion of non-protein sulfhydryl (NPSH) at doses of 20–50 mg/kg bw per day, while severe depletion of NPSH (25% of baseline concentration) was observed at doses of ≥100 mg/kg bw per day. In comparison, no significant depletion of NPSH was noted in animals receiving drinking-water containing ethyl acrylate. On the basis of the results of this study, the authors concluded that continued exposure to ethyl acrylate at low oral doses is not associated with severe tissue toxicity or carcinogenicity (Frederick et al., 1990).

Table 5. Studies of the acute toxicity of aliphatic, linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters administered orally

No.	Flavouring agent	Species	Sex	LD_{50} (mg/kg bw)	Reference
1349	2-Decenal	Rat	NR	5000	Moreno (1977a)
1350	2-Dodecenal	Rat	M	>5000	Moreno (1980a)
1351	Ethyl acrylate	Rat	F	0.83 ml/kg[a] (767 mg/kg bw)[b]	Smyth et al. (1970)
1351	Ethyl acrylate	Rat	M	1020	Pozzani et al. (1949)
1352	Ethyl 2-nonynoate	Rat	NR	2850	Moreno (1973a)
1353	2-Hexenal	Rat	NR	850	Moreno (1973b)
1353	2-Hexenal	Rat	M, F	780 (M) 1130 (F)	Gaunt et al. (1971)
1353	2-Hexenal	Mouse	M, F	1750 (M) 1550 (F)	Gaunt et al. (1971)
1356	Methyl 2-nonynoate	Rat	M, F	1180 (M) 870 (F)	Freeman (1980)
1356	Methyl 2-nonynoate	Rat	NR	2220	Moreno (1973c)
1357	Methyl 2-octynoate	Rat	M	2500	Moreno (1972)
1357	Methyl 2-octynoate	Rat	NR	1530	Bär & Griepentrog (1967)
1359	2-Tridecenal	Mouse	NR	>5000	Moreno (1980b)
1359	2-Tridecenal	Rat	NR	>5000	Moreno (1979)
1360	*trans*-2-Heptenal	Rat	NR	1300	Moreno (1980c)
1360	*trans*-2-Heptenal	Rat	NR	1300	Moreno (1982)
1362	2-Nonenal	Rat	NR	5000	Moreno (1977b)
1367	*trans*-2-Octen-1-yl acetate	Mouse	NR	>8000	Pellmont (1974a)
1368	*trans*-2-Octen-1-yl butanoate	Mouse	NR	>8000	Pellmont (1974b)
1369	*cis*-2-Nonen-1-ol	Rat	M, F	>5000	Mondino (1981)
1371	*(E)*-2-Butenoic acid	Rat	NR	1000	Bär & Griepentrog (1967)
1375	*trans*-2-Hexenyl butyrate	Rat	NR	>5000	Moreno (1978a)
1377	*trans*-2-Hexenyl isovalerate	Rat	NR	>5000	Moreno (1978b)
1378	*trans*-2-Hexenyl propionate	Rat	NR	>5000	Moreno (1976)
1381	*(E)*-2-hexenyl hexanoate	Rat	NR	>5000	Moreno (1978c)
1383	*(E)*-2-Hexenal diethyl acetal	Rat	NR	860	Moreno (1977c)

F, female; M, male; NR, not reported.

[a] Ethyl acrylate was provided as a mixture, together with ethyl acetate or formalin.

[b] Calculated using density of ethyl acrylate = 0.924 g/ml (available at www.sigmaaldrich.com).

Table 6. Results of short-term studies of toxicity and long-term studies of toxicity and carcinogenicity with aliphatic, linear, α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/ no. per group[b]	Route	Duration	NOEL (mg/kg bw per day)	Reference
Short-term studies of toxicity							
1351	Ethyl acrylate	Rat; M	6/10	Gavage	2 weeks	10	Frederick et al. (1990)
1351	Ethyl acrylate	Rat; M	4/10	Drinking-water	2 weeks	23	Frederick et al. (1990)
1351	Ethyl acrylate	Rat; M, F	4/40 M, 20 F	Drinking-water	13 weeks	20	Bernacki et al. (1987a)
1351	Ethyl acrylate	Rat; M	3/20[c]	Gavage	13 weeks	<20	Bernacki et al. (1987b)
1351	Ethyl acrylate	Rat; M	2/10 to 11[d]	Gavage	13 weeks	<100	Ghanayem et al. (1991a and 1991 b)
1353	2-Hexenal	Rat; M, F	4/30	Diet	13 weeks	80	Gaunt et al. (1971)
1353	2-Hexenal	Rabbit; F	1/10	Gavage	13 weeks	<200	Gaunt et al. (1971)
Long-term studies of toxicity and carcinogenicity (and range-finding studies)							
1351	Ethyl acrylate	Mouse; M, F	5/20	Gavage	13 weeks	25	National Toxicology Program (1986)
1351	Ethyl acrylate	Mouse; M, F	4/20	Gavage	13 weeks	100	National Toxicology Program (1986)
1351	Ethyl acrylate	Mouse; M, F	2/100	Gavage	103 weeks	<100	National Toxicology Program (1986)
1351	Ethyl acrylate	Rat; M, F	5/20	Gavage	13 weeks	55	National Toxicology Program (1986)
1351	Ethyl acrylate	Rat; M, F	2/100	Gavage	103 weeks	<100	National Toxicology Program (1986)
1351	Ethyl acrylate	Rat; M, F	3/50	Drinking-water	2 years	170 (M) 120 (F)	Borzelleca et al. (1964)
1351	Ethyl acrylate	Dog; M, F	3/4	Oral[e]	2 years	75	Borzelleca et al. (1964)

F, female; M, male.

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] A recovery group (10 rats) was also maintained.

[d] Additional recovery groups of 10 and 26–35 rats were also maintained.

[e] gelatine capsules.

Groups of 40 male and 20 female F344/N rats were given drinking-water containing ethyl acrylate at a concentration of 0, 200, 1000, 2000 or 4000 mg/kg for 7 days per week for 13 weeks. These concentrations correspond to average daily doses of 0, 20, 100, 200 and 400 mg/kg bw, respectively (Food and Drug Administration, 1993). Clinical signs of toxicity were monitored daily, while food and water consumption and body weight were monitored weekly. At weeks 1, 2, 4 and 13, a select number of rats from each group was necropsied and subjected to a complete histopathological examination. All treated animals survived until the end of the study period. No clinical signs of systemic toxicity were observed at any of the doses examined. Decreased consumption of water was observed in all treated animals. Although decreased food consumption and body weight were reported in all treated males and decreased food consumption was reported in females at 100, 200 and 400 mg/kg bw per day, the authors did not consider this to be an indication of systemic toxicity, but rather a secondary response to the decreased consumption of water. Similarly, although variations in weights of the kidney and liver were noted at weeks 4 and 13, these were deemed to be secondary to body-weight changes and toxicologically insignificant on the basis of the lack of a dose-dependent response and the magnitude of the changes. Increased weights of the stomach were reported in males and females at 100, 200 and 400 mg/kg bw per day throughout the study. This increase was accompanied by histopathological changes, including minimal (100 mg/kg bw per day) to moderate (400 mg/kg bw per day) hyperplasia and hyperkeratosis (200 and 400 mg/kg bw per day) of the forestomach. Acute irritation was present in the forestomach of males and females at 200 and 400 mg/kg bw per day during the first 2 weeks, but not after week 4. No histopathological changes were reported in the glandular stomach of males and females at any dose. No compound-related changes were reported at 20 mg/kg bw per day compared with the controls (Bernacki et al., 1987a).

Groups of 20 male F344/N rats were given ethyl acrylate (dissolved in corn oil) at a dose of 0, 20, 100 or 200 mg/kg bw per day by intragastric instillation, 5 days per week, for 13 weeks. An additional 'recovery' group of 10 rats was given ethyl acrylate at a dose of 200 mg/kg bw per day for the first 4 weeks of the study only (administration was subsequently discontinued for the remainder of the study period, i.e. 9 weeks). Animals were monitored daily for clinical signs of toxicity. Food consumption and body weights were monitored weekly. At weeks 4 and 13 (only at week 13 for the recovery group), 10 animals from each treatment group were necropsied and subjected to a complete histopathological examination. No clinical signs of toxicity and no early deaths were reported throughout the study. A slight decrease in body weight was reported at 200 mg/kg bw per day at week 13. Only incidental, non-dose-dependent liver and kidney weight changes were identified. Significantly increased ($p < 0.05$), generally in a dose-dependent manner, absolute and relative weights of the stomach were reported in the groups receiving a dose of 100 and 200 mg/kg bw per day after 4 weeks of exposure and in all treated groups after 13 weeks of exposure. The increased stomach weights were accompanied at every dose by histopathological changes in the forestomach, including diffuse hyperplasia and hyperkeratosis of the squamous epithelium. Evidence of sustained irritation of the forestomach was present at 100 (minimal only) and 200 mg/kg bw per day at week 4, and at 200 mg/kg bw per day at week 13.

Focal papillomatous hyperplasia, overlaying sites of focal inflammation, was reported at weeks 4 and 13, but only at the highest dose (200 mg/kg bw per day), as were incidences of focal eschar, epithelial haemorrhage and ulcers of the limiting ridge at week 4. No histopathological changes were reported in the glandular stomach of males and females at any dose. Increased stomach weights and the accompanying histopathological changes were not reported in males or females fed ethyl acrylate at a dose of 200 mg/kg bw per day for 4 weeks and allowed to recover for 9 weeks. On the basis of these results, the authors concluded that early histopathological changes in the forestomach of rats resulting from treatment with ethyl acrylate are reversible (Bernacki et al., 1987b).

In a stop-exposure study, groups of male F344/N rats were given ethyl acrylate (in corn oil) at a dose of 100 or 200 mg/kg bw per day by intragastric instillation 5 days per week, for 13 weeks. A concurrent control group was maintained on corn oil. At study termination, necropsies were performed on 10–11 rats from each group at 24 h after the last dose was administered, and complete gross and histopathological evaluations of the forestomach, glandular stomach and the liver were also carried out. Additional groups of 10 and 26–35 remaining rats were similarly examined at necropsy following 8-week and 19-month recovery periods, respectively, which followed immediately after the last dose of ethyl acrylate was administered. Results showed that compound-related toxicity occurred as a result of the interaction of ethyl acrylate with the tissues directly exposed to the test material. At the end of the 13 weeks of treatment, moderate (at 100 mg/kg bw per day) to severe (at 200 mg/kg bw per day) forestomach hyperplasia was reported in all treated rats, presented as a slight thickening accompanied by occasional foci at the lowest dose (100 mg/kg bw per day), and as focal and multifocal raised nodules at the highest dose (200 mg/kg bw per day). No lesions were observed in the glandular stomach or liver. Following the 8- and 19-month recovery periods, a significant decline in the incidence and severity of forestomach hyperplasia was reported. The authors noted that most of the treated rats had histologically normal forestomachs. These results indicate that sustained forestomach hyperplasia in rats resulting from prolonged exposure to high doses of ethyl acrylate administered by gavage can regress after cessation of dosing (Ghanayem et al., 1991a).

Groups of male F344 rats were given ethyl acrylate (in corn oil) at a dose of 100 or 200 mg.kg bw per day by intragastric instillation 5 days per week for 13 weeks. A concurrent control group was maintained on corn oil only. At week 13, 10–11 rats from each group were necropsied 24 h after the last dose was administered and their stomachs were subjected to histopathological evaluation. Additionally, 5 rats and 12–15 rats from each group were similarly examined following 8-week and 19-month recovery periods, respectively. At the end of the 13 weeks of treatment, a slight thickening of the forestomach mucosa accompanied by occasional foci was observed at the lowest dose and focal and multifocal raised nodular proliferations of the forestomach were found at the highest dose. After the 8-week and 19-month recovery periods, the incidences of forestomach hyperplasis were reported to be 20% and 0%, respectively, at the lowest dose and 100% and 27%, respectively, at the highest dose. Therefore, regression of the forestomach hyperplasia induced by ethyl acrylate occurred upon cessation of dosing (Ghanayem et al., 1991b).

(ii) 2-Hexenal (No. 1353)

Rats

Groups of 15 male and 15 female CFW rats were maintained on diets containing 2-hexenal at a concentration of 0, 260, 640, 1600 or 4000 mg/kg for 13 weeks (Gaunt et al., 1971). These concentrations correspond to average daily doses of 0, 13, 32, 80 and 200 mg/kg bw, respectively (Food and Drug Administration, 1993). Body weights and food intake were measured weekly. Samples of blood and urine were collected at weeks 6 and 7, respectively, and again at the end of the study. At termination, gross examinations were carried out on all treated rats and a range of organs (brain, pituitary, thyroid, heart, liver, spleen, adrenal glands, kidneys, and gonads) were weighed. Tissue sections taken from weighed organs and other tissues (lymph nodes, thymus, urinary bladder, stomach, duodenum, ileum, colon, caecum, rectum, pancreas, uterus, and skeletal muscle) of the control rats and the rats at 200 mg/kg bw per day were stained for microscopic examination. No difference in general health and behaviour was reported in any of the treated animals. The slight, not statistically significant, decrease in growth rate at 200 mg/kg bw per day was associated with a 10% reduction in food intake attributable to decreased palatability of the diet. In males, statistically significant but not dose-dependent decreases were reported in concentrations of haemoglobin at 80 mg/kg bw per day and in erythrocytes at 32 and 80 mg/kg bw per day; these, however, were not associated with increased kidney weights or any changes in histology. No changes in haematology were reported in any treated females. The results of urine analysis were unremarkable, with the exception of a significant reduction in the specific gravity of the urine observed in male at the highest dose (200 mg/kg bw per day). Compared with controls, a 20–30%, non-dose dependent increase in relative and absolute weights of the ovary was reported in females at every dose, without any accompanying changes in ovarian histology. Subsequent to these results, additional groups of female rats (10 per group) were fed diets containing 2-hexenal at a concentration of 0 or 4000 mg/kg (approximately 200 mg/kg bw per day) from the sample used in the main study or from a different sample for 13 weeks. The effects observed in the first study were not observed in the second study regardless of the source sample of 2-hexenal. Furthermore, histopathological examination of the ovaries, uterus, pituitary, and adrenal glands revealed no adverse effects. The authors concluded that the increased ovary weight seen in the first study was accidental and that the no-observed-effect-level (NOEL) for 2-hexenal in rats was approximately 80 mg/kg bw per day (Gaunt et al., 1971).

Rabbits

In a subsequent study at the same laboratory, groups of 10 female New Zealand white rabbits were given *trans*-2-hexenal at a dose of 0 or 200 mg/kg bw per day by gavage in corn oil (2 ml/kg) for 13 weeks. A single early death, attributed to gavage error, was reported. Weekly measurement of body weights showed that treated animals gained less weight compared with controls early in the study, but the difference was not statistically significant. At necropsy, blood samples for haematological evaluation were obtained at the same intervals as in the study in rats,

and organ weight measurements and histopathological examinations were performed on the same organs and tissues as in the study in rats. Weights of stomachs of treated rabbits were significantly increased ($p < 0.001$) compared with those of controls, but there were no differences in the weights of any other organs, including the ovaries, between test and control animals. Microscopic examination revealed haemorrhage and small acute ulcers in three of the treated animals. The authors concluded that the gastric damage in rabbits treated by gavage was due to the route of administration, which produced high stomach concentrations (600 mg/100 ml) of an irritating aldehyde, providing conditions for ulceration of the gastric mucosa. The lack of ulcerative effects in the stomachs of rats fed diets containing 2-hexenal at a dose of 200 mg/kg bw per day supported this conclusion. No other histopathological changes were reported. Mild anaemia was reported in treated rabbits, as evidenced by a statistically significant decrease in concentrations of haemoglobin, which the authors associated with the presence of stomach ulcers (Gaunt et al., 1971).

(c) Long-term studies of toxicity and carcinogenicity

The results of long-term studies of toxicity and carcinogenicity with ethyl acrylate (No. 1351) are described below and summarized in Table 6.

(i) Ethyl acrylate (No. 1351)

Several studies were conducted in mice and rats to determine a mode of administration that would deliver sufficient amounts of ethyl acrylate to produce systemic toxicity without producing severe irritation at the site of administration (National Toxicology Program, 1986). Histopathological changes and lesions in the forestomach and glandular stomach have been reported in mice and rats given ethyl acrylate at doses of >100 mg/kg bw per day by gavage for 2–14 days (Ghanayem et al., 1986; National Toxicology Program, 1986; Ghanayem et al., 1991b).

Mice

No compound-related effects were seen in male and female B6C3F$_1$ mice given ethyl acrylate (in corn oil) at a dose of 0, 1.5, 3, 6, 12 or 25 mg/kg bw per day by intragastric instillation, 5 days per week for 13 weeks (National Toxicology Program, 1986). Consequently, a second study was undertaken in which groups of 10 male and 10 female B6C3F$_1$ mice were given ethyl acrylate (in corn oil) at a dose of 0, 12, 25, 50 or 100 mg/kg bw per day by gavage, 5 days per week for 13 weeks (National Toxicology Program, 1986). A concurrent control group was maintained. The animals were observed twice daily for clinical signs of toxicity and body weights were recorded weekly. At termination, necropsies were performed on all treated animals and complete histopathology was performed on animals in the group receiving the highest dose (100 mg/kg bw per day) and the control groups. No compound-related effects were reported in survival or body weight in either sex at any dose. No compound-related gross or microscopic changes were observed in any of the treated groups when compared with the controls (National Toxicology Program, 1986).

Groups of 50 male and 50 female B6C3F$_1$ mice were given ethyl acrylate (in corn oil) at doses of 0, 100 or 200 mg/kg bw per day by intragastric instillation, 5 days per week for 103 weeks. A concurrent control group was maintained. The animals were observed twice daily for mortality and signs of morbidity. Body weights were recorded weekly for the first 12 weeks and monthly thereafter. At termination, necropsies were performed on all the animals that survived to the end of the study and on all animals found dead during the study. No significant differences in survival were observed between any treated groups of the same sex. The mean body weights of all treated animals were similar to the controls throughout the study, except for an unexplained decrease in the females at the lowest dose (100 mg/kg bw per day). No compound-related clinical findings were observed in any treated animals. A dose-related increase in the incidences of hyperkeratosis, hyperplasia, and inflammation of the forestomach were reported in both sexes of treated mice (see Table 7). Statistically significant positive trends in the incidence of squamous cell neoplasms were reported in the forestomachs of treated male and female mice (National Toxicology Program, 1986).

On the basis of these findings, the National Toxicology Program concluded that:

> . . . under the conditions of these studies, ethyl acrylate was carcinogenic for the forestomach of B6C3F$_1$ mice causing squamous cell carcinomas in male mice, squamous cell papillomas in male mice, and squamous cell papillomas or carcinomas (combined) in male and female mice. Evidence for carcinogenicity was greater in males than in females. Ethyl acrylate also caused irritation of the forestomach mucosa in male and female mice.

Rats

Groups of 10 male and 10 female F344/N rats were given ethyl acrylate (in corn oil) at a dose of 0, 7, 14, 28, 55 or 110 mg/kg bw per day by gavage, 5 days per week for 13 weeks. A concurrent control group was maintained. The animals were observed twice daily for clinical signs of toxicity and body weights were recorded weekly. At termination, necropsies were performed on all treated animals and complete histopathology was performed on the animals at the highest dose (110 mg/kg bw per day) and in the control groups. No compound-related effects were reported in survival or body weight in either sex at any dose. Reddening in the duodenum and prominent blood vessels in the cardiac region of the stomach were seen in three males fed with ethyl acrylate at a dose of 110 mg/kg bw per day. No compound-related clinical signs of toxicity and no histopathological effects were observed in any of the treated groups when compared with the controls (National Toxicology Program, 1986).

Groups of 50 male and 50 female F344/N rats were given ethyl acrylate (in corn oil) at a dose of 0, 100 or 200 mg/kg bw per day by gavage, 5 days per week for 103 weeks. A concurrent control group was maintained. The animals were observed twice daily for mortality and signs of morbidity. Body weights were recorded weekly for the first 12 weeks and monthly thereafter. At termination, necropsies were performed on all animals that survived to the end of the study and on any animals found dead during the study. No significant differences in survival were observed between groups of the same sex. The mean body weights of

Table 7. Incidences of neoplasms and non-neoplastic lesions of the forestomach in B6C3F$_1$ mice given ethyl acrylate by gavage

Neoplasm or non-neoplastic lesion	Dose (mg/kg per day)		
	0 (vehicle control)	100	200
Males			
Hyperkeratosis, incidence/ No. of animals necropsied (%)	0/48 (0%)	19/47 (40%)	28/50 (56%)
Epithelial hyperplasia, incidence/ No. of animals necropsied (%)	0/48 (0%)	17/47 (36%)	26/50 (52%)
Squamous cell papilloma/ No. of animals necropsied (%)	0/48 (0%)	4/47 (9%)	9/50 (18%)
Squamous cell carcinoma/ No. of animals necropsied (%)	0/48 (0%)	2/47 (4%)	5/50 (10%)
Squamous cell papilloma or carcinoma/No. of animals necropsied (%)	0/48 (0%)	5/47 (11%)	12/50 (24%)
Females			
Hyperkeratosis, incidence/ No. of animals necropsied (%)	2/50 (4%)	14/49 (29%)	32/48 (67%)
Epithelial hyperplasia, incidence/ No. of animals necropsied (%)	3/50 (6%)	12/49 (24%)	30/48 (63%)
Squamous cell papilloma/ No. of animals necropsied (%)	1/50 (2%)	4/49 (8%)	5/48 (10%)
Squamous cell carcinoma/ No. of animals necropsied (%)	0/50 (0%)	1/49 (2%)	2/48 (4%)
Squamous cell papilloma or carcinoma/ No. of animals necropsied (%)	1/50 (2%)	5/49 (10%)	7/48 (15%)

From National Toxicology Program (1986).

the treated groups were comparable to those of the controls and no compound-related clinical signs of toxicity were observed in the treated animals throughout the study. Dose-related increases in the incidence of non-neoplastic lesions were observed in the forestomach of males and females (see Table 8). Statistically significant positive trends in the incidence of squamous cell papillomas were reported in males and females. Statistically significant positive trends in the incidence of squamous cell carcinomas were reported in males (National Toxicology Program, 1986).

On the basis of these findings, the National Toxicology Program concluded that:

> under the conditions of these studies, ethyl acrylate was carcinogenic for the forestomach of F344/N rats, causing squamous cell carcinomas in male rats, squamous cell papillomas in male and female rats, and squamous cell papillomas or carcinomas (combined) in male and female rats. Evidence for carcinogenicity was greater in males than in females. Ethyl acrylate also caused irritation of the forestomach mucosa in male and female rats.

Studies have indicated that concentrations of GSH are depleted in the rat forestomach after dosing with ethyl acrylate by gavage (DeBethizy et al., 1987; Frederick et al., 1990). Saturation of the GSH pathway is suggested from the observation that, as dose of ethyl acrylate increase, excretion of mercapturic acid conjugates decreases (DeBethizy et al., 1987; Frederick et al., 1990).

Tissue toxicity as a result of the accumulation of ethyl acrylate is a function of the rate of ethyl acrylate delivery to the tissue and the efficiency of the metabolic detoxication process in the tissue. Studies have shown that the rapid delivery of large quantities of ethyl acrylate to a tissue depletes GSH faster than it can be re-synthesized. This method of dosing also depletes the available non-lethal protein-binding sites (Frederick et al., 1992). The depletion of GSH observed in the rat forestomach is associated with the toxicity observed in rats given ethyl acrylate in large doses by gavage (Frederick et al., 1990). Previous studies have also shown

Table 8. Incidences of neoplasms and non-neoplastic lesions of the forestomach in F344/N rats given ethyl acrylate by gavage

Neoplasm or non-neoplastic lesion	Dose (mg/kg per day)		
	0 (vehicle control)	100	200
Males			
Hyperkeratosis, incidence/ No. of animals necropsied (%)	0/50 (0%)	37/50 (74%)	46/50 (92%)
Epithelial hyperplasia, incidence/ No. of animals necropsied (%)	1/50 (2%)	41/50 (82%)	46/50 (92%)
Squamous cell papilloma/ No. of animals necropsied (%)	1/50 (2%)	15/50 (30%)	29/50 (58%)
Squamous cell carcinoma/ No. of animals necropsied (%)	0/50 (0%)	5/50 (10%)	12/50 (24%)
Squamous cell papilloma or carcinoma/ No. of animals necropsied (%)	1/50 (2%)	18/50 (36%)	36/50 (72%)
Females			
Hyperkeratosis, incidence/ No. of animals necropsied (%)	0/50 (0%)	24/50 (48%)	46/50 (92%)
Epithelial hyperplasia, incidence/ number animals necropsied (%)	0/50 (0%)	34/50 (68%)	49/50 (98%)
Squamous Cell Papilloma/ Number animals necropsied (%)	1/50 (2%)	6/50 (12%)	9/50 (18%)
Squamous cell carcinoma/ number animals necropsied (%)	0/50 (0%)	0/50 (0%)	2/50 (4%)
Squamous cell papilloma or carcinoma/ number animals necropsied (%)	1/50 (2%)	6/50 (12%)	11/50 (22%)

From National Toxicology Program (1986).

no changes in the histopathology of the forestomach at doses of ≤10 mg/kg per day, mild hyperplasia at doses of between 20 and 50 mg/kg bw per day and severe hyperplasia, inflammation and ulceration at doses of ≥100 mg/kg. These toxic responses were attributed to inefficient metabolic detoxification at higher doses (Frederick et al., 1990).

No carcinogenic effects on the skin were reported when 100% ethyl acrylate as 25 µl doses was applied to the skin of a group of 40 male C3H/HeJ mice three times per week in a lifetime study (408 days) (DePass et al., 1984) or when mice or rats were exposed to vapours of ethyl acrylate at concentrations of ≤75 mg/l for 6 h per day, 5 days per week for 27 months and 225 mg/l for 6 h per day, 5 days per week for 6 months (Miller et al., 1985). These results support the conclusion that the carcinogenic potential of ethyl acrylate is associated with the route of administration (i.e. in corn oil, by gavage) when doses administered to the test animals exceed the maximum tolerated dose.

Groups of 25 female and 25 male young albino Wistar rats were given drinking-water containing ethyl acrylate at a concentration of at 0, 6, 60 or 2000 mg/l for 2 years (Borzelleca et al., 1964). The National Toxicology Program determined that the concentration of 2000 mg/l in drinking-water corresponded to a dose of approximately 170 or 120 mg/kg bw per day for male and female rats, respectively (National Toxicology Program, 1986). Decreased body weights were reported in male and female rats receiving ethyl acrylate at 2000 mg/l. Haematological evaluations (i.e. erythrocyte volume fraction, haemoglobin, total white and differential white cell counts) and urine analysis (i.e. protein, reducing substances) conducted at 3-month intervals showed normal ranges for the parameters studied in all treated animals throughout the study. At termination, histopathology revealed no compound-related neoplastic or non-neoplastic lesions in treated animals of either sex.

Dogs

Groups of two male and two female pure-bred beagle dogs were given corn oil gelatin capsules containing ethyl acrylate at a 'dietary equivalent' concentration of 0, 10, 100 or 1000 mg/kg for 2 years. This corresponds to average daily intakes of 0.75, 7.5 and 75 mg/kg bw, respectively (Food and Drug Administration, 1993). Reduced body-weight gains reported in dogs receiving ethyl acrylate at a dose of 75 mg/kg bw per day were correlated with decreased food consumption. Haematological evaluations (i.e. erythrocyte volume fraction, haemoglobin, total and differential leukocyte counts) and urine analysis (i.e. protein, reducing substances) conducted at 3-month intervals showed normal ranges for the parameters studied in all treated animals throughout the study. At termination, histopathology revealed no compound-related neoplastic or non-neoplastic lesions in either sex of treated animals (Borzelleca et al., 1964).

(ii) Related substance, trans,trans-2,4-hexadienal (No. 1175)

Trans,trans-2,4-Hexadienal (No. 1175), which was previously evaluated by the Committee at its sixty-first meeting (Annex 1, reference *166*), is structurally similar

to *trans*-2-hexenal (No. 1353). The two substances differ only in the presence of an additional double bond at the 3,4 position of hexadienal. Being α,β-unsaturated aldehydes of the same carbon chain length, both substances are rapidly absorbed and metabolized via the same pathways. At high concentrations, both are irritating aldehydes that exhibit toxic effects upon repeated direct contact with tissues such as the gastric mucosa of the forestomach.

The structurally related α,β-unsaturated aldehyde, *trans,trans*-2,4-hexadienal, has been the subject of a recent National Toxicology Program bioassay (National Toxicology Program, 2001a) in mice and rats. The results of these studies were presented in a previous review (Annex 1, reference *166*). Therefore, a concise summary of the results of these studies and the evaluation of the effects in the context of 2-hexenal (No. 1353) will be presented.

Groups of 50 male and 50 female $B6C3F_1$ mice and 50 male and 50 female F344/N rats were given 2,4-hexadienal (in corn oil) at a dose of 0, 30, 60 or 120 and 0, 22.5, 45 or 90 mg/kg bw per day, respectively, by gavage 5 days per week for 104 weeks (National Toxicology Program, 2001a). On the basis of these findings, the National Toxicology Program concluded that:

> . . . there was clear evidence of carcinogenic activity of 2,4-hexadienal in male and female $B6C3F_1$ mice and in male and female F344/N rats based on increased incidences of squamous cell neoplasms of the forestomach.

Relevance of forestomach tumours

The relevance of the appearance of forestomach tumours in rodents to potential carcinogenic targets in humans has been the subject of much investigation (Grice, 1988; Wester & Kroes, 1988; Clayson et al., 1990). An International Agency for Research on Cancer working group (IARC, 2003) concluded that in evaluating the relevance of the induction of forestomach tumours in rodents for human cancer, the exposure conditions in the experiments have to be considered. The exposure conditions during oral administration are unusual (particularly if dosing by gavage is employed) in that physical effects may result in high local concentrations of test substances in the forestomach and prolonged exposure of the epithelial tissue. Agents that only produce tumours in the forestomach in rodents after prolonged treatment through non-DNA reactive mechanisms may be of less relevance to humans, since human exposure to such agents would need to surpass time-integrated dose thresholds in order to elicit the carcinogenic response.

Therefore, the appearance of forestomach lesions in the 2-year bioassays in rodents in which ethyl acrylate (No. 1351) or *trans,trans*-2,4-hexadienal (No. 1175) were administered at high concentrations by gavage has no relevance to humans, given that the results are attributable to the irritating effect of high bolus doses of ethyl acrylate or *trans,trans*-2,4-hexadienal delivered to the contact site (forestomach) by gavage, and not the effects of systemic concentrations in the whole animal. On biochemical grounds and in analogy to *trans,trans*-2,4-hexadienal, *trans*-2-hexenal (No. 1353), therefore, would also be expected not to have any carcinogenic potential in humans.

(d) Genotoxicity

(i) In vitro

Testing in vitro for genotoxicity has been performed with representative members of the group of aliphatic, linear, α,β-unsaturated alcohols, aldehydes, carboxylic acids and related esters used as flavouring agents (see Table 9). Testing for mutagenicity in bacteria with α,β-unsaturated aldehydes is problematic owing to their high bacterial toxicity. The cytotoxicity of these substances is believed to arise from their interactions with protein sulfhydryl and amino groups (Marnett et al., 1985; Eder et al., 1992). Owing to the nature of the GSH conjugation pathway, studies of genotoxicity in which α,β-unsaturated aldehydes are applied at high concentrations are likely to promote oxidative stress. It is anticipated that cells exposed to α,β-unsaturated aldehydes at high concentrations will rapidly deplete GSH, eventually leading to cellular damage and decreased cell viability, and subsequent reaction with cellular proteins and DNA.

Since the majority of assays were performed with ethyl acrylate, a series of homologous α,β-unsaturated aldehydes, or unsaturated acids and esters, the discussion of the results of these tests are organized according to substance (e.g. ethyl acrylate) or group of substances (2-pentenal, 2-hexenal, 2-heptenal, 2-octenal).

Ethyl acrylate (No. 1351)

Negative results were reported in Ames assays when *Salmonella typhimurium* strains (TA97, TA98, TA100, TA1535, TA1537 and TA1538) were incubated with ethyl acrylate (No. 1351) at a concentration of up to 10 000 μg/plate (Ishidate et al., 1981; Waegemaekers & Bensink, 1984; Tennant et al., 1987; Zeiger et al., 1992).

No evidence of mitotic chromosomal loss was obtained when ethyl acrylate at concentrations of up to 1095 μg/ml were incubated with *Saccharomyces cerevisiae* strain D61.M. Under a cold shock regimen, evidence of mitotic recombination was reported (Zimmermann & Mohr, 1992).

In the standard assay for forward mutation in mouse lymphoma cells, ethyl acrylate gave uniformly positive results in the absence of metabolic activation when incubated with mouse lymphoma L5178Y Tk^{+} cells at cytotoxic concentrations (37.5–50 μg/ml) (Tennant et al., 1987; McGregor et al., 1988; Moore et al., 1988; Ciaccio et al., 1998). In the most recent study (Ciaccio et al., 1998), the relationship between cytotoxicity and mutation frequency in the assay for forward mutation in mouse lymphoma cells, the ethyl acrylate-induced mutagenic response was found to be directly related to the time- and concentration-dependent decrease in non-protein sulfhydryl levels and subsequent mitochondrial membrane impairment.

In assays for clastogenicity, ethyl acrylate elicited increases in sister chromatid exchanges (SCE) and chromosomal aberrations (CA) in Chinese hamster ovary cells with metabolic activation, but showed no evidence of clastogenicity in the

Table 9. Results of studies of the genotoxicity of aliphatic, alicyclic, linear, α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters used as flavouring agents

No.	Flavouring agent	End-point	Test system	Dose or concentration	Result	Reference
In vitro						
1351	Ethyl acrylate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, and TA1538	30–2 000 μg/plate	Negative[a]	Waegemaekers & Bensink (1984)
1351	Ethyl acrylate	Reverse mutation	*S. typhimurium* TA97, TA98, TA100 and TA1535	33–3 333 μg/plate	Negative[a,b]	Zeiger et al. (1992)
1351	Ethyl acrylate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, and TA1537	100–10 000 μg/plate	Equivocal[a,b,c]	Haworth et al. (1983)
1351	Ethyl acrylate	Reverse mutation	*S. typhimurium* TA98, TA100, and TA1537	NR	Negative[a,b]	Ishidate et al. (1981)
1351	Ethyl acrylate	Reverse mutation	*S. typhimurium*	≤10 000 μg/plate	Negative[a]	Tennant et al. (1987)
1351	Ethyl acrylate	Mitotic chromosomal loss	*Saccharomyces cerevisiae* D61.M	≤1 095 μg/ml	Negative[d,e]	Zimmermann & Mohr (1992)
1351	Ethyl acrylate	Mitotic recombination	*S. cerevisiae* D61.M	≤1 095 μg/ml	Positive[d]	Zimmermann & Mohr (1992)
1351	Ethyl acrylate	Mitotic recombination	*S. cerevisiae* D61.M	≤914 μg/ml	Negative[e]	Zimmermann & Mohr (1992)
1351	Ethyl acrylate	Forward mutation	Mouse lymphoma L5178Y $Tk^{+/-}$ cells	20 μg/ml	Positive[f]	Tennant et al. (1987)
1351	Ethyl acrylate	Forward mutation	Mouse lymphoma L5178Y $Tk^{+/-}$ cells	10, 15, 20, 25, 27.5, 30, 32.5, 35, 40 or 50 μg/ml	Positive[f,g]	Ciaccio et al. (1998)
1351	Ethyl acrylate	Forward mutation	Mouse lymphoma L5178Y $Tk^{+/-}$ cells	20, 25, 30 or 37.5 μg/ml	Positive[f,h]	Moore et al. (1988)

Table 9. (Contd)

No.	Flavouring agent	End-point	Test system	Dose or concentration	Result	Reference
1351	Ethyl acrylate	Forward mutation	Mouse lymphoma L5178Y $Tk^{+/-}$ cells	2.5–40 μg/ml	Positive[f,i]	McGregor et al. (1988)
1351	Ethyl acrylate	Chromosomal aberration	Chinese hamster ovary cells	299 μg/ml ≤29.9 μg/ml	Positive[j] Negative[f,h]	Loveday et al. (1990)
1351	Ethyl acrylate	Chromosomal aberration	Mouse lymphoma L5178Y $Tk^{+/-}$ cells	20.0, 25, 30 or 37.5 μg/ml	Positive[f,h]	Moore et al. (1988)
1351	Ethyl acrylate	Chromosomal aberration	Chinese hamster CHL cells	0.0098 mg/ml[k] (9.8 μg/ml)	Positive[a]	Ishidate et al. (1981)
1351	Ethyl acrylate	Chromosomal aberration	Chinese hamster ovary cells	299 μg/ml	Positive[j] Negative[f]	Tennant et al. (1987)
1351	Ethyl acrylate	Sister chromatid exchange	Chinese hamster ovary cells	150 μg/ml	Positive[j] Negative[f]	Tennant et al. (1987)
1351	Ethyl acrylate	Sister chromatid exchange	Chinese hamster ovary cells	150 μg/ml ≤5 μg/ml	Weak Positive[j] Negative[f,h]	Loveday et al. (1990)
1351	Ethyl acrylate	Sister chromatid exchange	Mouse lymphoma L5178Y $Tk^{+/-}$ cells	20.0–37.5 μg/ml	Positive[f,h]	Moore et al. (1988)
1351	Ethyl acrylate	Sister chromatid exchange	Mouse splenocytes	10–80 μg/ml	Negative[l]	Kligerman et al. (1991)
1351	Ethyl acrylate	Sister chromatid exchange	Mouse splenocytes	1–20 μg/ml	Negativem	Kligerman et al. (1991)
1353	2-Hexenal	Reverse mutation	*S. typhimurium* TA104	≤2 μmol/plate[n] (196.3 μg/plate)[o]	Positive[f,p]	Marnett et al. (1985)
1353	2-Hexenal	Reverse mutation	*S. typhimurium* TA104	>5 μmol/plate (>490.7 μg/plate)[o]	Positive[f,p,q]	Marnett et al. (1985)
1353	2-Hexenal	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535 and TA1537	3 μmol/plate (294.4 μg/plate)[o]	Negative[a]	Florin et al. (1980)
1353	2-Hexenal	Reverse mutation	*S. typhimurium* TA98, TA100, and TA104	NR	Positive[a,b,r]	Kato et al. (1989)

1353	2-Hexenal	Reverse mutation	*S. typhimurium* TA100	0.01–0.50 µl/plate	Negative[a,s] Positive[j,t]	Eder et al. (1992)
1353	2-Hexenal	SOS chromotest	*E. coli* PQ37 and PQ243	70–435 nmol/plate (6.9–42.7 µg/plate)°	Negative[h]	Eder et al. (1992)
1353	2-Hexenal	Mutation	*E. coli* WP2*uvrA*/ pKM101	NR	Positive[a,b,r]	Kato et al. (1989)
1353	2-Hexenal	Mutation induction	Chinese hamster V79 cells	0.03, 0.10 or 0.30 mmol/l (2.9, 9.8 or 29.4 µg/ml)°	Positive[f,u] Negative[f,v]	Canonero et al. (1990)
1353	2-Hexenal	Micronucleus formation	Human blood lymphocytes	5–250 µmol/l (0.5–24.5 µg/ml)°	Positive	Dittberner et al. (1995)
1353	2-Hexenal	Micronucleus formation	Lymphoblastoid Namalva	5–250 µmol/l cells (0.5–24.5 µg/ml)°	Positive[w]	Dittberner et al. (1995)
1353	2-Hexenal	Chromosomal aberration	Human blood lymphocytes	5–250 µmol/l (0.5–24.5 µg/ml)°	Negative	Dittberner et al. (1995)
1353	2-Hexenal	Chromosomal aberration	Lymphoblastoid Namalva	5–150 µmol/l cells (0.5–14.7 µg/ml)°	Positive[x]	Dittberner et al. (1995)
1353	2-Hexenal	Sister chromatid exchange	Human blood lymphocytes	5–250 µmol/l (0.5–24.5 µg/ml)°	Positive	Dittberner et al. (1995)
1353	2-Hexenal	Sister chromatid exchange	Lymphoblastoid Namalva cells	5–200 µmol/l (0.5–19.6 µg/ml)°	Positive	Dittberner et al. (1995)
1353	2-Hexenal	DNA single strand break	Mouse leukaemia L1210	100, 250 or 500 µmol cells (9814, 24535 or 49070 µg)°	Positive[h]	Eder et al. (1993)
1353	2-Hexenal	DNA repair	Rat hepatocytes	60–600 nmol (5.9–58.9 µmol)°	Positive	Griffin & Segall (1986)
1356	Methyl 2-nonynoate	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98 and TA100	≤3.6 mg/plate (3600 µg/plate)	Negative[a]	Wild et al. (1983)
1357	Methyl 2-octynoate	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98 and TA100	≤3.6 mg/plate (3600 µg/plate)	Negative[a]	Wild et al. (1983)

Table 9. (Contd)

No.	Flavouring agent	End-point	Test system	Dose or concentration	Result	Reference
1360	2-Heptenal	Reverse mutation	*S. typhimurium* TA104	≤0.9 μmol/plate (101 μg/plate)[y]	Negative[f,p]	Marnett et al. (1985)
1360	2-Heptenal	Reverse mutation	*S. typhimurium* TA104	≤4.4 μmol/plate (493.5 μg/plate)[y]	Negative[f,p,q]	Marnett et al. (1985)
1360	2-Heptenal	Reverse mutation	*S. typhimurium* TA100	0.005–0.400 μl/plate	Negative[a,z]	Eder et al. (1992)
1360	2-Heptenal	SOS chromotest	*E. coli* PQ37 and PQ243	35–270 nmol/plate (3.9–30.3 μg/plate)[y]	Negative[h]	Eder et al. (1992)
1360	2-Heptenal	Mutation induction	Chinese hamster V79 cells	0.01, 0.03 or 0.10 mmol/l (1.1, 3.4 or 11.2 μg/ml)[y]	Positive[f,1]	Canonero et al. (1990)
1360	2-Heptenal	DNA single strand break	Mouse leukaemia L1210	200, 400 or 500 μmol (22 434, cells 44 868, or 56 085 μg)[y]	Positive	Eder et al. (1993)
1362	2-Nonenal	Reverse mutation	*S. typhimurium* TA104	≤0.007 μmol/plate (1.0 μg/plate)[2]	Negative[f,p]	Marnett et al. (1985)
1362	2-Nonenal	Mutation induction	Chinese hamster V79 cells	0.003 or 0.01 mmol/l (0.4 or 1.4 μg/ml)[2]	Positive[f,u] Negative[3]	Canonero et al. (1990)
1362	2-Nonenal	Micronucleus formation	Rat hepatocytes	0.1, 1, 10 or 100 μmol/l (0.01, 0.1, 1.4 or 14.0 μg/ml)[2]	Positive	Esterbauer et al. (1990)
1362	2-Nonenal	Micronucleus formation	Rat hepatocytes	0.1, 10 or 100 μmol/l (0.01, 1.4 or 14.0 μg/ml)[2]	Negative	Eckl et al. (1993)
1362	2-Nonenal	Chromosomal aberration	Rat hepatocytes	0.1, 1, 10 or 100 μmol/l (0.01, 0.1, 1.4 or 14.0 μg/ml)[2]	Negative	Esterbauer et al. (1990)
1362	2-Nonenal	Chromosomal aberration	Rat hepatocytes	0.1, 10 or 100 μmol/l (0.01, 1.4 or 14.0 μg/ml)[2]	Negative	Eckl et al. (1993)
1362	2-Nonenal	Sister chromatid exchange	Rat hepatocytes	0.1, 10 or 100 μmol/l (0.01, 1.4 or 14.0 μg/ml)[2]	Positive	Eckl et al. (1993)
1353	2-Nonenal	DNA repair	Rat hepatocytes	60–600 nmol (8.4–84.1 μg/plate)[2]	Positive	Griffin & Segall (1986)

1363	2-Octenal	Reverse mutation	*S. typhimurium* TA104	≤0.8 μmol/plate (101.0 μg/plate)[4]	Negative[f,p]	Marnett et al. (1985)
1363	2-Octenal	Reverse mutation	*S. typhimurium* TA104	≤4 μmol/platen (504.8 μg/plate)[4]	Negative[f,p,q]	Marnett et al. (1985)
1363	2-Octenal	Mutation induction	Chinese hamster V79 cells	0.01, 0.03 or 0.10 mmol/l (1.3, 3.8 or 12.6 μg/ml)[4]	Positive[f] Negative[3]	Canonero et al. (1990)
1363	2-Octenal	DNA single strand break	Mouse leukaemia L1210	350 μmol cells (44 170 μg/plate)[4]	Positive	Eder et al. (1993)
1364	2-Pentenal	Reverse mutation	*S. typhimurium* TA100	0.01–0.75 μl	Positive[a,b,5]	Eder et al. (1992)
1364	2-Pentenal	SOS chromotest	*E. coli* PQ37 and PQ243	60–435 nmol/plate (5.0–36.7 μg/plate)[6]	Negative[h]	Eder et al. (1992)
1364	2-Pentenal	Mutation induction	Chinese hamster V79 cells	0.03, 0.10 or 0.30 mmol/l (2.5, 8.4 or 25.2 μg/ml)[6]	Positive[f,u,7] Negative[3,7]	Canonero et al. (1990)
1364	2-Pentenal	DNA single strand break	Mouse leukaemia L1210	400, 600 or 800 μmol (33 648, cells 50 472 or 67 296 μg)[6]	Positive[h]	Eder et al. (1993)
1371	*(E)*-2-Butenoic acid	Reverse mutation	*S. typhimurium* TA100	0.1–1 000 μg/plate	Negative	Rapson et al. (1980)
1371	*(E)*-2-Butenoic acid	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98, and TA100	≤1 000 μg/plate	Negative[a]	Lijinsky & Andrews (1980)
1371	*(E)*-2-Butenoic acid	Reverse mutation	*S. typhimurium* TA100	≤1 000 μg/plate	Positive[a,p,8]	Lijinsky & Andrews (1980)
1371	*(E)*-2-Butenoic acid	Sister chromatid exchange	Human lymphocytes	2.5, 5.0 or 10.0 mmol/l (215.2, 430.4 or 860.9 μg/ml)[9]	Positive[10]	Sipi et al. (1992)
In vivo						
1351	Ethyl acrylate	Sister chromatid exchange	Male C57BL/6 mice	125, 250, 500 or 1 000 mg/kg[11]	Negative	Kligerman et al. (1991)
1351	Ethyl acrylate	Chromosomal aberration	Male C57BL/6 mice	125, 250, 500 or 1 000 mg/kg[11]	Negative	Kligerman et al. (1991)
1351	Ethyl acrylate	Sex-linked recessive lethal mutations	*D. melanogaster*	20 000 mg/kg	Negative[12]	Valencia et al. (1985)
1351	Ethyl acrylate	Sex-linked recessive lethal mutations	*D. melanogaster*	18 000, 20 000 or 40 000 mg/kg	Negative[13]	Valencia et al. (1985)

Table 9. (Contd)

No.	Flavouring agent	End-point	Test system	Dose or concentration	Result	Reference
1351	Ethyl acrylate	Micronucleus formation	Male C57BL/6 mice	125, 250, 500 or 1 000 mg/kg[11]	Negative[14]	Kligerman et al. (1991)
1351	Ethyl acrylate	Micronucleus formation	Male BALB/c mice	225–1 800 mg/kg[15]	Positive	Przubojewska et al. (1984)
1351	Ethyl acrylate	Micronucleus formation	Male and female C57BL/6 mice	461[11,16] or 738 mg/kg[11,17]	Negative	Ashby et al. (1989)
1351	Ethyl acrylate	Micronucleus formation	Male BALB/c mice	812 mg/kg[18]	Negative	Ashby et al. (1989)
1351	Ethyl acrylate	Micronucleus formation	Male C57BL/6 mice	738 mg/kg[18]	Negative	Ashby et al. (1989)
1351	Ethyl acrylate	Micronucleus formation	Male BDF_1 mice	188, 375 or 750 mg/kg[16,19]	Negative	Hara et al. (1994)
1351	Ethyl acrylate	Micronucleus formation	Male BDF_1 mice	188, 375 or 750 mg/kg[20]	Negative[21]	Hara et al. (1994)
1351	Ethyl acrylate	Micronucleus formation	Male BDF_1 mice	375, 500 or 750 mg/kg[11,16]	Negative[21]	Hara et al. (1994)
1351	Ethyl acrylate	Micronucleus formation	Male BDF_1 mice	188, 375, 750 or 1 000 mg/kg[11,16,19]	Negative[22]	Morita et al. (1997)
1351	Ethyl acrylate	DNA alkaline elution	Male F344 rat forestomach squamous epithelium	0.1–4.0% (50–2 000 mg/kg)[19,23]	Negative	Morimoto et al. (1990)
1353	2-Hexenal	Micronucleus formation	Human buccal mucosa cells	10 mg/kg	Positive[24]	Dittberner et al. (1997)
1356	Methyl 2-nonynoate	Micronucleus formation	NMRI mouse bone marrow	168, 336 or 505 mg/kg[11]	Negative	Wild et al. (1983)
1356	Methyl 2-nonynoate	Sex-linked recessive lethal mutation	*D. melanogaster*	2.5 mmol/l (420.6 µg/ml)[25]	Negative	Wild et al. (1983)
1357	Methyl 2-octynoate	Micronucleus formation	NMRI mouse bone marrow	154, 231 or 308 mg/kg[11]	Negative	Wild et al. (1983)

1357	Methyl 2-octynoate	Sex-linked recessive lethal mutation	*D. melanogaster*	1 mmol/l (154.2 μg/ml)[26]	Negative	Wild et al. (1983)

NR, not reported.
[a] With and without metabolic activation.
[b] Pre-incubation method.
[c] The Ames tests were performed by two different laboratories, resulting in positive and negative results in the first and second experiments, respectively. The authors considered the results from the confirmation (second) experiment to be more definitive.
[d] Cold shock regimen (i.e. 4-h incubation at 28 °C, followed by a 16-h storage in an ice bath, and a 4-h incubation at 28 °C).
[e] Uninterrupted 16-h incubation at 28 °C.
[f] Without metabolic activation.
[g] Relative cell growth was 20% and 13%, respectively, at the two highest doses tested (40 and 50 μg/ml, respectively).
[h] Cytotoxicity was observed at the highest dose tested.
[i] Statistically significant increases in mutant fraction were observed at doses of 20 and 40 μg/ml.
[j] With metabolic activation.
[k] Dose at which chromosomal aberrations were detected in 20% of metaphase cells.
[l] Exposure during the G_o phase of the cell cycle. Cytotoxicity was observed at doses of >30 μg/ml.
[m] Exposure during the G-S phase of the cell cycle. Cytotoxicity was observed at doses of >10 μg/ml.
[n] Maximum non-toxic dose.
[o] Calculated using the relative molecular mass of 2-hexenal = 98.14.
[p] Liquid pre-incubation procedure.
[q] Addition of glutathione at 10 mmol/l.
[r] According to the authors, 2-hexenal was 'suspected to be positive' (Kato et al., 1989); however, no further details were provided.
[s] Without metabolic activation, in a threefold bacterial cell density assay.
[t] Conducted in a threefold bacterial cell density assay.
[u] A dose-dependent increase in the number of 6-thioguanine mutants was observed. However, a significant increase in mutation frequency relative to controls was noted only at the highest dose tested.
[v] No significant increase in the number of ouabain mutants was observed relative to controls.
[w] Significantly increased relative to controls only at doses of ≥150 μmol/l.
[x] Significantly increased relative to controls only at doses of ≥100 μmol/l.
[y] Calculated using the relative molecular mass of 2-heptenal = 112.17.
[z] Dose-dependent increases in mutation frequency were noted in standard and threefold bacterial cell density assays; however, these increases were never twofold higher than the spontaneous mutation frequency.

Table 9. (Contd)

1. Dose-dependent increases in the number of 6-thioguanine and ouabain mutants were observed; however, these increases were significantly different from controls only at the highest dose tested (0.10 mmol/l).
2. Calculated using the relative molecular mass of 2-nonenal = 140.22.
3. No significant increase relative to controls was observed in the number of ouabain mutants.
4. Calculated using the relative molecular mass of 2-octenal = 126.20.
5. In standard and threefold bacterial cell density assays.
6. Calculated using the relative molecular mass of 2-pentenal = 84.12.
7. Relative cell viabilities were reduced from 0.92 to 0.20 and from 0.83 to 0.17, respectively, in the tests for 6-thioguanine and ouabain mutation.
8. Positive results were first observed at doses as low as 10 μg/plate in the absence of metabolic activation, and as high as 250 μg/plate in the presence of metabolic activation. Cytotoxicity was reported at doses greater than 50 and 500 μg/plate in the absence and presence of metabolic activation, respectively.
9. Calculated using the relative molecular mass of *(E)*-2-butenoic acid = 86.09.
10. A slight dose-dependent increase in the induction of sister chromatid exchange was observed; however, a significant increase relative to controls was noted only at the highest dose tested (10 mmol/l). At the highest dose, the pH of the medium was decreased by 0.4–.68 pH units relative to that of controls.
11. Administered intraperitoneally.
12. Injection experiment.
13. Feeding experiment.
14. A slight but significant increases in the frequency of micronucleus formation was observed at the highest dose tested (1000 mg/kg), which was thought to be due to an elevated frequency in one of the four treated mice.
15. Administered intraperitoneally in two doses within 24 h.
16. Assessment of bone marrow for formation of micronuclei 24 h after dosing.
17. Assessment of bone marrow for formation of micronuclei 24, 48 or 72 h after dosing.
18. Administered intraperitoneally at 0 and 24 h, followed by assessment of bone marrow for formation of micronuclei 6 h later.
19. Administered orally.
20. Administered orally twice within 24-h.
21. A significant decrease in the reticulocyte ratio was observed at the highest dose tested compared with vehicle controls.
22. Mortality was observed at the highest dose tested (1000 mg/kg).
23. Calculated using Food and Drug Administration (1993).
24. Increases of about threefold in formation of micronuclei were observed on days 6 and 7 after administration.
25. Calculated using the relative molecular mass of methyl 2-nonynoate = 168.24.
26. Calculated using the relative molecular mass of methyl 2-octynoate = 154.21.

absence of metabolic activation (Loveday et al., 1990). The clastogenic potential was unaffected by changes in harvest time (Loveday et al., 1990). In a dose-dependent manner, beginning at 20 μg/ml, ethyl acrylate induced an increase in SCE and CA in mouse lymphoma cells (Moore et al., 1988) in the absence of metabolic activation. An increase in SCE and CA in Chinese hamster ovary cells was reported with ethyl acrylate at concentrations of 150 and 299 μg/ml, respectively, with metabolic activation (Tennant et al., 1987). There was no evidence of clastogenicity in the absence of metabolic activation (Tennant et al., 1987). Increases in CA were reported at 9.8 μg/ml in Chinese hamster cells with or without metabolic activation (Ishidate et al., 1981).

Linear α,β-unsaturated aldehydes

In a study using tester strains (TA104) of *S. typhimurium* that are more sensitive than other strains in identifying α,β-unsaturated aldehydes as mutagens, a series of α,β-unsaturated aldehydes were incubated with TA104. In this modified Ames assay using liquid pre-incubation protocols (i.e. addition of a GSH chase at the end of an incubation of 20 min in TA104), significant increases in reverse mutations in the absence of metabolic activation were reported when *S. typhimurium* strain TA104 was incubated with 2-hexenal (No. 1353) at concentrations of >196 μg/plate (Marnett et al., 1985). α,β-Unsaturated aldehydes of higher relative molecular mass were too toxic to test. At the concentrations tested, 2-heptenal (No. 1360) (up to 101 μg/plate), 2-octenal (No. 1363) (up to 101 μg/plate), and 2-nonenal (No. 1362) (up to 1 μg/plate) gave no evidence of mutagenicity when incubated with TA104 without metabolic activation. *S. typhimurium* strain TA104 contains a nonsense mutation (–TAA–) at the site of reversion and is much more sensitive to carbonyl mutagenesis than standard *Salmonella* strains. Increased TA104 sensitivity is related to the deletion of the *uvrB* gene, which encodes for an error-free DNA excision repair and incorporation of the pKM101 plasmid, which encodes for an error-prone DNA polymerase involved in bypass replication of lesions (Marnett et al., 1985). TA104 is also sensitive to cytotoxicity. To reduce cytotoxicity, GSH was incorporated into the Ames assay. The maximum non-toxic dose of 2-hexenal tested increased from 196 to >491 μg/plate after the addition of reduced GSH at a concentration of 10 mmol/l at the end of the pre-incubation period; however, its mutagenic potential remained unaltered. The authors proposed that the addition of GSH reduced toxicity by preventing excess aldehyde, present after incubation, from reacting with protein sulfhydryl groups. No mutagenicity was reported for 2-heptenal (No. 1360) (up to 494 μg/plate) or 2-octenal (No. 1363) (up to 505 μg/plate) when GSH at 10 mmol/l was added. Also, no evidence of mutagenicity was reported when the six 2-alkenals were incubated with TA102, which contains the *uvrB* gene that encodes for an error-free DNA excision repair (Marnett et al., 1985).

In other modified Ames assays, changes in methodology have been used to evaluate mutagenic potential in the presence of significant cytotoxicity. In Ames pre-incubation assays, using strain TA100, α,β-unsaturated aldehydes were incubated with the standard bacterial cell density or three times the standard bacterial cell density (Eder et al., 1992, 1993). Under usual conditions involving a

pre-incubation period of 30 min, and a standard cell density, the high cytotoxicity demonstrated by simple linear aldehydes may limit the detection of mutagenic responses (e.g. butenal, pentenal, hexenal, heptenal); however, at three times the standard cell density and an increased pre-incubation time of 90 min, butenal, pentenal, hexenal, or hexadienal incubation, with or without metabolic activation, produced a spontaneous reversion frequency of at least twice that observed under standard conditions. Under the specified conditions, results obtained using *S. typhimurium* strain TA100 were found to be consistent with reports of mutagenicity caused by α,β-unsaturated aldehydes as demonstrated in tester strain TA104 in the presence of GSH (Marnett et al., 1985). Among the aldehydes investigated, increased cytotoxicity and mutagenicity correlated with increased lipophilicity. The effect of detoxication upon addition of metabolic activation was indicated by a shift to higher non-cytotoxic doses and higher peak revertant frequencies.

2-Hexenal (No. 1353) was tested for mutagenicity in the Ames assay using different strains of *S. typhimurium* (e.g. TA98, TA100, TA1535, TA1537) in the presence or absence of metabolic activation. No evidence was found for mutagenicity at concentrations up to 3 μmol/plate (294 μg/plate) (Florin et al., 1980).

Negative results were reported with *E. coli* strains PQ37 and PQ243 (SOS chromotest) incubated in the presence of 2-pentenal (No. 1364), 2-hexenal (No. 1353), or 2-heptenal (No. 1360) at concentrations up to 37, 43 or 30 μg/plate, respectively (Eder et al., 1992). The authors noted that high bacterial toxicity interfered with the performance of the test.

The ability of α,β-unsaturated aldehydes to induce SCE, numerical and structural CA, and formation of micronuclei was investigated in cell lines that are low in GSH and detoxication enzymes (i.e. human blood lymphocytes and Namalva cell lines) (Dittberner et al., 1995). *trans*-2-Butenal at 5–250 μmol/l, 2-hexenal (No. 1353) at 5–250 μmol/l, or *trans*-2-*cis*-6-nonadienal at 5–50 μmol/l were separately incubated with human lymphocyte and Namalva cells. The number of SCE increased significantly ($p < 0.05$) at concentrations of 10 μmol/l (0.7 μg/ml), 40 μmol/l (3.9 μg/ml) and 20 μmol/l (2.8 μg/ml) for 2-butenal, 2-hexenal, and *trans*-2-*cis*-6-nonadienal, respectively, in lymphocytes, and 20 μmol/l for 2-butenal (1.4 μg/ml) and 2-hexenal (2.0 μg/ml), and 10 μmol/l for *trans*-2-*cis*-6-nonadienal (1.4 μg/ml) in Namalva cells. In the CA experiment using the same ranges of concentrations, the number of structural chromosomal aberrations in human blood lymphocytes significantly increased only for 2-butenal at concentrations of ≥10 μmol/l. In Namalva cells, which contain lower concentrations of GSH and detoxication enzymes, increases in chromosomal aberrations were reported at concentrations of 100 μmol/l (7.0 μg/ml) for 2-butenal, 100 μmol/l (9.8 μg/ml) for 2-hexenal (No. 1353), and 5 μmol/l (0.7 μg/ml) for *trans*-2-*cis*-6-nonadienal. The incidence of micronuclei was significantly increased at minimum concentrations of 50 μmol/l for 2-butenal and 2-hexenal (No. 1353) in lymphocytes, and 40 μmol/l for 2-butenal and 150 μmol/l for 2-hexenal in Namalva cells. The incidence of formation of micronuclei in blood lymphocytes and Namalva cells was significantly increased at minimum concentrations of *trans*-2-*cis*-6-nonadienal of 20 μmol/l (2.8 μg/ml) and 40 μmol/l (5.5 μg/ml), respectively. *trans*-2-*cis*-6-Nonadienal exhibited severe toxicity at concentrations of >50 μmol/l. The authors concluded that under the conditions of the experiment,

2-butenal is clastogenic. On the basis of the observations that chromosome breaks were not significantly increased and that micronuclei were positive for a centromere-specific DNA, 2-hexenal (No. 1353) and *trans*-2-*cis*-6-nonadienal were classified as aneugens, not clastogens. In the above study, no attempts were made to assess at what concentrations lysosomal breakdown occurred in the assays for SCE and CA. It has been previously established that increases in the incidence of SCE and CA near or at observable levels of cytotoxicity may be reflective of secondary effects resulting from lysosome breakdown and release of DNAase (Zajac-Kaye & Ts'o, 1984; Bradley et al., 1987).

The potential mutagenicity of 2-pentenal (No. 1364), 2-hexenal (No. 1353), 2-heptenal (No. 1360), 2-octenal (No. 1363), and 2-nonenal (No. 1362) was tested in Chinese hamster V79 cells at concentrations ranging from 0.003 mmol/l to 0.3 mmol/l (Canonero et al., 1990). All five alkenals induced a dose-dependent increase in the frequency of 6-thioguanine-resistant mutants and their mutagenic potency was found to increase with the length of the carbon chain. 2-Heptenal produced an increase in the number of mutations to ouabain resistance, but these increases were significantly different from controls only at the highest dose tested (0.10 mmol/l) (Canonero et al., 1990).

Cultured rat hepatocytes were incubated with 0.1, 1.0, 10 or 100 μmol/l of *trans*-2-nonenal (No. 1362) for 3 h (Esterbauer et al., 1990). Significant increases in the incidence of micronuclei formation were reported at 10 and 100 μmol/l, but not at 0.1 or 1.0 μmol/l. There was no statistically significant increase in the incidence of chromosomal aberrations at any concentration tested. In a similar study conducted by Eckl et al. (1993), significant increases in SCEs were reported with *trans*-2-nonenal at concentrations of 0.1, 10, and 100 μmol/l; however, no significant induction of chromosomal aberrations or micronuclei formation was demonstrated.

In an assay for unscheduled DNA synthesis, cultured rat hepatocytes (60 to 600 nmol/10^6 cells) were incubated with *trans*-2-hexenal (No. 1353) or *trans*-2-nonenal (No. 1362) for 20 h (Griffin & Segall, 1986). Cytotoxicity was evaluated by measurement of lactate dehydrogenase release. Increases in unscheduled DNA synthesis activity, as measured by an increase in net grain counts (nuclear-cytoplasmic grain counts), increased in a dose-dependent manner beginning at 120 nmol/10^6 cells for 2-hexenal and 60 nmol/10^6 cells for 2-nonenal. The increases correlated closely with increased release of LDH.

High concentrations of a series of α,β-unsaturated aldehydes induced single strand breaks as determined by the alkaline elution assay using mouse leukaemia L1210 cells (Eder et al., 1993). In almost all cases, strand breaks occurred at or near cytotoxic concentrations: 600–800 μmol for 2-pentenal (No. 1364), 250–500 μmol for 2-hexenal (No. 1353), 400–500 μmol for 2-heptenal (No. 1360), and 350 μmol for 2-octenal (No. 1363). With the exception of 2-pentenal at 600 μmol, 2-hexenal at 250 μmol, and 2-heptenal at 400–500 μmol, cytotoxicity was observed at all concentrations inducing strand breaks. Additionally, *trans*-2-pentenal and *trans*-2-hexenal reacted with nucleosides and nucleotides. When the DNA adducts were investigated, both aldehydes produced 1,2-cyclic deoxyguanosine, but no 7,8-cyclic guanosine adducts or evidence of cross-linked adducts were observed.

The authors concluded that α,β-unsaturated aldehydes may induce strand breaks either by direct DNA interaction, or by programmed cell death, which involves the release of endonucleolytic enzymes (Eder et al., 1993).

Subsequent studies investigated the influence of GSH and detoxication enzymes on 2-alkenal-induced DNA damage in primary rat hepatocytes and human Namalva cells, the latter having lower GSH content and GST activity (Eisenbrand et al., 1995). DNA single strand breaks were induced at lower concentrations in Namalva cells than in hepatocytes.

Methyl 2-nonynoate (No. 1356) and methyl 2-octynoate (No. 1357)

In standard assay for mutation in *S. typhimurium*, methyl 2-nonynoate (No. 1356) and methyl 2-octynoate (No. 1357) were not mutagenic in *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538 when tested at concentrations of up to 3600 μg/plate, with and without metabolic activation (Wild et al., 1983).

(E)-2-Butenoic acid (No. 1371)

(*E*)-2-Butenoic acid (No. 1371) was tested for mutagenicity in the Ames assay using *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538 in the presence or absence of metabolic activation. There was no evidence of mutagenicity at concentrations of up to 1000 μg/plate (Lijinsky & Andrews, 1980). However, using the liquid pre-incubation method, positive results were obtained for (*E*)-2-butenoic acid in *S. typhimurium* strain TA100 with or without metabolic activation (Lijinsky & Andrews, 1980). In the absence of metabolic activation, positive results were first observed with (*E*)-2-butenoic acid at concentrations as low as 10 μg/plate, while in the presence of metabolic activation, significant mutagenic activity was first observed with (*E*)-2-butenoic acid at a concentration of 250 μg/plate. According to the authors, the addition of the metabolic activation system (S9) partially detoxifies the compound, producing a mutagen that is different from that detected without the added activation. In a similar assay, there was no evidence for mutagenicity at concentrations ranging from 0.1 μg/plate to 1000 μg/plate in strain TA100 (Rapson et al., 1980).

A slight dose-dependent increase in SCEs was observed in vitro for (*E*)-2-butenoic acid in human lymphocytes, at concentrations ranging from 2.5 to 10.0 mmol/l (215 to 861 μg/ml) (Sipi et al., 1992). However, a significant increase in SCEs relative to controls was noted only at the highest dose tested (10 mmol/l); at this dose, a decrease in the pH of the medium (by 0.4–0.68 pH units) compared with that of controls was also observed.

(ii) In vivo

Ethyl acrylate (No. 1351)

Single oral doses of ethyl acrylate at concentrations of up to 4% were administered to male F344 rats (Morimoto et al., 1990). The forestomachs exhibited

oedema and inflammation, but no DNA damage was detected by alkaline elution.

In an in vivo-in vitro assay for clastogenicity, C57BL/6 male mice were given ethyl acrylate at a dose of 0, 125, 250, 500 or 1000 mg/kg bw by intraperitoneal injection (Kligerman et al., 1991). Twenty-four h later, animals were sacrificed, splenocytes were isolated, and concanavalin A was added to stimulate cell division. Analysis for chromosomal aberrations in first division cells and SCE in second division cells revealed no evidence of clastogenicity. At the highest dose (1000 mg/kg bw), ethyl acrylate did induce a small increase in binucleated cell micronuclei; however, this dose was fivefold higher than the highest dose used in the National Toxicology Program study (see below) (Kligerman et al., 1991).

In an assay for mutagenicity in vivo in *Drosophila melanogaster*, there was no evidence of an increase in sex-linked recessive lethals in three successive broods obtained from Basc virgin females mated with male Canton-S wild-type males either injected with ethyl acrylate at a concentration of 20 000 mg/kg or fed a solution containing ethyl acrylate at a concentration of 40 000 mg/kg for 3 days (Valencia et al., 1985). In a second experiment, there was no evidence of mutagenicity when *D. melanogaster* were fed a solution containing ethyl acrylate at a concentration of 18 000 or 20 000 mg/kg (Valencia et al., 1985).

Although an early report (Przybojewska et al., 1984) indicated that ethyl acrylate was genotoxic in a standard assay for micronucleus formation in mice, subsequent studies (Ashby et al., 1989; Kligerman et al., 1991; Hara et al., 1994; Morita et al., 1997) confirmed that ethyl acrylate exhibits no genotoxic potential in this assay. An increase in the incidence of micronuclei in bone-marrow polychromatic erythrocytes was reported when BALB/C male mice were given ethyl acrylate at doses of 225–1800 mg/kg bw by intraperitoneal injection in two separate doses (Przybojewska et al., 1984). However, there was no evidence of an increase in micronuclei collected 24 h after groups of six BDF_1 male mice were given ethyl acrylate as a single dose at 0, 188, 375 or 750 mg/kg bw by oral gavage. There were also no clastogenic effects observed when ethyl acrylate at a dose of 0, 188, 375 or 750 mg/kg bw was administered by double oral gavage, or when ethyl acrylate as a single dose at 0, 375, 500 or 750 mg/kg bw was administered by intraperitoneal injection (Hara et al., 1994). In another assay for micronucleus formation, groups of five male and five female C57BL/6 mice were given ethyl acrylate as a single intraperitoneal dose at 461 or 738 mg/kg bw and samples were collected at 24, 48 (738 mg/kg bw dose only), and 72 h (738 mg/kg bw dose only) (Ashby et al., 1989). In subsequent experiments duplicating conditions used in an earlier study (Przybojewska et al., 1984), groups of 5–10 male C57BL/6 and BALB/c mice were given ethyl acrylate at a dose of 738 or 812 mg/kg bw in two doses administered by intraperitoneal injection within 24 h, and erythrocytes were sampled at 30 h. In none of these experiments was there any evidence of an increase in the formation of micronuclei in bone marrow of mice (Ashby et al., 1989). Negative results were obtained for micronucleus induction when groups of six male BDF_1 mice were given ethyl acrylate either as a single oral dose (188, 375 or 750 mg/kg bw) or a single intraperitoneal dose (188 or 375 mg/kg bw) and samples of bone marrow were collected after 24 h (Morita et al., 1997).

2-Hexenal (No. 1353)

Human volunteers rinsed their mouths with 100 ml of an aqueous solution of *trans*-2-hexenal (No. 1353) at a concentration of 10 mg/kg four times per day for 3 consecutive days and exfoliated buccal mucosa cells were then evaluated for induction of micronuclei (Dittberner et al., 1997). An increase of about threefold in the frequency of formation of micronuclei was observed on days 6 and 7 after administration. No increases were observed on preceding days. Slowly eating five to six completely yellow bananas, which contain hexenal, produced a similar, but weaker effect.

Methyl 2-nonynoate (No. 1356) and methyl 2-octynoate (No. 1357)

The potential of methyl 2-nonynoate and methyl 2-octynoate to induce sex-linked recessive lethal mutations in adult *D. melanogaster* were studied in the Basc test for genotoxicity. Mutation frequency was unaffected when solutions of methyl 2-nonynoate and methyl 2-octynoate, at 2.5 and 1.0 mmol/l (421 and 154 μg/ml, respectively) respectively, were fed to the flies for 3 days (Wild et al., 1983).

In a test for micronucleus formation, groups of four male and female NMRI mice were given methyl 2-nonynoate as a single intraperitoneal dose at 168, 336 or 505 mg/kg bw, or methyl 2-octynoate at 154, 231 or 308 mg/kg bw. No increase in micronucleated erythrocytes in bone marrow samples obtained 30 h after administration was observed for either substance (Wild et al., 1983).

(iii) Conclusion

Testing of α,β-unsaturated aldehydes in standardized Ames assays using a variety of strains (TA97, TA98, TA100, TA102, TA104, TA1535, TA1537 and TA1538) has shown no evidence for mutagenicity in bacteria (Florin et al., 1980; National Toxicology Program, 2001a). However, alternative protocols have been developed to avoid competing cytotoxicity of α,β-unsaturated aldehydes. In these studies, positive results were reported in modified Ames assays with pre-incubation conditions conducive to depletion of metabolic detoxication pathways (Eder et al., 1992, 1993). Positive evidence of genotoxicity also was reported in other assays (SCE, CA, micronucleus formation) performed in cell lines low in detoxication capacity (Namalva cells and human lymphocytes) (Dittberner et al., 1995). The high concentrations of α,β-unsaturated aldehydes (20–40 μmol/l) used in these studies resulted in single DNA strand breaks but no cross-linking. The conditions of the experiments (high concentrations of aldehyde in cell lines poor in detoxication capacity) provided opportunity for either direct interaction of α,β-unsaturated aldehydes with DNA or indirect formation of DNA adducts because of oxidative stress. It is now well recognized that high concentrations of α,β-unsaturated aldehydes deplete GSH, leading to release of nucleocytolytic enzymes that induce DNA fragmentation, cellular damage and apoptosis (see discussion in section 2.2.1(c)). Nonetheless, evidence also has indicated that at low concentrations, such as those resulting from intake of flavouring substances, α,β-unsaturated aldehydes are rapidly metabolized in the high-capacity β-oxidation pathway. In addition, there

is no convincing evidence that α,β-unsaturated aldehydes exhibit significant genotoxic potential in vivo.

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MONOCYCLIC AND BICYCLIC SECONDARY ALCOHOLS, KETONES AND RELATED ESTERS

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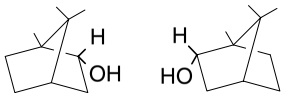
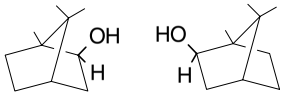
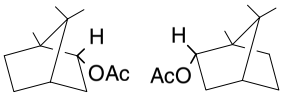
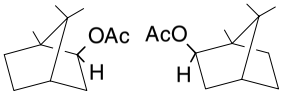
1. EVALUATION

1.1 Introduction

The Committee evaluated a group of 32 monocyclic and bicyclic secondary alcohols, ketones and related esters (see Table 1) by the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, p 192). The Committee has not previously evaluated any of the members of this group.

Nineteen of the 32 flavouring agents (Nos 1385–1389, 1391, 1394–1400, 1403, 1404, 1407, 1412, 1414 and 1416) have been reported to occur naturally in foods. They have been detected in butter, beef, beer, parmesan and other cheeses, wine, fruit, herbs, spices, mints, and cocoa (Nijssen et al., 2003).

Table 1. Summary of the results of safety evaluations of monocyclic and bicyclic secondary alcohols, ketones and related esters[a] used as flavouring agents

Flavouring agent	No.	CAS No. and structure	Step A3[a,b] Does intake exceed the threshold for human intake?	Comments	Conclusion based on current intake
Structural class I					
Borneol	1385	507-70-0  (-)-Borneol (+)-Borneol	No Europe: 155 USA: 23	See note 1	No safety concern
Isoborneol	1386	124-76-5  (-)-Isoborneol (+)-Isoborneol	No Europe: 24 USA: 0.07	See note 1	No safety concern
Bornyl acetate	1387	76-49-3  (-)-Bornyl acetate (+)-Bornyl acetate	No Europe: 21 USA: 3	See notes 1 and 2	No safety concern
Isobornyl acetate	1388	125-12-2  (-)-Isobornyl acetate (+)-Isobornyl acetate	No Europe: 1039 USA: 236	See notes 1 and 2.	No safety concern

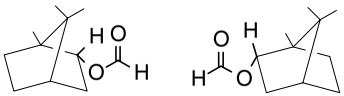
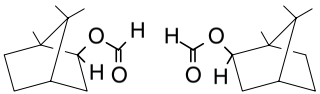
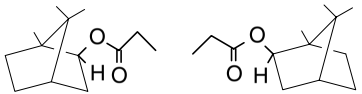
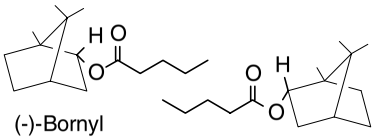
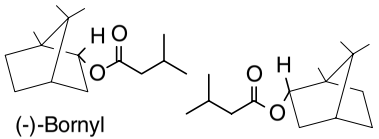
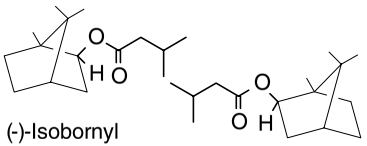
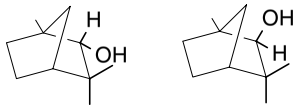
Bornyl formate	1389	7492-41-3  (-)-Bornyl formate (+)-Bornyl formate	No Europe: 1 USA: 0.09	See notes 1 and 2	No safety concern
Isobornyl formate	1390	1200-67-5  (-)-Isobornyl formate (+)-Isobornyl formate	No Europe: 0.7 USA: 0.4	See notes 1 and 2.	No safety concern
Isobornyl propionate	1391	2756-56-1  (-)-Isobornyl propionate (+)-Isobornyl propionate	No Europe: 3 USA: 0.007	See notes 1 and 2	No safety concern
Bornyl valerate	1392	7549-41-9  (-)-Bornyl valerate (+)-Bornyl valerate	No Europe: ND USA: 5	See notes 1 and 2	No safety concern

Table 1. (Contd)

Flavouring agent	No.	CAS No. and structure	Step A3[a,b] Does intake exceed the threshold for human intake?	Comments	Conclusion based on current intake
Bornyl isovalerate (endo-)	1393	76-50-6  (-)-Bornyl isovalerate (+)-Bornyl isovalerate	No Europe: 0.1 USA: 0.5	See notes 1 and 2	No safety concern
Isobornyl isovalerate	1394	7779-73-9  (-)-Isobornyl isovalerate (+)-Isobornyl isovalerate	No Europe: 0.01 USA: 0.08	See notes 1 and 2	No safety concern
Fenchyl alcohol	1397	1632-73-1  *alpha*-Fenchol *beta*-Fenchol	No Europe: 64 USA: 17	See note 1	No safety concern

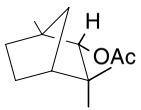
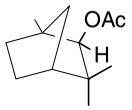
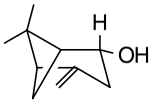
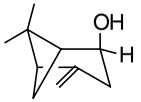
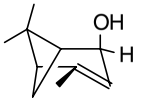
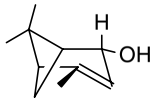
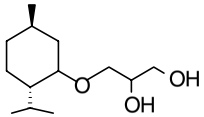
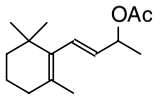
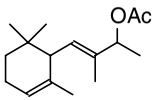
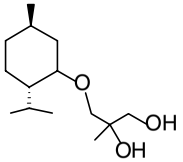
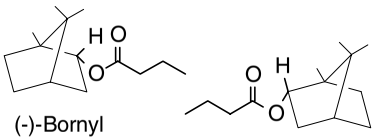
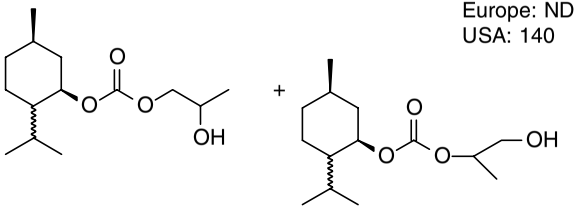
1,3,3-Trimethyl-2-norbornanyl acetate	1399	13851-11-1  *alpha-form*  *beta*-form	No Europe: 3 USA: 0.07	See notes 1 and 2	No safety concern
2(10)-Pinen-3-ol	1403	5947-36-4  *trans*-form  *cis*-form	No Europe: 0.01 USA: 0.01	See notes 1 and 3	No safety concern
Verbenol	1404	473-67-6  *cis*-form  *trans*-form	No Europe: 0.3 USA: 0.2	See notes 1 and 3	No safety concern
3-*l*-Menthoxypropane-1,2-diol	1408	87061-04-9 	No Europe: ND USA: 789	See notes 1 and 4	No safety concern
β-Ionyl acetate	1409	22030-19-9 	No Europe: ND USA: 9	See notes 1 and 2	No safety concern

Table 1. (Contd)

Flavouring agent	No.	CAS No. and structure	Step A3[a,b] Does intake exceed the threshold for human intake?	Comments	Conclusion based on current intake
α-Isomethylionyl acetate	1410	68555-61-3 	No Europe: ND USA: 9	See notes 1 and 2	No safety concern
3-(*l*-Menthoxy)-2-methyl —propane-1,2-diol	1411	195863-84-4 	No Europe: ND USA: 88	See notes 1 and 4	No safety concern
Bornyl butyrate	1412	13109-70-1  (-)-Bornyl butyrate (+)-Bornyl butyrate	No Europe: ND USA: 9	See notes 1 and 2	No safety concern
d,l-Menthol(±)-propylene glycol carbonate	1413	156324-82-2 	No Europe: ND USA: 140	See notes 1 and 2	No safety concern

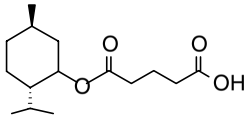
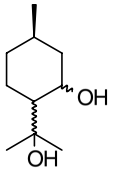
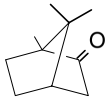
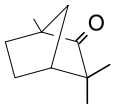
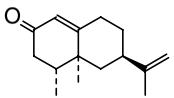
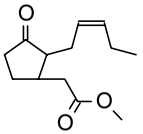
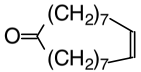
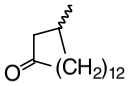
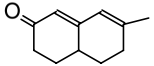
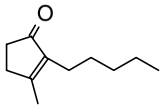
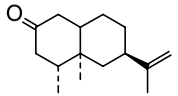
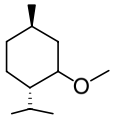
l-Monomenthyl glutarate	1414	220621-22-7 	No Europe: ND USA: 132	See notes 1 and 2	No safety concern
p-Menthane-3,8-diol	1416	42822-86-6 	No Europe: ND USA: 18	See note 1	No safety concern
Structural class II					
d-Camphor	1395	464-49-3  (*d*)-(+)-Camphor	No Europe: 58 USA: 396	See notes 1, 5, and 6	No safety concern
d-Fenchone	1396	4695-62-9  (*d*)-(+)-Fenchone	No Europe: 7 USA: 5	See notes 1, 5, and 6	No safety concern
Nootkatone	1398	4674-50-4 	No Europe: 152 USA: 20	See notes 1, 5, 6, and 7	No safety concern

Table 1. (Contd)

Flavouring agent	No.	CAS No. and structure	Step A3[a,b] Does intake exceed the threshold for human intake?	Comments	Conclusion based on current intake
Methyl jasmonate	1400	1211-29-6 	No Europe: 31 USA: 0.4	See notes 1 and 2	No safety concern
Cycloheptadeca-9-en-1-one	1401	542-46-1  *trans*-isomer	No Europe: 0.3 USA: 0.05	See notes 1, 5, and 6	No safety concern
3-Methyl-1-cyclopentadecanone	1402	541-91-3 	No Europe: 0.4 USA: 0.009	See notes 1, 5, and 6	No safety concern
7-Methyl-4,4a,5,6-tetrahydro-2(3*H*)-naphthalenone	1405	34545-88-5 	No Europe: ND USA: 0.04	See notes 1, 5, and 6	No safety concern
3-Methyl-2-(*n*-pentanyl)-	1406	1128-08-1	No	See notes 1	No safety

2- cyclopenten-1-one			Europe: 0.4 USA: 0.2	and 5	concern
Dihydronootkatone	1407	20489-53-6 	No Europe: 0.7 USA: 0.9	See notes 1, 5, 6, and 7	No safety concern
Structural class III					
l-Menthyl methyl ether	1415	1565-76-0 	No Europe: ND USA: 53	See notes 1 and 8	No safety concern

CAS: Chemical Abstracts Service; ND: No intake data reported.

[a] Step 2: All the agents in this group can be predicted to be metabolized to innocuous products.

[b] The threshold for human intake for structural classes I and II is 1800 and 540 μg/person per day, respectively. All intake values are expressed in μg/person per day. The combined intake of flavouring agents in structural class I is 1311 μg/per person per day in Europe and 1479 μg/person per day in the USA. The combined intake of flavouring agents in structural class II is 250 μg/person per day in Europe and 423 μg/person per day in the USA. The intake for the flavouring agent in structural class III is 53 μg/person per day in the USA.

Notes:

1. Formation of glucuronic acid conjugates directly or after metabolism, which are subsequently excreted in the urine.
2. Ester hydrolysis to liberate the corresponding alcohol and carboxylic acid.
3. Ring cleavage to polar excretable metabolites.
4. Oxidation of the primary alcohol to the corresponding carboxylic acid
5. Reduced to yield the corresponding alcohol
6. Hydroxylation of alkyl ring-substituents and ring positions
7. Oxidation and hydration of exocyclic and, to a lesser extent, endocyclic double bonds
8. Oxidized by *O*-demethylation to yield corresponding alcohol

1.2 Estimated daily intake

The total annual volume of production of the 32 monocyclic and bicyclic secondary alcohols, ketones and related esters in this group is approximately 11 000 kg in Europe (International Organization of the Flavor Industry, 1995) and 14 000 kg in the USA (National Academy of Sciences, 1970, 1982, 1987; Lucas et al., 1999) (Table 2). Approximately two-thirds of the total annual volume of production in Europe is accounted for by one agent in the group, isobornyl acetate (No. 1388), while borneol (No.1385) and nootkatone (No. 1398) account for an additional 20% of the total volume. Approximately 80% of the total annual volume of production in the USA is accounted for by three agents, isobornyl acetate (No. 1388), *d*-camphor (No. 1395) and 3-*l*-menthoxypropane-1,2-diol (No. 1408). Daily intakes in Europe and the USA were calculated to be 1039 and 236 μg/person for isobornyl acetate (No. 1388), 155 and 23 μg/person for borneol (No. 1385), 152 and 20 μg for nootkatone (No. 1398), and 58 and 396 μg/person for *d*-camphor (No. 1395), respectively. For 3-*l*-menthoxypropane-1,2-diol (No. 1408) and *d*,*l*-menthol-propylene glycol carbonate (No. 1413), the daily intakes in the USA are calculated to be 789 and 140 μg/person, respectively. The daily intakes of the other flavouring agents in the group were in the range of 0 to 132 μg/person, with most of the values being at the lower end of this range. The estimated daily per capita intake of each agent in Europe and in the USA is reported in Table 1.

1.3 Absorption, distribution, metabolism and elimination

Studies in humans, dogs, and rabbits have shown that the mono- and bicyclic secondary alcohols and ketones in this group are rapidly absorbed, distributed, metabolized and excreted mainly in the urine. Small amounts may be eliminated in exhaled air. In humans, the esters within this group are expected to be hydrolysed to their component secondary alcohol and carboxylic acid.

The major metabolic pathway for the ketones involves reduction to the corresponding secondary alcohols, which are subsequently excreted, primarily as the glucuronic acid conjugates (Williams, 1959; Lington & Bevan, 1994; Topping et al., 1994). Metabolites containing a double bond that are excreted into the bile may be reduced to the corresponding dihydro derivatives by the gut microflora (Krasavage et al., 1982). In addition to reductive pathways, alicyclic ketones and, to a lesser extent, secondary alcohols containing an alkyl side-chain, undergo oxidation of the side-chain to form polar poly-oxygenated metabolites that are excreted mainly in the urine, either unchanged or as the glucuronide or sulfate conjugates.

For more lipophilic ketones (e.g. nootkatone, No. 1398) or those with sterically hindered functional groups (e.g. *d*-camphor, No. 1395), oxidation of a ring position by cytochrome P450 (CYP) may compete with reduction of the ketone group or oxidation of the alcohol group (Asakawa et al., 1986; Nelson et al., 1992). For example, bicyclic ketones tend to show greater lipophilicity and steric hindrance of the carbonyl function than do short-chain aliphatic or monocyclic ketones. As such, bicyclic ketones are expected to be poor substrates for cytosolic reducing enzymes. Consequently, the predominant detoxication route is CYP-mediated ring hydroxylation to yield polar, excretable poly-oxygenated metabolites.

Table 2. Annual volumes of production of monocyclic and bicyclic secondary alcohols, ketones and related esters used as flavouring agents in Europe and the USA

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		μg/day	μg/kg bw per day		
Borneol (1385)					
Europe	1084	155	3		
USA	172	23	0.4	863	5
Isoborneol (1386)					
Europe	170	24	0.4		
USA	0.5	0.07	0.001	+	NA
Bornyl acetate (1387)					
Europe	146	21	0.3		
USA	23	3	0.05	424	18
Isobornyl acetate (1388)					
Europe	7278	1039	17		
USA	1792	236	4	+	NA
Bornyl formate (1389)					
Europe	10	1	0.02		
USA[f]	0.5	0.09	0.001	+	NA
Isobornyl formate (1390)					
Europe	5	0.7	0.01		
USA[f]	2	0.4	0.006	–	NA
Isobornyl propionate (1391)					
Europe	21	3	0.05		
USA	0.05	0.007	0.0001	+	NA
Bornyl valerate (1392)					
Europe	ND	ND	ND		
USA[f]	30	5	0.09	–	NA
Bornyl isovalerate, *endo*- (1393)					
Europe	1	0.1	0.002		
USA[f]	3	0.5	0.009	–	NA
Isobornyl isovalerate (1394)					
Europe	0.1	0.01	0.0002		
USA[f]	0.5	0.08	0.001	+	NA
d-Camphor (1395)					
Europe	408	58	1		
USA	3007	396	7	+	NA
d-Fenchone (1396)					
Europe	52	7	0.1		
USA	40	5	0.09	+	NA
Fenchyl alcohol (1397)					
Europe	451	64	1		
USA	132	17	0.3	873	7
Nootkatone (1398)					
Europe	1067	152	3		
USA	154	20	0.3	1051	7
1,3,3-Trimethyl-2-norbornanyl acetate (1399)					
Europe	24	3	0.06		
USA	0.5	0.07	0.001	+	NA

Table 2. (Contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		μg/day	μg/kg bw per day		
Methyl jasmonate (1400)					
Europe	217	31	0.5		
USA	3	0.4	0.007	37	12
Cycloheptadeca-9-en-1-one (1401)					
Europe	2	0.3	0.005		
USA	0.4	0.05	0.0009	–	NA
3-Methyl-1-cyclopentadecanone (1402)					
Europe	3	0.4	0.01		
USA[f]	0.05	0.009	0.0001	–	NA
2(10)-Pinen-3-ol (1403)					
Europe	0.1	0.01	0.0002		
USA	0.1	0.01	0.0002	+	NA
Verbenol (1404)					
Europe	2	0.3	0.005		
USA[f]	1	0.2	0.003	+	NA
7-Methyl-4,4a,5,6-tetrahydro-2(3*H*)-naphthalenone (1405)					
Europe	ND	ND	ND		
USA	0.3	0.04	0.0007	–	NA
3-Methyl-2-(*n*-pentanyl)-2-cyclopenten-1-one (1406)					
Europe	3	0.4	0.007		
USA	1.4	0.2	0.003	–	NA
Dihydronootkatone (1407)					
Europe	5	0.7	0.01		
USA[e]	5	0.9	0.01	+	NA
3-*l*-Menthoxypropane-1,2-diol (1408)					
Europe	ND	ND	ND		
USA	5987	789	13	–	NA
β-Ionyl acetate (1409)					
Europe	ND	ND	ND		
USA[e]	50	9	0.1	–	NA
α-Isomethyllionyl acetate (1410)					
Europe	ND	ND	ND		
USA[e]	50	9	0.1	–	NA
3-(*l*-Menthoxy)-2-methylpropane-1,2-diol (1411)					
Europe	ND	ND	ND		
USA[e]	500	88	1	–	NA
Bornyl butyrate (1412)					
Europe	ND	ND	ND		
USA[e]	50	9	0.1	+[g]	NA
d,l-Menthol-(±)-propylene glycol carbonate (1413)					
Europe	ND	ND	ND		
USA[e]	800	140	2	–	NA
l-Monomenthyl glutarate (1414)					
Europe	ND	ND	ND		
USA[e]	750	132	2	+[h]	NA
l-Menthyl methyl ether (1415)					
Europe	ND	ND	ND		
USA[e]	300	53	0.9	–	NA

Table 2. (Contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		μg/day	μg/kg bw per day		
p-Menthane-3,8-diol (1416)					
Europe	ND	ND	ND		
USA[e]	100	18	0.3	+[i]	NA
Total					
Europe	10949				
USA	13955				

NA, not available; ND, no intake data reported; +, reported to occur naturally in foods (Nijssen et al., 2003), but no quantitative data; –, not reported to occur naturally in foods

[a] From International Organization of the Flavour Industry (1995) and Lucas et al. (1999) or National Academy of Sciences (1970, 1982, 1987).

[b] Intake expressed as μg/person per day was calculated as follows: [(annual volume, kg) × (1×10^9 μg/kg)/(population × survey correction factor × 365 days)], where population (10%, 'eaters only') = 32×10^6 for Europe and 26×10^6 for the USA. The correction factor = 0.6 for Europe and 0.8 for the USA representing the assumption that only 60% and 80% of the annual production volume of the flavour, respectively, was reported in the poundage surveys (International Organization of the Flavour Industry, 1995; Lucas et al., 1999; National Academy of Sciences, 1970, 1982, 1987) or in the anticipated annual volume of production.
Intake expressed as μg/kg bw per day was calculated as follows: [(μg/person per day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.

[c] Quantitative data for the USA reported by Stofberg & Grundschober (1987).

[d] The consumption ratio is calculated as follows: (annual consumption via food, kg)/(most recently reported volume as a flavouring agent, kg)

[e] The volume cited is the anticipated annual volume, which was the maximum amount of flavouring agent estimated to be used annually by the manufacturer at the time the material was proposed for flavour use.

[f] Annual volume reported in previous USA surveys (National Academy of Sciences, 1970; 1982; 1987).

[g] Frattini et al. (1981)

[h] Natural occurrence data reported in a private communication to Flavor and Extract Manufacturers Association (2003)

[i] Nishimura et al. (1984)

The pathways by which fused ring and macrocyclic ketones are detoxified are similar to those for the bridged bicyclic substances. Activated ring positions (e.g. tertiary and allylic positions) and ring substituents are oxidized primarily by CYP, introducing additional polar groups into the molecule. The resulting metabolites are then excreted, mainly in the urine.

1.4 Application of the procedure for the safety evaluation of flavouring agents

Step 1. In applying the Procedure, the Committee assigned 22 (Nos 1385–1394, 1397, 1399, 1403, 1404, 1408–1414, 1416) of the 32 agents to structural class I. Nine of these agents (Nos 1395, 1396, 1398, 1400–1402, 1405–1407) were assigned to structural class II, and the remaining agent (No. 1415) was assigned to structural class III (Cramer et al., 1978).

Step 2. All the flavouring agents in this group are expected to be metabolized to innocuous products. Their evaluation therefore proceeded via the A-side of the decision-tree.

Step 3. The estimated daily intakes of all 22 of the flavouring agents in structural class I, all nine of the agents in structural class II and the agent in structural class III are below the thresholds of concern (i.e. 1800 μg/person for class I, 540 μg/person for class II, and 90 μg/person for class III). According to the Procedure, the safety of these 32 flavouring agents raises no concern when they are used at estimated current intakes.

The intake considerations and other information used to evaluate the 32 monocyclic and bicyclic secondary alcohols, ketones and related esters in this group according to the Procedure are summarized in Table 1.

1.5 Consideration of secondary components

Six members (Nos 1386, 1398, 1407, 1409, 1413 and 1414) of this group of flavouring agents have minimum assay values of <95%. Information on the safety of the secondary components of these six compounds is summarized in Annex 5 (Summary of the safety evaluation of secondary components for flavouring agents with minimum assay values of less than 95%). The secondary components of No. 1407 (acetic acid and β-ionol) were evaluated by the Committee at its forty-ninth meeting and fifty-first meetings (Annex 1, references *131* and *137*), respectively. The secondary components of No. 1413, *d,l*-menthol 2-propylene glycol carbonate, and of No. 1414, dimenthyl glutarate and glutaric acid, have not been previously evaluated. However, *d,l*-menthol 2-propylene glycol carbonate and dimenthyl glutarate are structurally related to the primary flavouring agents in this group and are expected to share the same metabolic fate. Glutaric acid is structurally related to valeric acid, which was evaluated by the Committee at its forty-ninth meeting (Annex 1, reference *131*). The secondary components of Nos 1386, 1398 and 1407 (borneol, dihydronootkatone and nootkatone) were evaluated as flavouring agents by the Committee at its current meeting. On the basis of these evaluations, the secondary components for these six flavouring agents were considered not to present a safety concern at current estimated intakes.

1.6 Consideration of combined intakes from use as flavouring agents

In the unlikely event that all 22 agents in structural class I were consumed concurrently on a daily basis, the estimated combined intake would not exceed

the human intake threshold for class I (1800 μg/person per day). In the unlikely event that all nine agents in structural class II were consumed concurrently on a daily basis, the estimated combined intake would not exceed the human intake threshold for class II (540 μg/person per day). Overall evaluation of the data indicated that combined intake of the agents in this group would not present a safety concern.

1.7 Conclusions

The Committee concluded that none of the flavouring agents in this group of monocyclic and bicyclic secondary alcohols, ketones and related esters would raise a safety concern at current estimated intakes. Available data on the toxicity and metabolism of these substances were consistent with the results of the safety evaluation.

2. RELEVANT BACKGROUND INFORMATION

2.1 Additional considerations on intake

The majority of members in this group of monocyclic and bicyclic secondary alcohols and ketones are terpene monocyclic and bicyclic ketones (e.g. camphor; No. 1395), secondary alcohols (e.g. borneol; No. 1385), and related esters (e.g. isobornyl acetate; No. 1388). Therefore, it is expected that many are common constituents of plants. In some cases, different stereoisomers of a substance may be major constituents of the volatile portion of different plants. For instance, (+)-borneol (No. 1385) is found as a constituent of thyme oil and rosemary oil at concentrations of up to 40%, while (–)-borneol (No. 1385) and its acetate ester (No. 1387) are present in fir and pine oils (*Abies*, *Pinus*, and *Picea* species) at concentrations of up to 45% (Nijssen et al., 2003).

2.2 Biological data

2.2.1 Biochemical data

(a) Hydrolysis

The esters within this group are expected to be hydrolysed in humans to their component alcohols and aliphatic carboxylic acids. Subsequently, the carboxylic acids are completely metabolized through recognized biochemical pathways (Nelson & Cox, 2000). Ester hydrolysis is catalysed by classes of enzymes recognized as carboxylesterases (Heymann, 1980; White et al., 1990), the most important of which are the B-esterases. In mammals, these enzymes occur in most tissues (Heymann, 1980; Anders, 1989), but predominate in hepatocytes (Heymann, 1980).

In rabbits, >90% of an oral dose of *d*-, *l*-, or *dl*-bornyl acetate (No. 1387) was excreted in the urine as the glucuronic acid conjugate of hydrolysed borneol (Williams, 1959). In two separate in vitro hydrolysis studies l-menthol ethylene

glycol carbonate (No. 443) and *l*-menthol propylene glycol carbonate (No. 1413) were hydrolysed following incubation with rat liver homogenate (Emberger, 1998). Incubation of β-ionyl acetate (No. 1409) in the presence of simulated gastric juice or intestinal fluid resulted in 43% and >60% hydrolysis to β-ionol within 4 h, respectively (Bennett, 1998). Approximately 75% of *d,l*-menthol propylene glycol carbonate (No. 1413) was hydrolysed to menthol when incubated for 4 h with liver homogenate (Emberger, 1998). It is anticipated that *d,l*-mentholethylene glycol carbonate would be hydrolysed in a similar manner. Incubation of the mandelic acid ester of 3,3,5,5-tetramethylcyclohexanol, a structurally related ester, with rat liver microsomes resulted in >80% hydrolysis within 2 min (White et al., 1990). *cis*- and *trans-p*-1(7),8-Menthadien-2-yl acetate (No. 1098) was also rapidly hydrolysed in vitro in the presence of rat liver homogenate. Incubation of the ester resulted in 92% hydrolysis after 15 min and 100% after 60 min (Salzer, 1998). On the basis of these data, it is anticipated that the esters of this group will be rapidly hydrolysed in the digestive tract or in the liver.

(b) Absorption, distribution and excretion

(i) Bicyclic derivatives

Studies in humans, dogs and rabbits, have shown that the secondary alcohols and ketones of this group are rapidly absorbed, distributed, metabolized, and excreted mainly in the urine as glucuronide conjugates. Small amounts may be expired in exhaled air. Previously reviewed (Annex 1, reference *138*) data for other cyclic terpene secondary alcohols and ketones including menthol (No. 427), menthone (No. 429), and carvone (Nos 380a, 380b) support this conclusion.

Case reports, in which ingestion of camphor (No. 1395) resulted in toxicity in both adults and children within minutes of exposure (Jacobziner & Raybin, 1962; Phelan, 1976; Kopelman et al., 1979; Gibson et al., 1989), demonstrate rapid absorption of this substance. Rabbits given *d*-camphor (No. 1395) at a dose of 1.9–3.5 mmol/kg bw (289–533 mg/kg bw) by gavage excreted 59.1% of the administered dose conjugated with glucuronic acid in the urine within 24 h (Robertson & Hussain, 1969). A group of 50 Sprague-Dawley rats was given 40% camphor in cottonseed oil as a single dose at 1000 mg/kg bw (approximately 400 mg of camphor) by gavage, and killed at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, or 10.0 h after treatment. Blood samples were taken before death. Peak blood concentration of camphor occurred at 96 min, with an absorption half-life of 38 min and a plasma elimination half-life of 142 min. The authors considered that these data compared favourably with those in humans (Dean et al., 1992).

The toxicokinetics of *d,l*-camphor (No. 1395 for the *d*-form) were studied in B6C3F$_1$ mice and F344 rats. In mice, camphor was rapidly eliminated from the plasma after a single intravenous injection at 50 mg/kg bw with an elimination rate constant of 0.0337 and 0.0335 min^{-1} for males and females, respectively, and a half-life of 21 min. In rats, camphor underwent biphasic elimination from plasma after a single intravenous injection at 6 mg/kg bw with an elimination rate constant of 0.0038 and 0.0059 min^{-1} for males and females, respectively, and half-lives of 185 and 118 min for males and females, respectively (Grizzle et al., 1996).

In a case report, a pregnant woman (week 40 of gestation) accidentally ingested 12 g of camphorated oil (% camphor not specified) and 36 h later gave birth to a cyanotic baby exhibiting no respiration. The baby died within 30 min. The presence of camphor was noted at 15 min in maternal circulation, at 20 h in amniotic fluid, and at 36 h in cord blood, infant brain, liver and kidneys (Riggs et al., 1965).

Approximately 80% of an orally administered dose of 2000 mg of *d*-borneol (No. 1385) given to humans (sex and number not specified) was excreted within 10 h (Williams, 1959).

(ii) Monocyclic derivatives

Monocyclic ketones and alcohols in this group follow a fate similar to that of bicyclic derivatives. Five female and five male Sprague-Dawley rats pre-treated with 3-*l*-menthoxypropane-1,2-diol (No. 1408) at a daily oral dose of 29.4 mg/kg bw for 7 days were given [3-^{14}C]3-*l*-menthoxypropane-1,2-diol as a single oral dose at 30 mg/kg bw on day 8, and urine, faeces, expired air, and cage washes were collected over the next 120 h. Total recovery of the radiolabelled substance was 95.2% for males and 94.4% for females with most of the dose (72.0% for males and 64.6% for females) being recovered within the first 24 h. The primary routes of excretion were the urine (56.4% for males and 61.7% for females) and the faeces (34.5% for males and 26.5% for females). Less than 3% was recovered as radiolabelled carbon dioxide for both sexes (Ferdinandi, 1993a).

Four male beagle dogs pre-treated with 3-*l*-menthoxypropane-1,2-diol (No. 1408) at daily oral doses of 49.9 mg/kg bw for 7 days, were given [3-^{14}C]3-*l*-menthoxypropane-1,2-diol as a single oral dose of 49.6 mg/kg bw on day 8, and the urine, faeces, expired air, and cage washes were collected over the next 120 h. Total recovery of the radiolabelled substance was 91.9%, with most of the dose (63.7%) being recovered within the first 24 h. As in rats, the primary routes of excretion were the urine (58.2%) and the faeces (28.1%) (Ferdinandi, 1993b).

In summary, the esters of monocyclic and bicyclic secondary alcohols are readily hydrolysed. The resulting secondary alcohols and the corresponding ketones are then rapidly absorbed, metabolized, and excreted primarily as glucuronic acid conjugates in the urine.

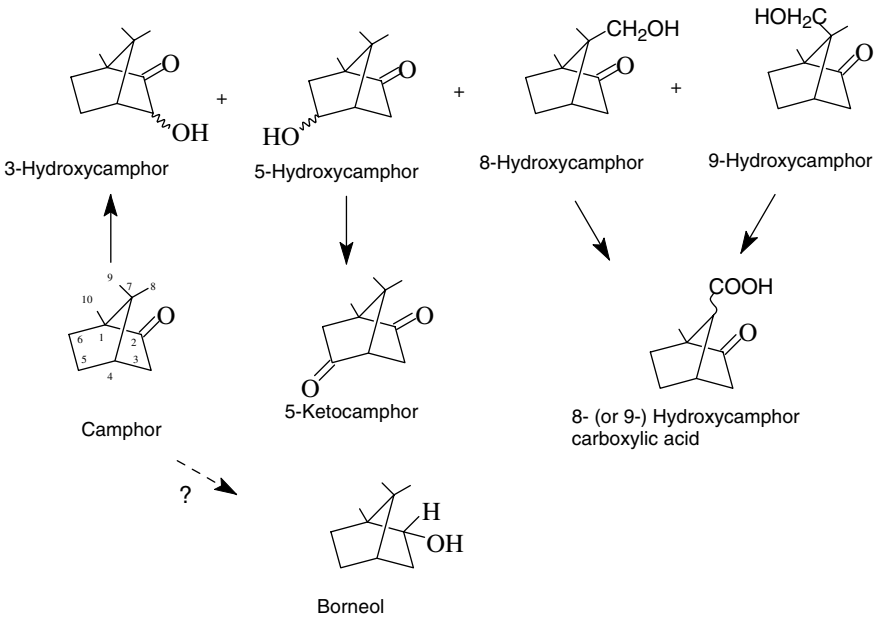
(c) Metabolism

The major metabolic pathway for the ketones involves reduction to the corresponding secondary alcohols, which are subsequently excreted primarily as the glucuronic acid conjugates (Williams, 1959; Lington & Bevan, 1994; Topping et al., 1994). Metabolites that are excreted into the bile and that contain a double bond may be reduced to the corresponding dihydro derivatives by the gut microflora (Krasavage et al., 1982). In addition to reductive pathways, alicyclic ketones and, to a lesser extent, secondary alcohols containing an alkyl side-chain undergo oxidation of the side-chain to form polar poly-oxygenated metabolites that are excreted either unchanged or as the glucuronide, or sulfate conjugates mainly in the urine.

For more lipophilic ketones (e.g. nootkatone, No. 1398) or those with sterically hindered functional groups (e.g. *d*-camphor, No. 1395) oxidation of a ring position by nonspecific CYP mixed function oxidases may compete with reduction of the ketone functional group or oxidation of the alcohol functional group (Asakawa et al., 1986; Nelson et al., 1992). For example, bicyclic ketones tend to show greater lipophilicity and steric hindrance of the carbonyl function than do short-chain aliphatic or monocyclic ketones, which are primarily reduced to the corresponding secondary alcohol. As such, bicyclic ketones are expected to be poor substrates for cytosolic reducing enzymes. Consequently, the predominant detoxication route is CYP-mediated ring hydroxylation to yield polar, excretable poly-oxygenated metabolites.

As shown in Figure 1, in humans ingestion of 6000–10 000 mg of camphor (No. 1395) resulted in urinary excretion of 3-, 5-, 8-, and 9-hydroxycamphor, 5-ketocamphor and the carboxylic acid of either 8- or 9-hydroxycamphor, unconjugated or conjugated with glucuronic acid (Köppel et al., 1982). A minor amount was exhaled in expired air. Hydroxylation products, predominantly 5-endo- and 5-exo-hydroxycamphor and a compound resembling 3-endo-hydroxycamphor, have also been reported when camphor was administered orally to dogs (1000 mg per animal, in gelatin capsules, four times per day for 7 days) or rabbits (300 mg per animal, single dose administered by gavage) (Leibman & Ortiz, 1973). The same camphor hydroxylation products, with a small amount of 2,5-bornanedione, were similarly identified in vitro after incubation with rat and rabbit liver fractions (Leibman

Figure 1. Metabolism of camphor in humans



& Ortiz, 1973). Similar hydroxylation products (4- and 5-hydroxyfenchone and *p*-apofenchone-3-carboxylic acid) were detected in the urine of dogs fed *d*-fenchone (No. 1396) (Reinartz & Zanke, 1936). The metabolism of *d*-fenchone also demonstrates that hydroxylation of ring methyl substituents leads to the corresponding carboxylic acid derivatives.

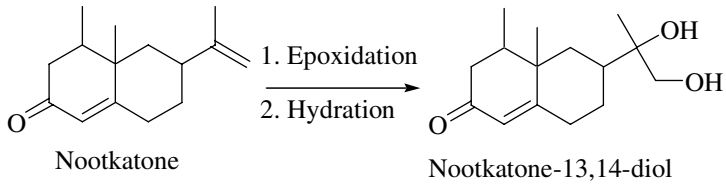
In rabbit liver cytosol, *d*-camphor (No. 1395) was reduced via an NADPH-dependent pathway to borneol and a small amount of isoborneol (Robertson & Hussain, 1969; Leibman & Ortiz, 1973). In rat liver, camphor induced members of the CYPIIB subfamily, which were most likely to be CYPb and/or CYPe (Austin et al., 1988). Female Swiss albino mice given camphor at a dose of 50, 150, or 300 mg/kg bw per day in olive oil by gavage for 20 days showed a statistically significant increase in CYP and cytochrome b_5, aryl hydrocarbon hydrolase, and glutathione *S*-transferase activities only at the highest dose (Banerjee et al., 1995).

Data for bicyclic ketones structurally related to *d*-camphor indicate that ring hydroxylation is a major pathway of metabolism for such compounds. For example, at 18 h after oral administration of *cis*-3-pinanone (100 mg/kg bw) to male albino Swiss-Webster mice, the major metabolites excreted in the urine were conjugated (glucuronide or sulfate) 2-hydroxy-*cis*-3-pinanone, two other hydroxylated *cis*-3-pinanones, and unconjugated 2(8)-dehydro-*cis*-3-pinanone. Mouse or human liver microsomes or human CYP3A4 containing NADPH were incubated with either *cis*-3-pinanone or *trans*-3-pinanone. For *cis*-3-pinanone, 2-hydroxy-*cis*-3-pinanone, the major metabolite, and two other minor hydroxylated *cis*-3-pinanone metabolites were identified in incubations with microsomal fractions and with CYP3A4. Mouse liver microsomes produced more 2-hydroxy-*cis*-3-pinanone than did human microsomes or CYP3A4. For *trans*-3-pinanone, two hydroxy-*trans*-3-pinanones were identified. The *cis*-3-pinanone metabolites were identical to those obtained in vivo. Mice were given a lethal dose of *cis*-3-pinanone or *trans*-3-pinanone at 250 mg/kg bw by intraperitoneal injection and sacrificed at different times up to 80 min. The brain tissue contained 2-hydroxy-*cis*-3-pinanone as the major metabolite of *cis*-3-pinanone. The maximum amount of metabolite was reached within 10–20 min of dosing. Metabolites identified for *trans*-3-pinanones in vivo were the same as those identified in the study in liver microsomes in vitro (Höld et al., 2002).

Fused ring and macrocyclic ketones are detoxicated by pathways similar to those for the bridged bicyclic substances. Activated ring positions (e.g. tertiary and allylic positions) and ring substituents are oxidized primarily by CYP to introduce additional polar functionalities into the molecule. The resulting metabolites are then excreted mainly in the urine.

Gas–liquid chromatography (GLC) analysis of 3-day urine samples taken from rabbits given nootkatone (No. 1380) in large doses (6000 mg) by oral administration, identified nootkatone-13,14-diol and nootkatone-13,14-diol monoacetate as metabolites (Asakawa et al., 1986). Nootkatone-13,14-diol is a neutral metabolite, which is most likely to be the result of epoxidation of the side-chain isopropenyl group followed by hydration, as shown in Figure 2, while nootkatone-13,14-diol monoacetate probably results from the subsequent acetylation of nootkatone-13,14-diol during isolation of the diol metabolite (Asakawa et al., 1986).

Figure 2. Metabolism of nootkatone in rabbits



Nootkatone — Nootkatone-13,14-diol

Although nootkatone (No. 1380) contains α,β-unsaturation, no glutathione conjugation in a Michael-type addition has been observed for this fused ring ketone or other moncyclic α,β-unsaturated ketones. The presence of ring carbons or alkyl substituents at the β-position inhibits glutathione conjugation (Portoghese et al., 1989). Structurally related α,β-unsaturated monocyclic ketones isophorone (No. 1112) and carvone (No. 380) have been evaluated previously by the Committee (Annex 1, references *138* and *161*, respectively) and metabolism studies indicate little evidence of extensive glutathione conjugation. Rather, side-chain oxidation and ketone reduction are the reported metabolic pathways leading to poly-oxygenated metabolites similar to that reported for nootkatone.

Samples of urine collected over 4 days from rabbits given isophorone (a structurally related substance; No. 1112, 3,5,5-trimethyl-2-cyclohexen-1-one) at a dose of 1000 mg/kg bw by gavage contained several metabolites: the major metabolite, 5,5-dimethyl-1-cyclohexene-3-one-1-carboxylic acid formed by oxidation of the methyl group at an exocyclic allylic position; 3,5,5-trimethyl-2-cyclohexen-1-ol (isophorol) formed by reduction of the ketone group and then conjugation with glucuronic acid; 3,5,5-trimethylcyclohexanone (dihydroisophorone) formed by hydrogenation of the endocyclic double bond; *cis*- and *trans*-3,5,5-trimethylcyclohexanol formed by hydrogenation of the endocyclic double bond and reduction of the ketone group (see Figure 3) (Truhaut et al., 1970; Dutertre-Catella, 1978).

Carvone (No. 380, 2-methyl-5-(1-methylethenyl)-2-cyclohexen-1-one), a structurally related α,β-unsaturated ketone, was partially excreted as the parent compound in both humans and rats (Tamura et al., 1962; Zlatkis et al., 1973). Allylic oxidation products, namely 9-hydroxycarvone, have also been detected in rats, (Williams, 1959; Ishida et al., 1989). In mice, carvone induces cytosolic glutathione transferase activity in mice (Zheng et al., 1992) suggesting that carvone may undergo some detoxication via glutathione conjugation at the β-position (Portoghese et al., 1989). In rabbits, carvone was mainly reduced to yield carveol, which was then converted to the glucuronic acid conjugate and excreted in the urine (Fisher & Bielig, 1940). Unchanged dihydrocarveol (Fisher & Bielig, 1940) and the glucuronic acid conjugate of dihydrocarveol (Hämäläinen, 1912) were additional metabolites of carvone detected in the urine of rabbits treated with carvone.

Structurally related fused ring ketones also undergo oxidation of ring positions that are remote from the ketone function (Asakawa et al., 1986). An example of this is cyclocolorenone, a tricyclic, α,β-unsaturated ketone, which is metabolized to yield two hydroxyketone metabolites (Asakawa et al., 1986). The C9 (methylene) and C10 (methane) ring positions are hydroxylated, as shown in Figure 4. Similar

Figure 3. Metabolic fate of isophorone in rabbits

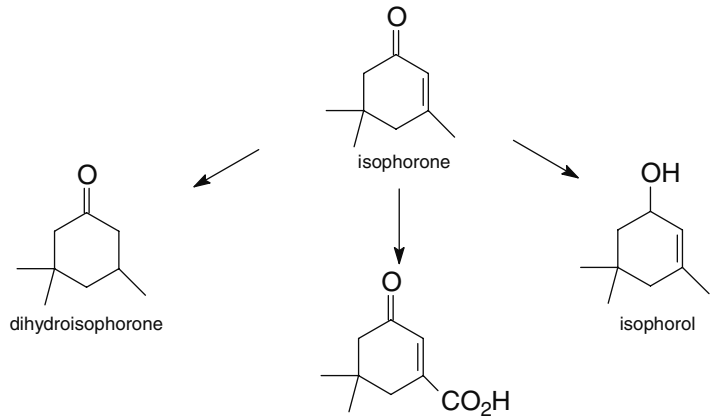
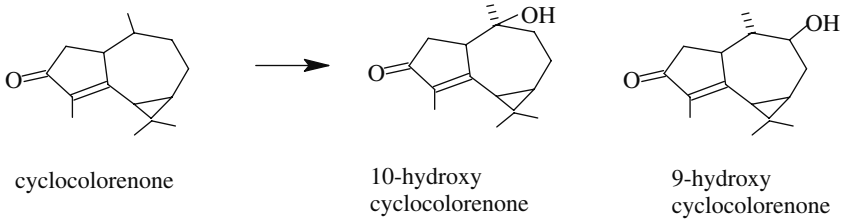


Figure 4. Metabolism of cyclolorenone in rabbits



cyclocolorenone

10-hydroxy cyclocolorenone

9-hydroxy cyclocolorenone

pathways of oxidation of the double-bond ring positions and ring alkyl substituents are expected in humans.

The bicyclic secondary alcohols are rapidly conjugated with glucuronic acid in humans, dogs, and rabbits and excreted via the urine. In humans (Figure 5), 81% and 94% of the orally administered dose of borneol (No. 1385) at 1000 and 2000 mg, respectively, were excreted as the glucuronic acid conjugate within 24 h (Wagreich et al., 1941). At 10 h after ingestion of 2000 mg of borneol, 81% of the administered dose was detected as the glucuronic acid conjugate in human urine (Quick, 1928). At a higher dose (i.e. 3500 mg of borneol), 69% of the administered dose was detected in human urine after 6 h (Quick, 1928). Similar conjugation has been reported in dogs (Quick, 1927; Pryde & Williams, 1934). An increased level of β-glucuronidase activity has been reported in several tissues of dogs given borneol by oral administration (Fishman, 1940). At oral doses of ≥100 mg/kg per day, rats fed borneol over 10 days showed an increase in the urinary concentrations of total glucuronic acid, *o*-glucuronide, and ascorbic acid (Tamura et al.,

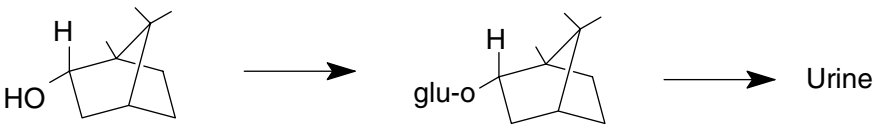
1962). Fenchyl alcohol (No. 1397) administered by gavage to rabbits was also excreted via urine as a glucuronide conjugate (Hämäläinen, 1912). Glucuronic conjugates of verbenol (No. 1404) and 2(10)-pinen-3-ol (No. 1403) were identified in the urine of rabbits given α-pinene by oral administration (Ishida et al., 1981). *Cis*- and *trans*-verbenol (No. 1404) have also been detected in human urine after occupational inhalation exposure to α- and β-pinene and δ-3-carene (Eriksson & Levin, 1990).

In rats, treatment with borneol (No. 1385) for 3 days (intraperitoneal or dietary exposure), causes increases (of approximately 25%) in the activities of biphenyl 4-hydroxylase, glucuronyl transferase, 4-nitrobenzoate reductase, and in hepatic CYP (Parke & Rahman, 1969). In another study, groups of four rats given *l*-borneol at a dose of 250 mg/kg bw per day by intraperitoneal injection for 3 days showed no significant increase in liver UDP-glucuronosyltransferase (UDPGT) activity. After daily treatment for up to 4 weeks, slight increases in this activity were observed. The authors concluded that over short periods of exposure, detoxication of borneol does not require the induction of UDPGT; however, longer exposure periods, at high doses, necessitate induction of UDPGT (Boutin et al., 1983). Conversely, in rats intubated with borneol at a dose of 3 mmol/kg bw (463 mg/kg bw, in olive oil), the activity of hepatic *S*-3-hydroxy-3-methylglutaryl coenzyme A reductase was decreased by approximately 50% at 17 h after dosing (Clegg et al., 1980).

CYP2B1 was induced in liver microsomes isolated from rats injected intraperitoneally with borneol at a dose of 300 mg/kg bw (Hiroi et al., 1995), indicating that oxidation may occur to a limited extent. Rats injected intraperitoneally with isobornyl acetate (No. 1388) at a dose of 1000 mg/kg bw for 3 days showed a minimum increase of twofold in the activities of *N*-demethylase and NADPH cytochrome *c* reductase, and in CYP content, indicating that isobornyl acetate induces the microsomal mixed-function oxidase system (Cinti et al., 1976), which also suggests that oxidation of ring positions and ring substituents may occur.

Other minor routes of metabolism of the bicyclic secondary alcohols include hydroxylation of an allylic position and oxidative cleavage of the strained ring in the bicyclic substance. Verbenol (No. 1404) contains both an allylic methyl group and a strained (cyclobutane) ring system. Therefore, verbenol may undergo oxidation of the allylic methyl group in a manner similar to that reported for *trans*-sobrerol, a structurally related terpenoid (Ventura et al., 1985), as well as cleavage

Figure 5. Metabolism of borneol in humans



Borneol

Glu = Glucuronic acid

of the cyclobutane ring to yield monocyclic polar metabolites, as has been reported for structurally related ring-strained bicyclic aldehydes (Ishida et al., 1989).

As with bicyclic alcohols, the metabolism of the menthol derivatives included in the group demonstrates that conjugation with glucuronic acid is a major pathway of excretion. Five female and five male Sprague-Dawley rats, pre-treated with 3-*l*-menthoxypropane-1,2-diol at an oral dose of 29.4 mg/kg per day for 7 days, were given [3-^{14}C]3-*l*-menthoxypropane-1,2-diol (No. 1408) as a single oral dose at 30 mg/kg bw on day 8. The major urinary metabolite in both sexes (56.5% in males and 73.3% in females) was the glucuronic acid conjugate of the parent diol (Ferdinandi, 1993a). Four male beagle dogs, pre-treated with 3-*l*-menthoxypropane-1,2-diol (No. 1408) at an oral dose of 49.9 mg/kg bw per day for 7 days, were given [3-^{14}C]3-*l*-menthoxypropane-1,2-diol as a single oral dose at 49.6 mg/kg bw on day 8. The parent diol and its glucuronic acid conjugate accounted for 78.9% of the radiolabelled substance recovered within the first 48 h (Ferdinandi, 1993b).

The above data demonstrate that the esters in this group are readily hydrolysed to the corresponding mono- or bicyclic secondary alcohols and are subsequently conjugated with glucuronic acid and excreted in the urine. Other minor metabolic routes include oxidation of ring positions and substituents to yield poly-oxygenated metabolites that are also readily excreted. The mono- and bicyclic ketones in the group undergo reduction of the corresponding secondary alcohol followed by conjugation with glucuronic acid and excretion in the urine. However, if the bicylic ketone is sterically hindered and exhibits increased lipophilicity, then oxidation of the ring positions and substituents competes favourably with the reduction of the ketone functional group. In the case of fused ring (nootkatone; No. 1398) and macrocyclic ketones, oxidation of side-chain alkyl group substituents and reduction of the ketone function yield polar excretable metabolites. These pathways are also operative for α,β-unsaturated ketones.

2.2.2 Toxicological studies

(a) Acute toxicity

Oral median lethal doses (LD_{50}) have been reported for 18 of the 32 substances in this group and are summarized in Table 3. In rats, LD_{50} values ranged from 1220 mg/kg bw for 7-methyl-4,4a,5,6-tetrahydro-2(3*H*)-naphthalenone (No. 1405) to >10 000 mg/kg bw for isobornyl acetate (No. 1388), demonstrating that the acute toxicity of these monocyclic and bicyclic secondary alcohols and ketones when administered orally is low (Fogleman & Margolin, 1970; Keating, 1972; Denine, 1973; Moreno, 1973, 1974; Levenstein, 1975; Moreno, 1975, 1976a, 1976b, 1977a, 1977b, 1977c; Gabriel, 1980; Mallory et al., 1982; Sedlacek, 1985; Watanabe & Kinosaki, 1989; Driscoll, 1993; Kajiura & Kinosaki, 1995; Oh et al., 1997; Gilman, 1998; Yajima & Tanaka, 2001).

For 3-methyl-2-(pentanyl)-2-cyclopenten-1-one (No. 1406), an oral LD_{50} of between 4000 and 8000 mg/kg bw was reported in mice (Engler & Bahler, 1983), and >2000 mg/kg bw in dogs (You et al., 1997), confirming the low acute toxicity of the substances in this group when administered orally.

Groups of eight rabbits were given a single dose of camphor (No. 1395) at 1000, 1300, 1400, 1600, 1800, 2000, 3000, or 4000 mg/kg bw in cottonseed oil by gavage. Additionally, groups of two rabbits were given camphor at a dose of 1600 or 1800 mg/kg bw in alcohol by gavage. All treated rabbits exhibited tonic (rigidity and hyperextension of the forelegs) and clonic (violent shaking motions of entire body) convulsions within 5–40 min of treatment. Time to convulsion was related to dose. Surviving rabbits were killed and examined microscopically, revealing no significant lesions of the kidneys, lungs, heart, liver, pancreas, spleen, brain, or spinal cord. Congestion and small focal haemorrhages were reported in the oesophageal and gastric mucosa of several rabbits (Smith & Margolis, 1954).

In an assay for competitive binding in vitro, which was developed to predict male rat-specific $\alpha_{2\mu}$globulin nephropathy, four male and four female rats were intubated daily with borneol (No. 1385) at a dose of 1 mmol/kg bw (154 mg) for 3 days, after which the rats were killed, and their kidneys removed, weighed and examined histologically for hyaline droplets (Lehman-McKeeman & Caudill, 1999). Treatment with borneol was reported to increase hyaline droplet formation significantly at an incidence that was approximately half of that reported for *d*-limonene (evaluated by the Committee in 1993; Annex 1, reference *107*), which was administered under the same conditions.

(b) Short-term studies of toxicity

Short-term studies of toxicity conducted to examine the potential toxicity of the monocyclic and bicyclic secondary alcohols, ketones and related esters were available for seven representative members of this group (Nos 1385, 1388, 1395, 1396, 1402, 1408, and 1411). The results of these studies are summarized in Table 4 and described below.

(i) Borneol (No. 1385)

Dogs

In a study on the metabolism of glucuronic acid, three dogs were given borneol at a dose of approximately 526 mg/kg bw per day in 1% agar by gavage for 31 days. No adverse effects were reported (Miller et al., 1933).

In another study, a group of three dogs was fed borneol at a dose of approximately 312 mg/kg bw per day, which was gradually increased to 1300 mg/kg bw per day within 2 months. Mucin (approximately 625 mg/kg bw per day) was added to the diet in order to offset any toxic effects of administration of borneol at high doses. During the third month, the dogs were fed borneol at a dose of 1300 mg/kg bw per day for 24 days, but mucin was not added to the diet. One dog developed distemper and was killed. After 17 days, a second dog died after a drop in the level of glucuronic acid excreted and its death was considered by the authors to be due to toxicity caused by borneol. The third dog was fasted for 7 days after 21 days of treatment with borneol at a high dose and died within 3 days after fasting. The dog showed signs of toxicity, including a drop in the level of glucuronic acid excreted. The authors considered that the results indicated that mucin, as a source of glucuronic acid, did provide protective properties against toxicity attributable to

Table 3. Studies of the acute toxicity of monocyclic and bicyclic secondary alcohols, ketones and related esters administered orally

No.	Flavouring agent (No.)	Species	Sex	LD_{50} (mg/kg bw)	Reference
1386	Isoborneol	Rat	NR	5200	Moreno (1977a)
1388	Isobornyl acetate	Rat	NR	>10000	Fogleman & Margolin (1970)
1390	Isobornyl formate	Rat	NR	>5000	Levenstein (1975)
1391	Isobornyl propionate	Rat	NR	>5000	Moreno (1973)
1393	Bornyl isovalerate (endo-)	Rat	NR	>5000	Denine (1973)
1395	*d*-Camphor	Rat	NR	>5000	Moreno (1976a)
1397	Fenchyl alcohol	Rat	NR	ND	Moreno (1976b)
1398	Nootkatone	Rat	NR	>5000	Moreno (1977b)
1398	Nootkatone	Rat	M, F	>2000	Gilman (1998)
1399	1,3,3-Trimethyl-2-norbornanyl acetate	Rat	NR	>5000	Moreno (1975)
1400	Methyl jasmonate	Rat	M, F	>5000	Gabriel (1980)
1401	Cycloheptadeca-9-en-1-one	Rat	NR	>5000	Moreno (1974)
1402	3-Methyl-1-cyclopentadecanone	Rat	NR	>5000	Moreno (1977c)
1402	3-Methyl-1-cyclopentadecanone	Dog	M, F	>2000	You et al. (1997)
1402	3-Methyl-1-cyclopentadecanone	Rat	M, F	>5000	Oh et al. (1997)
1405	7-Methyl-4,4a,5,6-tetrahydro-2(3*H*)-naphthalenone	Rat	M, F	1220	Mallory et al. (1982)
1406	3-Methyl-2-(*n*-pentanyl)-2-cyclopenten-1-one	Rat	NR	2500	Keating (1972)
1406	3-Methyl-2-(*n*-pentanyl)-2-cyclopenten-1-one	Mouse	M	>4000, but <8000	Engler & Bahler (1983)
1407	Dihydronootkatone	Rat	NR	>5 ml/kg	Sedlacek (1985)
1408	3-*l*-Menthoxypropane-1,2-diol	Rat	M, F	5800 (M); 5600 (F)	Watanabe & Kinosaki (1989)
1408	3-*l*-Menthoxypropane-1,2-diol	Rat	M, F	>2000	Yajima & Tanaka (2001)
1413	*d,l*-Menthol-(±)-propylene glycol carbonate	Rat	M, F	>2000	Driscoll (1993)
1416	*p*-Menthane-3,8-diol	Rat	M, F	>2000	Kajiura & Kinosaki (1995)

F, female; M, male; NR, not reported

high doses of borneol and even with the withdrawal of mucin from the diet, the body was capable of storing large quantities of glucuronic acid, which provided some extended ability to detoxify borneol (Miller et al., 1933).

Finally, a third group of five dogs was fasted and fed 5 g of borneol daily (approximately 500 mg/kg bw per day) for 37 days. One pregnant dog died after 2

Table 4. Results of short-term studies of toxicity with monocyclic and bicyclic secondary alcohols, ketones and related esters used as flavouring agents

No.	Flavouring agent	Species; sex[a]	No. test groups[b]/no. per group[a]	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
1385	Borneol	Dog; NR	1/3	Gavage	31	526[d]	Miller et al. (1933)
1385	Borneol	Dog; NR	1/5	Diet	37	<500	Miller et al. (1933)
1385	Borneol	Dog; NR	1/3	Diet	90	<1300[e]	Miller et al. (1933)
1388	Isobornyl acetate	Rat; M, F	3/30	Gavage	91	15 (M) 90 (F)[f]	Gaunt et al. (1971)
1395	*d*-Camphor	Rat; NR	4/5	Gavage	56	75[g]	Skramlik (1959)
1398	Nootkatone	Rat; M, F	1/10	Gavage	28	10	Jones et al. (2004)
1402	3-Methyl-1-cyclopentadecanone	Rat; M, F	3/20	Gavage	30	1000[d]	Oh et al. (1997)
1402	3-Methyl-1-cyclopentadecanone	Dog; M, F	3/6	Gavage	28	20[d]	You et al. (1997)
1408	3-*l*-Menthoxypropane-1,2-diol	Rat; M, F	1/10	Diet	14	738 (M)[d] 809 (F)[d]	Weaver & Van Miller (1989)
1408	3-*l*-Menthoxypropane-1,2-diol	Rat; M, F	5/10	Diet	28	250 (M) 1000 (F)	Wolfe (1992a)
1408	3-*l*-Menthoxypropane-1,2-diol	Rat; M, F	3/40	Diet	91	30 (M) 200 (F)	Wolfe (1992b)
1408	3-*l*-Menthoxypropane-1,2-diol	Dog; M, F	5/4	Oral/capsule	28	1000 (M) 500 (F)	Dalgard (1993)
1408	3-*l*-Menthoxypropane-1,2-diol	Dog; M, F	3/8	Oral/capsule	91	50	Dalgard (1994)
1411	3-(*l*-menthoxy)-2-methylpropane-1,2-diol	Rat; M, F	1/10	Diet	28	<1000	Madarasz & Bolte (1997)

[a] F, female; M, male; NR, not reported

[b] Total number of test groups does not include control animals.

[c] Total number per test group includes both male and female animals.

[d] Study was performed with either a single dose or multiple doses that produced no adverse effect. The value is therefore not a true NOEL, but is the highest dose tested that produced no adverse effects. The actual NOEL may be higher.

[e] Animals were gradually introduced to the final dose of 1300 mg/kg bw per day over a 2-month period.

[f] Author specified a single NOEL of 15 mg/kg bw per day, without distinguishing between male and female rats.

[g] Study performed using sage oil at doses of 0, 250, 500, 1000 or 1200 mg/kg bw per day. With an estimated camphor content of approximately 30%, these doses provide approximately 0, 75, 150, 300 or 360 mg/kg bw per day of camphor.

weeks of fasting. A second dog showed signs of toxicity after 2 weeks and was then given a diet supplemented with mucin. The third dog survived the 37-day treatment period without supplementation with mucin. The two other dogs were fasted, but also fed 10 g of mucin daily. At necropsy, the dogs showed signs of gastritis (more marked in the pyloric area) and marked duodenitis (Miller et al., 1933).

(ii) Isobornyl acetate (No. 1388)

Rats

Groups of 15 male and 15 female CFE rats were orally intubated with isobornyl acetate at a dose of 0 (control), 15, 90 or 270 mg/kg bw per day in corn oil for 13 weeks. Additional groups of five male and five female rats were orally intubated with isobornyl acetate at a dose of 0, 90 or 270 mg/kg bw per day for 2 or 6 weeks. Animals were observed throughout the study for signs of toxicity and overall condition. Weekly measurements of body weight, and food and water intake were obtained. Haematological evaluation was performed at the end of the study period, while complete urine analysis was conducted at week 6 and at study termination. At week 2, only urinary volume and specific gravity were determined. At study termination, blood samples were taken for clinical chemistry evaluation and animals were killed and necropsied. Animals were examined macroscopically, and selected organs were weighed and tissues preserved for histopathological evaluation. No deaths or abnormal appearance or behaviour were reported. Body weight measurements revealed no differences in body-weight gain between controls and treated rats, but a slight decrease in the weight of males at the highest dose was significant after the 24-h fast before death at both 6 and 13 weeks. Compared with controls, water consumption in males at the highest dose was increased throughout the study ($p < 0.01$). No differences in food intake were reported. Haematological examination at week 2 showed statistically significant increases in haemoglobin concentration in females at the highest dose ($p < 0.01$) and in total leukocyte counts in males at the highest dose ($p < 0.05$). Additionally, significantly elevated erythrocyte counts were observed in and males at the intermediate and highest doses. These changes were not reported at 6 or 13 weeks. Reticulocyte counts in all (control and treated) younger rats were higher than in their older counterparts and erythrocytes showed a marked polychromasia. Serum chemistry results were similar in treated and control animals, showing no significant dose–response effects. Urine analysis results showed that urine was of normal colour and was free from glucose, blood, bile, and ketones. In terms of concentration and results of tests for urinary cell excretion, treated females at weeks 2, 6, and 13 and treated males at week 2 were not different from controls throughout the study; however, males treated for 6 or 13 weeks had significantly increased cell excretion at the highest dose and males treated for 13 weeks also showed this effect at 90 mg/kg bw per day. Males at the highest dose also exhibited impairment of urine concentrating ability; seen at week 6 only after 16–20 h of water deprivation and at week 13 after dehydration for 6 h. Absolute weights of the liver ($p < 0.001$), kidney ($p < 0.05$) and caecum ($p < 0.001$) were significantly increased in females at the highest dose after 13 weeks. Significantly increased absolute weight of the caecum ($p < 0.05$) also was reported in groups of males treated at the highest dose. At

week 2, relative brain weight was significantly increased in females at the highest dose ($p < 0.01$). At week 6, relative weight of the kidney ($p < 0.01$) was significantly increased in males at the highest dose and relative weight of the gonads ($p < 0.05$) was significantly increased in females at the highest dose. At week 13, relative weights of the liver ($p < 0.05$), kidney ($p < 0.01$) and caecum ($p < 0.001$) in rats at the highest dose (both sexes) were significantly increased. Histological examination revealed a mild pulmonary infection in all rats (control and treated). The only histological findings attributed by the authors to exposure to isobornyl acetate were reported in the kidney of animals at the highest dose animals and consisted of an increased incidence of focal tubular degeneration and atrophy (both sexes) and a vacuolation of the tubular epithelium (males only). In addition, vacuolation of the epithelial cells of the intrahepatic bile ducts of males at the highest dose was reported. On the basis of renal and hepatic effects observed in males at the intermediate and highest doses and the absence of any effect in females at the lowest and intermediate doses, the no-observed-effect level (NOEL) was 15 and 90 mg/kg bw per day for males and females, respectively (Gaunt et al., 1971).

(iii) d-Camphor (No. 1395)

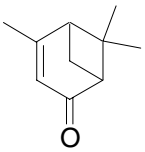
Rats

Sage oil, which is estimated to contain approximately 30% camphor (Millet et al., 1981), was orally administered to groups of five rats (strain and sex not reported) at a dose of 0, 250, 500, 1000 or 1200 mg/kg bw per day (approximately equivalent to camphor at a dose of 0, 75, 150, 300 and 360 mg/kg bw per day, respectively) for 8 weeks. Administration of 250 mg/kg bw per day of sage oil was reported to be well tolerated by the rats, as they showed no clinical signs of toxicity and their body weights were comparable to those of the controls. Rats in the group receiving a dose of 500 mg/kg bw per day exhibited occasional clonic seizures immediately after dosing, as well as slightly lower body-weight gains compared with those of controls. One rat given a dose of 500 mg/kg bw per day died on day 22 of treatment during one of these cramp-like attacks. Deaths were also reported in the majority of the animals at 1000 mg/kg bw per day, and in all the rats at the highest dose (1200 mg/kg bw per day) (Skramlik, 1959).

(iv) Nootkatone (No. 1398)

Rats

In a 28-day study designed to investigate the systemic toxicity of two α,β-unsaturated ketones, nootkatone and verbenone[1], groups of 10 male and 10 female Sprague-Dawley Crl:CD (SD) IGS BR rats were given nootkatone or ver-

[1] 

verbenone

benone (in Arachis oil BP) at a dose of 10 mg/kg bw per day by gavage for 28 days. Ten male and 10 female rats served as vehicle controls. All animals were examined for clinical signs of toxicity immediately before dosing and 1–5 h after dosing during the working week and 1 h after dosing on weekends. Body weights were recorded on day 0 and weekly thereafter. Food consumption was measured at weekly intervals and water intake was measured daily. Haematology and blood chemical investigations were performed at day 28 for all animals. At termination all animals were subjected to gross examination. Organ weights were obtained and histopathological examination was performed for all major tissue types and on any lesions. No signs of clinical toxicity were observed throughout the study. Water consumption, food consumption, food efficiency and weight gain were similar in test and control groups. Blood biochemistry revealed no differences between test groups and controls. Females in both study groups showed increased concentrations of total protein. The authors viewed this intergroup difference, which did not occur in males, to be of no toxicological importance. No gross abnormalities were attributed to the administration of nootkatone or verbenone. There were no treatment-related changes to organ weights.

Histopathology findings revealed glomular accumulations of eosinophillic material in the kidney tubular epithelium of male rats treated with nootkatone and verbenone. The authors noted that the observations were consistent with the presence of $\alpha_{2\mu}$globulin nephropathy.

Given that $\alpha_{2\mu}$globulin is a well characterized phenomenon that is specific to male rats (Capen et al., 1999), the authors concluded that no adverse effects occurred at a dose of 10 mg/kg bw per day for nootkatone or verbenone when orally administered daily for 28 days (Jones et al., 2004).

(v) 3-Methyl-1-cyclopentadecanone (No. 1402)

Rats

Groups of 10 male and 10 female Sprague-Dawley rats were given 3-methyl-1-cyclopentadecanone at a dose of 0 (vehicle control), 10, 100 or 1000 mg/kg bw per day in Tween 80 by gavage for 30 days. Rats were observed daily for clinical signs of toxicity and were weighed twice per week. Intakes of feed and water were measured two to three times per week. At study termination, samples of blood and urine were taken for serum chemistry, haematology, and urine analysis determinations. Subsequently, all the rats were killed, necropsied, and examined histopathologically. No significant differences from controls were reported in any of the parameters examined. Relative to controls, the only statistically significant finding was an increase in the absolute and relative weight of the liver in males and females at the highest dose. According to the authors, this effect may be due to enzyme induction, since hepatic CYP and associated activities were elevated in rats at the highest dose. However, owing to the absence of histopathological or haematological findings to support compound-related toxicity, these effects were not considered to be toxicologically significant. The NOEL for 3-methyl-1-cyclopentadecanone assigned by the authors was therefore >1000 mg/kg bw per day (Oh et al., 1997).

Dogs

Nine male and nine female beagle dogs were given 3-methyl-1-cyclopentadecanone at a dose of 0, 0.2, 2.0 or 20 mg/kg bw per day in Tween 80 by oral administration (specific route not stated) for 4 weeks. Dogs were observed daily for clinical signs of toxicity, and body weights were recorded twice per week. Intakes of feed and water were measured daily. Ophthalmoscopic examinations were conducted at the start of the study and then weekly thereafter. Blood samples were taken before the start of the study and at study termination for determination of haematological parameters and serum chemistry. Urine samples were collected on the first and last days of treatment. At study termination, dogs were killed, necropsied, and examined histopathologically. Some temporary and sporadic decreases in feed intake and an increased incidence of diarrhoea were noted in treated rats; however, no compound-related effects were reported in any of the parameters tested, including organ weight changes (You et al., 1997).

(vi) 3-l-Menthoxypropane-1,2-diol (No. 1408)

Rats

In a 14-day screen for toxicity, groups of five male and five female F344 rats were fed diets containing 3-*l*-menthoxypropane-1,2-diol, to provide an intended dose of 1000 mg/kg bw per day. On the basis of feed consumption, the mean dose of 3-*l*-menthoxypropane-1,2-diol was calculated to be approximately 738.1 and 809.2 mg/kg bw per day for males and females, respectively. Rats were observed daily for clinical signs of toxicity, and body weights were recorded 1 day before study initiation, as well as on days 6 and 14 of the study. Feed intake was determined on days 7 and 14. At study termination, rats were killed, necropsied and examined for gross pathology and renal and hepatic histopathology. With the exception of a statistically significant increase in absolute and relative weights of the liver in rats treated with 3-*l*-menthoxypropane-1,2-diol relative to controls, no compound-related effects were reported in any of the parameters examined. In addition, no compound-related macroscopic or microscopic lesions were noted in the liver or any other organs of treated rats (Weaver & Van Miller, 1989).

Groups of five male and five female Sprague-Dawley rats were given diets containing 3-*l*-menthoxypropane-1,2-diol at a dose of 0, 250, 500, 1000, 2000 or 5000 mg/kg bw per day for 28 days. Parameters evaluated included daily clinical observation, weekly measurement of body weights and food consumption, and ophthalmoscopic, electrocardiographic and clinical pathology (i.e. haematology, serum chemistry, and urine analysis) examinations at week 4. All animals were sacrificed at the end of the study, and subjected to macroscopic and microscopic histopathological examination, and absolute and relative organ weight measurements. A dose-related decrease in mean body weights was noted in treated rats; however, relative to controls, this difference reached statistical significance only at the highest dose (5000 mg/kg bw per day). Compared with controls, mean weekly and total food consumption was significantly decreased in males at the highest dose (5000 mg/kg bw per day). No compound-related changes were reported in the clinical, haematological, and ophthalmological examinations of the animals.

While no compound-related alterations in serum chemistry and urine analysis were reported in female rats, male rats exhibited significant dose-related decreases in serum glucose and blood urea nitrogen concentrations compared with those of controls. At the highest dose, rats exhibited significantly decreased mean absolute weights of the heart compared with controls. In addition, significant dose-related increases in absolute and relative weights of the liver were reported in animals of both sexes. It was suggested that the reductions in serum concentrations of glucose observed in male rats were associated with the dose-related increases in the incidence and severity of diffuse hepatocellular enlargement and eosinophilic inclusions reported at all doses except 250 mg/kg bw per day. Diffuse hepatocellular enlargement was also reported in females at 2000 and 5000 mg/kg bw per day. No other histopathological effects were noted that could be attributed to the compound administered (Wolfe, 1992a). The livers of males and females at the highest dose were later examined by electron microscopy (Cockrell, 1992a). Myelinoid bodies, and moderate proliferation of the smooth endoplasmic reticulum were observed in the liver of males at the highest dose, while moderate proliferation and minor whorling of the smooth endoplasmic reticulum was noted in the liver of females at the highest dose. Formation of myelinoid bodies was presumed to be the result of metabolic adaptation in which continuous enzyme induction stimulated proliferation of the smooth endoplasmic reticulum, similar observations have been reported in the liver of dogs treated with butylated hydroxyanisole (Ikeda et al., 1986). The laboratory reported that these effects appeared to be reversible upon cessation of treatment (Ghadially, 1982), and should not be considered to be a degenerative change in the hepatocyte (Cockrell, 1992a).

In a subsequent 91-day study, groups of 20 male and 20 female CD rats were maintained on diets containing 3-*l*-menthoxypropane-1,2-diol at a dose of of 0 (control), 30, 200 or 1000 mg/kg bw. Daily clinical observation, weekly measurement of body weights and food consumption, measurement of water consumption every 2 weeks, and ophthalmoscopic and electrocardiographic examinations at week 12 were performed. All animals were reported to survive the entire duration of the study. Male and female rats showed hunched posture, and females at 1000 mg/kg bw per day showed significant decreases in body weight and body-weight gain. In the females at the highest dose, the body-weight variations were accompanied by slight decreases in food consumption. Sporadic, statistically significant differences in food consumption were observed at various time-points in most other groups. Haematological examinations, blood chemical determinations, and urine analyses were performed at weeks 4 and 13, and with the exception of a significant increase in γ-glutamyl transferase activity reported in males at the highest intake, no other variations were observed. In males and females at the intermediate and highest doses, significant increases were reported in absolute weights of the liver. Relative weights of the liver and ratios of liver to brain weight were increased at all three doses in females, and at 200 and 1000 mg/kg bw per day in males. The increases observed in weight of the liver were dose-dependent in both sexes. Slight to severe diffuse hepatocellular enlargement was reported in males at 1000 mg/kg bw per day and midzonal and centrilobular enlargement was reported in females at 1000 mg/kg bw per day. Eosinophilic cytoplasmic inclusions were noted in the livers of two and ten males at the intermediate and highest doses,

respectively. Absolute and relative weights of the kidney were significantly increased in males at 1000 mg/kg bw per day and in females at 200 mg/kg bw per day. Relative weights of the kidney were also significantly elevated in females at the highest dose. Increased ratios of kidney to brain weight were significant in males at the highest dose and in females at 30 and 200 mg/kg bw per day. In males, the increase in absolute weights of the kidney was noted to be dose-related, as was the increase in relative weights of the kidney in females. Granular casts were noted in the kidneys of two males at the highest dose; however, this was not associated with an increased presence of hyaline droplets in stained tissues. Overall, histopathology did not reveal any compound-related variations in animals of either sex at the lowest dose or in females at the intermediate dose. On the basis of histopathological examination, the NOEL was 30 mg/kg bw per day in males and 200 mg/kg bw per day in females (Wolfe, 1992b).

Dogs

Groups of two male and two female beagle dogs were given gelatin capsules containing 3-*l*-menthoxypropane-1,2-diol at a dose of 0, 250, 500, 1000, 2000 or 5000 mg/kg bw per day by oral administration for 28 days. Parameters evaluated included daily clinical observation, weekly measurement of body weights and food consumption, and electrocardiographic examinations at weeks 2 and 4. Clinical pathology evaluations (haematology, serum chemistry, and urine analysis) were performed on all animals before initiation of the study, on all dogas at the highest dose (5000 mg/kg bw per day) during week 2, on one female dog at 2000 mg/kg bw per day during week 3, and on all surviving dogs at the end of the study. Statistical evaluation of the clinical pathology data was not performed owing to the limited sample size. All surviving animals were sacrificed at the end of the study, and subjected to macroscopic and microscopic histopathological examination, and measurement of absolute and relative organ weights. Clinical signs of toxicity, including soft or mucoid faeces, emesis, and salivation, were noted primarily in dogs given 3-*l*-menthoxypropane-1,2-diol at a dose of 1000, 2000 and 5000 mg/kg bw per day. Treated dogs also exhibited lacrimation, tremors, nasal discharge, ataxia, and decreased activity. Food consumption and mean body weights of all surviving treated dogs were reported to be similar to those of controls throughout the study, with the exception of one female at 500 mg/kg bw per day that exhibited progressive body-weight reduction with accompanying decrease in food consumption. No compound-related effects on electrocardiographic tracings, haematology, and urine analysis were observed. Dogs at 200, 500, 1000, 2000 and 5000 mg/kg bw per day exhibited increased mean concentrations of cholesterol, and slight, dose-related reductions in serum concentrations of glucose. Increased absolute and relative weights of the liver were reported in both sexes at 250, 500, 1000 and 2000 mg/kg bw per day. Male dogs at 1000 and 2000 mg/kg bw per day showed decreased absolute and relative weights of the thymus. Relative to controls, decreased absolute and relative weights of the prostate and testis of males at 250, 500, 1000 and 2000 mg/kg bw per day, and of the uterus in females at 1000 and 2000 mg/kg bw per day were observed. All dogs at the highest dose, two dogs at 2000 mg/kg bw per day, and one female at 1000 mg/kg bw per day were sacrificed in extremis before the end of the study. These dogs were reported to show clinical

signs of lethargy, depressions in food consumption with progressive body-weight losses, elevated concentrations of total cholesterol and triglyceride consistent with the emesis noted clinically, and increased alkaline phosphatase activity and concentrations of total protein, albumin, sodium, potassium, and chloride, indicative of dehydration. The cause of the moribund condition of the three females (at 1000, 2000 and 5000 mg/kg bw per day) sacrificed in extremis was not determined; however, the death of three males (at 2000 and 5000 mg/kg bw per day) was attributed to pulmonary inflammation caused by aspiration of vomit. While the death of one female at the highest dose was attributed to severe liver necrosis, no such liver alterations were observed in any of the other treated animals. Clinical pathological and histopathological examination of all of the treated animals was reported to reveal no organ effects or morphological tissue alterations that could be related to the administration of 3-*l*-menthoxypropane-1,2-diol. The NOEL for 3-*l*-menthoxypropane-1,2-diol administered orally was 1000 mg/kg bw per day in males and 500 mg/kg bw per day in females (Dalgard, 1993).

Groups of four male and four female beagle dogs were given capsules containing 3-*l*-menthoxypropane-1,2-diol at a dose of 0, 10, 50 or 250 mg/kg bw per day orally for 91 days. Daily clinical observation, weekly measurement of body weights and food consumption, measurement of water consumption every 2 weeks, and ophthalmoscopic and electrocardiographic examinations at week 12 were performed and revealed no significant difference between test and control animals. Haematological examinations, blood chemical determinations and urine analyses were performed at weeks 4 and 13, and showed a slight (less than twofold greater than that of controls) but significant ($p \leq 0.05$) increase in alkaline phosphatase activity for the group receiving the highest dose (250 mg/kg bw per day). A non-specific increase in absolute and relative weights of the liver was reported for the group at the highest dose, accompanied by a diffuse hepatocellular enlargement in five out of eight dogs in this group. A dose-related increase in weights of the thyroid and/or parathyroid and decrease in weights of the prostate were noted; however, these observations did not reach statistical significance, and were not accompanied by histopathological evidence. Therefore, these alterations were not considered to be biologically significant. The NOEL was 50 mg/kg bw per day (Dalgard, 1994). Electron microscopy performed on livers of males and females a the highest dose revealed proliferation of smooth endoplasmic reticulum associated with the presence of myelinoid bodies (i.e. lipid cytosomes) (Cockrell, 1992b). The same laboratory observed myelinoid bodies and proliferation of smooth endoplasmic reticulum in the liver of dogs treated with butylated hydroxyanisole (Ikeda et al., 1986). The laboratory reported that these changes appear to be reversible (Ghadially, 1982), and should not be considered a degenerative change in the hepatocyte (Cockrell, 1992b).

(vii) 3-l-Menthoxy-2-methylpropan-1,2-diol (No. 1411)

Rats

In a 28-day single dose screening study, groups of five male and five female Sprague-Dawley CD rats were given diets containing 3-*l*-menthoxy-2-methylpropan-1,2-diol at a dose of 1000 mg/kg bw per day. Rats were individually

housed and given access to food and water ad libitum Rats were observed twice daily for clinical signs of toxicity, body weights and food consumption were recorded weekly, and feed efficiency was calculated. Necropsy was performed on day 29, and brain, kidney and liver were weighed. Tissues from these organs as well as those exhibiting any macroscopic abnormalities were preserved for histopathological examination. All animals were reported to survive until the end of the study and physical examination did not reveal any compound-related adverse effects. Although mean body weights were noted to be generally lower in treated animals than in controls (i.e. at study termination, mean body weights of treated females were 8% lower than those of controls), no statistically significant differences were observed. However, at weeks 3 and 4, females exhibited a significantly reduced body-weight gain compared with that of control animals. Decreased food consumption was reported in treated males and females during the first week of the study and was partially attributed to the initial poor palatability of the diet. Mean values for food consumption were comparable to those for the controls during the remainder of the study. Similarly, feed utilization in treated animals was reduced during the first week, but was comparable to that of controls thereafter. The authors reported a statistically significant increase in the mean weight of the liver ($p < 0.01$) of treated males, and in ratios of liver to terminal body weight and brain weight ($p < 0.01$) compared with those for the controls. The statistically significant decrease in mean weight of the brain, as well as the increases in ratios of kidney to terminal body weight and brain weight were reported to be reflective of normal variability and were, therefore, considered to be unrelated to compound administration. Histopathology revealed hepatocellular hypertrophy in both sexes. In females, it was central lobular and minimal to slight in severity, while in males it was diffuse (pan-lobular) and moderate to moderately severe. Hepatocellular hypertrophy is a common response associated with the increased metabolic requirements (Crampton et al., 1977; O'Neill et al., 2003). On the basis of microscopic variations observed in both sexes, the NOEL was <1000 mg/kg bw per day (Madarasz & Bolte, 1997).

(c) Long-term studies of toxicity and carcinogenicity

No information on long-term studies of toxicity and carcinogenicity was available.

(d) Genotoxicity

Tests for genotoxicity in vitro and in vivo using standardized protocols have been used to study seven representative members (Nos 1385, 1391, 1395, 1408, 1411, 1413 and 1416) of the monocyclic and bicyclic secondary alcohols, ketones and related esters group used as flavouring agents (see Table 5).

(i) In vitro

Seven members of this group (borneol, No. 1385; isobornyl propionate, No. 1391; *d*-camphor, No. 1395; 3-*l*-menthoxypropane-1,2-diol, No. 1408; 3-*l*-menthoxy-2-methylpropan-1,2-diol, No. 1411; *d,l*-menthol 1- and 2-propylene

glycol carbonate; No. 1413 and *p*-menthan-3,8-diol; No. 1416) consistently gave negative results in the Ames assay when incubated at a concentration of up to 5000 μg/plate with a variety of *Salmonella typhimurium* strains including TA97, TA98, TA100, TA102, TA1535, TA1537 and TA1538 with or without metabolic activation (Simmon et al., 1977; Anderson & Styles, 1978; Wild et al., 1983; Watanabe & Morimoto, 1989; National Toxicology Program, 1992a; King, 1993; Azizan & Blevins, 1995; Kajiura, 1995, 1996; Marzin, 1998; Shirai & Sasaki, 2000).

Borneol (No. 1385), 3-*l*-menthoxypropane-1,2-diol (No. 1408), 3-*l*-menthoxy-2-methylpropan-1,2-diol (No. 1411) and *p*-menthan-3,8-diol (No. 1416) showed no mutagenic activity when tested in *Escherichia coli* WP2 *uvrA* at concentrations of up to 5000 μg/plate (Yoo, 1986; Watanabe & Morimoto, 1989; Kajiura, 1995; Kajiura, 1996; Shirai & Sasaki, 2000), although cytotoxicity was reported at concentrations exceeding 1250 μg/plate (Shirai & Sasaki, 2000).

In the Rec$^-$ assay, borneol (No. 1385) was reported to induce growth inhibition in *Bacillus subtilis* strain M45$^-$ when tested at concentrations of up to 10 mg/disc (Yoo, 1986).

When tested by the National Toxicology Program, *d*-camphor (No. 1395) at concentrations reaching 1500 μg/ml did not induce sister chromatid exchanges in Chinese hamster ovary cells in the presence or absence of metabolic activation derived from the livers of rats induced with Aroclor 1254 (National Toxicology Program, 1992a). Cytotoxicity was reported at concentrations exceeding 750 μg/ml without metabolic activation and at concentrations exceeding 550 μg/ml with metabolic activation.

Structurally related α,β-unsaturated alicyclic ketones isophorone (No. 1112) and carvone (No. 380) showed no mutagenic potential in the standard Ames assay when incubated at concentrations of 3.3–333 μg/plate with various strains of *S. typhimurium* (TA98, TA100, TA1535, TA1537) with or without metabolic activation from S9 (Mortelmans et al., 1986). When incubated with *Bacillus subtilis* H17 (rec$^+$) or M45 (rec$^-$) in the rec$^-$ assay at a dose of 0.6 ml/disc, carvone (unspecified stereochemistry) was negative both in the presence and absence of metabolic activation from S9 (Matsui et al., 1989).

The results of the assay for forward mutation in mouse lymphoma cells in vitro were negative with isophorone at concentrations of 130–1300 μg/ml without metabolic activation from S9 and with isophorone at concentrations of 67–890 μg/ml with metabolic activation (McKee et al., 1987; O'Donoghue et al., 1988). An increase in the frequency of mutation was reported with isophorone at concentrations of 400 and 800 μg/ml in mouse lymphoma L5178Y *Tk*$^{+/-}$ cells without metabolic activation (McGregor et al., 1988).

Isophorone, tested in Chinese hamster ovary cells, produced equivocal results. In one study, no chromosomal aberrations were induced at concentrations up to 1600 μg/ml with or without metabolic activation (Gulati et al., 1989). In another study, isophorone, at a concentration of 1250 μg/ml without metabolic activation or at a concentration of 1500 μg/ml with metabolic activation, induced chromosomal aberrations (Matsuoka et al., 1996); however, lower concentrations of 250–1000 μg/

Table 5. Results of studies of genotoxicity with monocyclic and bicyclic secondary alcohols, ketones and related esters used as flavouring agents

No.	Flavouring agent	End-point	Test system	Dose or concentration	Result	Reference
In vitro						
1385	Borneol	Reverse mutation	*S. typhimurium* TA98, TA100, TA97	1 mg/ml (1000 μg/ml)	Negative[a]	Azizan & Blevins (1995)
1385	Borneol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	≤5 mg/plate (5000 μg/plate)	Negative[a]	Simmon et al. (1977)
1385	Borneol	DNA repair	*B. subtilis* M45^{-} and H17^{+}	≤10 mg/disc	Positive	Yoo (1986)
1385	Borneol	Mutation test	*E. coli* WP2 *uvrA* (trp-)	0.4–3.2 mg/plate	Negative	Yoo (1986)
1391	Isobornyl propionate	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	≤3.6 mg/plate (3600 μg/plate)	Negative[a]	Wild et al. (1983)
1395	*d*-Camphor	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1538	4, 20, 100, 500 and 2500 μg/plate	Negative[b]	Anderson & Styles (1978)
1395	*d*-Camphor	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA102	≤50 μg/plate	Negative[c]	Marzin (1998)
1395	*d*-Camphor	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA102	≤150 μg/plate	Negative[a]	Marzin (1998)
1395	*d*-Camphor	Reverse mutation	*S. typhimurium* TA97, TA100, TA1535, TA98	10, 33, 100, 333 and 667 μg/plate	Negative[a]	National Toxicology Program (1992a)
1395	*d*-Camphor	Sister chromatid exchange	Chinese hamster ovary cells	250, 500, 750, 1000 and 1500 μg/ml	Negative[a,c]	National Toxicology Program (1992a)
1395	*d*-Camphor	Sister chromatid exchange	Chinese hamster ovary cells	500, 525, 550, 575 and 600 μg/ml	Negative[b]	National Toxicology Program (1992a)
1408	3-*l*-Menthoxypropane-1,2-diol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	10, 50, 100, 500, 1000 and 5000 μg/plate	Negative[a]	Watanabe & Morimoto (1989)
1408	3-*l*-Menthoxypropane-1,2-diol	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	39.1, 78.1, 156, 313, 625 and 1250 μg/plate	Negative[a]	Shirai & Sasaki (2000)

1408	3-*l*-Menthoxypropane-1,2-diol	Mutation test	*E. coli* WP2 *uvrA* (trp-)	10, 50, 100, 500, 1000 and 5000 μg/plate	Negative[a]	Watanabe & Morimoto (1989)
1408	3-*l*-Menthoxypropane-1,2-diol	Reverse mutation	*E. coli* WP2 *uvrA* (trp-)	39.1, 78.1, 156, 313, 625 and 1250 μg/plate	Negative[a]	Shirai & Sasaki (2000)
1411	3-*l*-Menthoxy-2-methylpropan-1,2-diol	Reverse mutation	*S. typhimurium* TA100, TA1535, TA98, TA1537	50, 100, 500, 1000 and 5000 μg/plate	Negative[a,d]	Kajiura (1996)
1411	3-*l*-Menthoxy-2-methylpropan-1,2-diol	Reverse mutation	*E. coli* WP2 *uvr*A	50, 100, 500, 1000 and 5000 μg/plate	Negative[a]	Kajiura (1996)
1413	*d,l*-Menthol 1-and 2-propylene glycol carbonate	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98, TA100	5, 15, 50, 150, 500, 1500 and 5000 μg/plate	Negative[a,d]	King (1993)
1416	*p*-Menthan-3,8-diol	Reverse mutation	*S. typhimurium* TA100, TA1535, TA 98, TA1537	156, 313, 625, 1250, 2500 and 5000 μg/plate	Negative[a,d]	Kajiura (1995)
1416	*p*-Menthan-3,8-diol	Reverse mutation	*E. coli* WP2 *uvr*A	156, 313, 625, 1250, 2500 and 5000 μg/plate	Negative[a,d]	Kajiura (1995)
In vivo						
1391	Isobornyl propionate	Somatic mutation and recombination	*D. melanogaster*	10 mmol/l (2103 μg/ml)	Negative[f]	Wild et al. (1983)
1391	Isobornyl propionate	Micronucleus formation	Mouse bone marrow cells	841, 1893 and 2944 mg/kg bw	Negative[g]	Wild et al. (1983)
1395	*d,l*-Camphor	Micronucleus induction	Mouse peripheral blood erythrocytes	200, 400, 600, 800 and 1000 mg/kg bw	Negative[h]	National Toxicology Program (1999)

Data on genotoxicity for isophorone (No. 1112; Annex 1, reference *160*) and carvone (No. 380; Annex 1, references *137*) were used to assist in the evaluation of flavouring agents Nos 1398, 1405 and 1406, for which no dats on genotoxicity were available

[a] Tested with and without metabolic activation

[b] Tested with metabolic activation

[c] Tested without metabolic activation

[d] Pre-incubation method

[e] Without S9 bactericidal activity at 500 μg/plate for strains TA1537, TA1538 and TA98, at 1500 μg/plate for strain TA1535 and with 5000 μg/plate for strain TA100

[f] Dose calculated based on the relative molecular mass of substance = 210.32

[g] Administered via intraperitoneal injection

[h] Administered topically

ml without metabolic activation did not induce chromosomal aberrations. Isophorone only induced sister chromatid exchanges when tested at concentrations of 500–1000 μg/ml in Chinese hamster ovary cells without metabolic activation, and then only after a delayed harvest, due to the cytostatic effect of isophorone (Gulati et al., 1989). At lower concentrations without metabolic activation, or at concentrations of up to 1600 μg/ml with metabolic activation, isophorone did not induce sister chromatid exchanges (Gulati et al., 1989). In an assay for unscheduled DNA synthesis in rat hepatocytes, there was no evidence for genotoxicity with isophorone at concentrations of up to 0.2 μl/ml (McKee et al., 1987; O'Donoghue et al., 1988).

d-Carvone at concentrations of 0.167–502 μg/ml induced slight increases in sister chromatid exchange and chromosomal aberration in Chinese hamster ovary cells either with or without metabolic activation from S9 (liver from male Sprague-Dawley rats induced with Aroclor 1254). The results of this assay were statistically positive in two out of three assays for sister chromatid exchanges, but there was no correlation of dose with response. Also, the number of sister chromatid exchanges per cell was less than 1.5-times that of solvent controls. A dose–response relationship was not confirmed in a second trial of the assay for chromosome aberration, conducted without metabolic activation; chemical-induced cell cycle delay was reported in this trial (National Toxicology Program, 1990).

(ii) In vivo

The potential of isobornyl propionate (No. 1391) to induce sex-linked recessive lethal mutations in adult *Drosophila melanogaster* was studied in a Basc test. No increased frequency of mutation was observed in flies fed with isobornyl propionate (No. 1391) in a 10 mmol/l solution for 3 days (Wild et al., 1983).

In the test for micronucleus formation, groups of NMRI mice given isobornyl propionate (No. 1391) at a dose of 841, 1893 or 2944 mg/kg bw by intraperitoneal administration showed no increase in micronucleated erythrocytes in samples of bone marrow, 30 h after administration (Wild et al., 1983).

Topical application of *d,l*-camphor at a dose of up to 1000 mg/kg bw over 90 days (a total of 65 treatments) did not induce micronucleus formation in the peripheral blood erythrocytes of B6C3F_1 mice (National Toxicology Program, 1999).

When the structurally related monocyclic α,β-unsaturated ketone, isophorone (No. 1112) was fed to adult *D. melanogaster* for 3 days, no mutations were observed (Foureman et al., 1994). In addition, negative results were obtained when *D. melanogaster* were injected with a single dose of 12 500 μg of isophorone (Foureman et al., 1994).

There was no increase in the frequency of micronucleated polychromatic erythrocytes in the bone marrow of male or female CD-1 mice given isophorone at a dose of 540 μg/kg bw by intraperitoneal injection (McKee et al., 1987; O'Donoghue et al., 1988).

(iii) Conclusion

The testing of these representative monocyclic and bicyclic secondary alcohols, ketones and related esters in bacterial (Ames assay) and mammalian (micronucleus formation) in-vivo systems showed no evidence of genotoxic potential, and these results are further supported by the lack of positive findings in the *Drosophila* Basc test. These data are fortified by the lack of genotoxic potential of related α,β-unsaturated monocyclic ketones, isophorone and carvone.

(e) Reproductive toxicity

(i) d-*Camphor (No. 1395)*

Rats

Groups of 20 pregnant Sprague-Dawley rats were given *d*-camphor[2] at a dose of 0 (vehicle control), 216, 464 or 1000 mg/kg bw per day in propylene glycol by gavage during days 6–17 of gestation. Dams were observed for any signs of toxicity, and body weight and food intake were recorded daily. On day 20 of gestation, dams were killed and examined macroscopically. Fetuses were examined for external, skeletal and visceral anomalies. No adverse effects were reported in dams given *d*-camphor at a dose of 216 mg/kg bw per day. At the two higher doses, salivation and reduced feed intake were reported, with clonic convulsion, piloerection, reduced motility, and reduced body-weight gain reported in dams at the highest dose. Necropsy revealed ulcers in the cardiac region of the stomach of two dams at the intermediate dose and five dams at the highest dose. In addition, one dam at the highest dose was reported to have a thickened rough cardiac epithelium. In the fetuses, no effect on prenatal development was reported and no variations, retardations, or malformations were reported at any dose, even those causing maternal toxicity (Leuschner, 1997).

Groups of 26–29 pregnant Sprague-Dawley rats were given *d*-camphor at a dose of 0 (vehicle control), 100, 400 or 800 mg/kg bw per day in corn oil by gavage during days 6–15 of gestation. The dams were observed for clinical signs of toxicity, and body weights, feed and water consumption were recorded. On day 20 of gestation, dams were killed and body, liver and intact uterus weights were recorded, and corpora lutea were counted. Numbers of implantation sites, resorptions, dead fetuses, and live fetuses were determined. Live fetuses were weighed, and examined for external, visceral and skeletal abnormalities. No maternal deaths were reported. Initially, food consumption was significantly decreased at the two higher doses, but was reported to recover by the end of the study. Water intake was increased in all treated groups, reaching statistical significance at the two higher doses at various time-points during the study. In the group receiving the lowest dose, water intake was significantly increased only on days 6–9 of gestation. During the treatment period, a dose-dependent reduction in weight gain was

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[2] The authors described the test material as 'D-camphor', CAS No. 76-22-2. The CAS No. for *d*-camphor [(+)-camphor] is 464-49-3; while the CAS No. for (±)-camphor is 76-22-2.

reported, which reached levels of statistical significance in dams at the highest dose. Additionally, slight (≤10%) but significant and dose-dependent increases in absolute and relative weights of the liver were reported at the intermediate and highest doses. Exposure to *d*-camphor produced no effect on fetal growth, viability, or morphological development, even at doses causing maternal toxicity (National Toxicology Program, 1992b).

Rabbits

Groups of 12 pregnant Himalayan rabbits were given *d*-camphor at a dose of 0 (vehicle control), 147, 316 or 681 mg/kg bw per day in propylene glycol by gavage during days 6–18 of gestation. Does were observed for toxicity, and body weight and feed intake were recorded daily. On day 29 of gestation, does were killed and examined macroscopically. Fetuses were examined for external and skeletal anomalies. No effects were reported in does at the lowest (147 mg/kg bw per day) and intermediate (316 mg/kg bw per day) doses; while reduced body-weight gain and food intake were reported in does at the highest dose (681 mg/kg bw per day). At necropsy, no pathological findings were reported at any dose. In the fetuses, no effect on prenatal development was reported and no variations, retardations, or malformations were reported at any dose, even those causing maternal toxicity (Leuschner, 1997).

Groups of 26 pregnant New Zealand white rabbits were given *d*-camphor at a dose of 0 (vehicle control), 50, 200 or 400 mg/kg bw per day in corn oil by gavage during days 6–19 of gestation. Two does were removed from the group receiving the highest dose owing to gavage error. Does were observed for clinical signs of toxicity and fetuses were examined for possible effects on growth, viability, and morphological development. No compound-related maternal deaths were reported. Maternal body weights and feed intake were comparable to those of controls; however, maternal body-weight gain tended to decrease (significant trend test) in a dose-related manner: 13, 5 and 59% reduction in the 50, 200 and 400 mg/kg bw per day groups, respectively, compared with controls. Gravid uterine and liver weights (absolute and relative) for does treated with *d*-camphor were not significantly different from those of controls. No effects on resorptions per litter (%), late fetal deaths per litter (%), non-live implants per litter (%), adversely affected implants per litter (%), the proportion of litters with one or more resorptions, late fetal deaths, non-live implants or adversely affected implants, live litter size, average fetal body weight, the overall incidence of malformations, the incidence of external, skeletal and visceral malformations, and the incidence of anatomical variations or defects were reported. The NOEL for *d*-camphor for developmental and maternal toxicity was 400 mg/kg bw per day, the highest dose tested.

(ii) 3-l-Menthoxypropane-1,2-diol (No. 1408)

Rats

Groups of 30 pregnant Charles River CD rats were given 3-*l*-menthoxypropane-1,2-diol (No. 1408) as a single daily oral doses at 0, 100, 500 or 1500 mg/kg bw in

corn oil by gavage on days 6–15 of gestation. Caesarean sections were performed on all females surviving to day 20 of gestation. Two mortalities were reported in the group receiving the highest dose (1500 mg/kg bw per day); one of these rats was sacrificed in extremis on day 6 of gestation. No gross lesions were observed upon necropsy examination of this rat; however, discolouration of the lung was noted upon necropsy of a second dam at the highest dose that died on day 13 of gestation. Clinical observations noted in these two animals include prostration, loss of righting reflex, gasping, increased salivation, convulsions, and moribundity. Absolute and relative weights of the liver were significantly higher in dams at the highest dose than in the controls; however, no compound-related liver lesions were noted at necropsy. Body-weight gain of dams at the highest dose, which was slightly decreased on days 6–9 of gestation, was significantly higher on days 9–12 of gestation than in control animals. Relative to controls, food consumption was significantly decreased at the highest from days 6–12 of gestation. No treatment-related differences in maternal or fetal parameters were observed. No fetal malformations or developmental variations were reported. The NOEL for maternal toxicity was 500 mg/kg bw per day and the NOEL for developmental toxicity was >1500 mg/kg bw per day (York, 1993).

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AMINO ACIDS AND RELATED SUBSTANCES

First draft prepared by

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of 20 flavouring agents comprising amino acids and related substances. The group included 16 α-amino acids (some L-form and some D,L-form) (Nos 1419–1424, 1426, 1428–1432, 1434, 1437–1439) and one α-imino acid (No. 1425, L-proline), which are normally found in protein, and two β-amino acids (β-alanine, No. 1418, and taurine, No. 1435) and the *S*-methyl sulfonium salt of methionine (D,L-(3-amino-3-carboxypropyl)dimethylsulfonium chloride, No. 1427), which are not normally found in protein (see Table 1). L-Glutamic acid (No. 1420) was evaluated by the Committee at its thirty-first meeting (Annex 1, reference *77*) and an ADI 'not specified' was established for L-glutamic

acid and its ammonium, calcium, magnesium, monosodium, and potassium salts.

The Committee was of the opinion that the use of the Procedure for the Safety Evaluation of Flavouring Agents (Annex 1, reference *131*) was inappropriate for 12 members of this group, namely, the 11 L-form α-amino acids (L-cysteine, No. 1419; L-glutamic acid, No. 1420; glycine, No. 1421; L-leucine, No. 1423; L-phenylalanine, No. 1428; L-aspartic acid, No. 1429; L-glutamine No. 1430; L-histamine, No. 1431; L-tyrosine, No. 1434; L-arginine, No. 1438; L-lysine, No. 1439) and the one α-imino acid (L-proline, No. 1425). These substances are macronutrients and normal components of protein and, as such, human exposure through food is orders of magnitude higher than the anticipated level of exposure from use as flavouring agents.

The Committee also noted that amino acids may react with other food constituents upon heating. The mixtures thus formed are commonly referred to as 'process flavours'. The safety of process flavours has not been reviewed during this evaluation and may be considered at a future meeting. The present evaluation is therefore on the basis that these flavouring agents are present in an unchanged form at the point of consumption.

For the remaining eight members of the group, namely, the D,L-amino acids (D,L-isoleucine, No. 1422; D,L-methionine, No. 1424; D,L-valine, No. 1426; D,L-phenylalanine, No. 1432; D,L-alanine, No. 1437), the two β-amino acids (β-alanine, No. 1418, and taurine, No. 1435) and the *S*-methyl sulfonium salt of methionine (D,L-(3-amino-3-carboxypropyl)dimethylsulfonium chloride, No. 1427) (see Table 1), the evaluations were conducted according to the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, p 192). Although the D-form of the α-amino acids and the other three compounds are not found in protein, they are natural components of food. For these eight members of the group, the evaluation has been conducted only in relation to their use as flavouring agents leading to the current estimated intakes.

1.2 Estimated daily intake

The total annual volume of production for use as flavouring agents only of the 20 substances in this group is approximately 11 200 kg in Europe (International Organization of the Flavor Industry, 1995) and 21 100 kg in the USA (National Academy of Sciences, 1987; Lucas et al., 1999). The annual volumes of production are equivalent to a total daily intake of 1600 μg/person in Europe and 2800 μg/person in the USA.

Approximately 74% of the total annual volume of production in Europe is accounted for by four flavouring agents: L-cysteine (No. 1419), 40%; L-glutamic acid (No. 1420), 20%; glycine (No. 1421), 10%; and D,L-alanine (No. 1437), 8%. Approximately 83% of the total annual volume of production in the USA is accounted for by five flavouring agents in the group: L-cysteine (No. 1419) 11%; L-glutamic acid (No. 1420), 10%; L-aspartic acid (No. 1429), 45%; L-histidine (No. 1431), 9%); and taurine (No. 1435), 8%. The estimated daily per capita intake of each flavouring agent is reported in Table 1.

1.3 Absorption, distribution, metabolism and elimination

Amino acids are absorbed readily through the intestinal mucosa, distributed through the bloodstream and transported into cells by a variety of carrier systems. The D-isomers and those L-amino acids that are not needed for new protein synthesis undergo catabolism, primarily in the liver. There is no mechanism for storage of amino acids in humans. Amino acids undergo oxidative deamination, in which amino acids are deaminated to yield α-ketoacids that are either completely oxidized to carbon dioxide (CO_2) and water, or provide three or four carbon units that are converted via gluconeogenesis to yield glucose, or undergo ketogenesis to yield ketone bodies.

The *S*-methyl sulfonium salt of methionine (No. 1427) is demethylated to methionine or converted to homoserine by the loss of dimethylsulfide.

1.4 Application of the procedure for the safety evaluation of flavouring agents

Step 1. In applying the Procedure, the Committee assigned seven of the eight flavouring agents (D,L-isoleucine, No. 1422; D,L-methionine, No. 1424; D,L-valine, No. 1426; D,L-phenylalanine, No. 1432; D,L-alanine, No. 1437) and the two β-amino acids (β-alanine, No. 1418, and taurine, No. 1435) to structural class I. The remaining flavouring agent (D,L-(3-amino-3-carboxypropyl)dimethylsulfonium chloride, No. 1427) was assigned to structural class III (Cramer et al., 1978).

Step 2. The eight flavouring agents evaluated using the Procedure were all predicted to be metabolized to innocuous products. Their evaluation therefore proceeded via the A-side of the decision-tree.

Step A3. The estimated daily intakes of all the flavouring agents in structural class I and that of the one flavouring agent in structural class III are below the thresholds for daily human intake for their respective classes (1800 μg/person per day for class I, and 90 μg/person per day for class III). According to the Procedure, the use of these eight flavouring agents raises no safety concerns at estimated current intakes.

The intake considerations and other information used to evaluate the 20 amino acids and related substances are summarized in Table 1.

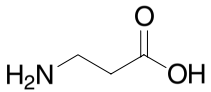
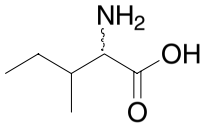
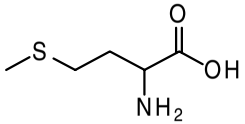
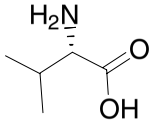
1.5 Consideration of secondary components

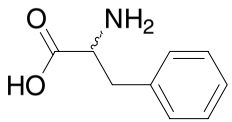
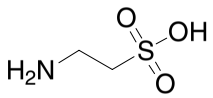
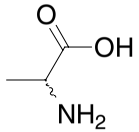
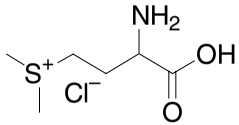
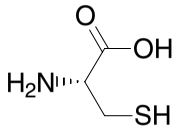
No flavouring agents in this group have minimum assay values of <95%.

1.6 Consideration of combined intakes from use as flavouring agents

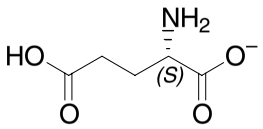
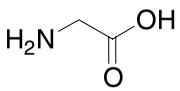
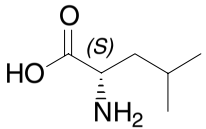
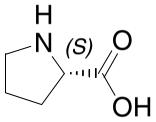
The eight flavouring agents evaluated using the Procedure are efficiently metabolized and eliminated, and the overall evaluation of the data indicates that combined intake would not raise any safety concerns at estimated current intakes.

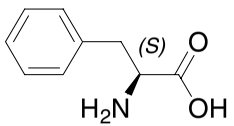
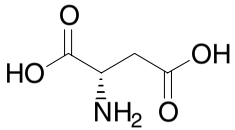
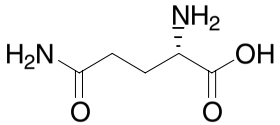
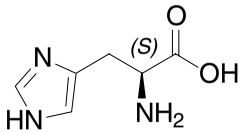
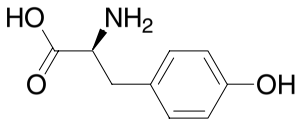
Table 1. Summary of the results of safety evaluations of amino acids and related substances[a]

Flavouring agent	No.	CAS No. and structure	Step A3 Does intake exceed the threshold for human intake?[a,b]	Comments	Conclusion based on current intake
Structural class I					
β-Alanine	1418	107-95-9 	No Europe: ND USA: 13	See note 1	No safety concern
D,L-Isoleucine	1422	443-79-8 	No Europe: 6 USA: 22	See note 2	No safety concern
D,L-Methionine	1424	59-51-8 	No Europe: 97 USA: 35	See note 3	No safety concern
D,L-Valine	1426	516-06-3 	No Europe: 41 USA: 48	See note 4	No safety concern

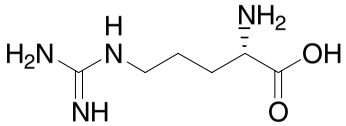
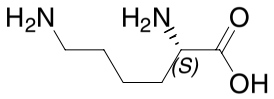
D,L-Phenylalanine	1432	150-30-1 	No Europe: 2 USA: 0.7	See note 5	No safety concern
Taurine	1435	107-35-7 	No Europe: ND USA: 217		No safety concern
D,L-Alanine	1437	302-72-7 	No Europe: 134 USA: 1	See note 6	No safety concern
Structural class III					
D,L-(3-Amino-3-carboxypropyl)dimethylsulfonium chloride	1427	1115-84-0 	No Europe: ND USA: 75	See note 7	No safety concern
L-Cysteine	1419	52-90-4 	Europe: 642 USA: 293		No safety concern

Amino acids not evaluated by the Procedure

Flavouring agent	No.	CAS No. and structure	Daily per capita intake[b]	Conclusion based on current intake
L-Glutamic acid	1420	56-86-0 	Europe: 313 USA: 273	No safety concern
Glycine	1421	56-40-6 	Europe: 158 USA: 5	No safety concern
L-Leucine	1423	61-90-5 	Europe: 14 USA: 24	No safety concern
L-Proline	1425	147-85-3 	Europe: 49 USA: 115	No safety concern

L-Phenylalanine	1428	63-91-2 	Europe: 20 USA: 28	No safety concern
L-Aspartic acid	1429	56-84-8 	Europe: 79 USA: 1 240	No safety concern
L-Glutamine	1430	56-85-9 	Europe: 16 USA: 10	No safety concern
L-Histidine	1431	71-00-1 	Europe: 11 USA: 259	No safety concern
L-Tyrosine	1434	60-18-4 	Europe: 12 USA: 4	No safety concern

Amino acids not evaluated by the Procedure (Contd)

Flavouring agent	No.	CAS No. and structure	Daily per capita intake[b]	Conclusion based on current intake
L-Arginine	1438	74-79-3 	Europe: ND USA: 57	No safety concern
L-Lysine	1439	56-87-1 	Europe: ND USA: 57	No safety concern

CAS: Chemical Abstracts Service; ND: No intake data reported.

[a] Step 2: All eight flavouring agents in this group evaluated using the Procedure are expected to be metabolized to innocuous products. The evaluation of these flavouring agents therefore proceeded via the A-side of the decision-tree.

[b] The threshold for human intake is 1800 μg per day for structural class I and 90 μg/peson per day for class III. All intake values are expressed in μg/person per day. The combined intake of the flavouring agents in structural class I is 1594 μg/person per day in Europe and 2701 μg/person per day in the USA. The intake of the flavouring agent in structural class III is 75 μg/person per day in the USA.

Notes:

1 Deaminated to formylacetic acid and metabolized in the citric acid cycle.
2 Deaminated to form acetyl coenzyme A (CoA), propionyl CoA, succinyl CoA, and metabolized in citric acid cycle.
3 Deaminated to form homocysteine, propionyl CoA, succinyl CoA, and metabolized in citric acid cycle.
4 Deaminated to form propionyl CoA, succinyl CoA, and metabolized in citric acid cycle.
5 Converted to tyrosine, then deaminated to form acetoacetyl CoA and metabolized in the citric acid cycle.
6 Deaminated to pyruvate and acetyl CoA and metabolized in the citric acid cycle.
7 Demethylated to methionine or deaminated to homoserine by loss of diemethylsulfide.

1.7 Conclusion

In view of the fact that the L-form of the 11 α-amino acids and the one α-imino acid in this group are macronutrients and normal components of protein, the use of these substances as flavouring agents would not raise any safety concerns at estimated current intakes. The Committee also concluded that the use of the other eight substances in the group leading to the estimated current intakes would not raise any safety concerns.

The ADI 'not specified' for L-glutamic acid and its ammonium, calcium, magnesium, monosodium and potassium salts was maintained.

2. RELEVANT BACKGROUND INFORMATION

2.1 Explanation

The background information summarizes key data relevant to the safety evaluation of the 20 amino acids and related substances used as flavouring agents.

2.2 Additional considerations on intake

The total annual volume of production of the 19 amino acids (Nos 1418–1426, 1428–1432, 1434, 1435, 1437–1439) and *S*-methyl sulfonium salt of methionine (No. 1427) obtained from industry-wide surveys is approximately 180 600 kg in Europe (International Organization of the Flavor Industry, 1995), and 316 100 kg in the USA (National Academy of Sciences, 1989; Lucas et al., 1999) (see Table 2). However, most of the total annual volume of production of amino acids is used as starting material in the production of process flavourings. Process flavours are polyheteroaromatic substances produced by the heated reaction of amino acids and simple sugars and these flavours do not contain significant amounts of free amino acids. The percentage of use of an individual amino acid as flavouring agents will vary, but overall it is estimated that <10% of the total reported annual volume of production of amino acids is intended for use as flavouring agents per se. Based on estimates of the relative amount of each amino acid used exclusively as a flavouring agent, the total annual volume of production of amino acids for use as flavouring agents is 11 200 kg in Europe and 21 000 kg in the USA (see Table 2). The estimated total daily intake of amino acids added to food as flavouring agents is approximately 1.5 and 3 mg/person per day in Europe and the USA, respectively.

α-L-Amino acids are normal constituents of protein and therefore dietary intake can be estimated from analysis of the protein content of the normal diet. Dietary reference intakes for amino acids have been determined by the Institute of Medicine (2002). The dietary intake from food varied from 1010 mg/day for cysteine to 15 270 mg/day for glutamic acid. Dietary intakes for the other amino acids were between 2000 and 6000 mg/day. Dietary exposure to the 15 amino acids analysed was significantly greater (by three to four orders of magnitude) in the normal diet than from their use as flavouring agents.

Eight amino acids cannot be synthesized by the adult body and are considered to be essential in the human diet — those used as flavouring agents are: L-phenylalanine, L-methionine, L-valine, L-leucine, L-isoleucine, L-histidine and L-lysine. The recommended daily requirements for these amino acids are in the range of 1330 to 2940 mg/day (Institute of Medicine, 2002). These levels of intake are significantly greater than the daily per capita intake from their use as flavouring agents.

Amino acids are used as flavouring agents in a variety of food categories (Hall & Oser, 1965; Oser & Hall, 1972; Oser & Ford, 1975; 1978; Oser et al., 1984; Smith et al., 1996; Newberne et al., 1998). Typical use levels range from 5 mg/kg for L-arginine in processed vegetables to 4000 mg/kg for L-alanine in seasonings and flavours (Newberne et al., 1998). It is claimed that amino acids such as glycine, alanine, valine, leucine, isoleucine, and lysine reduce the unpalatable astringent flavour of zinc and aluminium salts in food (Godfrey, 1987, 1993).

Amino acids are also used as dietary supplements. In the USA, amino acids can be used to a maximum percentage of the diet — this ranges from 2.3% for L-cysteine/L-cystine to 8.8% for L-leucine, as specified in the Code of Federal Regulations of the USA (Title 21, 21CFR172.320, 2003). These levels are considerably higher than the comparable recommended levels of use of the same amino acids as flavouring agents, namely, 0.01% for L-cysteine/L-cystine and 0.005% for L-leucine (Oser & Hall, 1972).

2.3 Biological data

2.3.1 Biochemical data

α-Amino acids are present in animal- and plant-based foods and are normal constituents of a healthy diet. α-Amino acids are referred to as either essential or non-essential. Animals do not synthesize the essential amino acids and must ingest them as part of a normal diet. Essential amino acids are metabolic precursors to the synthesis of other amino acids. The essential amino acids are isoleucine (No. 1422), leucine (No. 1423), methionine (No. 1424), valine (No. 1426), phenylalanine (Nos 1428 and 1432), histidine (No. 1431), arginine (No. 1438)[1], lysine (No. 1439), threonine, and tryptophan.

Excess dietary amino acids are neither stored nor excreted; rather, they undergo conversion to common metabolic intermediates (e.g. pyruvate, oxaloacetate, α-ketoglutarate). There are numerous studies on the absorption, distribution, metabolism, and excretion of amino acids in the literature, only some of which are summarized below.

(a) Absorption, distribution, and excretion

Free α-amino acids, whether ingested as such in the form of flavouring agents or released after the digestion of proteins by proteolytic enzymes, are absorbed

[1] Arginine is only an essential amino acid for young, growing animals, not for adults.

Table 2. Annual volumes of production of amino acids and related substances used as flavouring agents in Europe and the USA

Flavouring agent (No.)	Total annual volume of production (kg)[a]	Estimated % of total annual volume used as a flavouring agent[b]	Annual volume used as a flavouring agent (kg)[c]	Intake ('eaters only')[d]		Intake from foods (mg/day)[e]	Consumption ratio[f]
				µg/day from use as a flavour	µg/kg bw per day from use as a flavour		
β-Alanine (1418)							
Europe	N/D	N/D	N/D	N/D	N/D		
USA	100	100	100	13	0.2	3640	NA
L-Cysteine (1419)							
Europe	89933	5	4497	642	11		
USA	44497	5	2225	293	5	1010	2000
L-Glutamic acid (1420)							
Europe	43873	5	2194	313	5		
USA	41413	5	2071	273	5	15270	49000
Glycine (1421)							
Europe	7385	15	1108	158	3		
USA	263	15	39	5	0.09	3210	20000
D,L-Isoleucine (1422)							
Europe	71	60	43	6	0.1		
USA	1637	10	164	22	0.4	3550	161000
L-Leucine (1423)							
Europe	475	20	95	14	0		
USA	3701	5	185	24	0.4	6100	254000
D,L-Methionine (1424)							
Europe	4519	15	678	97	2		
USA	295	90	266	35	0.6	1770	18000
L-Proline (1425)							
Europe	2275	15	341	49	0.8		
USA	5806	15	871	115	2	5210	45000

Table 2. (Contd)

Flavouring agent (No.)	Total annual volume of production (kg)[a]	Estimated % of total annual volume used as a flavouring agent[b]	Annual volume used as a flavouring agent (kg)[c]	Intake ('eaters only')[d]		Intake from foods (mg/day)[e]	Consumption ratio[f]
				µg/day from use as a flavour	µg/kg bw per day from use as a flavour		
D,L-Valine (1426)							
Europe	482	60	289	41	0.7		
USA	612	60	367	48	0.8	3990	83000
D,L-(3-Amino-3-carboxypropyl)dimethylsulfonium chloride (1427)							
Europe	N/D	N/D	N/D	N/D	N/D		
USA	567	100	567	75	1	NA	NA
L-Phenylalanine (1428)							
Europe	229	60	137	20	0.3		
USA	526	40	210	28	0.5	3400	121000
L-Aspartic acid (1429)							
Europe	11138	5	557	79	1		
USA	188241	5	9412	1240	21	6540	5000
L-Glutamine (1430)							
Europe	1090	10	109	16	0.3		
USA	776	10	78	10	0.2	NA	NA
L-Histidine (1431)							
Europe	78	100	78	11	0.2		
USA	1964	100	1964	259	4	2200	9000
D,L-Phenylalanine (1432)							
Europe	161	10	16	2	0.04		
USA[g]	5	100	5	0.7	0.01	NA	NA
L-Tyrosine (1434)							
Europe	85	100	85	12	0.2		
USA	27	100	27	4	0.06	2790	233000

Taurine (1435)							
Europe	N/D	N/D	N/D	N/D	N/D		
USA[h]	8250	20	1650	217	4	NA	NA
D,L-Alanine (1437)							
Europe	18845	5	942	134	2		
USA	204	5	10	1	0.02	3640	27000
L-Arginine (1438)							
Europe	N/D	N/D	N/D	N/D	N/D		
USA[h]	8600	5	430	57	0.9	4180	73000
L-Lysine (1439)							
Europe	N/D	N/D	N/D	N/D	N/D		
USA[h]	8600	5	430	57	0.9	5270	93000
Total							
Europe	180639		11169	1594			
USA	316084		21071	2775			

NA, not available; N/D, no intake data reported

[a] From International Organization of the Flavour Industry (1995) and Lucas et al. (1999) or National Academy of Sciences (1970, 1982, 1987). The total annual volume is reported for the combined use as a flavouring agent and as a starting material used in the preparation of process flavours.

[b] Estimated percentage of total annual volume used as a flavouring agent reported to the Flavor and Extract Manufacturers Association (Private communication to Flavor and Extract Manufacturers Association, 2003).

[c] Most recent annual volume (kg) used as a flavouring agent = [Most recent total annual volume (kg)] × [Estimated % of total annual volume used a flavouring agent]

[d] Intake expressed as μg/person per day was calculated as follows:
[(annual volume, kg) × (1 × 109 μg/kg)]/[population × survey correction factor × 365 days], where population (10%, 'eaters only') = 32×10^6 for Europe and 26×10^6 for the USA. The correction factor = 0.6 for Europe and 0.8 for the USA, representing the assumption that only 60% and 80% of the annual production volume of the flavour, respectively, was reported in the poundage surveys (Lucas et al., 1999; International Organization of the Flavour Industry, 1995; National Academy of Sciences, 1970, 1982, 1987). Intake expressed as μg/kg bw per day) was calculated as follows:
[(μg/person per day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.

[e] Institute of Medicine (2002)

[f] Intake from food, mg/day)/(Added flavour intake, mg/day)

[g] Annual volume reported in previous USA surveys (National Academy of Sciences, 1987).

[h] The volume cited is the anticipated annual volume, which was the maximum amount of flavouring agent estimated to be used annually by the manufacturer at the time the material was proposed for flavour use. National surveys (National Academy of Sciences, 1970, 1982 or 1987; Lucas et al., 1999), if applicable, revealed no reported use as a flavouring agent.

primarily through the intestinal mucosa and enter the portal blood. Once absorbed, a variety of carrier systems transport α-amino acids into cells (Kilberg, 1982). These amino-acid carriers are mostly sodium ion-dependent systems that are specific to a particular class of α-amino acids (e.g. neutral amino acids with short side-chains, neutral amino acids with branched or aromatic side-chains, basic amino acids, and dicarboxylic amino acids). The carrier systems are adaptive and under hormonal regulatory control. Although small amounts of protein and polypeptides may be absorbed by a transport system involving membrane-bound γ-glutamyl transferase, most amino acids enter the cells unchanged (Nelson & Cox, 2000).

After absorption, α-amino acids are used in protein synthesis or rapidly metabolized to intermediates in the citric acid cycle, as evidenced by the presence of only trace amounts of α-amino acids in the plasma. The excretion of α-amino acids is regulated by renal tubular reabsorption, in which the proximal tubules conserve α-amino acids. The daily excretion of α-amino acids in the urine amounts to only 20–150 mg/day in humans[2] (Tietz, 1986). Minimal loss of α-amino acids occurs in the urine and faeces.

β-Amino acids are also rapidly absorbed but they are not incorporated into proteins — rather they are metabolised via oxidative deamination to yield shorter chain acids that are either completely oxidized in the fatty acid pathway and tricarboxylic acid cycle, or excreted primarily in the urine.

(i) α-Amino acids (Nos 1419–1426, 1428–1432, 1434, 1437–1439)

The simplest α-amino acid, glycine, is rapidly absorbed and distributed to cells where it is either metabolized or enters the amino acid pool. In a metabolic study, 20 healthy volunteers consumed a fruit beverage containing glycine at a dose of 0.14 mg/kg bw. A maximum plasma concentration of glycine of 4.18 mg/100 ml of plasma was attained in 45 min. Subsequently, glycine was rapidly removed from the plasma and at 180 min was detected at concentrations of only 0.06 mg/100 ml of plasma (Craft et al., 1968). In another study with glycine, patients were given 8 mg (100 μC_i) of [^{14}C-carboxy]glycine via intravenous injection; after 3 h, an average of 25% (13–36%) of the radiolabel was eliminated in expired CO_2. Over the following 55 days, 83–92% of the remaining radiolabelled glycine was removed. Only 5% was excreted in the urine during the first 14 days of the study, while 2% was retained by erythrocytes for the duration of their lifespan (approximately 120 days) (Berlin et al., 1951).

In a study comparing liver function in healthy volunteers and in patients with hepatic cirrhosis, control volunteers and cirrhotic patients received L-alanine (No. 1437) intravenously at an infusion rate of 0.007 mg/kg bw per min for 2 h after a priming dose of 0.623 mg/kg bw of [^{15}N]L-alanine. Ten min after the 2-h perfusion,

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[2] During pregnancy in humans, excretion of protein may increase harmlessly to 200–300 mg/day (Tietz, 1986).

the rate at which radiolabelled alanine appeared in the control volunteers was slightly greater than 50% of the rate of infusion (3.8 ± 0.2 μmol/kg per min or 0.004 mg/kg per min) and the rate of clearance was approximately four times (16 ± 1.0 μmol/kg per min) the rate of appearance, indicating rapid clearance of L-alanine from the blood (Schricker et al., 1995).

Rates of intestinal absorption of leucine (No. 1423) and valine (No. 1426) were measured in 11 normal human volunteers (Adibi, 1969). The intestinal absorption of leucine (10–100 mmol/l) and valine (5–100 mmol/l) was reported to be directly proportional to the concentration of amino acid, with saturation of intestinal absorption occurring with leucine at >50 mmol/l (Adibi, 1969).

In another metabolic study, mice were given 2.0 mg of ^{14}C-labelled leucine (No. 1423) via intravenous injection. Almost no leucine could be detected in the blood 10 min after injection, while 47% was accounted for in the viscera after 30 min, 100% was distributed throughout the carcass within 4 h, and 57% was exhaled as $^{14}CO_2$ within the same period of 4 h (Borsook et al., 1950).

In a study in 12 healthy, fasted volunteers, peak serum concentrations of glutamate were reported 30–45 min after they were given a bouillon drink containing glutamic acid sodium salt at a dose of 43 mg/kg bw. When the dose was increased to 64 mg/kg bw, the peak serum concentration of glutamic acid also increased and the area under the curve (AUC) more than doubled, suggesting near saturation of distribution and metabolic processes (Ghezzi et al., 1985).

In a study of three cystinuria phenotypes differentiated by urinary excretion of amino acids, three human volunteers were given lysine (No. 1439) or arginine (No. 1438) as a single oral dose at 0.5 mmol/kg bw. Plasma concentrations of lysine, arginine and ornithine, an arginine metabolite, increased by 110, 75, and 100%, respectively, over baseline levels. Concentrations of lysine and arginine in the urine remained constant over the 6 h after administration. However, urinary excretion of ornithine, an amino acid intermediate in urea synthesis, increased by 175% (de Sanctus, 2001).

In a study in mice, [^{14}C]lysine (No. 1439) or [^{14}C]histidine (No. 1431) at a dose of 1.46 mg or 1.47 mg, respectively, were given to mice via intravenous injection. Most of the radioactivity was removed from the blood within the first 10 min. Histidine and lysine were detected at 30 and 45%, respectively, in the non-protein fraction of the viscera after 10 min, while 58 and 92% were distributed throughout the carcass within 4 h, respectively. In the 4 h after administration, 28% of histidine and 43% of lysine were exhaled as $^{14}CO_2$ (Borsook et al., 1950).

In a multi-species study, a single dose of [2-^{14}C]L-histidine was administered intravenously to Macaque monkeys (2.5 mg), orally to 23 normal and schizophrenic human volunteers (14 mg), or intraperitoneally to Sprague-Dawley rats (7.4 mg). Less than 10% of the radiolabel was excreted in the urine within 5 h in both monkey and humans, and within 24 h in rats. Measurement of tissue radioactivity in the monkey indicated that L-histidine concentrated in the liver after 2 h, the liver and muscle after 7 days, and was then widely distributed to all tissues after 11 days (Brown et al., 1960).

Two healthy volunteers, one male and one female, were given L-[3,3-$^{2}H_{2}$,1′,3′-$^{15}N_{2}$]histidine as a single oral dose at 100 mg (approximately 1.5 mg/kg bw). Radiolabelled L-histidine was rapidly absorbed and maximum plasma concentrations (C_{max}) (1.06 and 1.64 μg/ml) were reached at 30 and 60 min in the male and female, respectively. The plasma half-life ($t_{1/2}$) for labelled L-histidine was 1.0 and 1.9 h for the male and female, respectively and the AUC was 2.4 times greater in the female than in the male. The total amount of radiolabelled histidine recovered from the 24-h urine sample was minor (Furuta et al., 1996).

Groups of five male Sprague-Dawley or Holzman-Rolfsmeyer albino rats given diets containing D,L-methionine (No. 1424) at 0, 0.6, or 2.5 % for 4 weeks excreted only minor amounts in their daily urine (64, 667, or 1833 μg of D,L-methionine per day, respectively) (DeBey et al., 1952).

In another metabolic study, Wistar rats (two of each sex) were given a diet supplemented with 4.7% L-methionine and either 6% or 12% casein for 18 days. Urine analysis showed that only 2.1 and 3.4% of the calculated intake of methionine remained unchanged for males and females, respectively. Both groups excreted minor amounts of L-methionine in the faeces (Hoshino & Miyazaki, 1964).

(ii) D,L-(3-amino-3-carboxypropyl)dimethylsulfonium chloride (No. 1427)

In a metabolic study in humans, four male volunteers were given D,L-(3-amino-3-carboxypropyl)dimethylsulfonium chloride (MMSC), the *S*-methyl sulfonium salt of methionine (No. 1427), as a single oral dose at 750 mg/kg bw. Plasma concentrations of radiolabelled MMSC were 7.1 μg/ml at 30 min, 9.2 μg/ml after 2 h, and then decreased by more than 50% to 3.93 μg/ml over the next 4 h. At 2 h, the maximum concentration of labelled MMSC in the blood was reached and represented approximately 12% of the administered dose (Suzue et al., 1975).

(iii) β-Amino acids (Nos 1418 and 1435)

In a study to examine the absorption and excretion of β-alanine, an unspecified number of albino rats were given β-alanine labelled with ^{14}C at position 1, 2, or 3 at a dose of 6.25–7.78 mg/kg bw by intraperitoneal injection. Exhaled air revealed that the carboxyl carbon atom (C_1) was excreted rapidly, with maximal rate at 30 min after injection. Within 2 h, 89% of the 1-^{14}C was eliminated as exhaled CO_2. This is consistent with metabolic oxidative decarboxylation of β-alanine. The α-carbon (C2), had the slowest rate of excretion, with maximum rate reported at 60 min after injection. The β-carbon (C3), was reported to display intermediate kinetics of elimination. The cumulative excretion of ^{14}C at 5 h was 93, 60, and 77% of the total dose of [1-, 2-, or 3-^{14}C]β-alanine, respectively (Pihl & Fritzson, 1955).

As part of a study of the metabolism of malondialdehyde, an intermediary metabolite of β-alanine, an unspecified number of male and female Swiss albino mice were injected with [1-^{14}C]- or [3-^{14}C]-labelled β-alanine at 200 or 600 μmol. It

was reported that male and female mice expired the carboxy-labelled carbon as CO_2 within the first 1.5 h. After 4 h, male and female mice injected with [1-^{14}C]β-alanine at 200 µmol eliminated 89 and 90%, respectively, of the total radiolabel as expired CO_2. In addition, male and female mice eliminated 66 and 74% of the [3-^{14}C]β-alanine, respectively, as expired CO_2 within a period of 3 h after injection. The rapid oxidation of β-alanine was accompanied by limited excretion of unchanged β-alanine in the urine (Marnett et al., 1985). On the basis of these data, it was concluded that β-alanine is rapidly absorbed and completely oxidized to CO_2, water, and ammonia.

(b) Metabolism

Under normal conditions, α-amino acids that are not required for new protein synthesis undergo catabolism primarily in the liver, with the exception of the branched amino acids, which undergo degradation in muscle, adipose, kidney and brain tissues. There is no storage of amino acids in humans. The amino acids undergo a process called oxidative deamination in which most amino acids are transformed to α-ketoacids that are completely oxidized to CO_2 and water or provide three or four carbon units that are converted via gluconeogenisis to yield glucose, or via ketogenesis to yield ketone bodies (Nelson & Cox, 2000).

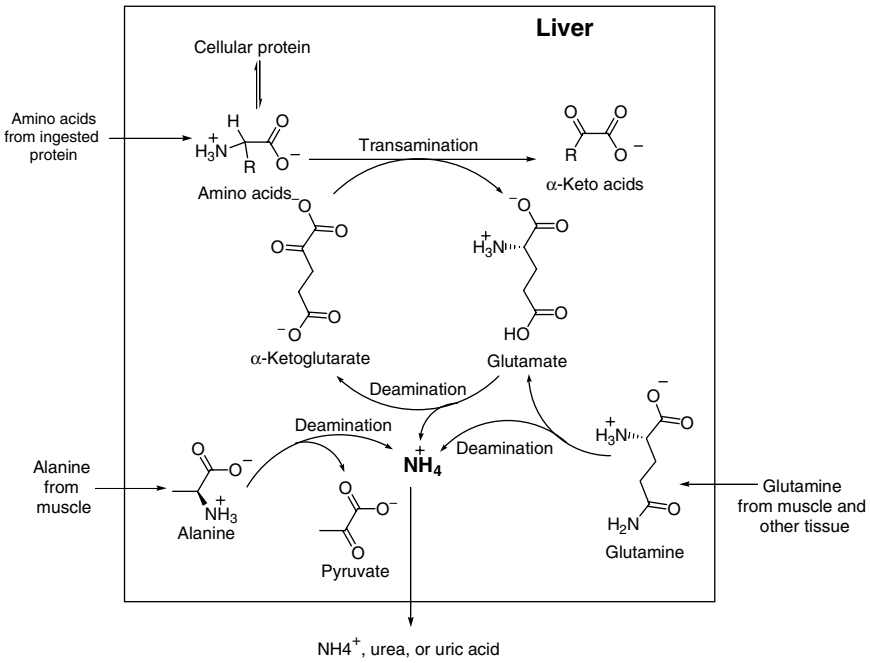
The amino groups resulting from transamination of most of the amino acids are transferred by transaminases to α-ketoglutarate to form L-glutamate in the cytosol of hepatocytes. L-Glutamate then undergoes deamination in the mitochondria yielding NH_4^+ via L-glutamate dehydrogenase (see Figure 1). The ammonium ion is either used in other metabolic pathways or converted to urea for excretion.

The carbon unit skeletons of the amino acids are all broken down to one of five products, each of which enters the citric acid cycle. These five products are acetyl-coenzyme A (acetyl-CoA), α-ketoglutarate, succinyl-CoA, fumarate, and oxaloacetate. These pathways are illustrated in Figures 2–5 and discussed below.

(i) D,L-Alanine (No. 1437), L-cysteine (No. 1419) and glycine (No. 1421)

These amino acids are degraded to acetyl-CoA via pyruvate (see Figure 2). D-Alanine is converted to pyruvate upon transamination. Cysteine is converted to pyruvate in two steps, the first being removal of sulfur and the second transamination to remove the amino group. Glycine is converted first to the amino acid serine via serine hydroxymethyltransferase, or undergoes oxidative cleavage yielding CO_2, NH_4^+, and a methylene group (Nelson & Cox, 2000).

Figure 1. Catabolism of amino groups

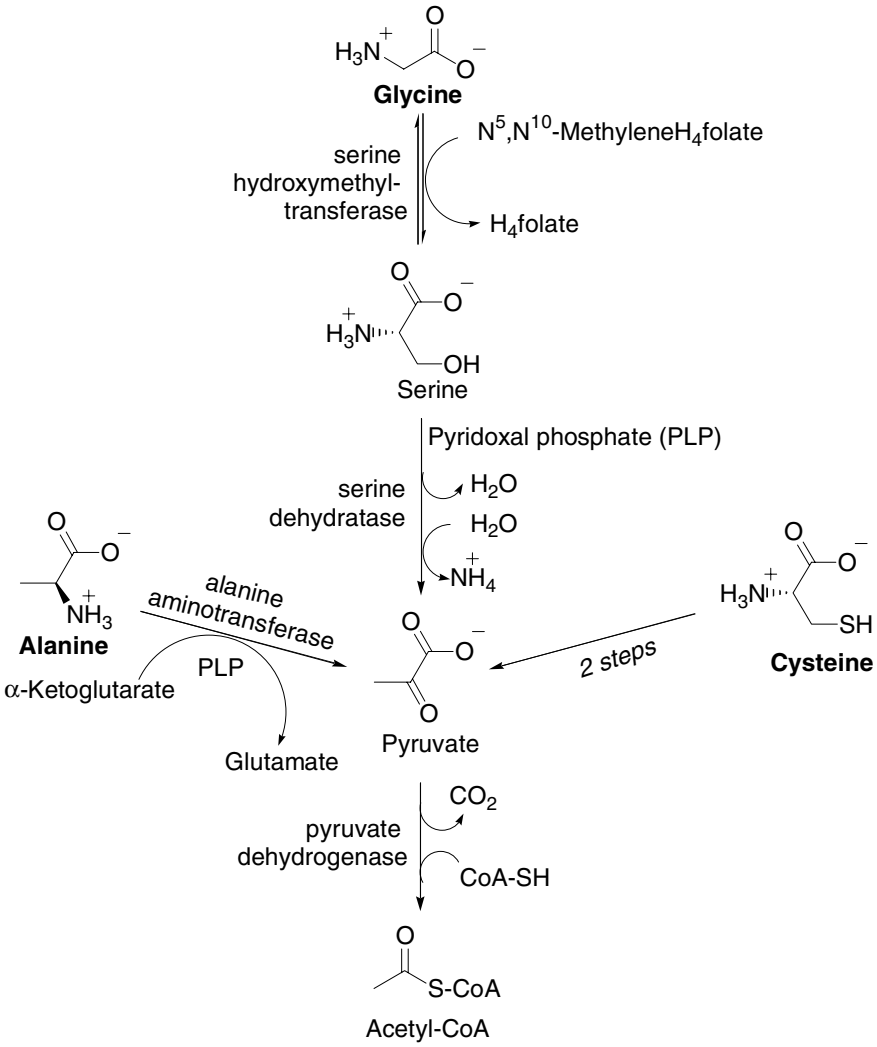


From Nelson & Cox (2000)

(ii) D,L-Isoleucine (No. 1422), L-leucine (No. 1423), L-phenylalanine (No. 1428), L-tyrosine (No. 1434), L-lysine (No. 1739)

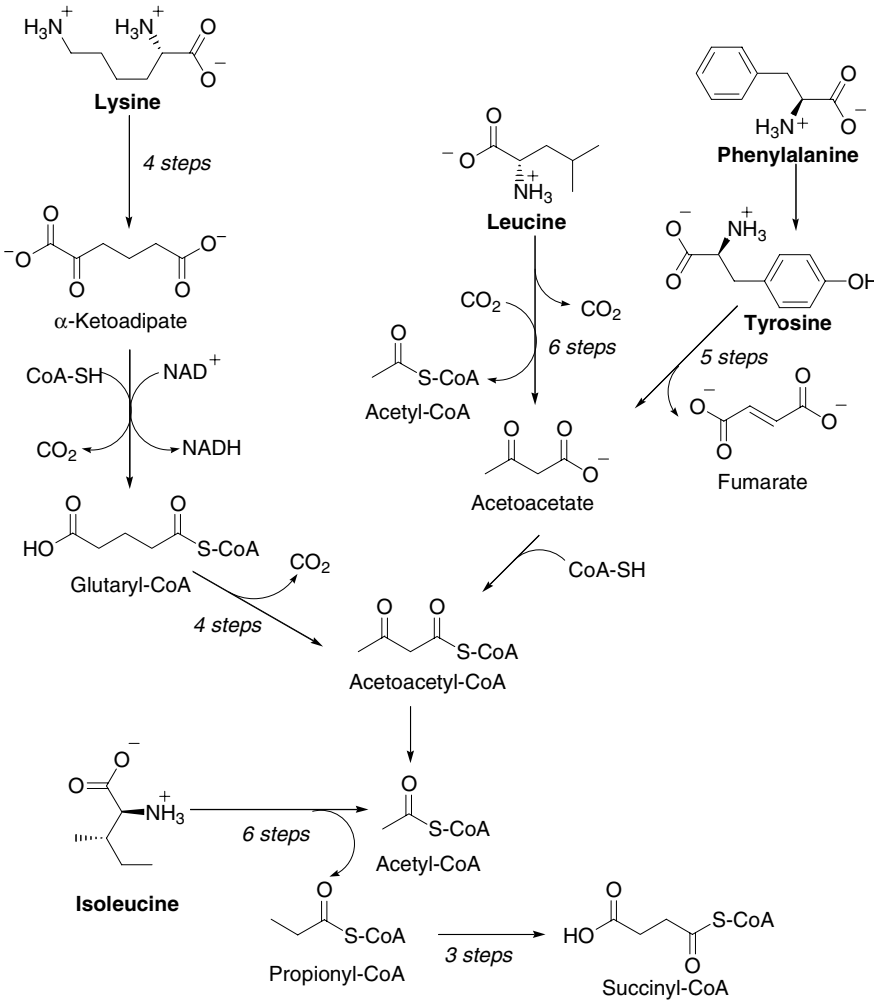
These five amino acids enter the citric acid cycle via acetyl-CoA (see Figure 3) following deamination. Unlike phenylalanine, tyrosine and lysine, leucine and isoleucine are degraded in extrahepatic tissues that contain a specific aminotransferase that removes the amino group of branched chain amino acids to produce the corresponding α-ketoacid. In addition to contributing carbon atoms to acetyl-CoA, phenylalanine, tyrosine, and isoleucine also donate carbon atoms to pyruvate or to other intermediates in the citric acid cycle. For example, phenylalanine is hydroxylated to form tyrosine that is subsequently broken down into two carbon units, the four-carbon unit yielding free acetoacetate and another four-carbon unit yielding fumarate, with one carbon being converted to CO_2 (Nelson & Cox, 2000).

Figure 2. Catabolic pathways for alanine, cysteine and glycine



From Nelson & Cox (2000)
CoA, coenzyme A; PLP, pyridoxal phosphate

Figure 3. Catabolic pathways for lysine, phenylalanine, tyrosine, leucine and isoleucine



From Nelson & Cox (2000)

(iii) L-Glutamic acid (No. 1420), L-proline (No. 1425), L-glutamine (1430), L-histidine (No. 1431), and L-arginine (No. 1438)

These five amino acids enter the citric acid cycle as α-ketoglutarate (see Figure 4). Proline, which has a five-membered ring, is oxidized to the Schiff base, which is then hydrolysed to yield glutamate γ-semialdehyde. This aldehyde undergoes further oxidation to glutamate, which is subsequently deaminated to yield α-ketoglutarate. Glutamine is converted to glutamate via the loss of its amide nitrogen (Nelson & Cox, 2000).

Although arginine and histidine are C6 amino acids, they are also converted to C5 α-ketoglutarate via the transamination of glutamate, but the removal of the additional carbon attached requires additional steps. Arginine is converted to ornithine in the urea cycle, which is subsequently deaminated to glutamate γ-semialdehyde. Histidine is degraded by non-oxidative deamination to urocanate followed by hydration of both double bonds and hydrolytic ring opening resulting in formiminoglutamate. The formimino moiety is transferred to tetrahydrofolate and the glutamate is deaminated to α-ketoglutarate (Nelson & Cox, 2000).

A recent study illustrates the pathway for catabolism of histidine. Two healthy volunteers, one male and one female received L-[3,3-$^{2}H_2$,1′,3′-$^{15}N_2$]histidine as a single oral dose at 1.5 mg/kg bw after a 12-h overnight fast. Urocanic acid was detected in the plasma within 5 min for the male volunteer and within 15 min for the female volunteer. Trace amounts of urocanic acid were excreted in the urine (Furuta et al., 1996).

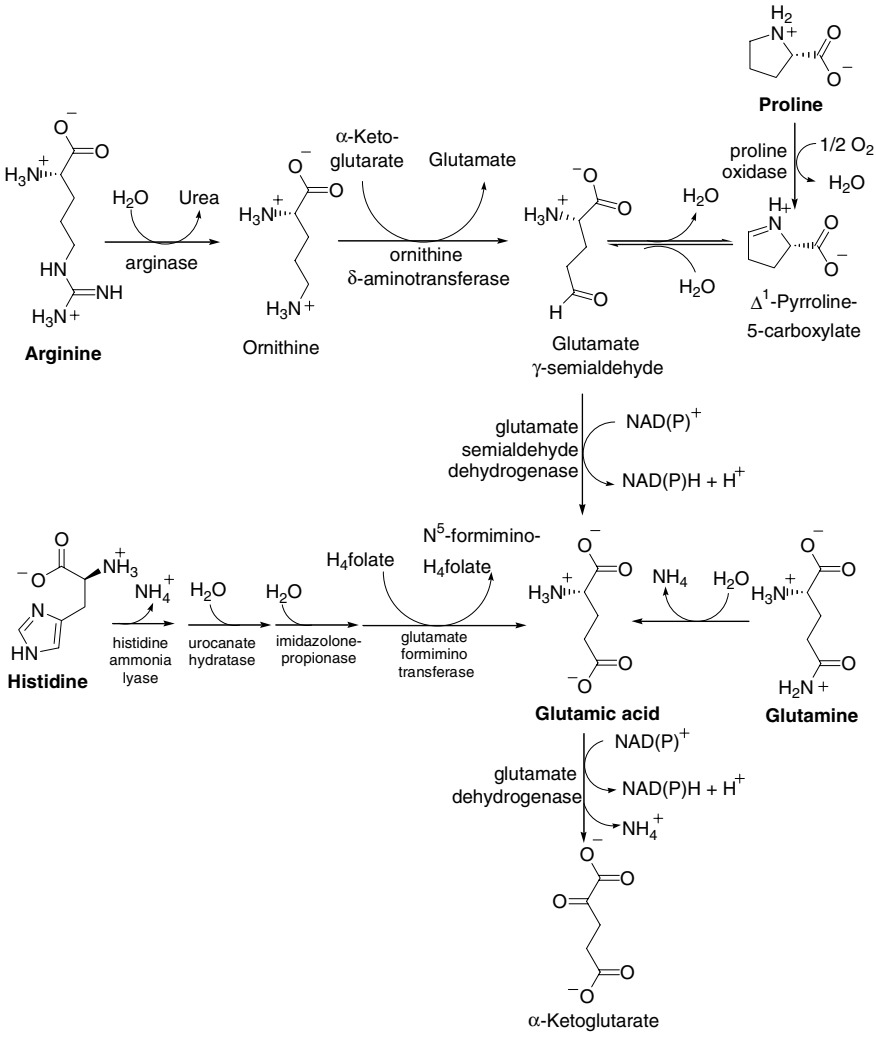
(iv) D,L-Isoleucine (No. 1422), D,L-methionine (No. 1424), and D,L-valine (No. 1426)

The degradation products of these amino acids yields succinyl-CoA, which is an intermediate in the citric acid cycle (see Figure 5). After deamination, the branched amino acid isoleucine undergoes oxidative decarboxylation yielding CO_2, acetyl-CoA and propionyl-CoA, which is a precursor of succinyl-CoA. Methionine donates a methyl group through *S*-adenosylmethionine, while the remaining carbons are converted to α-ketobutyrate which is subsequently decarboxylated to form propionyl-CoA. Valine, another branched chain amino acid, undergoes deamination in extrahepatic tissues followed by decarboxylation and oxidation eventually yielding propionyl-CoA.

(v) D,L-(3-amino-3-carboxypropyl)dimethylsulfonium chloride (No. 1427)

In a study of the metabolism of the *S*-methyl sulfonium salt of methionine, male volunteers were given the racemic form of (3-amino-3-carboxypropyl)dimethylsulf onium chloride (No. 1427) at a dose of 750 mg/kg bw orally and samples of blood were taken at 0.5, 1, 2, 3, and 6 h. Only small amounts (0.5–2%) of the demethylation metabolite, methionine, and the metabolite, homoserine, formed by loss of dimethylsulfide were measured in the blood at 2 h, this was expected since

Figure 4. Catabolic pathways for proline, glutamic acid, glutamine, arginine and histidine



From Nelson & Cox (2000)

methionine formed in the liver would be quickly incorporated into proteins, and homoserine further metabolized (Suzue et al., 1975).

(vi) Aspartic acid (No. 1429)

Aspartic acid undergoes transamination with α-ketoglutarate to oxaloacetate, which is an intermediate in the citric acid cycle.

(vii) β-Alanine (No. 1418)

β-Alanine has been reported to be deaminated to formylacetic acid and then metabolized rapidly and extensively to CO_2 and water (Pihl & Fritzson, 1955; Marnett et al., 1985). It is also incorporated into two dipeptides, carnosine[3] (β-alanyl-L-histidine) and anserine[4] (β-alanyl-1-methyl-L-histidine), in vertebrate muscle tissue at reported concentrations of 0.50 and 1.90 mg/g, respectively. When five partially hepatectomized rats were given β-alanine at a dose of 22 μmol/l per 100 g, 22 h after surgery, synthesis of carnosine and anserine increased (Harms & Winnick, 1954).

2.4 Toxicological studies

Numerous studies of toxicity with amino acids were available, the majority being of short duration, employing limited protocols and conducted in the 1950s and 1960s. These studies were designed to evaluate nutritional factors related to intake of amino acids rather than potential toxicity. This monograph focuses on studies of longer duration and those that provide more detailed descriptions of protocol.

2.4.1 Acute toxicity

Oral median lethal dose (LD_{50}) values have been reported for seven of the 20 flavouring agents in this group (Table 3). In rats, LD_{50} values were in the range of 1890 to 12 400 mg/kg bw (Breglia et al., 1973; Takasaki et al., 1973; Sprince et al., 1974). In mice, oral LD_{50} values have been reported for four flavouring agents of the 20 in this group. The values ranged from greater than 1000 to 8380 mg/kg bw (Bersin et al.,1956; Doull et al., 1962; Takasaki et al., 1973; Llobet et al., 1988). These LD_{50} values indicate that the oral acute toxicity of these flavouring agents is low.

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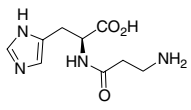
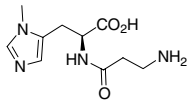
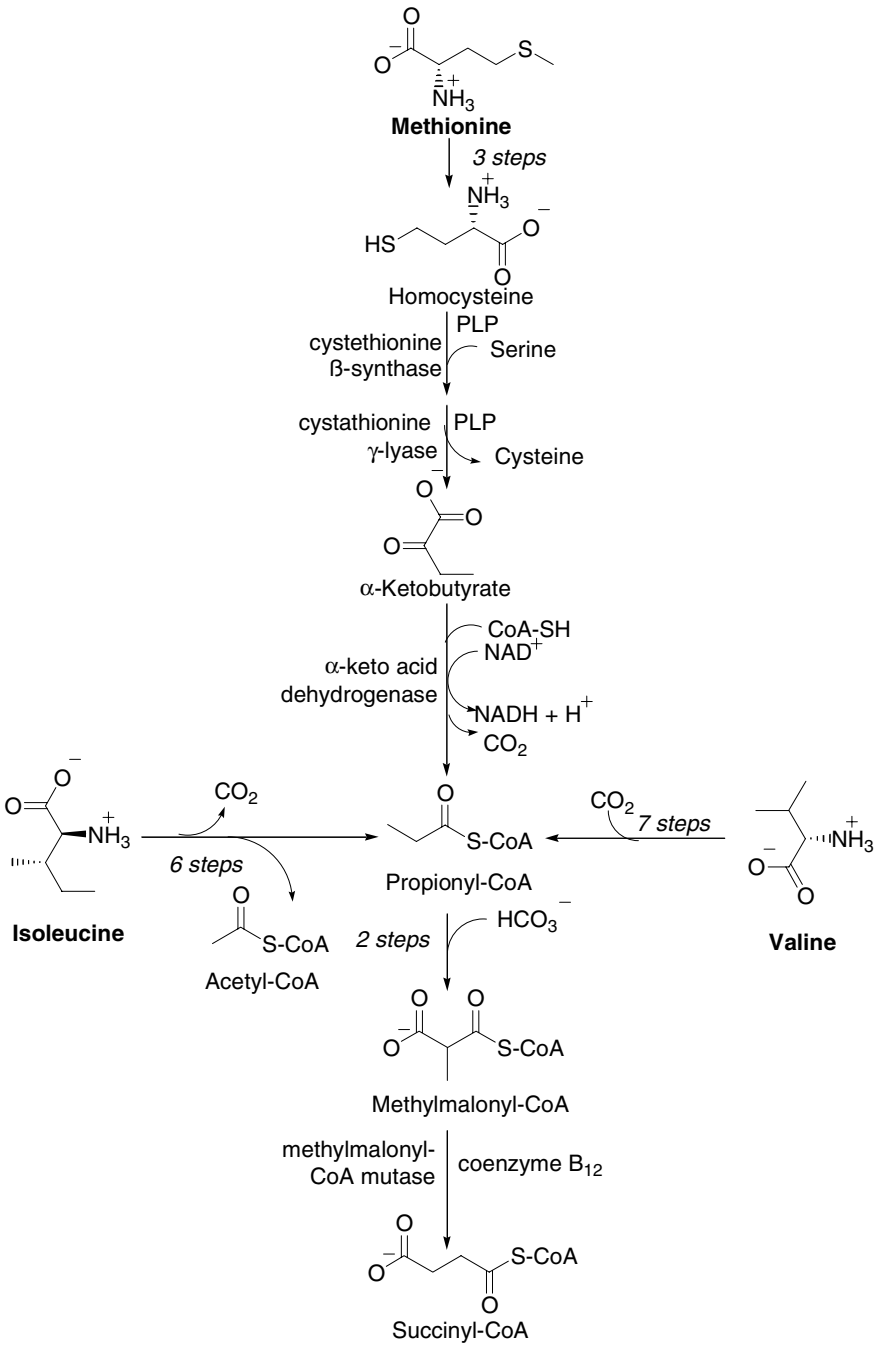
[3] 

carnosine (β-alanyl-L-histidine)

[4] 

anserine (β-alanyl-1-methyl-L-histidine)

Figure 5. Catabolic pathways for isoleucine, methionine and valine



From Nelson & Cox (2000)
CoA, coenzymze A; PLP, pyridoxal phosphate

Table 3. Studies of acute toxicity with amino acids and related substances administered orally

No.	Flavouring agent	Species	Sex	LD_{50} (mg/kg bw)	Reference
1419	L-Cysteine	Mouse	M, F	3550 (M) 4200 (F)	Takasaki et al. (1973)
1419	L-Cysteine	Rat	M, F	6350 (M) 5580 (F)	Takasaki et al. (1973)
1419	L-Cysteine	Rat	NR	1890	Sprince et al. (1974)
1419	L-Cysteine	Mouse	M	1502	Llobet et al. (1988)
1421	Glycine	Rat	M, F	3340	Breglia et al. (1973)
1424	D,L-Methionine	Mouse	M	>2000	Doull et al. (1962)
1427	DL-(3-Amino-3-carboxypropyl) dimethylsulfoinium chloride	Mouse	NR	8380	Bersin et al. (1956)
1435	Taurine	Mouse	M	>1000	Doull et al. (1962)
1438	L-Arginine	Rat	M, F	12400	Breglia et al. (1973)
1439	L-Lysine	Rat	M, F	10130	Breglia et al. (1973)

F, female; M, male; NR, not reported

(a) Short-term studies of toxicity

Short-term studies of toxicity have been undertaken for a number of the amino acids and related substances in this group. In those cases where a no-observed-effect level (NOEL) can be derived, the results are provided in Table 4.

(i) β-Alanine (No. 1418) and L-glutamic acid (No. 1420)

Rats

In a study designed to examined the effect of β-alanine on the natural levels of taurine in heart and retinal tissues, groups of 15 or 18male Sprague-Dawley rats were given drinking-water containing β-alanine at 0 or 3% (approximately 3000 mg/kg bw per day) for up to 6 weeks. Four treated and three control rats were killed at the end of weeks 1, 2 and 3, and the remaining six treated and six control rats were killed at the end of week 6. Treated rats exhibited a transient, but significant decrease in levels of taurine in the heart at weeks 1, 2, and 3. No difference between the test and control group was noted at week 6. Retinal content of taurine was unaffected by treatment with β-alanine. Compared with the age-matched controls, body-weight gain was reduced among the treated animals at all time-points; however, the decrease was not statistically significant. Orally administered β-alanine was not considered to have a toxicologically significant effect on taurine levels (Lake & De Marte, 1988).

Two weanling male albino rats were given a diet containing a mixture of L-glutamic acid (No. 1420) at 0.7% (700 mg/kg bw per day) and β-alanine at 0.4%

Table 4. Results of short-term studies of toxicity and long-term studies of toxicity and carcinogenicity with amino acids and related substances used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/no. per group[b]	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
Short-term studies of toxicity							
1419	L-Cysteine	Mouse; M, F	3/16–24	Gavage	30	<200	Takasaki et al. (1973)
1419	L-Cysteine	Rat; M, F	3/18–24	Gavage	30	<200	Takasaki et al. (1973)
1419	L-Cysteine	Rat; M	3/10–12	Gavage	180	<100	Takasaki et al. (1973)
1422	D,L-Isoleucine	Rat; M, F	4/20	Diet	90	1 250	Kawabe et al. (1996)
1425	L-Proline	Rat; F	1/7	Drinking-water	30	50	Kampel et al. (1989)
1435	Taurine	Rat; M	1/6	Diet	56	5 000	Hwang et al. (2000)
1437	D,L-Alanine	Rat; M, F	3/16	Diet	182	10 000	Chow et al. (1976)
1439	L-Lysine	Rat; F	1/6	Drinking-water	30	<50	Kampel et al. (1989)
Long-term studies of toxicity and carcinogenicity							
1421	Glycine	Rat; M, F	2/100	Drinking-water	756	<2 500	Kitahori et al. (1994)
1435	Taurine	Rat; M, F	2/16	Diet	540	500	Takahashi et al. (1972)
1420	L-Glutamate, sodium salt[c]	Rat; M, F	3/40	Diet	728	4 000	Owen et al. (1978)
1420	L-Glutamic acid[c]	Rat; M, F	6/70	Diet	728	4 000	Ebert et al. (1979)

F, female; M, male

[a] Total number of test groups does not include control animals

[b] Total number per test group includes both male and female animals

[c] Previously considered by the Committee (Annex 1, Reference 77)

(400 mg/kg bw per day) for 56 days. Compared with a control group of four rats, maintained for only 45 days, treated animals showed a reduction in weight gain. No skeletal or aortic lesions were observed as a result of the addition of 0.7% L-glutamic acid and 0.4% β-alanine to the diet (Wawzonek et al., 1955).

(ii) L-Cysteine (No. 1419)

Mice

In a 30-day study, groups of male and female ICR-JCL mice (8–12 of each sex per group) were given suspensions of L-cysteine in 1% CMC solution at a dose of 0, 200, 1000 or 3000 mg/kg bw per day orally via gavage. Throughout the course of the experiment, general condition, body-weight gain, and food and water consumption were monitored. After 30 days, all surviving mice were subjected to clinical chemistry tests and then necropsied. All animals were examined macroscopically, and selected organs were weighed and tissues preserved for histological evaluation. No clinical symptoms of toxicity and no early deaths were reported at the lowest dose (200 mg/kg bw per day). Two mice (one of each sex) in the group receiving the intermediate dose (1000 mg/kg bw per day) died before the end of the study. Animals in the group receiving the highest dose (3000 mg/kg bw per day) exhibited lethargy and only three mice (two female and one male) survived to the completion of the study. These surviving mice were reported to be two-thirds the weight of the control mice, as a result of an initial weight loss. At the lowest dose (200 mg/kg bw per day), animals were reported to gain slightly less weight than controls while weight gain of animals at the intermediate dose (1000 mg/kg bw per day) was comparable to that of controls. Water and food consumption of treated animals was generally less than that of the controls. Clinical chemistry indicated a significant decrease in concentrations of blood urea nitrogen and glucose, transaminase activity in males at the intermediate dose (1000 mg/kg bw per day), and an increase in total protein concentrations in females at the intermediate dose. Haematology analysis indicated no differences between the control and test animals. Analysis of organ weights indicated a decrease in absolute weights of the adrenal and an increase in absolute weights of the thymus in females at the intermediate and lowest doses, respectively, and a decrease in absolute weight of the seminal vesicles in males at the lowest and intermediate doses. Necropsy of the surviving animals revealed no significant changes; however, haemorrhage and blood congestion in the internal organs and in the brain were reported in the animals that died. Histopathology indicated all treated mice had blood congestion and haemorrhage in the lungs. Mice receiving cysteine at a dose of 1000 or 3000 mg/kg bw per day showed congestion of the tissues surrounding the follicles in the spleen, accompanied by atrophy of the follicles at the highest dose. Approximately 25–30% of mice at the lowest dose and <25% of the mice at 3000 mg/kg bw per day exhibited disappearance of hepatic cell nuclei. Congestion of the liver was also reported in the control group (Takasaki et al., 1973).

Rats

In a 30-day study, groups of male and female SD-JCL rats (9–12 of each sex per group) were given L-cysteine at a dose of 0, 200, 1000 or 5000 mg/kg bw per day orally via gavage. The protocol was the same as for the study in mice, described above. All males and females at the highest dose died before the end of the study. Food consumption for animals at the highest dose was slightly decreased. Water consumption of both sexes was decreased at the intermediate dose. With the exception of an increase in blood urea nitrogen and glucose concentrations, and a decrease in total protein concentrations in males at the lowest and intermediate doses, respectively, the results of tests for haematology and liver function were otherwise unremarkable. Absolute weights of the liver were significantly elevated in females at the lowest and intermediate doses and in males at intermediate dose. In both males and females at the intermediate dose, a significant increase in relative weights of the kidney was observed. Absolute weights of the thymus were increased in females at 5000 mg/kg bw per day. At necropsy, no obvious changes were observed in the rats that survived the experiment. Those that died prematurely showed blood congestion and haemorrhage in the internal organs. More specifically, histological evaluation revealed blood congestion in the lungs, liver, kidneys, and cerebrum of rats at 5000 mg/kg bw per day. At the intermediate dose (1000 mg/kg bw per day), congestion of the lungs and liver also were reported. Animals at 200 mg/kg bw per day were reported to have blood congestion in the liver. The structure of the fascicular layer of the adrenal cortex was reported to be indefinite in animals at 200 or 1000 mg/kg bw per day, and also in the control group (Takasaki et al., 1973).

Groups of 10–12 male SD-JCL rats were given L-cysteine at a dose of 0, 100, 500 or 2000 mg/kg bw per day as a 20% suspension with 1% CMC via gavage, 6 days a week, for 6 months. All rats survived to the end of the study, and no significant differences in behaviour, body-weight gain or food and water consumption were observed. A slight, but significant decrease in blood urea nitrogen and total protein concentrations were reported in rats at 500 and 2000 mg/kg bw per day. A reduction in total protein concentrations was limited to the intermediate dose group. A slight enlargement of the liver was observed in rats at 100 and 500 mg/kg bw per day, while an increase in absolute weights of the kidney was reported at the intermediate and highest doses (500 and 2000 mg/kg bw per day, respectively). At necropsy, histological examination of treated rats showed blood congestion in the liver, lungs, spleen and kidneys, indefinite structure of the fascicular layer of the adrenal glands, and haemosiderin pigment in the spleen. However, these same changes, except for the congestion of the kidneys, also were observed in the control animals. Indefinite structure of liver cells also was reported in rats receiving L-cysteine at a dose of 500 or 2000 mg/kg bw per day. No obvious changes were noted in other internal organs and the authors found it difficult to assign any significance to the findings discussed above (Takasaki et al., 1973).

(iii) D,L-Isoleucine (No. 1422)

Rats

Groups of 10 male and 10 female F344 rats (aged 4 weeks) were given diets containing 0, 1.25, 2.5, 5.0, or 8.0% D,L-isoleucine (equivalent to a dose of approximately 0, 1250, 2500, 5000 or 8000 mg/kg bw per day) for 13 weeks. Body weights and food consumption were measured weekly. No clinical signs of toxicity or mortality related to administration of isoleucine were observed throughout the test period. Body weights and food consumption were comparable in test and control animals. Urine analysis at week 13 revealed a dose-related increase in urinary pH in males receiving diets containing ≥2.5% D,L-leucine, and significantly increased urine volume in animals receiving diet containing 8% D,L-leucine. Haematological examination and blood chemical determinations at week 13 revealed a decrease in glutamic pyruvic transaminase activity at dietary concentrations of 2.5, 5.0 and 8.0% in both sexes, a slight increase in alkaline phosphatase activity in males at 5 and 8%, a statistically significant decrease in total protein in females at 5 and 8% ($p < 0.05$), and several changes in serum electrolytes in both males and females at 2.5% and greater. Significant increases in relative kidney weights were observed in both males and females at the 8% dietary level. Gross pathology was conducted at necropsy and histological examination performed on all major organs and all grossly visible lesions. Histological examination revealed no treatment-related abnormalities. The NOEL was 1250 mg/kg bw per day in rats (Kawabe et al., 1996).

(iv) D,L-Methionine (No. 1424)

Rats

Twelve male Holtzman rats were given a diet providing 2% D,L-methionine (equivalent to approximately 1000 mg/kg bw per day) for up to 12 weeks. Necropsies were performed after 2, 8 and 12 weeks of exposure. Significantly decreased weight gain was observed at 2 weeks ($p < 0.01$), but was comparable to that in control animals by 8 and 12 weeks. A significant, but transient enlargement of the kidneys ($p < 0.01$) and of the adrenals ($p < 0.05$) was reported at 2 weeks. At weeks 8 and 12, however, organ weights did not differ from those of the controls (Horger & Gerheim, 1958).

Male Sprague-Dawley rats were given diets containing 0 or 4% D,L-methionine (approximately 2000 mg/kg bw per day) for 10 weeks. The treated rats were compared with the controls at week 5 and at study termination. Body weights of treated animals were slightly decreased at both time-points. After 10 weeks, the animals were sacrificed and the enzyme activities and fat content of the liver were analysed. There was a marked depression of growth in treated animals compared with the controls. The levels of neutral fat in the livers increased along with the activities of tryptophan pyrrolase, arginase, glutamate pyruvate transaminase and glutamate oxaloacetate transaminase, however there was a marked decrease in the concentration of nicotinamide adenine dinucleotide (NAD^+). The decrease in NAD^+ was

attributed to the accumulation of lipids, as NAD^+ is a necessary cofactor for the oxidation of lipids (Klain et al., 1963).

(v) L-Proline (No. 1425)

Rats

In a 30-day study, a group of seven white female Sprague-Dawley rats were given L-proline at a dose of 50 mg/kg bw per day in water. A group of 10 rats served as the control group. After 30 days, all animals were weighed, necropsied, and subjected to a complete gross examination. Histopathology and microscopic examinations of the liver and kidneys were conducted. Samples of serum were obtained for determination of enzyme activities, and concentrations of creatinine and total protein. There were no treatment-related effects in rats given L-proline at 50 mg/kg bw per day compared with the controls (Kampel et al., 1989).

(vi) L-Phenylalanine (No. 1428)

Rats

In a limited study of toxicity in rats, young Wistar rats were given water containing 7% L-phenylalanine by gavage from postnatal days 1 to 21. At day 21, they were placed on a solid diet supplemented with 7% L-phenylalanine for 7 days. This dietary level corresponds to an average daily intake of of L-phenylalanine of approximately 7000 mg/kg bw. There were no concurrent controls in this study. A high rate of mortality was reported and the rats demonstrated signs of toxicity which included lesions of the eyes, swollen toes and some toe atrophy, as well as difficulty in urinating. Necropsy revealed swelling of the bladder and obstruction of the urethra in the more severe cases (number unspecified). The urine contained small white crystals, which were identified as primarily tyrosine, a metabolite of phenylalanine (Dolan & Godin, 1966).

(vii) Taurine (No. 1435)

Rats

In an 8-week feeding study, a group of six male weanling Wistar rats were given a diet containing either 0 or 5% taurine (corresponding to an average daily intake of 0 or 5000 mg/kg bw). Food and water were available ad libitum. Body weights of the taurine-treated rats, recorded after each feeding, were comparable to those of the controls throughout the entire study period. Blood samples drawn at 2-week intervals for analysis of aspartate transaminase, alanine transaminase and alkaline phosphatase activities revealed no significant differences between control and treated animals. At termination, no significant differences in the relative weights of liver and kidney were reported between the control and treated animals (Hwang et al., 2000).

(viii) D,L-Alanine (No. 1437)

Rats

In a 26-week feeding study, groups of eight male and eight female weanling Wistar rats were given diets containing 0, 5, 10 or 20% alanine (equivalent to 0, 5000, 10 000 or 20 000 mg/kg bw per day). Food and water consumption and body weights recorded every two weeks revealed a dose-related increase in food consumption in males. All rats appeared to be healthy throughout the study period. Rats at the highest dose gained 20–30% less weight than those at the lower doses. Pooled samples of urine collected at weeks 13–14 and at weeks 25–26 showed no significant differences between test and control groups; however, increased intake of alanine was associated with a 100–1000-fold increase in the urinary excretion of this amino acid. The authors suggested that groups given the highest dose may have experienced hyperalaninaemia, possibly leading to an increase in concentrations of serum glucose and insulin. At termination, blood samples were collected, a gross pathological examination was performed and the liver and kidney weights were recorded. Serum concentrations of alanine increased only in males and females fed 20% D,L-alanine. Increased concentrations of ammonia in males and a decrease in concentrations of pyruvate in both sexes at the highest dose were observed. At necropsy, gross examination did not reveal any abnormalities or lesions. Although absolute weights of the kidney in treated rats were comparable to those of controls, increased relative weights of the kidney were reported in males and females fed D,L-alanine at at dietary concentration of 20%. There were no treatment-related effects in rats fed diets containing up to 10% D,L-alanine (10 000 mg/kg bw per day) (Chow et al., 1976).

(ix) L-Lysine (No. 1439)

Rats

In a 30-day study, a group of six white female Sprague-Dawley rats were given L-lysine at a dose of 50 mg/kg bw per day in tap water. An additional group of 10 untreated, healthy rats served as the control group. All animals were provided with access to food and tap water ad libitum. After 30 days, all animals were weighed, necropsied, and subjected to a complete gross examination. Histopathology and microscopic examinations of the liver and kidneys were conducted. Samples of serum were obtained for determination of enzyme activities, and concentrations of creatinine and total protein. Compared with controls, no changes were observed in final body weight, and no gross abnormalities were identified. Histopathological examination of the liver was unremarkable; however, severe proximal tubular dystrophy and necrosis, accompanied by glomerular mesangial crescents, were reported in samples of kidney. Electron microscopic studies, however, indicated the extracellular matrix of the kidney was without pathological findings. Serum analysis revealed a statistically significant increase in alkaline phosphatase and in creatinine levels ($p < 0.05$) The style of reporting of this study makes a clear interpretation of the results difficult (Kampel et al., 1989).

(x) Monosodium glutamate

Short-term studies of toxicity with glutamic acid and its salt were previously considered by the Committee at its thirty-first meeting (Annex 1, reference 77). More recent studies are summarized below.

Rats

In a 13-week study, groups of 10 weanling SPF-bred male albino Wistar rats were given either a cereal-based 'stock diet' or an acid casein-based 'purified diet' containing 6% monosodium glutamate (equivalent to approximately 6000 mg/kg bw). To investigate the mechanism of toxicity attributable to monosodium glutamate, additional groups of rats were fed with diets containing 6% monosodium glutamate plus 1.6% $NaHCO_3$ or 1.0% NH_4Cl, or 2.5% $KHCO_3$ alone. Animals were provided with access to food and water ad libitum for the duration of the experiment. Food intake and body weights were monitored weekly, with water intake measured during weeks 3, 6, 8, and 11. Urine was collected for analysis during the first 2 h of the light/dark cycle at several time-points. All animals were sacrificed and examined for gross abnormalities at the end of week 13. The urinary tissues and organs were preserved for histopathological analysis. Rats fed the purified diet containing 6% monosodium glutamate exhibited a decrease in final body weights and in body weights evaluated on day 28, compared with animals not receiving monosodium glutamate. No variation in body weight was observed among any of the groups maintained on the stock diet, nor were any differences in food intake reported. Relative weights of the kidney were increased in groups feeding on diets supplemented with monosodium glutamate only, reaching levels of statistical significance in animals receiving the purified diet containing monosodium glutamate. Feeding with diets that containined monosodium glutamate caused the urine to become markedly more alkaline. However, rats fed the purified diet produced urine of higher acidity than the rats fed the stock diet, a finding that was attributed to the greater excess of base in the stock diet. Rats receiving monosodium glutamate in the stock diet showed a clearly increased incidence and degree of hyperplasia of the urinary bladder epithelium. Such hyperplastic changes also occurred in rats fed the purified diet supplemented with monosodium glutamate, but only in a single animal and only to a minimal or slight degree. Hyperplasia in rats fed the purified diet was not associated with cyst formation. Simultaneous feeding with NH_4Cl salt reduced the urinary bladder hyperplasia observed in rats fed the stock diet containing monosodium glutamate. Hyperplasia of the epithelium lining the renal pelvis or renal papilla was observed in several rats fed monosodium glutamate or monosodium glutamate plus $NaHCO_3$ in either of the two diets. The authors concluded that the urinary bladder changes induced by monosodium glutamate are attributable to its alkalizing properties rather than to monosodium glutamate per se (de Groot et al., 1988).

Groups of 10 male F344 rats (aged 4 weeks) were given a diet supplemented with 5.83% monosodium glutamate (equivalent to 5830 mg/kg bw per day) for 10 weeks. Rats were given access to food and water ad libitum throughout the course of the study. Water and food consumption, and body weight were monitored throughout the study period. Urine was collected daily during weeks 4 and 10 of

the study. After 10 weeks, animals were necropsied, and the urinary bladder, kidneys and stomach subjected to microscopic examination. At week 8, body weights of treated rats were significantly reduced compared with those of untreated controls. An amorphous precipitate containing calcium phosphate was observed in the urine of some rats, with a larger amount being present at week 4 than at week 10. The urinary pH of treated rats (pH 8.0) was higher than that of the controls (pH 7.0). A significant ($p < 0.05$) decrease in urinary concentrations of creatinine was reported in the group fed monosodium glutamate (95 ± 16 mg/dl) compared with the controls (257 ± 100 mg/dl), but this was concluded to be a dilutional effect corresponding to an increase in urinary volume (increase not statistically significant). One of 10 rats receiving monosodium glutamate showed a slight hyperplasia of the limiting ridge of the forestomach. Simple urothelial hyperplasia of the renal papilla at the fornix was observed in three rats given monosodium glutamate and was associated with calcification (Cohen et al., 1995). However, subsequent studies have demonstrated that the formation of urinary calculi in rats generally occurs after administration of a variety of sodium salts at high doses (Capen et al., 1999; Cohen, 1999).

(b) Long-term studies of toxicity and carcinogenicity

(i) Glycine (No. 1421)

Rats

Groups of 50 male and 50 female F344 DuCrj rats (aged 6 weeks) were given drinking-water containing 0, 2.5 or 5% glycine (equivalent to approximately 0, 2500 or 5000 mg/kg bw per day) for 108 weeks. The animals were weighed every 1 or 2 weeks and dietary consumption was determined every 4 weeks. Haematological examination, blood chemical determinations, and urine analysis performed at study termination revealed no significant differences between the treated and control animals. At the highest dose tested, concentrations of haemoglobin were significantly increased in males and decreased in females. In females, a significant decrease in erythrocyte volume fraction was also noted. Blood chemistry revealed a decrease in serum creatine phosphokinase activity at both doses in males and females, and an increase in blood urea nitrogen also in both males and females, but only at the highest dose. Males at both doses exhibited a reduction in concentrations of creatinine. The final mean body weights of males and females given drinking-water containing 5% glycine were significantly lower ($p < 0.05$) than those of the controls. Absolute weights of the liver were significantly lower in males at 2.5 and 5%. A complete histopathological evaluation performed at necropsy revealed a high incidence of renal calcification in treated and control groups of females (control, 16 out of 41; 2.5%, 15 out of 46; 5%, 13 out of 31). A low incidence of kidney papillomas was reported in females treated with glycine (2.5%, 4 out of 46; 5%, 2 out of 31), but not in males. A renal cell carcinoma was reported in one male (1 out of of 40) treated with 5% glycine. Further detailed histopathology of the urinary system revealed hyperplasia of the transitional epithelium of the renal pelvis in 2 out of 46 of the females at the 2.5% level only. An increased incidence of necrosis of the renal papillae was reported in treated males (2.5%, 2 out

of 45; 5%, 3 out of 40) and females (2.5%, 14 out of 46; 5%, 10 out of 31) compared with the controls (0 out of 40 in males; 0 out of 41 in females). A tumour of the transitional epithelium of the renal pelvis was seen in one male control rat (1 out of 40). Owing to the organ distribution and the histological characteristics of the neoplastic lesions observed in the treated animals, with the exception of those in the renal pelvis, the authors concluded that they were spontaneous and not related to administration of glycine. These spontaneous tumours are known to occur in this strain of rats (Kitahori et al., 1994). Lesions of the urinary tract generally occur as a result of the formation of urinary tract calculi induced by exposure at high doses, which causes subsequent chronic irritation and toxicity (Capen et al., 1999; Cohen, 1999).

(ii) Histidine (No. 1431)

Groups of 50 male and 50 female F344 rats were given diets containing L-histidine monohydrochloride at a concentration of 0, 1.25 or 2.5% for 104 weeks (equivalent to 0, 0.47 and 0.96 g/kg bw per day in males, and 0, 0.56 and 1.1 g/kg bw per day in females). In the group receiving 2.5% L-histidine, there was significant depression of body-weight gain during weeks 76–105 for males and in weeks 84–104 for females. There was a slight decrease in survival rate in both males and females at 2.5% compared with controls. In females, relative weights of the brain and adrenals were significantly higher at 2.5% than those of the controls, while the absolute weights were comparable in all groups. There were significant increases in erythrocyte count, concentration of haemoglobin, erythrocyte volume fraction and platelet count in males at 2.5%. There was an increase in incidence of tumours in all groups, but there was no treatment-related increase in the incidence of any tumour (Ikezaki et al. 1996).

(iii) Taurine (No. 1435)

Groups of nine male and seven female Wistar rats (aged 7 week) were given diets containing 0, 0.5 or 5.0% taurine daily (equivalent to approximately 0, 500 or 5000 mg/kg bw per day) for 18 months. No differences in appearance and behaviour were observed between treated and control animals throughout the study. A slight, statistically insignificant depression of growth was observed in the animals given diet containing 5% taurine when compared with the controls, but no difference in average food consumption was reported between these two groups. At study termination, the animals were necropsied and peripheral blood samples were collected for haematological examination. No significant differences in organ weights and haematological values, or histological changes were observed between the control and treated animals. The haematological values obtained for treated animals were comparable to those for the controls. Males and females given diet containing 5% taurine exhibited moderate proliferation of the bile ducts; however, this was not accompanied by any other histological changes. The NOEL was 500 mg/kg bw per day (Takahashi et al., 1972).

(iv) Monosodium glutamate

Long-term studies of toxicity with glutamic acid and its salts were considered previously by the Committee at its thirty-first meeting (Annex 1, reference 77). More recent studies are summarized below.

Rats

Groups of 50 male and 50 female Fisher 344 rats (aged 5 weeks) were fed diets containing 0, 0.6, 1.25, 2.5 or 5.0% monosodium glutamate daily (equal to 0, 231, 481, 975 and 1982 mg/kg bw per day in males, and 0, 268, 553, 1121 and 2311 mg/kg bw per day in females) for 104 weeks. All animals were examined twice daily for general health and signs of toxicity. Body weights were measured weekly for the first 14 weeks and every 2 weeks thereafter until study termination. Food consumption was measured over a 2-day period before each weighing. Ten rats from each group were randomly chosen for urine analysis at week 1, and months 1, 3, 6, 12, 18 and 24. At study termination, haematological evaluations were conducted on all surviving animals. At necropsy, a gross pathological examination was performed and the organ weights of major organs (i.e. brain, heart, liver, spleen, kidneys) were measured. Extensive microscopic examinations were performed on all major organ tissues obtained from animals in the control group and at the highest dose, as well as all animals that died or were found to be in a moribund condition during the study period. Microscopic evaluation of all other animals was limited to the stomach, liver, kidneys, urinary bladder, and all gross lesions. There were no differences in physical appearance, behaviour, food consumption, or mortality between treated animals and controls throughout the study. No differences between treated and control animals were reported in any haematological parameter evaluated. A decrease in final body weight was reported in males at 5% compared with concurrent controls. Urine analysis revealed increased pH and concentrations of Na^+, and decreased concentrations of K^+ in males and females at 2.5 and 5%. Many animal models have been used to demonstrate that long-term variations in concentrations of Na^+ or K^+ and in pH are related to cell proliferation and the eventual development of tumours of the urinary bladder. However, the changes in electrolytes and pH in this study were not associated with any evidence for the development of tumours of the urinary bladder. These urine conditions promote tumour development only under specific conditions. A significant increase ($p < 0.05$) in relative weight of the urinary bladder observed in males at 5% was attributed to distension of the bladder as a result of increased volume of urine. The significantly increased ($p < 0.05$) relative weights of the kidney observed in rats of both sexes fed with diets containing 5% monosodium glutamate were attributed to the increased intake of Na^+. Transitional cell hyperplasia of the renal pelvis associated with moderate to severe chronic nephropathy was increased in the males at 1.25 and 5%, but not at 2.5% monosodium glutamate. No difference in the incidence of tumours was reported in control and treated animals of either sex at any dietary level (Shibata et al., 1995).

(c) Genotoxicity

Testing for genotoxicity has been performed for 17 representative amino acids in this group. The results of these tests are summarized in Table 5 and are described below.[5]

(i) In vitro

In the assay for reverse mutation in *Salmonella typhimurium*, negative results were reported for L-glutamic acid (No. 1420), glycine (No. 1421), methionine (No. 1424), and L-proline (No. 1425) at concentrations of up to 1000 mg/ml, with and without metabolic activation, in several strains of *S. typhimurium* (TA92, TA97, TA98, TA100, TA102, TA1530, TA1531, TA1532, TA1535, TA1537, TA1538, and TA1964) (Green & Savage, 1978; Baker & Bonin, 1981; Brooks & Dean, 1981; Ichinotsubo et al., 1981a; MacDonald, 1981; Nagao & Takahashi, 1981; Richold & Jones, 1981; Rowland & Severn, 1981; Trueman, 1981; Vennitt & Crofton-Sleigh, 1981; Haworth et al., 1983; Fujita et al., 1994).

When evaluated in various strains of *Escherichia coli*, methionine (No. 1424) at concentrations of up to 6000 μg/plate (or 1 000 000 μg/ml), with or without metabolic activation did not show any evidence of mutagenic activity (Fluck et al., 1976; Ichinosubo et al., 1981b; Matsushima et al., 1981; Mohn et al., 1981; Venitt & Crofton-Sleigh, 1981). When *E. coli* WP2 *uvr*A was incubated with methionine at a concentration of 10, 100 or 1000 μg/ml in a non-standard microtitre assay for fluctuation, a higher number of revertants was observed in the absence of metabolic activation at the highest dose tested, but not in the presence of metabolic activation (Gatehouse, 1981). The authors suggested that excess methionine has an inhibitory effect on auxotrophs that require tryptophan, including this strain of *E. coli*, allowing spontaneous revertants to dominate the culture (Gatehouse, 1981). When *E. coli* strains WP2 and WP67 *uvr*A*pol*A, and CM871 *uvr*A*rec*A*lex*A were incubated with methionine at concentrations of up to 2500 μg/ml, slight increases in the number of revertants were reported in the presence of metabolic activation. The authors, however, were of the opinion that these results were an artefact of enhanced bacterial growth caused by the presence of a high concentration of methionine and the metabolic activation system, S9, and, therefore, concluded that the results of the test were negative (Green, 1981). In a similar study, using the same strains of *E. coli* (WP2, WP67 *uvr*A*pol*A and CM871 *uvr*A*rec*A*lex*A) D,L-methionine gave negative results with and without metabolic activation at concentrations of up to 1000 μg/ml (Tweats, 1981).

No evidence of mutagenicity was reported when various strains of *E. coli* were incubated with valine (No. 1426), L-histidine (No. 1431), or L-tyrosine (No. 1434) at concentrations of up to 5000 μg/plate without metabolic activation (Fluck et al., 1976; Martinez et al., 2000).

In a Rec assay using *Bacillus subtilis* H17 and M45, glycine (No. 1421), D,L-isoleucine (No. 1422), D,L-methionine (No. 1424), D,L-valine (No. 1426), and

[5] For conversions used, see Table 4.

D,L-alanine (No. 1437) consistently gave negative results when incubated at concentrations up to 10 000 µg/ml with and without metabolic activation (Kada, 1981; Kuroda et al. 1984).

In a test designed to investigate potential induction of aneuploidy in *Saccharomyces cerevisiae* strain D6, D,L-methionine (No. 1424) at a concentration of 50 µg/ml was considered to produce a false positive response (Parry & Sharp, 1981). The authors concluded that the effect was caused by selective stimulation of growth of monosomic cells in a methionine-rich environment, at the expense of the original diploid cells, owing to loss of the wild-type allele of the *Met*13 gene in the monosomic cells. Although methionine (dissolved in dimethylsulfoxide) at concentrations of up to and including 750 µg/ml caused a doubling of the number of revertants compared with control values in a test evaluating mitotic gene conversion in *S. cerevisiae* strain JD1; the difference was not statistically significant (Sharp & Parry, 1981). Additionally, when distilled water was used as the solvent, the numbers of revertants observed with methionine at concentrations of up to and including 750 µg/ml were comparable to those in the controls. Overall, the authors concluded that methionine does not induce mitotic aneuploidy or gene conversion in the yeast strains tested (Parry & Sharp, 1981; Sharp & Parry, 1981). In *S. cerevisiae* strains D7 and D4, D,L-methionine at concentrations up to 1600 µg/ml and 333 µg/plate, respectively, tested negative for mitotic gene conversion with and without metabolic activation (Jagannath et al., 1981; Zimmermann & Scheel, 1981).

In an assay for forward mutation in mouse lymphoma L5178Y $Tk^{+/-}$ cells, D,L-methionine (No. 1424) and L-tyrosine (No. 1434) did not induce mutations at concentrations up to 3000 µg/ml and 271.8 µg/ml, respectively (Jotz & Mitchell, 1981; Garberg et al., 1988).

Sixteen amino acids (L-cysteine, No. 1419; L-glutamic acid, No. 1420; glycine, No. 1421; L-isoleucine, No. 1422; leucine, No. 1423; L-methionine, No. 1424; L-proline, No. 1425; L-valine, No. 1426; L-phenylalanine, No. 1428; L-aspartic acid, No. 1429; L-glutamine, No. 1430; L-histidine, No. 1431; L-tyrosine, No. 1434; L-alanine, No. 1437; L-arginine, No. 1438; L-lysine, No. 1439) at concentrations of 10, 50 and 100 µg/ml produced slight increases in sister chromatid exchanges (SCEs) in human lymphocytes. The elevated frequencies of SCE were similar at all three doses and were considered to be metabolic rather than genotoxic responses (Xing & Na, 1996). Furthermore, D,L-methionine (100–5000 µg/ml) and taurine (No. 1435) (125 µg/ml) showed no evidence of SCE in Chinese hamster ovary cells, with or without metabolic activation (Evans & Mitchell, 1981; Natarajan & van Kesteren-van Leeuwen, 1981; Perry & Thomson, 1981; Cozzi et al., 1995). Also, no SCEs were observed when Chinese hamster V79 cells were incubated with L-cysteine (No. 1419) (12.1–121 µg/ml) (Speit et al., 1980).

L-Cysteine (No. 1419), D,L-methionine (No. 1424), L-glutamine (No. 1430), L-tyrosine (No. 1434), and taurine (No. 1435) produced uniformly negative results in assays for chromosomal aberration when incubated at concentrations of up to 5000 µg/ml in Chinese hamster ovary cells (Natarajan & van Kesteren-van Leeuwen, 1981; Stich et al., 1981; Cozzi et al., 1995; Tavares et al., 1998). Similarly, in Chinese hamster lung V79 fibroblasts incubated with methionine at concentrations

Table 5. Results of studies of genotoxicity with amino acids and related substances used as flavouring agents

No.	Flavouring agent	End-point	Test system	Dose or concentration	Result	Reference
1419	L-Cysteine	Sister chromatid exchange	Chinese hamster V79 cells	10^{-4}–10^{-3} mol/l TA98 (12.1–121 μg/ml)[a]	Negative[b]	Speit et al. (1980)
1419	L-Cysteine	Chromosomal aberrations	Chinese hamster ovary cells	5×10^{-4} mol/l TA98 (61 μg/ml)[a]	Negative	Stich et al. (1981)
1419	L-Cysteine	Sister chromatid exchange	Human lymphocytes	47, 87 or 137 μg/ml[c]	Negative[d,e]	Xing & Na (1996)
1420	L-Glutamic acid	Reverse mutation	*S. typhimurium* TA97 & TA102	10, 50, 100, 500 or TA98 1 000 μg/plate	Negative[f,g]	Fujita et al. (1994)
1420	L-Glutamic acid	Sister chromatid exchange	Human lymphocytes	25, 65 or 115 μg/ml	Negative[d,e]	Xing & Na (1996)
1421	Glycine	Reverse mutation	*S. typhimurium* TA97 & TA102	100, 500, 1 000 or TA98 10 000 μg/plate	Negative[f,g]	Fujita et al. (1994)
1421	Glycine	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA98 & TA100	≤10 000 μg/plate	Negative[f]	Haworth et al. (1983)
1421	Glycine	Recombination	*B. subtilis* H17 & M45	1660 μg/plate	Negative[b,g]	Kuroda et al. (1984)
1421	Glycine	Sister chromatid exchange	Human lymphocytes	17, 57 or 107 μg/ml	Negative[d,e]	Xing & Na (1996)
1422	D,L-Isoleucine	Recombination	*B. subtilis* H17 & M45	≤2 000 μg/plate	Negative[g]	Kuroda et al. (1984)
1422	D,L-Isoleucine	Recombination	*B. subtilis* H17 & M45	≤5 000 μg/ml	Negative[g]	Kuroda et al. (1984)
1422	L-Isoleucine	Sister chromatid exchange	Human lymphocytes	47, 87 or 137 μg/ml[c]	Negative[d,e]	Xing & Na (1996)
1423	L-Leucine	Sister chromatid exchange	Human lymphocytes	47, 87 or 137 μg/ml[c]	Negative[d,e]	Xing & Na (1996)
1424	Methionine (unspecified)	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98 & TA100	2–2 000 μg/plate	Negative[b]	Richold & Jones (1981)
1424	Methionine (unspecified)	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98 TA98& TA100	0.1–2 000 μg/plate	Negative[c]	Rowland & Severn (1981)

1424	Methionine (unspecified)	Reverse mutation	*S. typhimurium* TA98 & TA100	≤1 000 000 µg/ml	Negative[e]	Ichinosubo et al. (1981a)
1424	Methionine (unspecified)	Reverse mutation	*S. typhimurium* TA1537, TA98 & TA100	5000 µg/plate	Negative[b]	MacDonald (1981)
1424	Methionine (unspecified)	Reverse mutation	*S. typhimurium* TA1537, TA98 & TA100	NR	Negative[b]	Nagao & Takahashi (1981)
1424	Methionine (unspecified)	Reverse mutation	*S. typhimurium* TA1535, TA1537,TA1538, TA98 & TA100	≤10 000 µg/plate	Negative[b]	Baker & Bonin (1981)
1424	Methionine (unspecified)	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98, TA92 & TA100	0.2, 2, 20, 200 or 2000 µg/plate	Negative[b,c]	Brooks & Dean (1981)
1424	Methionine (unspecified)	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98 & TA100	4, 20, 100, 500 or 2500 µg/plate	Negative[b]	Trueman (1981)
1424	Methionine (unspecified)	Reverse mutation	*S. typhimurium* TA98 & TA100	10, 20, 50, 100, 200 or 500 µg/plate	Negative[b]	Venitt & Crofton-Sleigh (1981)
1424	Methionine (unspecified)	Mutation	*E. coli* WP2 & WP2 *uvr*A	≤500 µg/plate	Negative[b]	Venitt & Crofton-Sleigh (1981)
1424	Methionine (unspecified)	Mutation	*E. coli* JC2921, JC9239, JC8471, JC5519, JC7689, JC7623	≤1 000 000 µg/ml	Negative[f]	Ichinosubo et al. (1981b)
1424	Methionine (unspecified)	Mutation	*E. coli* WP2 *uvr*A & WP2 *uvr*A(pKM101)	NR	Negative[c,h]	Matsushima et al. (1981)
1424	Methionine (unspecified)	Mutation	*E. coli* 343/113/*uvr*B & 343/113/*uvr*B/*leu*8	≤4 000 µg/ml	Negative[b]	Mohn et al. (1981)
1424	Methionine (unspecified)	Mutation	*E. coli* WP2, WP67 *uvr*A*pol*A & CM871 *uvr*A*rec*A*lex*A	250, 500 or 1 000 µg/ml	Negative[b]	Tweats (1981)
1424	Methionine (unspecified)	Mutation	*E. coli* WP2, WP67 *uvr*A*pol*A & CM871 *uvr*A*rec*A*lex*A	2500 µg/ml	Negative[b]	Green (1981)
1424	D,L-Methionine	Mutation	*E. coli* P3478	6000 µg/plate	Negative[e]	Fluck et al. (1976)

Table 5. (Contd)

No.	Flavouring agent	End-point	Test system	Dose or concentration	Result	Reference
1424	D,L-Methionine	Mutation	*E. coli* WP2 *uvr*A	10–1 000 μg/ml	Positive[e,f,i]	Gatehouse (1981)
1424	D,L-Methionine	Mutation	*E. coli* WP2 *uvr*A	10–1 000 μg/ml	Negative[f,i]	Gatehouse (1981)
1424	D,L-Methionine	Recombination	*B. subtilis* H17*rec*$^+$ & M45*rec*$^-$	20 μl/disk	Negative[j]	Kada (1981)
1424	D,L-Methionine	Mitotic gene conversion	*S. cerevisiae* D4	0.3–333 μg/plate	Negative[b]	Jagannath et al. (1981)
1424	D,L-Methionine	Aneuploidy	*S. cerevisiae* D6	50 μg/ml	Negative[b,k]	Parry & Sharp (1981)
1424	D,L-Methionine	Mitotic gene conversion	*S. cerevisiae* D7	1600 μg/ml	Negative[f]	Zimmerman & Scheel (1981)
1424	D,L-Methionine	Mitotic gene conversion	*S. cerevisiae* JD1	≤750 μg/ml	Negative[b]	Sharp & Parry (1981)
1424	D,L-Methionine	Forward mutation	Mouse lymphoma L5178Y cells	46.9–3 000 μg/ml	Negative[b]	Jotz & Mitchell (1981)
1424	D,L-Methionine	Forward mutation	Mouse lymphoma L5178Y *Tk*$^{+/-}$	0.0005–0.015 mol/l (74.6–2 238 μg/ml)[m]	Negative [f,i]	Garberg et al. (1988)
1424	L-Methionine	Sister chromatid exchange	Human lymphocytes	21, 61 or 111 μg/ml[c]	Negative[d,e]	Xing & Na (1996)
1424	D,L-Methionine	Sister chromatid exchange	Chinese hamster ovary cells	0.1, 1, 10 or 100 μg/ml	Negative[b]	Perry & Thomson (1981)
1424	D,L-Methionine	Sister chromatid exchange	Chinese hamster ovary cells	1670, 3300 or 5 000 μg/ml	Negative[g]	Natarajan & van Kesteren-van Leeuwen (1981)
1424	D,L-Methionine	Sister chromatid exchange	Chinese hamster ovary cells	21.88–350 μg/ml	Negative[b]	Evans & Mitchell (1981)
1424	D,L-Methionine	Chromosomal aberrations	Chinese hamster ovary cells	1670, 3300 or 5 000 μg/ml	Negative[f]	Natarajin & van Kesteren-van Leeuwen (1981)
1424	D,L-Methionine	Chromosomal aberration	Rat hepatocytes	50, 100 or 200 μg/ml	Inconclusive	Dean (1981)
1424	D,L-Methionine	Chromosomal aberration	Chinese hamster lung fibroblast V79 cells	0.3, 1, 3, or 10 mmol/l (45–1 494 μg/ml)[m]	Negative[b]	Swenberg et al. (1976)
1424	D,L-Methionine	Unscheduled DNA synthesis	Human fibroblast cells	≤1 000 μg/ml	Negative[f]	Agrelo & Amos (1981)

1424	D,L-Methionine	Unscheduled DNA synthesis	Human WI-38 fibroblasts	63–1 000 μg/ml	Negative[e]	Robinson & Mitchell (1981)
1424	D,L-Methionine	Unscheduled DNA synthesis	Human WI-38 fibroblasts	125–2 000 μg/ml	Positive[f]	Robinson & Mitchell (1981)
1424	D,L-Methionine	Unscheduled DNA synthesis	HeLa S3 cells	0.1–100 μg/ml	Negative[b]	Martin & McDermid (1981)
1425	L-Proline	Reverse mutation	*S. typhimurium* TA1530, TA1531, TA1532, & TA1964	0.5 mol/l (57 550 μg/plate)[n]	Negative[b]	Green & Savage (1978)
1425	L-Proline	Sister chromatid exchange	Human lymphocytes	25, 65 or 115 μg/ml[c]	Negative[d,e]	Xing & Na (1996)
1426	Valine (unspecified)	Mutation	*E. coli* P3478	500 μg/plate	Negative[e]	Fluck et al. (1976)
1426	D,L-Valine	Recombination	*B. subtilis* H17 & M45	≤2 000 μg/plate	Negative[g]	Kuroda et al. (1984)
1426	D,L-Valine	Recombination	*B. subtilis* H17 & M45	≤10 000 μg/ml	Negative[g]	Kuroda et al. (1984)
1426	L-Valine	Sister chromatid exchange	Human lymphocytes	25, 65 or 115 μg/ml[c]	Negative[d,e]	Xing & Na (1996)
1428	L-Phenylalanine	Sister chromatid exchange	Human lymphocytes	21, 61 or 111 μg/ml[c]	Negative[d,e]	Xing & Na (1996)
1429	L-Aspartic acid	Sister chromatid exchange	Human lymphocytes	25, 65 or 115 μg/ml	Negative[d,e]	Xing & Na (1996)
1430	L-Glutamine	Sister chromatid exchange	Human lymphocytes	229, 269 or 319 μg/ml[c]	Negative[d,e]	Xing & Na (1996)
1430	L-Glutamine	Chromosomal aberrations	Chinese hamster ovary cells	292.2 μg/ml	Negative	Tavares et al. (1998)
1431	L-Histidine	Mutation	*E. coli* WP2 *uvrA*/pKM101 *oxyR*+ & *oxyR*– (IC203)	5000 μg/plate	Negative[e]	Martinez et al. (2000)
1431	L-Histidine	Sister chromatid exchange	Human lymphocytes	21, 61 or 111 μg/ml[c]	Negative[d,e]	Xing & Na (1996)
1434	L-Tyrosine	Mutation	*E. coli* WP2 *uvrA/*pKM101 *oxyR*+ & *oxyR*– (IC203)	1000 μg/plate	Negative[e]	Martinez et al. (2000)
1434	L-Tyrosine	Forward mutation	Mouse L5178Y/Tk+/–	0.00005–0.0015 mol/l (9.06–271.8 μg/ml)[o]	Negative[b]	Garberg et al. (1988)

Table 5. (Contd)

No.	Flavouring agent	End-point	Test system	Dose or concentration	Result	Reference
1434	L-Tyrosine	Sister chromatid exchange	Human lymphocytes	25, 65 or 115 μg/ml[c]	Negative[d,e]	Xing & Na (1996)
1434	L-Tyrosine	Chromosomal aberrations	Chinese hamster ovary cells	10^{-3} mol/l (181.2 μg/ml)[o]	Negative	Stich et al. (1981)
1435	Taurine	Sister chromatid exchange	Chinese hamster ovary cells	10^{-3} mol/l (125 μg/ml)[p]	Negative[b]	Cozzi et al. (1995)
1435	Taurine	Chromosomal aberrations	Chinese hamster ovary cells	10^{-3} mol/l (125 μg/ml)[o]	Negative[b]	Cozzi et al. (1995)
1437	D,L-Alanine	Recombination	*B. subtilis* H17 & M45	≤2 000 μg/plate	Negative[g]	Kuroda et al. (1984)
1437	D,L-Alanine	Recombination	*B. subtilis* H17 & M45	≤10 000 μg/ml	Negative[g]	Kuroda et al. (1984)
1437	L-Alanine	Sister chromatid exchange	Human lymphocytes	10, 50 or 100 μg/ml[c]	Negative[d,e]	Xing & Na (1996)
1438	L-Arginine	Sister chromatid exchange	Human lymphocytes	156, 196 or 246 μg/ml	Negative[d,e]	Xing & Na (1996)
1439	L-Lysine	Sister chromatid exchange	Human lymphocytes	39, 79 or 129 μg/ml[c]	Negative[d,e]	Xing & Na (1996)
In vivo						
1424	Methionine (unspecified)	Sister chromatid exchange	Mouse	1, 10, 100 or 1 000 mg/kg bw	Negative[q]	Paika et al. (1981)
1424	Methionine (unspecified)	Micronucleus formation	Mouse	3.7, 17.5 or 35 mg/kg bw	Negative[q]	Salamone et al. (1981)
1224	Methionine (unspecified)	Micronucleus formation	Mouse	250, 500 or 1 000 mg/kg bw	Negative[q]	Tsuchimoto & Matter (1981)
1430	L-Glutamine	Chromosomal aberration	Rat	600 mg/kg bw	Negative[r]	Tavares et al. (1998)

NR, not reported.

[a] Calculated using relative molecular mass of L-cysteine = 121.2.
[b] With or without metabolic activation.
[c] Cumulative concentrations comprised of background levels present in the medium and test compound added at concentrations of 10, 50 or 100μg/ml.
[d] A slight increase in sister chromatid exchanges was attributed to the resulting metabolic imbalance.
[e] Without metabolic activation.
[f] With metabolic activation.
[g] Article in Japanese.
[h] Pre-incubation method.
[i] Microtitre fluctuation test.
[j] Owing to the selective growth inhibitory effect of methionine on auxotrophs that require tryptophan (Gatehouse, 1981).
[k] With and without the spore method of metabolic activation.
[l] Owing to selective growth of cells (Parry & Sharp, 1981).
[m] Calculated using relative molecular mass of DL-methionine = 149.2.
[n] Calculated using relative molecular mass of L-proline = 115.1.
[o] Calculated using relative molecular mass for L-tyrosine = 181.2.
[p] Calculated using relative molecular mass for taurine = 125.1.
[q] Administered by the intraperitoneal route.
[r] Administered by gavage.v

of 45 to 1494 μg/ml, no increase in the frequency of chromosome aberrations was reported (Swenberg et al., 1976). In a study evaluating the potential induction of chromosomal aberrations in rat hepatocytes treated with methionine at doses of up to 200 μg/ml, results were reported to be inconclusive (Dean, 1981).

No unscheduled DNA synthesis (UDS) was observed when human fibroblast cells were incubated with D,L-methionine (No. 1424) at a concentration of up to 1000 μg/ml with metabolic activation (Agrelo & Amos, 1981). HeLa S3 cells incubated with D,L-methionine at a concentrations of up to 100 μg/ml also showed no evidence of UDS (Martin & McDermid, 1981). Furthermore, no UDS was observed in WI-38 human fibroblasts incubated with D,L-methionine at concentrations of 63 to 1000 μg/ml in the absence of metabolic activation. However, in the presence of metabolic activation, a weak UDS response was observed, which appeared to be dose-related at concentrations of 125 to 2000 μg/ml (Robinson & Mitchell, 1981).

(ii) In vivo

Three male and three female Wistar rats were given glutamine (No. 1430) as a single dose at 600 mg/kg bw by gavage. Preparations of metaphase cells were obtained from samples of bone marrow. When compared with untreated controls, rats receiving glutamine did not exhibit an increase in the number of chromosomal aberrations (Tavares et al., 1998).

Male CBA/J mice were given methionine (No. 1424) at a dose of 0, 1, 10, 100 or 1000 mg/kg bw by intraperitoneal injection. Partial hepatectomy was performed on half of the animals, which were then given up to 54 h to allow for liver regeneration. At termination, the mice were sacrificed and bone marrow and hepatocytes were harvested for analysis. It was reported that, overall, methionine did not induce SCE in the bone marrow of intact or partially hepatectomized mice (Paika et al., 1981).

In a modified assay for micronucleus formation in bone marrow, B6C3F$_1$ mice were injected intraperitoneally with methionine at a dose of 35 mg/kg bw at 0 and 24 h. Samples of bone marrow were harvested at 48, 72 and 96 h, and analysed for induction of micronuclei. Methionine was reported to cause increased formation of micronuclei in samples harvested at 72 h only. However, in a subsequent test conducted under the same conditions (samples of bone marrow obtained only at 48 and 72 h), methionine at a dose of 3.7, 17.5 or 35 mg/kg bw did not increase the formation of micronuclei (Salamone et al., 1981). In another test for induction of micronuclei, CD-1 mice were given methionine at a dose of 0, 250, 500 or 1000 mg/kg bw as two intraperitoneal injections separated by an interval of 24 h. The mice were sacrificed 6 h after the last injection, and the bone marrow was harvested for analysis. There was no increase in the frequency of micronucleated polychromatic erythrocytes (Tsuchimoto & Matter, 1981).

(iii) Conclusion

The amino acids tested were found to be non-genotoxic in vitro and in vivo in a variety of test systems.

(d) Reproductive toxicity

A study was performed to determine the effect of excess L-cysteine on reproduction in rats. Groups of four pregnant CD Norwegian hooded inbred Middle Aston rats were fed diets containing L-cysteine at a concentration of either 0 or 3500 mg/kg of diet daily (equivalent to approximately 0 and 175 mg/kg bw per day, respectively) for the duration of gestation and throughout weaning of the pups. At weaning, the dams and all except two male and three female offspring (retained for breeding) from each litter were sacrificed. The number of pups born, litter weight at birth and weaning, and numbers at weaning were recorded. After sacrifice, the weights of the carcass, liver and kidney were recorded, and an examination for gross lesions was performed. This protocol was followed for the second and third generations. At the fourth generation, 12 males and 24 females were retained from each diet and bred. The sixth generation was divided into four groups that were given L-cysteine at a concentration of 0, 35, 350 or 3500 mg/kg of diet daily (equivalent to approximately 0, 1.75, 17.5 and 175 mg/kg bw per day, respectively). The study was terminated at the seventh generation. Gross and microscopic examination of animals from the fifth generation revealed no evidence of pathology. There was an increase in absolute weights of the liver and kidney for animals in the group receiving the highest dose, but no significant differences in relative weights of the liver and kidney were reported for this group. The authors concluded that no significant abnormalities related to the administration of cysteine over six generations were observed (Frape et al, 1971).

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TETRAHYDROFURAN AND FURANONE DERIVATIVES

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of 18 tetrahydrofuran and furanone flavouring agents (Table 1) by the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, p 192). The Committee has not previously evaluated any member of the group.

Twelve of the 18 flavouring agents in this group (Nos 1443, 1446, 1448–1457) have been reported to occur naturally in various foods. They have been detected in strawberries, pineapple, mango, other fruits, shoyu, cooked beef and pork, fried

chicken, roasted hazelnuts and peanuts, cocoa, *maté*, black and green teas, smoked fish, popcorn, and Swiss cheese (Nijssen et al., 2003).

1.2 Estimated daily intake

The total annual volume of production of the 18 tetrahydrofuran and furanone derivatives is approximately 40 000 kg in both Europe (International Organization of the Flavor Industry, 1995) and in the USA (National Academy of Sciences, 1982, 1987; Lucas et al., 1999) (see Table 2). Approximately 92% of the total annual volume of production in Europe and approximately 98% in the USA is accounted for by 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF; No. 1446). The estimated daily intake of DMHF was 5300 μg/person in Europe and 5200 μg/person in the USA. The daily intakes of all the other flavouring agents in this group were in the range of 0.001 to 238 μg/person, with most values being at the lower end of this range (National Academy of Sciences, 1982, 1987; Lucas et al., 1999). The estimated daily per capita intake of each agent in Europe and the USA is reported in Table 1.

1.3 Absorption, distribution, metabolism and elimination

The four esters in this chemical group (Nos 1442, 1444, 1445 and 1447) are expected to be hydrolysed to tetrahydrofurfuryl alcohol and the corresponding carboxylic acids. Tetrahydrofuran derivatives are rapidly absorbed and eliminated primarily in the urine in laboratory animals.

Hydroxyl-substituted tetrahydrofuran and furanone derivatives are predicted to form glucuronic acid conjugates, which are primarily excreted in the urine. In humans fed with fresh strawberries, DMHF (No. 1446) is rapidly absorbed, conjugated in the liver with glucuronic acid and excreted in the urine. The metabolism of the tetrahydrofurfuryl alcohol derivatives is anticipated to be similar to that of the furfuryl alcohol derivatives. These compounds will not form epoxides. After hydrolysis of the tetrahydrofurfuryl esters, the resulting primary alcohol is oxidized to the corresponding carboxylic acid, conjugated and excreted in the urine (Nomeir et al., 1992). The remaining tetrahydrofurfuryl alcohol, linalool oxide (No. 1454) is a tertiary alcohol that is conjugated with glucuronic acid and excreted in the urine (Parke et al., 1974).

The alkyl-substituted tetrahydrofuran derivatives are subjected to ring or side-chain hydroxylation catalysed by human cytochrome P450 (CYP) to yield ring or side-chain-substituted alcohols that may be conjugated with glucuronic acid and excreted, or further oxidized, conjugated, and excreted in the urine (White et al., 1979; Guengerich et al., 1984; Kremers & Beaune, 1987; Ortiz de Montellano, 1995).

Genotoxicity observed with some members of the group (Nos 1446, 1449 and 1450) was considered to be an effect caused by high dose and related to a mechanism involving reactive oxygen species, rather than the generation of a reactive metabolite, such as an epoxide. DMHF (No 1446) showed no evidence of carcinogenicity in a 2-year study in which rats were given a dose of up to 400 mg/kg bw per day (Kelly & Bolte, 2003).

Table 1. Summary of the results of safety evaluations of tetrahydrofuran and furanone derivatives[a] used as flavouring agents

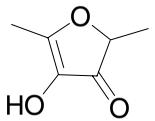
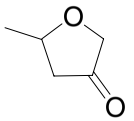
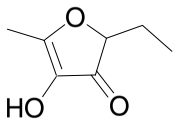
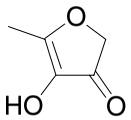
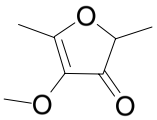
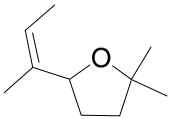
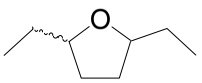
Flavouring agent	No.	CAS No. and structure	Step A3[b] Does intake exceed the threshold for human intake?	Step A4 Is the flavouring agent or are its metabolites endogenous?	Step A5 Adequate margin of safety for the flavouring agent or related substance?	Comments	Conclusion based on current intake
Structural class II							
4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	1446	3658-77-3 	Yes Europe: 5254 USA: 5203	No	Yes. The NOEL of 200 mg/kg bw per day for 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (Kelly and Bolte, 2003) is >2300 times the estimated daily intake when used as flavouring agent.	See note 2	No safety concern
2-Methyltetrahydrofuran-3-one	1448	3188-00-9 	No Europe: 24 USA: 9	NR	NR	See note 1	No safety concern
2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone	1449	27538-09-6 	No Europe: 238 USA: 13	NR	NR	See note 2	No safety concern

Table 1. (Contd)

Flavouring agent	No.	CAS No. and structure	Step A3[b] Does intake exceed the threshold for human intake?	Step A4 Is the flavouring agent or are its metabolites endogenous?	Step A5 Adequate margin of safety for the flavouring agent or related substance?	Comments	Conclusion based on current intake
4-Hydroxy-5-methyl-3(2*H*)-furanone	1450	19322-27-1 	No Europe: 56 USA: 0.07	NR	NR	See note 2	No safety concern
2,5-Dimethyl-4-methoxy-3(2*H*)-furanone	1451	4077-47-8 	No Europe: 14 USA: 0.7	NR	NR	See note 4	No safety concern
2,2-Dimethyl-5-(1-methylpropen-1-yl) tetrahydrofuran	1452	7416-35-5 	No Europe: 11 USA: 0.04	NR	NR	See note 5	No safety concern
2,5-Diethyltetrahydrofuran	1453	41239-48-9  *cis* and *trans*	No Europe: 0.01 USA: 0.09	NR	NR	See note 5	No safety concern

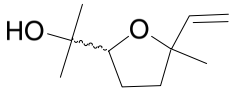
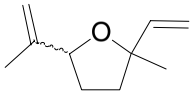
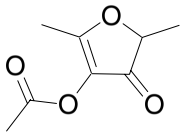
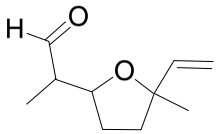
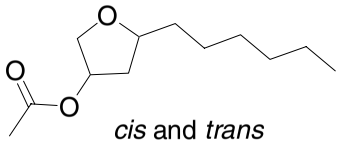
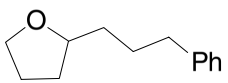
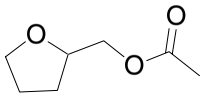
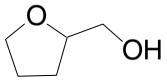
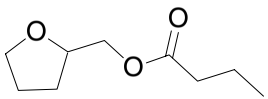
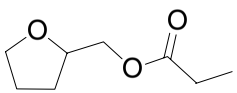
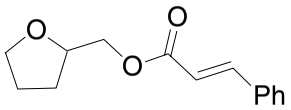
Linalool oxide	1454	1365-19-1  *cis* and *trans*	No Europe: 85 USA: 14	NR	NR	See note 2	No safety concern
5-Isopropenyl-2-methyl-2-vinyltetrahydrofuran	1455	13679-86-2  *cis* and *trans*	No Europe: 1 USA: 0.03	NR	NR	See note 5	No safety concern
4-Acetoxy-2,5-dimethyl-3(2*H*)-furanone	1456	4166-20-5 	No Europe: ND USA: 8	NR	NR	See note 3	No safety concern
(±)-2-(5-Methyl-5-vinyltetrahydrofuran-2-yl)propionaldehyde	1457	51685-39-3 	No Europe: ND USA: 0.9	NR	NR	See note 5	No safety concern
Structural class III							
2-Hexyl-4-acetoxytetrahydrofuran	1440	10039-39-1  *cis* and *trans*	No Europe: ND USA: 0.7	NR	NR	See note 8	No safety concern

Table 1. (Contd)

Flavouring agent	No.	CAS No. and structure	Step A3[b] Does intake exceed the threshold for human intake?	Step A4 Is the flavouring agent or are its metabolites endogenous?	Step A5 Adequate margin of safety for the flavouring agent or related substance?	Comments	Conclusion based on current intake
2-(3-Phenylpropyl) tetrahydrofuran	1441	3208-40-0 	No Europe: 0.001 USA: 0.7	NR	NR	See note 5	No safety concern
Tetrahydrofurfuryl acetate	1442	637-64-9 	No Europe: 0.7 USA: 8	NR	NR	See note 7	No safety concern
Tetrahydrofurfuryl alcohol	1443	97-99-4 	No Europe: 39 USA: 22	NR	NR	See note 6	No safety concern
Tetrahydrofurfuryl butyrate	1444	2217-33-6 	No Europe: 0.01 USA: 0.2	NR	NR	See note 7	No safety concern

Tetrahydrofurfuryl propionate	1445	637-65-0 	No Europe: 0.06 USA: 5	NR	NR	See note 7	No safety concern
Tetrahydrofurfuryl cinnamate	1447	65505-25-1 	No Europe: ND USA: 0.01	NR	NR	See note 7	No safety concern

CAS: Chemical Abstract Service; ND: No intake data reported; NR: Not required for evaluation because consumption of the substance was determined to be of no safety concern at step A3 of the decision-tree.

[a] Step 2: All 18 tetrahydrofuran and furanone derivatives (Nos 1440–1457) in this group are expected to be metabolized to innocuous products. The evaluation of these flavouring agents therefore proceeded via the A-side of the decision-tree.

[b] The thresholds for human intake for structural classes II and III are 540 and 90 μg/person per day, respectively. All intake values are expressed in μg/person per day. The combined intake of flavouring agents in structural class II is 5683 μg/person per day in Europe and 5249 μg/person per day in the USA. The combined intake of flavouring agents in structural class III is 40 μg/person per day in Europe and 37 μg/person per day in the USA.

Notes:

1. Reduced to corresponding alcohol, which is conjugated with glucuronic acid and excreted in the urine.
2. Conjugated with glucuronic acid and excreted in the urine.
3. The ester group is readily hydrolysed and the resulting furanone is conjugated with glucuronic acid and excreted in the urine.
4. The ether group is readily oxidized and the resulting furanone is conjugated with glucuronic acid and excreted in the urine.
5. Subjected to side-chain or ring oxidation by human cytochrome P450 to yield ring or side-chain alcohols that may be conjugated with glucuronic acid or further oxidized, conjugated and excreted in the urine.
6. Alcohol is oxidized to the corresponding carboxylic acid, conjugated and excreted in the urine.
7. The ester group is readily hydrolysed and the resulting alcohol is oxidized to the corresponding carboxylic acid, conjugated and excreted in the urine.
8. Ester group is readily hydrolysed or it undergoes side-chain or ring oxidation by human cytochrome P450 to yield ring or side-chain alcohols that may be conjugated with glucuronic acid or further oxidized, conjugated and excreted in the urine.

Table 2. Annual volumes of production of tetrahydrofuran and furanone derivatives used as flavouring agents in Europe and the USA

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		μg/day	μg/kg bw per day		
2-Hexyl-4-acetoxytetrahydrofuran (1440)					
Europe	ND	ND	ND		
USA[e]	4.00	0.7	0.01	–	NA
2-(3-Phenylpropyl)tetrahydrofuran (1441)					
Europe	0.01	0.001	0.00002		
USA	5	0.7	0.01	–	NA
Tetrahydrofurfuryl acetate (1442)					
Europe	5	0.7	0.01		
USA	64	8	0.1	–	NA
Tetrahydrofurfuryl alcohol (1443)					
Europe	270	39	0.6		
USA	168	22	0.4	+	NA
Tetrahydrofurfuryl butyrate (1444)					
Europe	0.1	0.01	0.0002		
USA[e]	1	0.2	0.003	–	NA
Tetrahydrofurfuryl propionate (1445)					
Europe	0.4	0.06	0.001		
USA	37	5	0.08	–	NA
4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone, DMHF (1446)					
Europe	36 818	5254	88		
USA	39 500	5203	87	45 601	1
Tetrahydrofurfuryl cinnamate (1447)					
Europe	ND	ND	ND		
USA[e]	0.05	0.01	0.0001	–	NA
2-Methyltetrahydrofuran-3-one (1448)					
Europe	171	24	0.4		
USA	68	9	0.1	2 409	35
2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone, HEMF (1449)					
Europe	1 666	238	4		
USA	100	13	0.2	+	NA
4-Hydroxy-5-methyl-3(2*H*)-furanone (1450)					
Europe	391	56	0.9		
USA	0.5	0.07	0.001	5	10
2,5-Dimethyl-4-methoxy-3(2*H*)-furanone (1451)					
Europe	100	14	0.2		
USA[f]	5	0.9	0.01	699	140
2,2-Dimethyl-5-(1-methylpropen-1-yl)tetrahydrofuran (1452)					
Europe	78	11	0.2		
USA[e]	0.2	0.04	0.0006	+	NA
2,5-Diethyltetrahydrofuran (1453)					
Europe	0.1	0.01	0.0002		
USA[f]	0.5	0.09	0.001	+	NA
Linalool oxide (1454)					
Europe	594	85	1		
USA	109	14	0.2	6 233	57

Table 2. (Contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b]		Annual volume in naturally occurring foods (kg)[c]	Consumption ratio[d]
		μg/day	μg/kg bw per day		
5-Isopropenyl-2-methyl-2-vinyltetrahydrofuran (1455)					
Europe	9	1	0.02		
USA[e]	0.2	0.04	0.0006	+	NA
4-Acetoxy-2,5-dimethyl-3(2*H*)furanone (1456)					
Europe	ND	ND	ND		
USA[f]	45	8	0.1	+[g]	NA
(±)-2-(5-Methyl-5-vinyltetrahydrofuran-2-yl)propionaldehyde (1457)					
Europe	ND	ND	ND		
USA[e]	5	0.9	0.01	+[h]	NA
Total					
Europe	40103				
USA	40112				

NA, not available; ND, no intake data reported; +, reported to occur naturally in foods (Nijssen et al., 2003), but no quantitative data; –, not reported to occur naturally in foods.

[a] From International Organization of the Flavor Industry (1995) and Lucas et al. (1999) or National Academy of Sciences (1982, 1987).

[b] Intake expressed as μg/person per day was calculated as follows: [(annual volume, kg) × (1×10^9 μg/kg)]/[population × survey correction factor × 365 days], where population (10%, 'eaters only') = 32×10^6 for Europe and 26×10^6 for the USA. The correction factor = 0.6 for Europe, and 0.8 for the USA, representing the assumption that only 60 and 80% of the annual volume of the flavour, respectively, was reported in the poundage surveys (National Academy of Sciences, 1982, 1987; International Organization of the Flavor Industry, 1995; Lucas et al., 1999).
Intake expressed as μg/kg bw per day was calculated as follows: [(μg/person per day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.

[c] Quantitative data for the USA reported by Stofberg & Grundschober (1987).

[d] The consumption ratio is calculated as follows: (annual consumption via food, kg)/(most recently reported volume as a flavouring agent, kg).

[e] The volume cited is the anticipated annual volume, which was the maximum amount of flavour estimated to be used annually by the manufacturer at the time the material was proposed for flavour use. Subsequent national surveys (National Academy of Sciences, 1982, 1987; Lucas et al., 1999), if applicable, revealed no reported use of the substance as a flavour ingredient.

[f] Annual volume reported in previous USA surveys (National Academy of Sciences, 1982, 1987).

[g] Personal communication from Flavor and Extract Manufacturers Association of the USA (1994).

[h] van Dort et al. (1993).

1.4 Application of the procedure for the safety evaluation of flavouring agents

Step 1. In applying the Procedure, the Committee assigned 11 of the 18 agents (Nos 1446, 1448–1457) to structural class II. Seven agents (Nos 1440–1445 and 1447) were assigned to structural class III (Cramer et al., 1978).

Step 2. All 18 tetrahydrofuran and furanone derivatives (Nos 1440–1457) in this group are expected to be metabolized to innocuous products. The evaluation of these 18 flavouring agents therefore proceeded via the A-side of the decision-tree.

Step A3. The estimated daily intakes in Europe and the USA of 10 of the 11 flavouring agents in structural class II, and of all seven of the flavouring agents in structural class III are below the threshold of concern (i.e. 540 μg/person for class II, and 90 μg/person for class III). According to the Procedure, the safety of these 17 flavouring agents raises no concern when they are used at their estimated current intakes. One of the flavouring agents in structural class II, DMHF (No. 1446), exceeds the threshold of concern for that class. The daily intake of DMHF was 5300 μg/person in Europe and 5200 μg/person in the USA. According to the Procedure, the evaluation of this flavouring agent proceeded to step A4.

Step A4. DMHF(No. 1446) or its metabolites are not endogenous. Therefore, the evaluation of this flavouring agent proceeded to step A5.

Step A5. For DMHF (No. 1446), the no-observed-effect level (NOEL) of 200 mg/kg bw per day from a 2-year dietary study in rats (Kelly & Bolte, 2003) is >2300 times greater than the estimated daily per capita intake of this agent from its use as a flavouring agent in Europe or the USA. The Committee therefore concluded that the safety of this agent would not be a concern at the estimated current intake.

The intake considerations and other information used to evaluate the 18 tetrahydrofuran and furanone derivatives in this group according to the Procedure are summarized in Table 1.

1.5 Consideration of secondary components

Two members of this group of flavouring agents (Nos 1456 and 1457) have minimum assay values of <95%. Information on the safety of the secondary components of these two compounds is summarized in Annex 5 (Summary of the safety evaluation of secondary components for flavouring agents with minimum assay values of less than 95%). The secondary component of No. 1456 (DMHF, No. 1446) was evaluated by the Committee at its present meeting and was considered not to be a concern at current estimated intakes. The secondary component of No. 1457 (6-hydroxy-2,6-dimethyl-2,7-octadienal) has not been previously evaluated by the Committee. The Committee did evaluate a structurally related compound (hydroxycitronellal, No. 611) at its fifty-third meeting (Annex 1, reference *143*) and concluded that it did not present a safety concern at estimated current

intakes. On this basis, the Committee considered that 6-hydroxy-2,6-dimethyl-2,7-octadienal did not pose a safety concern at current estimated intakes.

1.6 Consideration of combined intakes from use as flavouring agents

In the unlikely event that all seven agents in structural class III were consumed concurrently on a daily basis, the estimated combined intake would not exceed the intake threshold for class III (90 μg/person per day). In the unlikely event that all 11 agents in structural class II were consumed concurrently on a daily basis, the estimated combined intake would exceed the human intake threshold for class II (540 μg/person per day). Nevertheless, all these flavouring agents are expected to be efficiently metabolized and would not saturate metabolic pathways. Overall evaluation of the data indicated that combined intake would not raise a safety concern.

1.7 Conclusions

The Committee concluded that none of the flavouring agents in this group of tetrahydrofuran and furanone derivatives would present safety concerns at estimated current intakes. The Committee noted that the available data on the toxicity and metabolism of these tetrahydrofuran and furanone derivatives were consistent with the results of the safety evaluation using the Procedure.

2. RELEVANT BACKGROUND INFORMATION

2.1 Additional considerations on intake

Tetrahydrofuran and furanone derivatives have been detected in a variety of foods including strawberries, fruit, shoyu, cooked beef, fried chicken, cooked pork, smoked fish, coffee, green and black tea, cocoa, *maté*, roasted hazelnuts and peanuts, popcorn, and Swiss cheeses (Nijssen et al., 2003). As shown in Table 2, 12 of the flavouring agents in this group have been reported to occur naturally in foods (Nijssen et al., 2003). Quantitative data on natural occurrence and consumption ratios have been reported for five substances in the group and demonstrate that their consumption occurs predominantly from traditional foods (i.e. consumption ratio, >1) (Stofberg & Kirschman, 1985; Stofberg & Grundschober, 1987) (see Table 2).

The principal member of the group, 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone[1] (DMHF, No. 1446), is consumed as a naturally occurring constituent of approximately 20 foods. Quantitative data on natural occurrence are available for its presence in pineapple, raspberry, strawberry, mango, coffee, tea and Swiss cheese (Nijssen et al., 2003). On the basis of its presence in pineapples, raspberries, strawberries, and coffee (Stofberg & Grundschober, 1987), intake of DMHF from consumption of food is slightly greater than from its consumption as an added flavouring agent. On the basis of more recent data on natural occurrence (Nijssen et al., 2003), the intake from consumption of food exceeds that from intake as an added flavouring agent by at least 50%.

2.2 Biological data

2.2.1 Biochemical data

(a) Hydrolysis

The four esters in this chemical group (Nos. 1442, 1444, 1445 and 1447) are expected to be hydrolysed to tetrahydrofurfuryl alcohol and the corresponding carboxylic acids. In animals, the hydrolysis of esters is catalysed by classes of enzymes known as carboxylesterases or esterases (Heymann, 1980).

The half-lives of furfuryl esters incubated in artificial pancreatic fluid with pancreatin ranged from <0.01 min for aliphatic linear component carboxylic acids, such as furfuryl acetate, to 5.1 ± 0.4 min for aliphatic branched-chain carboxylic acids, such as furfuryl isopentanoate. The half-life for hydrolysis of furfuryl propionate in rat liver homogenate was also extremely rapid ($t_{1/2}$ < 0.01 min) (Buck & Renwick, 2000). By analogy, the tetrahydrofurfuryl esters in this group are anticipated to undergo rapid hydrolysis in vivo.

(b) Absorption, distribution and excretion

The tetrahydrofuran and furanone derivatives would be expected to be rapidly absorbed and eliminated primarily in the urine of animals. DMHF (No. 1446) in aqueous solution was administered intraperitoneally to four or five ICR mice as a single dose at 500 mg/kg bw, or orally as a single dose at 1000 mg/kg bw (Hiramoto et al., 1998). The maximum plasma concentration of DMHF, 170–270 μg/ml, for the group treated intraperitoneally was attained 15 min after dosing, and gradually disappeared after 2 h. Mice given DMHF orally showed a maximum plasma concentration of 100–150 μg/ml after 15–45 min, followed by complete disappearance after 2 h (Hiramoto et al., 1998).

In a similar experiment, 4-hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone[2] (HEMF, No. 1449) at a dose of 500 or 1000 mg/kg bw was given intraperitoneally or orally to a group of nine or five ICR mice, respectively (Hiramoto et al., 1998). Plasma concentrations reached a maximum 15 min after intraperitoneal administration (75–330 μg/ml) or 15–45 min after oral administration (50–100 μg/ml). Essentially all of the plasma HEMF disappeared within 2 h after either intraperitoneal or oral administration (Hiramoto et al., 1998). On the basis of these data, it can be concluded that the two furanone derivatives are rapidly cleared from the plasma of ICR mice.

Wistar rats were given DMHF at a dose of 500 mg/kg bw via intragastric tube (Li et al., 1990). The plasma concentration of DMHF reached a peak (43.1261 μg/ml) 30 min after dosing, and gradually decreased to about 35% (14.9626 μg/ml) of the maximum plasma concentrations after 8 h. Within 30 min and 8 h after dosing, it was noted that 72% and 95% of the administered dose of DMHF, respectively,

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[1] Synonym for 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, DMHF, No. 1446.
[2] Synonym for 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone, HEMF, No. 1449.

was absorbed into all organ tissues of the rats (particularly the kidney, heart, and liver). DMHF and/or its metabolites were excreted primarily in the urine, with most being excreted in the first 6 h after dosing (87% of the total amount of DMHF excreted). A minor amount (1.34% of the total amount of excreted DMHF) was eliminated in the faeces within the first 24 h. The authors provided no data as to what percentage of the administered dose of DMHF was excreted by the rats (Li et al., 1990).

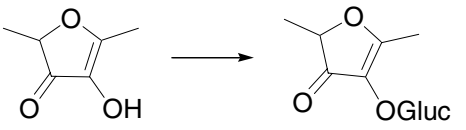
In groups of three male and female volunteers, 59–94% of an ingested dose of 65.5–180.6 mg of DMHF (total including free and glycoside conjugate), which is approximately equivalent to a dose of DMHF of 1.1–3.0 mg/kg bw, was excreted in the urine as the glucuronide conjugate within 24 h (Roscher et al., 1997). These data document the rapid absorption and elimination of DMHF and, by implication, other furanone derivatives, in animals.

(c) Metabolism

Furanone derivatives are predicted to form glucuronic acid conjugates, which are primarily excreted in the urine. Metabolic data on the principal furanone derivative in this group, DMHF, indicate that the furanones in this group would be rapidly conjugated with glucuronic acid and excreted (see Figure 1). The tetrahydrofurfuryl alcohol derivatives are anticipated to be metabolized in a manner similar to that for furfuryl alcohol derivatives. After hydrolysis of the tetrahydrofurfuryl esters, the resulting primary alcohol is oxidized to the corresponding carboxylic acid, conjugated, and excreted in the urine (Nomeir et al., 1992). The remaining tetrahydrofurfuryl alcohol, linalool oxide (No. 1454), is a tertiary alcohol that is conjugated with glucuronic acid and excreted in the urine (Parke et al., 1974). The alkyl-substituted tetrahydrofuran derivatives are subjected to ring or side-chain hydroxylation catalysed by human CYP to yield ring or side chain-substituted alcohols, which may be conjugated with glucuronic acid and excreted, or further oxidized, conjugated, and excreted in the urine (White et al., 1979; Guengerich et al., 1984; Kremers & Beaune, 1987; Ortiz de Montellano, 1995).

In a study of metabolism in humans, performed using fresh strawberries, a source of DMHF (No. 1446) (Roscher et al., 1997), two groups of three volunteers (two males and one female) fasted for 18 h and subsequently ingested either 2.5 kg of fresh strawberries from Spain, or 2.5–3.0 kg of fresh strawberries from Italy over the next 8 h. Analysis of the two batches of strawberries for free DMHF and gly-

Figure 1. Metabolic pathway for DMHF in humans



major urinary metabolite

coside conjugate content revealed that Spanish strawberries contained DMHF at a concentration of 26.2 mg/kg (ratio of free : glycoside DMHF, 2 : 13), while Italian strawberries contained DMHF at a concentration of 60.2 mg/kg (ratio of free : glycoside DMHF, 3 : 2). This corresponds to a total intake of DMHF of 65.5 mg for volunteers who ingested Spanish strawberries (approximately equivalent to 1.1 mg/kg bw), and 150.5–180.6 mg of DMHF for those who ingested Italian strawberries (approximately equivalent to 2.51–3.0 mg/kg bw). Samples of urine collected 24 h after the initial ingestion of strawberries revealed that all volunteers excreted 59–94% of the ingested DMHF as the glucuronic acid conjugate (4-hydroxy-2,5-dimethyl-3(2*H*)-furanone β-D-glucuronide). Urinary excretion of the DMHF glucuronic acid conjugate was greater in females (81–94% of the administered dose) than in males (59–69% of the administered dose), independent of the dose. No unchanged DMHF or glycosidically bound forms, DMHF glucoside or 6′-malonyl derivative, were detected in the urine of any of the subjects (Roscher et al., 1997).

In conclusion, furanone derivatives may undergo direct conjugation with glucuronic acid and rapid elimination via the urine. The esters of tetrahydrofurfuryl alcohol are predicted to undergo rapid hydrolysis, and the resulting tetrahydrofurfuryl alcohol is expected to be oxidized to the corresponding acid that is then conjugated and excreted in the urine. After undergoing ring or side-chain hydroxylation, the 4 alkyl-substituted tetrahydrofurans are conjugated and excreted, or further oxidized, conjugated, and excreted.

2.2.2 Toxicological studies

(a) Acute toxicity

Oral median lethal dose (LD_{50}) values have been reported for eight of the tetrahydrofuran and furanone derivatives in this group (see Table 3). In rats, LD_{50} values are in the range of 549 mg/kg bw for 2,5-dimethyl-4-methoxy-3(2*H*)-furanone (No. 1451) to 4500 mg/kg bw for tetrahydrofurfuryl alcohol (No. 1443), demonstrating that the acute toxicity of these compounds when administered orally is low (Gajewski & Alsdorf, 1949; Colaianni, 1967; Moreno, 1977; Cooper & Good, 1979; Levenstein, 1982; Reagan & Becci, 1984a, 1984b; Burdock & Ford, 1990). In mice, LD_{50} values range from 1000 mg/kg bw for 5-isopropenyl-2-methyl-2-vinyltetrahydrofuran (No. 1455) to >2000 mg/kg bw for 2,5-dimethyl-4-methoxy-3(2*H*)-furanone (No. 1451), also confirming the low acute toxicity of these compounds in this species (Moran & Easterday, 1980; Bonetti, 1983).

(b) Short-term studies of toxicity

The results of short-term studies with representative tetrahydrofuran and furanone derivatives are summarized in Table 4 and are described below.

Table 3. Studies of the acute toxicity of tetrahydrofuran and furanone derivatives administered orally

No.	Flavouring agent	Species	Sex	LD_{50} (mg/kg bw)	Reference
1443	Tetrahydrofurfuryl alcohol	Rat	NR	4500	Gajewski & Alsdorf (1949)
1448	2-Methyltetrahydrofuran-3-one	Mouse	M, F	1860	Moran & Easterday (1980)
1449	4-Hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone (HEMF)	Mouse	M, F	1932	Moran & Easterday (1980)
1451	2,5-Dimethyl-4-methoxy-3(2*H*)-furanone	Rat	NR	>0.5 ml/kg bw (>549 mg/kg bw)[a]	Levenstein (1982)
1452	2,2,-Dimethyl-5-(1-methylpropen-1-yl) tetrahydrofuran	Rat	M, F	3900	Cooper & Good (1979)
1453	2,5-Diethyltetrahydrofuran	Rat	M, F	3800	Burdock & Ford (1990)
1453	2,5-Diethyltetrahydrofuran	Rat	M, F	3754	Reagan & Becci (1984a)
1454	Linalool oxide	Rat	NR	1150	Moreno (1977)
1454	Linalool oxide	Rat	M, F	2210	Colaianni (1967)
1454	Linalool oxide	Rat	M, F	1924	Reagan & Becci (1984b)
1455	5-Isopropenyl-2-methyl-2-vinyltetrahydrofuran	Mouse	NR	1000–2000	Bonetti (1983)

F, female; M, male; NR, not reported.
[a] Calculated using density of 2,5-dimethyl-4-methoxy-3(2*H*)-furanone = 1.097 g/ml.

(i) 2-Hexyl-4-acetoxytetrahydrofuran (No. 1440)

Rats

In a 13-week feeding study, groups of 10–14 male and 10–14 female Charles River albino rats were given diets containing 2-hexyl-4-acetoxytetrahydrofuran (in gum arabic) at a concentration of 39, 65 and 78 mg/kg from weeks 0 to 4, 5 to 10, and 11 to 13. The control group was maintained on a basal diet containing only the vehicle, gum arabic, from weeks 0 to 13 of the study. The food consumption, food efficiency, and body weight of each rat were determined weekly. During week 7 of the study, haematological examination and clinical chemistry determinations were conducted on seven of the animals in each group. After 90 days, all the animals were killed and necropsies performed. The weights of the liver and kidney of each rat were measured, while a wide range of tissues and organs from seven of the animals in each group were subjected to histopathological examinations. Over the 13-week experimental period, the average daily intake of 2-hexyl-4-acetoxytetrahydrofuran by male and female rats was reported to be 4.45 and 5.32 mg/kg bw, respectively. Food efficiency, food consumption, body weights, and

Table 4. Results of short-term studies of toxicity and long-term studies of toxicity and carcinogenicity with tetrahydrofuran and furanone derivatives used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/ no. per group[b]	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
Short-term studies of toxicity							
1440	2-Hexyl-4-acetoxytetrahydrofuran	Rat; M, F	1/28	Diet	90	4.45[c] (M) 5.32[c] (F)	Rabinowicz (1963)
1441	2-(3-Phenylpropyl)tetrahydrofuran	Rat	1/10–16	Diet	90	42.98[c] (M) 48.58[c] (F)	Posternak et al. (1969)
1443	Tetrahydrofurfuryl alcohol	Rat; M, F	3/10	Diet	7	332[c] (M) 312[c] (F)	Arts & Lina (2003)
1443	Tetrahydrofurfuryl alcohol	Rat; M, F	3/10	Diet	28	60	Arts & Lina (2003)
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF)	Rat; M, F	1/10–16	Diet	90	6.15[c] (M) 7.04[c] (F)	Posternak et al. (1969)
1448	2-Methyltetrahydrofuran-3-one	Rat; M, F	1/46	Diet	90	91.6[c] (M) 91.2[c] (F)	Shellenberger (1970)
1449	2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone (HEMF)	Rat; M, F	1/30	Diet	93	1.43[c]	Cox & Re (1978)
1450	4-Hydroxy-5-methyl-3(2*H*)-furanone	Rat; M, F	6/6	Diet	21	146[c,d]	Munday & Kirkby (1971a)
1450	4-Hydroxy-,5-methyl-3(2*H*)-furanone	Rat; M, F	6/16	Diet	91	146[c,d]	Munday & Kirkby (1971b)
1456	4-Acetoxy-2,5-dimethyl-3(2*H*)-furanone	Rat	1/10	Diet	14	18[c,e]	Drummond (1993)
Long-term studies of toxicity and carcinogenicity							
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF)	Rat; M, F	3/120	Diet	730	200	Kelly & Bolte (2003)
1450	4-Hydroxy-5-methyl-3(2*H*)-furanone	Rat; M, F	1/96	Diet	365	146[c,f]	Munday & Kirkby (1973)

M, male; F, female; NR, not reported.

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] The study was performed with either a single dose or multiple doses that had produced no adverse effect. The value is therefore not a true NOEL, but is the highest dose tested that produced no adverse effects. The actual NOEL may be higher.

[d] Study performed using a a meat flavour cocktail (consisting of 74.1% 4-hydroxy-5-methyl-3(2*H*)-furanone, 23.2% 2,3-dimethyl-4-hydroxy-2,5-dihydrofuran-5-one, 1.8% 2-acetyl-2-thiazole, and 0.9% 2-acetylthiazoline) at a concentration of up to 3932 mg/kg of diet (a dose of approximately 197 mg/kg bw per day).

[e] Study performed using 4-acetoxy-2,5-dimethyl-3(2H)-furanone at a target dose of 18 mg/kg bw per day; however, the average dietary intakes of male and female rats were 25.2 and 23.8 mg/kg bw per day, respectively, during week 1, and 17.2 and 16.7 mg/kg bw per day, respectively, during week 2 of the study.

[f] Rats were maintained on diets containing a flavour cocktail (equivalent to a dose of 4-hydroxy-5-methyl-3(2*H*)-furanone of approximately 7, 18, 36, 73, 109 or 146 mg/kg bw per day) for the first 15 weeks, and subsequently only on diets containing -hydroxy-5-methyl-3(2*H*)-furanone at the highest dose of 146 mg/kg bw per day for an additional 37 weeks.

relative weightsof the kidney and liver were not significantly different between control and treated groups. In males, the percentage of haemoglobin and mean corpuscular concentration of haemoglobin were significantly increased during week 7, but only the percentage of haemoglobin in males was increased at week 13. The biological significance of this increase (7–10%) was not discussed by the authors. Histological examination of a wide range of tissues showed no evidence of any lesion that could be attributed to administration of the test substance (Rabinowicz, 1963).

(ii) 2-(3-Phenylpropyl)tetrahydrofuran (No. 1441)

Rats

In a 90-day dietary study, groups of 10–16 male and 10–16 female Charles River CD rats were given diets containing 2-(3-phenylpropyl)tetrahydrofuran at a concentration providing a dose of approximately 42.98 and 48.58 mg/kg bw per day to male and female rats, respectively. A control group, receiving a basal diet, also was studied. The concentration of the test material in the diet was adjusted during the study to maintain constant levels of dietary intake. Haematological examination and blood urea determinations were performed on 50% of the animals at week 7, and on all the animals at the end of the experimental period. After 90 days, all the animals were killed, and subjected to a detailed necropsy and gross histopathological examination. Clinical observations recorded daily and food consumption, food utilization, and body weights measured weekly revealed no significant differences between treated and control groups. There was no difference in absolute and relative weights of the liver or kidney between treated and control animals. No toxicologically significant effects on haematology and clinical chemistry were reported in any of the treated rats. Gross and histopathological examination of a wide range of tissues and organs from each animal showed no evidence of tissue alterations that could be associated with administration of 2-(3-phenylpropyl)tetrahydrofuran. The NOEL for 2-(3-phenylpropyl)tetrahydrofuran was 42.98 mg/kg bw per day in males and 48.58 mg/kg bw per day in females, respectively (Posternak et al., 1969).

(iii) Tetrahydrofurfuryl alcohol (No. 1443)

Rats

In a 7-day dose range-finding study, groups of five male and five female Fischer 344 rats (aged 5 weeks) were maintained on diets containing tetrahydrofurfuryl alcohol at a concentration of 0 (control), 50, 500 or 2500 mg/kg of diet. The animals were observed twice daily for clinical signs of toxicity. Body weights were recorded on days 0, 6 and 7 of the study. On day 7, the animals were killed by exsanguination through the abdominal aorta under carbon dioxide/oxygen anaesthesia. The brain, heart, kidneys, liver, spleen, ovaries, and testes were weighed. The actual consumption of tetrahydrofurfuryl alcohol was 0, 6, 62 or 332 mg/kg bw per day for male rats, and 0, 6, 62 or 312 mg/kg bw per day for female rats. There were

no compound-related clinical findings reported in any of the rats. Mean body weights, and relative and absolute organ weights were comparable beween test and control groups. No compound-related abnormalities were observed upon gross necropsy tetrahydrofurfuryl alcohol (Arts & Lina, 2003).

On the basis of the results of this 7-day range-finding study, groups of five male and five female Fischer 344 rats (aged 5–6 weeks) were given diets containing tetrahydrofurfuryl alcohol at a concentration of 0 (control), 60, 600 or 3000 mg/kg of diet for 28 days. These dietary levels were calculated by the authors to provide average daily intakes of of tetrahydrofurfuryl alcohol of 0, 6, 60 or 300 mg/kg bw. The animals were observed twice daily for clinical signs of toxicity. Measurements of body weight and food consumption were recorded weekly, and water intake was monitored during the first and third weeks of the study. Neurobehavioral observations and motor activity testing were performed on all rats after 28 days of exposure to the test substance. After 28 days, the animals were sacrificed by exsanguination from the abdominal aorta under carbon dioxide/oxygen anaesthesia. Complete haematology analyses were performed on all of the rats at the end of the study. Mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentrations were calculated. Routine analyses for plasma chemistry were performed, including enzymatic activities, and concentrations of protein, lipid and various inorganic salts. The major organs including, but not only, the liver, kidneys, lungs, heart, brain, and sex organs were weighed and necropsied. All major tissue types were preserved. The liver, lungs, and kidneys from all animals, in addition to any tissues showing gross abnormalities, were subjected to microscopic examination. No overt clinical signs of toxicity were noted in any of the treated rats. The results of neurobehavioral testing and body-weight measurements indicated no significant differences between control rats and rats treated with tetrahydrofurfuryl alcohol. Males at the highest dose (300 mg/kg bw per day) showed slightly lower food intake, food conversion efficiency and water intake by day 16 of the study. No significant changes in body weight, food consumption, and food conversion efficiency were noted in females at the highest dose, and no consistent changes were observed in the water consumption of treated females. Mean corpuscular volume ($p < 0.05$) and mean corpuscular haemoglobin ($p < 0.01$) values in males at the highest dose were significantly decreased compared to those of controls. While there was no significant difference in the number of lymphocytes for animals in the control and treated groups, a significantly decreased thrombocyte count ($p < 0.01$ and $p < 0.05$ for males and females, respectively) was observed in rats at the highest dose relative to controls. Males and females at the highest dose exhibited increased concentrations of plasma cholesterol, triglycerides, and phospholipids; however, these concentrations only reached statistical significance in females at the highest dose. No compound-related effects on absolute and relative organ weights, or macroscopic and microscopic effects were reported in any of the rats. In particular, the authors noted that there were no gross or microscopic changes to the liver to corroborate the clinical chemistry changes observed in treated rats. The NOEL for tetrahydrofurfuryl alcohol in Fischer F344 rats was 60 mg/kg bw per day (Arts & Lina, 2003).

(iv) 4-Hydroxy-2,5-dimethyl-3(2H)-furanone (DMHF; No. 1446)

Rats

In a 90-day dietary study, groups of 10–16 male and 10–16 female Charles River CD rats were given diets containing DMHF at a concentration providing a dose of approximately 6.15 mg/kg bw per day in males or 7.04 mg/kg bw per day in females. A control group, receiving a basal diet, also was studied. The concentration of the test material in the diet was adjusted during the study to maintain constant levels of dietary intake. Haematological examination and blood urea determinations were performed on 50% of the animals at week 7, and on all the animals at the end of the experimental period. After 90 days, all of the animals were killed, and subjected to a detailed necropsy and gross histopathological examination. Clinical observations recorded daily, and food consumption, food utilization, and body weights measured weekly revealed no significant differences between control groups and groups treated with DMHF. There were no significant differences in absolute and relative weights of the liver or kidney in control animals and in animals treated with DMHF. No toxicologically significant effects on haematology or clinical chemistry parameters were reported in any of the rats treated with DMHF. Gross and histopathological examination of a wide range of tissues and organs from each animal showed no evidence of tissue alterations that could be associated with administration of DMHF. The NOEL for DMHF was 6.15 mg/kg bw per day in male rats and and 7.04 mg/kg bw per day in female rats (Posternak et al., 1969).

(v) 2-Methyltetrahydrofuran-3-one (No. 1448)

Rats

Groups of 23 male and 23 female weanling Sprague-Dawley rats were given diets containing 2-methyltetrahydrofuran-3-one at a concentration providing an expected daily intake of 0 or 91.4 mg/kg bw for 90 days. Weekly measurements of weight and food consumption were obtained for each of the animals during the 13-week experimental period. Animals were observed daily for appearance, physiological responses, behaviour, any pharmacological or toxicological responses, and mortality. During weeks 6 and 13 of the study, urine from eight males and eight females in each group was collected for determination of pH, specific gravity, microscopic examination of sediment and qualitative estimates of concentrations of albumin, glucose, occult blood, ketones, and bilirubin. Additionally, at week 6 of the study, eight males and eight females in each group were killed and subjected to haematological examinations (erythrocyte volume fraction, haemoglobin, and erythrocyte, leukocyte and differential leukocyte counts) and blood chemical determinations (glucose, blood urea nitrogen, serum glutamic-oxaloacetic transaminase, serum glutamic-pyruvic transaminase and serum alkaline phosphatase). The remaining rats (15 of each sex per group) were necropsied after sacrifice at the end of the study, and subjected to the same haematological and blood chemistry determinations as those rats killed at week 6 of the study. All rats appeared normal throughout the study, and no compound-related physiological effects were observed. The averaged daily intake of DMHF in male and female rats was reported to be 91.6 and 91.2 mg/kg bw, respectively. There were no significant

differences between control and treated rats in parameters of growth, weight gain, feed consumption, or feed utilization. Results of urine analysis, blood chemistry, and haematological examinations of treated rats revealed no significant difference when compared with control rats. Measurement of serum concentrations of sodium, potassium, calcium, and chloride of treated rats at week 13 also revealed values that were not significantly different from those of controls. Gross pathological and histological examination of treated and control rats failed to reveal any consistent significant difference between the two groups. No adverse effects were observed in male and female rats given 2-methyltetrahydrofuran-3-one at a dose of 91.6 or 91.2 mg/kg bw per day, respectively, for 90 days (Shellenberger, 1970).

(vi) 2-Ethyl-4-hydroxy-5-methyl-3(2H)-furanone (HEMF; No. 1449)

Rats

Groups of 15 male and female weanling Sprague-Dawley rats were given HEMF at a concentration calculated to provide an average intake of 1.52 mg/kg bw per day for 93 days. However, it was reported that the actual average intake of HEMF by male and female rats was 1.43 mg/kg bw per day. The control group was given a basal diet containing only the vehicle, acetone, for a period of 93 days. Daily observation for symptoms and weekly measurement of body weight, food intake volume, and food utilization revealed no significant differences between treated and control groups. No compound-related effects were observed upon haematological examination (erythrocyte and leukocyte counts, erythrocyte volume fraction, or concentration of haemoglobin), blood chemical determination (glucose, blood urea nitrogen, glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, and alkaline phosphatase) and urine analysis during weeks 6 and 12 of the study. All of the surviving animals were killed at the end of the 93-day study, and gross and histopathological examination of the liver and kidneys were performed for all animals. In addition, histopathological examination of 25 tissues and organs was performed for eight animals of each sex in the control and test groups. Absolute and relative organ weights (liver, kidneys, adrenals, and spleen) were reported not to be significantly different between treated and control groups. There was also no evidence of any compound-related tissue alterations observed in rats treated with HEMF (Cox & Re, 1978).

(vii) 4-Hydroxy-5-methyl-3(2H)-furanone (No. 1450)

Rats

Groups of three male and female rats (strain not specified) were maintained on diets containing a meat flavour cocktail at a concentration of 197, 492, 983, 1966, 2949 or 3932 mg/kg of diet for 3 weeks. The cocktail included: 4-hydroxy-5-methyl-3(2*H*)-furanone (No. 1450), 74.1%; 2,3-dimethyl-4-hydroxy-2,5-dihydrofuran-5-one, 23.2%; 2-acetyl-2-thiazole, 1.8%; and 2-acetylthiazoline, 0.9%. These dietary concentrations provided average daily intakes of the flavour cocktail of approximately 10, 25, 49, 98, 147 or 197 mg/kg bw, equivalent to approximately 7, 18, 36, 73, 109 and 146 mg/kg bw of 4-hydroxy-5-methyl-3(2*H*)-furanone, respec-

tively (Food & Drug Administration, 1993). Groups of six male and six female rats were fed a control diet. Based on twice or thrice weekly measurements of body weight, food and water consumption, and food utilization, no consistent significant differences were observed between test and control animals over the 4-week study period. Haematological examination (erythrocyte volume fraction and total leukocyte counts) of all rats at the end of the study revealed a significant increase in erythrocyte volume fraction in males fed diets containing the flavour cocktail at 492, 983, 1966 or 2949 mg/kg of diet and in females fed diets containing the flavour cocktail at 492 or 983 mg/kg of diet. Total leukocyte counts of female rats given diets containing the flavour cocktail at 983 mg/kg of diet were also significantly greater than those of the controls. The authors concluded that, owing to the lack of a dose–response relationship in these effects, the findings must be regarded as a chance occurrence that was of no biological significance. Upon necropsy at study termination, absolute and relative organ weights (liver, spleen, heart, kidneys, testes) of male rats were observed not to be significantly different between test and control groups. Females at the highest dose (flavour cocktail, 3932 mg/kg of diet) exhibited relative weights of the liver that were significantly higher than those of the controls; however, macroscopic examination of the tissues of all animals at postmortem showed no significant findings attributable to the compound tested (Munday & Kirkby, 1971a).

In a follow-up study, groups of eight male and eight female weanling Colworth Wistar rats were fed diets containing the same meat flavour cocktail used in the previous study (Munday & Kirkby, 1971a) at a concentration of 197, 492, 983, 1966, 2949 or 3932 mg/kg of diet for 13 weeks. Groups of 16 males and females were maintained on a control diet throughout the duration of the study. These dietary concentrations provided average daily intakes of the meat flavour cocktail of approximately 10, 25, 49, 98, 147 or 197 mg/kg bw, equivalent to intakes of 4-hydroxy-5-methyl-3(2*H*)-furanone of approximately 7, 18, 36, 73, 109 and 146 mg/kg bw per day, respectively (Food and Drug Administration, 1993). Clinical chemistry tests were performed at 13 weeks for both sexes fed the diets containing the meat flavour cocktail at 492, 983 and 2949 mg/kg of diet. Haematological examination was performed at 6 and 13 weeks for groups of animals fed the diets containing the meat flavour cocktail at 197, 1966 and 3932 mg/kg of diet. All animals were subjected to post-mortem examination, measurement of organ weights, and histological examination of organs weighed at the conclusion of the study. In addition, groups of six males and six females were fed diets containing the meat flavour cocktail at a concentration of 197, 1966 or 3932 mg/kg of diet for 6 weeks, and biochemical studies were carried out on samples of blood and urine. A group of 12 males and 12 females were fed a basal diet for 6 weeks and served as controls. At the end of the 6 weeks, all the animals were sacrificed, and measurements of organ weight and macroscopic and microscopic examinations were performed.

Weekly measurement of body weight, food and water intake, and food utilization revealed a significantly decreased food intake in males and females at 2949 mg/kg, and a significantly increased water intake in males at 492 mg/kg and females at 983 mg/kg, when compared with controls. In the absence of any dose–response relationship, the authors regarded these changes as being unrelated to

the administration of the flavour compound. Measurement of urine refractive index and urine glutamic-oxalacetic transaminase activity for kidney function and qualitative urine analysis (pH, protein, glucose, and blood) in rats fed with diets containing the meat flavour cocktail at 197, 1966 or 3932 mg/kg for 6 weeks, and in those fed with diets containing the meat flavour cocktail at 492, 983 or 2949 mg/kg for 13 weeks failed to show any adverse effects attributable to the compound administered. Clinical chemistry measurements revealed normal values that were not significantly different between control groups and those given diets containing the flavour cocktail for 6 or 13 weeks. Results of serum protein electrophoresis (albumin, globulin, and fibrinogen) revealed no significant differences between animals treated for 6 or 13 weeks and the controls. While rats fed with diets containing the meat flavour cocktail at 492, 983 or 2949 mg/kg for 13 weeks exhibited no significant changes in haematological parameters when compared with the controls, rats fed with diets containing the meat flavour cocktail at 3932 mg/kg for 6 weeks exhibited significantly higher erythrocyte volume fraction and leukocyte counts than the controls. Additionally, rats fed diets containing the meat flavour cocktail at 197 mg/kg for 6 weeks showed significantly lower erythrocyte volume fractions than the controls. The authors considered these observations not to be related to the flavour compound administered.

For animals treated for 6 weeks, relative weights of the liver were increased ($p < 0.05$) in females at the two highest dietary concentrations (1966 and 3932 mg/kg), compared with controls. Other isolated increases in relative organ weights were found, but none was dose-related. The authors noted that the increase in relative weight liver observed in treated females was slight, and questioned the biological significance of the effect, given that the increase noted at 1966 mg/kg was not observed at 1966 mg/kg in the 13-week study. In addition, there was no evidence of gross and histopathological alterations to the liver or any other organs examined in rats in the 6-week study.

For animals treated for 13 weeks, the absolute weights of the kidney in males and females at 1966 and 3932 mg/kg, and the relative weights of the kidney in males at 1966 mg/kg and in females at 1966 and 3932 mg/kg were greater than ($p < 0.05$) those of the controls. Absolute and relative weights of the liver of males and females at 197, 1966 or 3932 mg/kg were increased; however, according to the authors, the increases were not marked, and were not supported by any evidence of histopathological changes in the liver. Likewise, histopathological examination of the remaining organs showed no evidence of alterations that could be associated with administration of the test material. Therefore, the authors concluded that no adverse effects were observed that could be attributed to the test compound (Munday & Kirkby, 1971b).

(viii) 4-Acetoxy-2,5-dimethyl-3(2H)-furanone (No. 1456)

Rats

Groups of five male and female Charles River Crl:CD BR rats were given diets containing 4-acetoxy-2,5-dimethyl-3(2*H*)-furanone at concentrations estimated to provide a dose of 0 or 18 mg/kg bw per day for 14 days. Animals were examined

for viability twice per day. Body weights and food consumption were recorded on days 0, 7 and 14. A gross necropsy was performed on each of the animals at the end of the study; this included examination of the external surface of the body, carcass, all orifices and the cranial, thoracic and abdominal cavities and their contents. Kidney and liver weights were recorded before fixation in 10% buffered formalin. All gross lesions were also fixed for histological examination. During week 1, the mean measured dietary intake of 4-acetoxy-2,5-dimethyl-3(2*H*)-furanone was 40% higher in males (25.2 mg/kg bw per day) and 32% higher in females (23.8 mg/kg bw per day) when compared to the target intake of 18 mg/kg bw per day. By week 2, the average dietary intakes of 4-acetoxy-2,5-dimethyl-3(2*H*)-furanone by males and females were 17.2 and 16.7 mg/kg bw per day, respectively. No statistically significant differences in any of the parameters tested were noted in treated and control animals. The authors concluded that dietary administration of 4-acetoxy-2,5-dimethyl-3(2*H*)-furanone produced no evidence of toxic effects under the conditions of this study (Drummond, 1993).

(c) Long-term studies of toxicity and carcinogenicity

The results of long-term studies of toxicity and carcinogenicity with representative tetrahydrofuran and furanone derivatives are summarized in Table 4 and are described below.

(i) 4-Hydroxy-5-methyl-3(2H)-furanone (No. 1450)

Rat

Groups of four male and female Colworth Wistar rats were fed diets containing a meat flavour cocktail at a concentration of 197, 492, 983, 1966, 2949 or 3932 mg/kg of diet (equivalent to a dose of 4-hydroxy-5-methyl-3(2*H*)-furanone) of approximately 7, 18, 36, 73, 109 and 146 mg/kg bw per day, respectively) for 13 weeks. Owing to the lack of organ weight changes attributable to the administration of the flavour mixture in a 13-week study discussed above (Munday & Kirkby, 1971b), all treatment groups (24 males and 24 females) were subsequently maintained on diets containing the flavour cocktail at the highest dietary concentration (3932 mg/kg) from week 15 of the study, for an additional 37 weeks (i.e. up to week 52). Evaluation of final mean body weights and haematological parameters revealed no significant differences between test and control animals at week 15 and at the end of the 1-year study. No compound-related effects on general health and survival were noted in any of the treated rats. At necropsy, there was no significant difference in absolute or relative organ weights (liver, spleen, heart, kidneys, brain, adrenals, pituitary, thyroid, and testes) between treated and control animals. Gross and histopathological examination of all treated and control rats revealed no significant macroscopic findings at the end of the study. Upon microscopic examination, lesions (subcutaneous sarcoma, chloroma, pituitary adenoma, and parafollicular thyroid adenoma) were observed in treated and control rats. Similar findings have been reported in previous studies using control rats of the same strain; therefore, these lesions were considered not to be related to the flavour cocktail administered. The authors concluded that administration of diets contain-

ing the flavour cocktail at a concentration of up to 3932 mg/kg (approximately equivalent to a dose of 4-hydroxy-5-methyl-3(2*H*)-furanone of 146 mg/kg bw per day) for up to 1 year produced no effects on the type, incidence, or time of development of tumours in Colworth Wistar rats (Munday & Kirkby, 1973).

(ii) 4-Hydroxy-2,5-dimethyl-3(2H)-furanone (DMHF; No. 1446)

Rat

Groups of 60 male and 60 female Sprague-Dawley rats given diets containing DMHF at a dose of 0 (control), 100, 200 or 400 mg/kg bw per day for 24 months. Observation for signs of mortality or clinical toxicity was conducted twice per day. Measurements of body weight and food consumption were recorded twice per week for the first 16 weeks of the study and twice per month thereafter. Haematology and coagulation parameters were evaluated at 12, 18 and 24 months. At sacrifice, absolute and relative organ weights were recorded. Complete macroscopic and histopathological examinations of selected tissues and gross lesions were performed.

No significant compound-related effects were reported in any of the animals at 100 and 200 mg/kg bw per day. The mean body weights and body-weight gains of males and females at the highest dose (400 mg/kg bw per day) were significantly lower than those of control animals at 24 months. Haematological examination revealed significant differences in absolute or total leukocyte counts between rats treated with DMHF at a dose of 200 or 400 mg/kg bw per day and the controls, which the authors attributed to normal biological variability.

The mean survival rate for males in the group receiving the highest dose was significantly lower (approximately 20%, $p < 0.05$) than that of males in the control group at 24 months. The authors concluded that this finding was attributable to an increased incidence of adenomas of the pars distalis of the pituitary, with subsequent compression of the hypothalamic region within the brains of males at the highest dose. However, it was noted that the incidence of pituitary adenomas in males at the highest dose was within the range for historical controls, as well as within reported ranges in the literature for the age and strain of rat (McComb et al., 1984). Pituitary adenomas are common in ageing and aged rats, and generally appear between age 13 and 24 months. In this study, all of the decedent animals with adenomas of the pars distalis died during month 18 of the study or later. In addition, Peto analysis, which compares incidence of tumours and time to tumour formation, revealed no statistical difference between control and treated males and females in this study. Therefore, it was concluded that these adenomas were common, spontaneous tumours that were unrelated to the administration of DMHF. No other significant compound-related haematological, biochemical, macroscopic, histopathological, or neoplastic changes were reported. The NOEL for DMHF was 200 mg/kg bw per day on the basis of decreased body-weight gains in males and females, and decreased survival rate in males at 400 mg/kg bw per day (Kelly & Bolte, 2003).

The incidence of pituitary adenoma of the par distalis is a spontaneous neoplasm, which also occurs in strains of rats other than Sprague-Dawley (Haseman et al., 1985, 1998). For example, the incidence of pituitary adenomas of the par distalis in control male Fischer F344 rats in dietary studies performed by the National Toxicology Program is in the range of 14 to 60%, with a mean rate of 30%.

(d) Genotoxicity

The results of tests for genotoxicity in vitro and in vivo performed with representative tetrahydrofuran and furanone derivatives are summarized in Table 5 and described below.

(i) In vitro

No evidence was found for reverse mutation in tests in *Salmonella typhimurium* strains TA1535, TA1537, TA1538, TA100, TA98 and TA102 with tetrahydrofurfuryl alcohol (1–102 100 μg/plate) (No. 1443), tetrahydrofurfuryl propionate (≤3600 μg/plate) (No. 1445) or 2-(3-phenylpropyl)tetrahydrofuran (≤3600 μg/plate) (No. 1441) (Wild et al., 1983; Aeschbacher et al., 1989).

4-Hydroxy-5-methyl-3(2*H*)-furanone (No. 1450) (10–12 000 μg/plate), DMHF (No. 1446) (10–10 000 μg/plate), and HEMF (No. 1449) (up to 10 000 μg/plate) induced reverse mutations in standard and modified Ames assays. Positive results were obtained for DMHF (No. 1446) in *S. typhimurium* strains TA100, TA102, TA98 and TA97 at the highest dose tested (4000 μg/plate) with or without metabolic activation (Xing et al., 1988). In contrast, Gilroy et al. (1978) and Hiramoto et al. (1996b) reported positive results for this compound only in *S. typhimurium* strain TA100 when tested at concentrations of ≤10 000 μg/plate with or without metabolic activation. Similarly, HEMF (No. 1449) and 4-hydroxy-5-methyl-3(2*H*)-furanone (No. 1450) produced positive results in *S. typhimurium* strain TA100 with or without metabolic activation (Hiramoto et al., 1996a; Li et al., 1998).

The standard Rec assay with *Bacillus subtilis* H17 (rec^+) and M45 (rec^-) exposed to DMHF (No. 1446) at a concentration of 20, 40, 60, 80 and 120 μg/disc of yielded a dose-dependent DNA damage response (Xing et al., 1988).

(ii) In vivo

In an assay for genotoxicity in vivo, groups of five ICR mice were given a negative control, or DMHF (No. 1446) or HEMF (No. 1449) at a concentration of 1000, 2000 or 3000 mg/kg bw by oral administration. Blood was drawn at intervals of 15 min after administration for up to 120 min. For DMHF, the frequency of micronucleated peripheral reticulocytes was increased at a dose of 2000 and 3000 mg/kg bw, but not at 1000 mg/kg bw. For HEMF, the frequency was increased at all three doses (Hiramoto et al., 1998).

Kunming mice injected intraperitoneally with DMHF (No. 1446) at a dose of 0, 186, 232 or 309 mg/kg bw demonstrated a dose-dependent increase in erythrocyte

Table 5. Results of studies of genotoxicity with tetrahydrofuran and furanone derivatives used as flavouring agents

No.	Flavouring agent	End-point	Test system	Dose or concentration	Result	Reference
In vitro						
1441	2-(3-Phenylpropyl) tetrahydrofuran	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA100 and TA98	≤3600 μg/plate	Negative[a]	Wild et al. (1983)
1443	Tetrahydrofurfuryl alcohol	Reverse mutation	*S. typhimurium* TA100, TA102 and TA98	1–102 100 μg/plate[b]	Negative[a,c]	Aeschbacher et al. (1989)
1445	Tetrahydrofurfuryl propionate	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA100 and TA98	≤3600 μg/plate	Negative[d]	Wild et al. (1983)
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF)	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA100 and TA98	10.0, 33.3, 100.0, 333.3, 1 000, 2 000, 3 300, 4 000, 6 000, 8 000 μg/plate	Positive[a,e]	Gilroy et al. (1978)
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF)	Reverse mutation	*S. typhimurium* TA100 and TA98	0–10 000 μg/plate	Positive[a,e,f]	Hiramoto et al. (1996b)
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF)	Reverse mutation	*S. typhimurium* TA100, TA102, TA98 and TA97	500–4 000 μg/plate	Positive[a,g]	Xing et al. (1988)
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF)	Reverse mutation	*E. coli* WP2 *uvr*A$^-$	10.0, 33.3, 100.0, 333.3, 1 000, 3 300 μg/plate	Negative	Gilroy et al. (1978)
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (DMHF)	DNA damage	*B. subtilis* H17(Rec$^+$) and M45 (Rec$^-$)	20, 40, 60, 80, 120 μg/disc	Positive	Xing et al. (1988)
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	DNA strand breaks	pBR322 DNA	2.6–780 μmol/l (0.3–100 mg/l)[h]	Positive	Hiramoto et al. (1996b)
1449	4-Hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone (HEMF)	Reverse mutation	*S. typhimurium* TA100 and TA98	0–10 000 μg/plate	Positive[a,e]	Li et al. (1998)
1449	4-Hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone (HEMF)	DNA strand breaks	pBR322 DNA	20–2 000 μmol/l (2.8–284.3 mg/l)[i]	Positive[j]	Li et al. (1998)
1450	4-Hydroxy-5-methyl-3(2*H*)-furanone	Reverse mutation	*S. typhimurium* TA100 and T98	0–5 000 μg/plate	Positive[a,e]	Hiramoto et al. (1996a)
1450	4-Hydroxy-5-methyl-3(2*H*)-furanone	DNA strand breaks	pBR322 DNA	0–900 μmol/l (0–103 mg/l)[k]	Positive[a,l]	Hiramoto et al. (1996a)

Table 5. Results of studies of genotoxicity with tetrahydrofuran and furanone derivatives used as flavouring agents

No.	Flavouring agent	End-point	Test system	Dose or concentration	Result	Reference
In vivo						
1445	Tetrahydrofurfuryl propionate	Micronucleus formation	Male and female mouse, bone marrow[m]	316, 632, 949 mg/kg bw	Negative	Wild et al. (1983)
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	Micronucleus formation	Mouse, bone marrow[m]	0, 186, 232 or 309 mg/kg bw	Positive[n]	Xing et al. (1988)
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	Sister chromatid exchange	Mice[o]	200, 400 or 800 mg/kg bw	Positive	Tian et al. (1992)
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	Chromosomal aberration	Mouse spermatocytes[m]	0, 232, 464 or 928 mg/kg bw	Positive	Xing et al. (1988)
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	Micronucleus formation	Mouse spermatogonial cells[o]	200, 400 or 800 mg/kg bw	Positive[p]	Tian et al. (1992)
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	Micronucleus formation	Mouse peripheral blood cells[o]	1 000, 2 000, 3 000 mg/kg bw	Negative[q] Positive	Hiramoto et al. (1998)
1446	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	Micronucleus formation	Male mice[m]	500, 1 000, 1 500[r] mg/kg bw	Positive	Hiramoto et al. (1996b)
1449	4-Hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone	Micronucleus formation	Male mouse[m]	500, 1 000, 1 500[r] mg/kg bw	Positive	Li et al. (1998)
1449	4-Hydroxy-2-ethyl-5-methyl-3(2*H*)-furanone	Micronucleus formation	Mouse peripheral blood reticulocytes[o]	1 000, 2 000, 3 000 mg/kg bw	Positive	Hiramoto et al. (1998)

[a] With or without metabolic activation provided by S9 (9000 × *g* supernatant from rodent liver).
[b] Calculated based on the relative molecular mass of tetrahydrofurfuryl alcohol = 102.1.
[c] Modified pre-incubation method.
[d] Without metabolic activation.
[e] Positive results only observed in TA100.
[f] Preiincubation method.
[g] Positive results in all strains at the highest dose tested.
[h] Calculated based on the relative molecular mass of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone = 128.0.
[i] Calculated based on the relative molecular mass of 4-hydroxy-2-ethyl-5-methyl-3(2H)-furanone = 142.2.

[j] Without inhibitor.
[k] Calculated based on the relative molecular mass of 4-hydroxy-5-methyl-3(2*H*)-furanone = 114.1.
[l] With or without inhibitor.
[m] Administered intraperitoneally.
[n] Dose-dependent increase in the frequency of formation of micronuclei in bone marrow erythrocytes was observed, reaching a maximum increase of 2.6-fold.
[o] Administered orally.
[p] A clear dose–response relationship could not be established; however, the increases in formation of erythrocyte micronuclei were significant relative to the negative control.
[q] Negative at the lowest dose administered (1000 mg/kg bw).
[r] Highest dose (1500 mg/kg bw) resulted in death of experimental animal.

micronucleus formation in the bone marrow, reaching a maximum increase of 2.6-fold (Xing et al., 1988). Also, male mice injected with DMHF at a dose of 0, 232, 464 or 928 mg/kg bw exhibited increases in spermatocyte chromosome aberrations (Xing et al., 1988). In a similar assay in male Kunming mice given DMHF at a dose of 200, 400 or 800 mg/kg bw by intragastric instillation, a significant increase in sister chromatid exchanges in spermatogonial cells compared to controls was reported at all three doses (Tian et al., 1992). Positive results were also obtained in an assay for micronucleus formation in male mice given DMHF at a dose of 200, 400 or 800 mg/kg bw by intraperitoneal injection (Tian et al., 1992). The increases observed at each dose did not establish a clear dose–response relationship, although increases were significantly higher than for the negative control.

Groups of five or six male ICR mice were given DMHF at a dose of 0, 500, 1000 or 1500 mg/kg bw by intraperitoneal injection. Blood samples were drawn at 24, 48 and 72 h after injection. The frequency of micronucleated peripheral erythrocytes was significantly increased at ≥500 mg/kg bw, with the maximum frequency of 1.6% being obtained at 48 h after dosing (Hiramoto et al., 1996b).

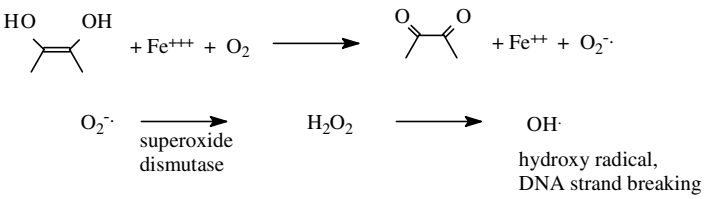
Groups of five or six male ICR mice were given HEMF (No. 1449) at a dose of 500, 1000 or 1500 mg/kg bw by intraperitoneal injection and samples of peripheral blood were taken at 24, 48 and 72 h after injection. All the mice in the group given HEMF at a dose of 1500 mg/kg bw died before 24 h. The frequency of micronucleated peripheral erythrocytes was significantly higher than that in the controls in groups given HEMF at a dose of 500 or 1000 mg/kg bw. The maximum number of micronucleated peripheral erythrocytes was observed at 48 h at 1000 mg/kg bw group (0.58%) and at 500 mg/kg bw (approximately 0.3%). The frequency of micronucleated peripheral erythrocytes reported in mice given the positive control substance, mitomycin C, at a dose of 1 mg/kg bw, was 3.1% (Li et al., 1998).

In summary, positive results were obtained in several assays for genotoxicity in vivo in mice given DMHF via intraperitoneal injection at doses as low as 196 mg/kg bw (Xing et al., 1988). Similarly, positive results were also obtained for DMHF administered orally; however, there are conflicting data pertaining to the lowest dose at which DMHF elicits a positive response: 200 mg/kg bw according to Tian et al. (1992); ≥2000 mg/kg bw according to Hiramoto et al. (1998).

Putative mechanism of genotoxicity of furanone derivatives

Furanones induce DNA damage in vitro by generating free radicals that induce strand scission. In the presence of metals (e.g. Fe^{3+}) and dissolved oxygen, the enolic hydroxyl group (OH) of the furanone is oxidized by single electron transfer to yield the corresponding carbon-centered radical and a reduced metal ion (e.g. Fe^{2+}). The carbon-centered radical can couple to molecular oxygen to produce a peroxyl radical that may damage DNA. Alternately, the reduced metal ion can auto-oxidize to form a superoxide radical anion. The superoxide radical then dismutates into hydrogen peroxide (H_2O_2). It is well recognized that reduced metals react with H_2O_2 to form a hydroxyl radical, which is a powerful oxidizing agent (see Figure 2). Hydrogen peroxide also oxidizes glutathione leading to decreased glu-

Figure 2. Mechanism of oxidation of furanone derivatives in vitro



tathione *S*-transferase/oxidized glutathione and an increase in cellular oxidative stress.

In the case of DMHF, experimental evidence for this H_2O_2-producing pathway includes the following:

—Fe^{3+} or Cu^{2+} is readily reduced to Fe^{2+} or Cu^{+} in the presence of DMHF;

—DNA strand-breaking of supercoiled plasmid DNA into an open circular form in the presence of DMHF is inhibited in the presence of superoxide dismutase and catalase, enzymes used to detoxicate superoxide to form oxygen and water;

—hydroxyl radical scavengers such as potassium iodine, sodium azide, or ethanol also inhibit DNA strand-breaking;

—free radical spin-trapping agents (e.g. 5,5-dimethyl-1-pyrroline *N*-oxide) also inhibit DNA strand-breaking;

—oxygen radical trapping agents such as 2-mercaptoethanol and cysteine are also inhibitory;

—removal of dissolved oxygen by nitrogen purge decreases DNA strand-breaking and H_2O_2 formation;

—addition of metal chelating agents also inhibits DNA strand-breaking by depleting the metal ions required for this process;

—DNA strand-breaking by DMHF was much faster in the presence of Fe^{3+} than in its absence; and

—electron spin resonance of a solution of DMHF and 5,5-dimethyl-1-pyrroline *N*-oxide showed the presence of hydroxyl radicals and bicarbonate radicals (Hiramoto et al., 1996a, 1996b; Yamashita et al., 1998).

On the basis of these observations, cellular oxidative stress is related to the dose-dependent oxidation of DMHF and structurally related furanones, yielding H_2O_2 and eventually hydroxyl radicals (Hiramoto et al., 1996a, 1996b; Li et al., 1998; Yamashita et al., 1998).

The ability of DMHF to induce oxygen radical formation and DNA strand breaks is reminiscent of similar activities observed for vitamin C. Vitamin C (ascorbic acid) contains an enediol that is superficially related to the enol of DMHF. Being both

an enol ether and an α,β-unsaturated ketone, DMHF is subject to hydrolytic ring opening, to yield an enediol. Like DMHF, vitamin C also reduces metal ions and produces superoxide anions to generate hydroxyl radicals that cleave DNA. As anticipated, vitamin C exhibits genotoxicity in test systems similar to those in which furanones give positive results. In standard Ames assays, ascorbic acid (vitamin C) induces reverse mutations in *S. typhimurium* strains TA104, TA102, TA100 and TA98 at concentrations of 352–1761 μg/plate (Ichinotsubo et al., 1981; D'Agostini et al., 2000). In the *E. coli* Mutoxitest, positive results were obtained when ascorbic acid at a concentration of 200, 300 or 400 μg/plate in the presence of Cu^{2+} was incubated with *E. coli* strain IC203 (Martinez et al., 2000). *E.coli* IC203 carries an *oxyR* mutation that effectively removes its ability to turn on the biosynthesis of H_2O_2-protective proteins and makes the strain sensitive to DNA damage under conditions of oxidative stress (Blanco et al., 1998).

Increased frequencies of micronucleus formation were observed when ascorbic acid (400, 500 or 600 μg/ml) was incubated with Chinese hamster cells (Miller et al., 1995). An increase in sister chromatid exchanges was observed in Chinese hamster ovary cells in the presence of ascorbic acid at 500 μg/ml without metabolic activation (Tennant et al., 1987). In a standard assay for micronucleus formation in mice, ascorbic acid at a dose of 1500 mg/kg bw induced a significant increase (Shelby et al., 1993).

(iii) Conclusion

Furanones are a class of substances present naturally in food and that are also added as flavouring agents. The principal furanone used as a flavouring agent is DMHF. In humans, DMHF is rapidly absorbed in the gastrointestinal tract and conjugated with glucuronic acid in the liver. Free DMHF is not detected in the blood of human volunteers to whom it is administered as a constituent of strawberries; its glucuronic acid conjugate is the principal urinary metabolite (Roscher et al., 1997). Thus, the potential for chemical reaction of DMHF with important cellular macromolecules, especially DNA, appears to be low.

Genotoxicity with 3-(2*H*)-furanone derivatives, notably DMHF and 2-ethyl-4-hydroxy-5-methyl-3-(2*H*)-furanone, was observed in standardized bacterial (Gilroy et al., 1978; Xing et al., 1988; Hiramoto et al., 1996a, 1996b; Li et al., 1998) and mammalian assays (Xing et al., 1988; Tian et al., 1992; Hiramoto et al., 1996b). A mechanism for genotoxicity involving dose-dependent formation of H_2O_2 and oxidized furanones has been extensively studied (Hiramoto et al., 1995, 1996a, 1996b); these studies indicate that, at high doses, DNA single-strand breaks result from the reaction of hydroxyl radicals with DNA.

Despite the fact that DMHF causes genotoxicity, it is not carcinogenic in rats. Two studies, one with DMHF and the other with a structurally related furanone, showed no evidence of carcinogenicity at intakes that are orders of magnitude greater than the intake of furanones added as flavouring agents (Munday & Kirkby, 1973; Kelly & Bolte, 2003). Furthermore, vitamin C, a structurally similar compound with a genotoxicity test profile similar to that of DMHF, does not demonstrate carcinogenicity (National Research Council, 1996). In a 2-year bioassay, the NOEL

for DMHF was 200 mg/kg bw per day in rodents. This intake is approximately 2000 times higher than the daily per capita intake ('eaters only') of 0.088 mg/kg bw per day from use of DMHF as a flavouring agent.

After consideration of all the available data, the Committee concluded that it is highly unlikely that DMHF, other furanones or tetrahydrofurans would pose any significant genotoxic risk to humans under the conditions of use as flavouring agents. Similarly, 2-ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone was considered not to pose a genotoxic risk.

(e) Other relevant studies: anti-tumour studies

Japanese-style fermented soy sauce (shoyu) contains significant amounts (230 mg/kg) of the antioxidant HEMF (No. 1449), which has been reported to have anticarcinogenic properties. In order to induce forestomach neoplasia, groups of 25–27 female ICR mice (aged 9 weeks), were given benzo[*a*]pyrene at a dose of 1.5 mg in corn oil by gavage once per week for 4 weeks (Nagahara et al., 1992). After the fourth dose of BP, the animals were fed an experimental diet containing HEMF at a dose of 0, 25, 50 or 75 mg/kg of diet for 120 days. These dietary concentrations provided an average daily intake of of HEMF of 3.75, 7.5 or 11.25 mg/kg bw (Food & Drug Administration, 1993). The animals were sacrificed at age 211 days. A significant reduction in the incidence of forestomach neoplasia induced by benzo[*a*]pyrene ($p \leq 0.05$) and the number of neoplasms per mouse was observed in all groups of mice given diets containing HEMF. In addition, dietary administration of HEMF produced no effect on food intake or body weight, indicating that the anticarcinogenic effect of HEMF was not caused by caloric restriction. The authors suggested that HEMF inhibits tumour promotion in mice (Nagahara et al., 1992).

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PHENYL-SUBSTITUTED ALIPHATIC ALCOHOLS AND RELATED ALDEHYDES AND ESTERS

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1. EVALUATION

1.1 Introduction

The Committee evaluated a group of 22 phenyl-substituted aliphatic alcohols and related aldehydes and esters (Table 1) using the Procedure for the Safety Evaluation of Flavouring Agents (see Figure 1, p 192). The Committee has not previously evaluated any member of the group.

Seven of the 22 flavouring agents (Nos 1465, 1467, 1472–1474, 1478 and 1479) have been reported to occur naturally in various foods. They have been

detected in roasted nuts, cooked potatoes, cheese, wine, fruit, vegetables, coffee, tea, and cocoa (Nijssen et al., 2003).

1.2 Estimated daily intake

The total annual volume of production of the 22 phenyl-substituted aliphatic alcohols and related aldehydes and esters in this group is approximately 1300 kg in Europe (International Organization of the Flavor Industry, 1995) and 3000 kg in the USA (National Academy of Sciences, 1970, 1982, 1987; Lucas et al., 1999) (see Table 2). Approximately 70% of the total annual volume of production in Europe is accounted for by 2-phenylpropionaldehyde (No. 1467), while approximately 87% of the total annual volume of production in the USA is accounted for by 2-methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465). The daily intake of 2-phenylpropionaldehyde (No. 1467) was calculated to be 125 and 6 μg/person in Europe and the USA, respectively. The daily intake of 2-methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465) was calculated to be 22 and 343 μg/person, in Europe and the USA, respectively. The daily per capita intake values for each agent are reported in Table 1.

1.3 Absorption, distribution, metabolism and elimination

The esters of phenyl-substituted flavouring agents (Nos 1458, 1460, 1461, 1464, 1469, 1470 and 1475) will be hydrolysed rapidly by carboxyesterases to the corresponding 2-phenyl substituted alcohol or acid (Heymann, 1980). Before absorption these esters, as well as 2-phenylpropionaldehyde dimethyl acetal (No. 1468), are predicted to undergo hydrolysis (Williams, 1959) in the gastrointestinal tract to yield compounds such as 2-phenylpropionaldehyde, β-methylphenethyl alcohol, 2-ethyl-3-phenylpropionic acid, 4-phenylbutyric acid, and 2-methyl-4-phenyl-2-butanol, which would be rapidly absorbed.

Once absorbed, the phenyl-substituted alcohols, aldehydes and acids may follow multiple metabolic pathways. The alcohols and aldehydes can be converted to phenyl-substituted carboxylic acids. These acids can be conjugated with glucuronic acid and excreted in the urine. They can also undergo β-oxidation to benzoic acid or phenylacetic acid derivatives, which are conjugated with glycine or glutamine before being excreted in the urine. Phenyl-substituted alcohols can also be conjugated directly with glucuronic acid before excretion (Williams, 1959).

2-Oxo-3-phenylpropionic acid (phenylpyruvate and its sodium salt, Nos 1478 and 1479) is a metabolite of phenylalanine. It is primarily decarboxylated to yield phenylacetate and is readily excreted in the urine (Nelson & Cox, 2000).

α,β-Unsaturated 2-phenylaldehyde derivatives (Nos 1472–1474) are electrophilic in nature and are predicted to be detoxified by glutathione conjugation. The structurally related substance 2-phenylpropenal (atropaldehyde) readily forms glutathione conjugates when incubated with glutathione in vitro (Thompson et al., 1996).

Table 1. Summary of the results of safety evaluations of phenyl-substituted aliphatic alcohols and related aldehydes and esters[a] used as flavouring agents

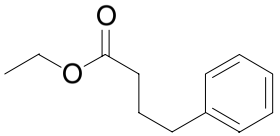
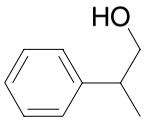
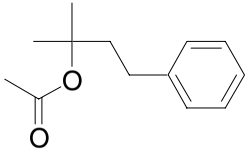
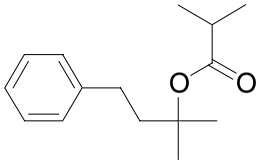
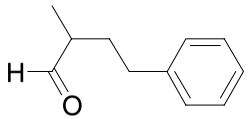
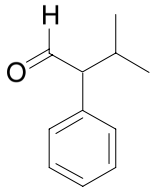
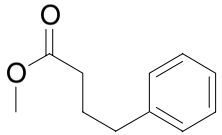
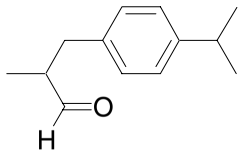
Flavouring agent	No.	CAS No. and structure	Step A3[a,b] Does intake exceed the threshold for human intake?	Comments	Conclusion based on current intake
Structural class I					
Ethyl 4-phenylbutyrate	1458	10031-93-3 	No Europe: ND USA: 0.01	See notes 4 and 1	No safety concern
ß-Methylphenethyl alcohol	1459	1123-85-9 	No Europe: 0.1 USA: 0.01	See note 1	No safety concern
2-Methyl-4-phenyl-2-butyl acetate	1460	103-07-1 	No Europe: 0.4 USA: 0.04	See notes 4 and 1	No safety concern
2-Methyl-4-phenyl-2-butyl isobutyrate	1461	10031-71-7 	No Europe: 2 USA: 1	See notes 4 and 1	No safety concern

Table 1. (Contd)

Flavouring agent	No.	CAS No. and structure	Step A3[a,b] Does intake exceed the threshold for human intake?	Comments	Conclusion based on current intake
2-Methyl-4-phenylbutyraldehyde	1462	40654-82-8 	No Europe: 0.4 USA: 0.4	See note 2	No safety concern
3-Methyl-2-phenylbutyraldehyde	1463	2439-44-3 	No Europe: ND USA: 0.07	See note 2	No safety concern
Methyl 4-phenylbutyrate	1464	2046-17-5 	No Europe: ND USA: 0.01	See notes 4 and 1	No safety concern
2-Methyl-3-(*p*-isopropylphenyl) propionaldehyde	1465	103-95-7 	No Europe: 22 USA: 343	See note 2	No safety concern

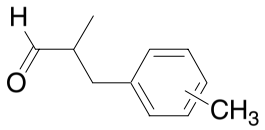
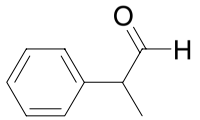
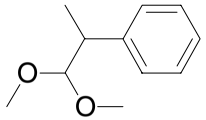
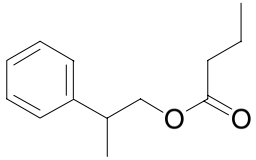
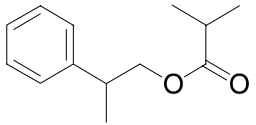
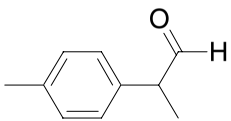
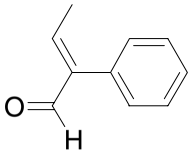
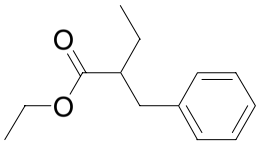
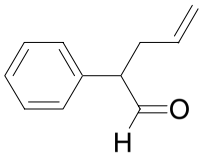
2-Methyl-3-tolylpropionaldehyde (*o*, *m*, and *p*)	1466	41496-43-9 	No Europe: 0.6 USA: 27	See note 2	No safety concern
2-Phenylpropionaldehyde	1467	93-53-8 	No Europe: 125 USA: 6	See note 2	No safety concern
2-Phenylpropionaldehyde dimethyl acetal	1468	90-87-9 	No Europe: 5 USA: 3	See notes 3 and 2	No safety concern
2-Phenylpropyl butyrate	1469	80866-83-7 	No Europe: 0.004 USA: 0.5	See notes 4 and 1	No safety concern
2-Phenylpropyl isobutyrate	1470	65813-53-8 	No Europe: 2 USA: 0.05	See notes 4 and 1	No safety concern

Table 1. (Contd)

Flavouring agent	No.	CAS No. and structure	Step A3[a,b] Does intake exceed the threshold for human intake?	Comments	Conclusion based on current intake
2-(*p*-Tolyl)propionaldehyde,	1471	99-72-9 	No Europe: 0.04 USA: 0.01	See note 2	No safety concern
2-Phenyl-2-butenal	1474	4411-89-6 	No Europe: 2 USA: 0.07	See note 7	No safety concern
Ethyl 2-ethyl-3-phenylpropanoate	1475	2983-36-0 	No Europe: ND USA: 0.9	See notes 4 and 1	No safety concern
2-Phenyl-4-pentenal	1476	24401-36-3 	No Europe: 0.03 USA: 0.04	See note 2	No safety concern

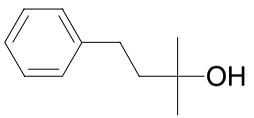
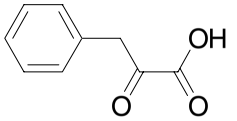
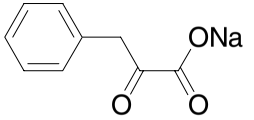
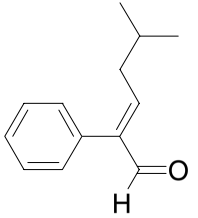
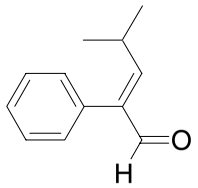
2-Methyl-4-phenyl-2-butanol	1477	103-05-9 	No Europe: 4 USA: 0.01	See note 1	No safety concern
2-Oxo-3-phenylpropionic acid	1478	156-06-9 	No Europe: ND USA: 0.09	See notes 5 and 6	No safety concern
Sodium 2-oxo-3-phenylpropionate	1479	114-76-1 			
Structural class II					
5-Methyl-2-phenyl-2-hexenal	1472	21834-92-4 	No Europe: 18 USA: 6	See note 7	No safety concern

Table 1. (Contd)

Flavouring agent	No.	CAS No. and structure	Step A3[a,b] Does intake exceed the threshold for human intake?	Comments	Conclusion based on current intake
4-Methyl-2-phenyl-2-pentenal	1473	26643-91-4 	No Europe: 0.4 USA: 5	See note 7	No safety concern

CAS: Chemical Abstracts Service; ND: No intake data reported.

[a] Step 2: All the agents in this group can be predicted to be metabolized to innocuous products.

[b] The thresholds for human intake for structural classes I and II are 1800 and 540 μg/person per day, respectively. All intake values are expressed in μg/person per day. The combined intake of flavouring agents in structural class I is 164 μg/person per day in Europe and 382 μg/person per day in the USA. The combined intake of flavouring agents in structural class II is 18 μg/person per day in Europe and 11 μg/person per day in the USA.

Notes:

1. Readily forms glucuronic acid conjugates, which are subsequently excreted in the urine.
2. Oxidized to the corresponding carboxylic acid and conjugated with glucuronic acid and is eliminated in the urine.
3. Rapidly hydrolysed to liberate the corresponding aldehyde and 2 equivalents of methanol.
4. Esters undergo rapid hydrolysis to liberate the corresponding alcohol and carboxylic acid.
5. Primarily decarboxylated to form phenylacetate which is excreted in the urine as such.
6. Readily undergoes transamination to form phenylalanine.
7. Readily forms glutathione conjugates and is rapidly eliminated in the urine.

Table 2. Annual volumes of production of phenyl-substituted aliphatic alcohols and related aldehydes and esters used as flavouring agents in Europe and the USA

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b] μg/day	Intake of		Annual volume in naturally occurring foods (kg)[d]	Consumption ratio[e]
			μg/kg bw per day	alcohol equivalents (μg/kg bw per day)[c]		
Ethyl 4-phenylbutyrate (1458)						
Europe	ND	ND	ND	ND		
USA	0.05	0.01	0.0001	0.00002	–	NA
β-Methylphenethyl alcohol (1459)						
Europe	0.7	0.10	0.002			
USA[f]	0.05	0.01	0.0001		–	NA
2-Methyl-4-phenyl-2-butyl acetate (1460)						
Europe	3	0.4	0.01	0.008		
USA[f]	0.2	0.04	0.0006	0.0005	–	NA
2-Methyl-4-phenyl-2-butyl isobutyrate (1461)						
Europe	12	2	0.03	0.02		
USA	8	1	0.02	0.01	–	NA
2-Methyl-4-phenylbutyraldehyde (1462)						
Europe	3	0.4	0.01			
USA	3	0.4	0.01		–	NA
3-Methyl-2-phenylbutyraldehyde (1463)						
Europe	ND	ND	ND			
USA	0.5	0.07	0.001		–	NA
Methyl 4-phenylbutyrate (1464)						
Europe	ND	ND	ND	ND		
USA	0.05	0.01	0.0001	0.00002	–	NA
2-Methyl-3-(*p*-isopropylphenyl)propionaldehyde (1465)						
Europe	153	22	0.4			
USA	2604	343	6		1380	0.5
2-Methyl-3-tolylpropionaldehyde (*o*, *m*, and *p*) (1466)						
Europe	4	0.6	0.01			
USA	204	27	0.4		–	NA
2-Phenylpropionaldehyde (1467)						
Europe	878	125	2			
USA	45	6	0.1		+	NA
2-Phenylpropionaldehyde dimethyl acetal (1468)						
Europe	32	5	0.08	0.03		
USA	20	3	0.04	0.01	–	NA
2-Phenylpropyl butyrate (1469)						
Europe	0.03	0.004	0.0001	0.00007		
USA	4	0.5	0.01	0.007	–	NA
2-Phenylpropyl isobutyrate (1470)						
Europe	13	2	0.03	0.02		
USA[f]	0.3	0.05	0.001	0.0007	–	NA
2-(*p*-Tolyl)propionaldehyde (1471)						
Europe	0.3	0.04	0.001			
USA	0.05	0.01	0.0001		–	NA
5-Methyl-2-phenyl-2-hexenal (1472)						
Europe	129	18	0.3			
USA	42	6	0.1		206	5

Table 2. (Contd)

Flavouring agent (No.)	Most recent annual volume (kg)[a]	Intake[b] μg/day	Intake of		Annual volume in naturally occurring foods (kg)[d]	Consumption ratio[e]
			μg/kg bw per day	alcohol equivalents (μg/kg bw per day)[c]		
4-Methyl-2-phenyl-2-pentenal (1473)						
Europe	3	0.4	0.01			
USA	41	5	0.1		+	NA
2-Phenyl-2-butenal (1474)						
Europe	11	2	0.03			
USA	0.5	0.07	0.001		902	1804
Ethyl 2-ethyl-3-phenylpropanoate (1475)						
Europe	ND	ND	ND	ND		
USA[g]	5	0.9	0.01	0.002	–	NA
2-Phenyl-4-pentenal (1476)						
Europe	0.2	0.03	0.0005			
USA[f]	0.2	0.04	0.001		–	NA
2-Methyl-4-phenyl-2-butanol (1477)						
Europe	25	4	0.06			
USA[f]	0.05	0.01	0.0001		–	NA
2-Oxo-3-phenylpropionic acid and 2-oxo-3-phenylpropionic acid sodium salt (1478 and 1479)						
Europe	ND	ND	ND			
USA[g]	0.5	0.09	0.001		+	NA
Total						
Europe	1267					
USA	2979					

NA, not available; ND, no intake data reported; +, reported to occur naturally in foods (Nijssen et al., 2003), but no quantitative data; –, not reported to occur naturally in foods

[a] From International Organization of the Flavour Industry (1995) and Lucas et al. (1999) or National Academy of Sciences (1970, 1982, 1987).

[b] Intake expressed as μg/person per day was calculated as follows: [(annual volume, kg) × (1×10^9 μg/kg)]/[population × survey correction factor × 365 days], where population (10%, 'eaters only') = 32×10^6 for Europe and 26×10^6 for the USA. The correction factor = 0.6 for Europe and 0.8 for the USA, representing the assumption that only 60% and 80% of the annual volume of production of the flavour, respectively, was reported in the poundage surveys (Lucas et al., 1999; International Organization of the Flavour Industry, 1995; National Academy of Sciences, 1970,1982, 1987). Intake expressed as μg/kg bw per day was calculated as follows: [(μg/person per day)/body weight], where body weight = 60 kg. Slight variations may occur from rounding.

[c] Calculated as follows: (Relative molecular mass of alcohol/Relative molecular mass of ester) × Daily per capita intake ('eaters only') ester.

[d] Quantitative data for the USA reported by Stofberg & Grundschober (1987).

[e] The consumption ratio is calculated as follows: (annual consumption via food, kg)/(most recently reported volume as a flavouring agent, kg).

[f] Annual volume reported in previous USA surveys (National Academy of Sciences, 1970, 1982, 1987).

[g] The volume cited is the anticipated annual volume of production, which was the maximum amount of flavouring agent estimated to be used annually by the manufacturer at the time the material was proposed for use as a flavour.

1.4 Application of the Procedure for the Safety Evaluation of Flavouring Agents

Step 1. In applying the Procedure, the Committee assigned 20 of the 22 flavouring agents in this group (Nos 1458–1471, 1474–1479) to structural class I. The other two flavouring agents (Nos 1472 and 1473) were assigned to structural class II (Cramer et al., 1978).

Step 2. All the flavouring agents in this group are expected to be metabolized to innocuous products. Their evaluation therefore proceeded via the A-side of the decision-tree.

Step A3. The estimated daily intakes of the 20 flavouring agents in structural class I and the two flavouring agents in structural class II, are below the respective thresholds of concern (i.e. 1800 μg/person for class I and 540 μg/person for class II). According to the Procedure, the safety of these 22 flavouring agents raises no concern when they are used at estimated current intakes.

The intake considerations and other information used to evaluate the 22 phenyl-substituted aliphatic alcohols and related aldehydes and esters in this group according to the Procedure are summarized in Table 1.

1.5 Consideration of secondary components

One member of this group of flavouring agents, 2-methyl-3-(*p*-isopropylphenyl)-propionaldehyde (No. 1465), has a minimum assay value of <95%. Information on the safety of the secondary component of this compound is summarized in Annex 5 (Summary of the safety evaluation of secondary components for flavouring agents with minimum assay values of less than 95%). The secondary component, 2-methyl-3-(*p*-isopropylphenyl)propionic acid, is structurally related to the primary flavouring agent and is expected to share the same metabolic fate. On this basis, the Committee considered that 2-methyl-3-(*p*-isopropylphenyl)propionaldehyde does not present a safety concern at estimated current intakes.

1.6 Consideration of combined intakes from use as flavouring agents

In the event that all 20 agents in structural class I were consumed concurrently on a daily basis, the estimated combined intake would not exceed the human intake threshold for class I (1800 μg/person per day). In the event that the two agents in structural class II were consumed concurrently on a daily basis, the estimated combined intake would not exceed the human intake threshold for class II (540 μg/person per day). Overall evaluation of the data indicated that combined intake of the agents in this group would not present a safety concern.

1.7 Conclusions

The Committee concluded that none of the flavouring agents in this group of phenyl-substituted aliphatic alcohols and related aldehydes and esters would raise a safety concern at estimated current intakes. Available data on the toxicity and

metabolism of these substances were consistent with the results of the safety evaluation.

2. RELEVANT BACKGROUND INFORMATION

2.1 Additional considerations on intake

The volumes of production and daily per capita intakes of each agent in this group are reported in Table 2.

Phenyl-substituted aliphatic alcohols and related substances have been detected in a variety of foods including nuts (i.e. nutmeg, roasted filberts, peanuts, macadamia nuts), cheeses (i.e. blue, provolone), sesame seeds, 'french-fried' potatoes, pork liver, wine, malt, starfruit, mushrooms, asparagus, okra, coffee, tea, and cocoa (Nijssen et al., 2003). As shown in Table 2, seven of the agents in this group have been reported to occur naturally in foods (Nijssen et al., 2003). Quantitative data on natural occurrence and consumption ratios have been reported for three agents in the group. Data on annual volumes of production for 2-phenyl-2-butenal (No. 1474), 5-methyl-2-phenyl-2-hexenal (No. 1472), and 2-methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465) demonstrate that their consumption occurs predominantly from traditional foods (i.e. consumption ratio, >1). 2-Methyl-3-(*p*-isopropylphenyl)propionaldehyde (No. 1465) is present in nutmeg; intake of this agent from this food is approximately 50% of the intake of 2-methyl-3-(*p*-isopropylphenyl)propionaldehyde as an added flavouring agent (Stofberg & Kirschman, 1985; Stofberg & Grundschober, 1987) (see Table 2).

2.2 Biological data

2.2.1 Biochemical data

(a) Hydrolysis

Phenyl-substituted esters are expected to be hydrolysed rapidly to the corresponding phenyl-substituted alcohols or carboxylic acids by classes of enzymes known as carboxylesterases (Heymann, 1980). In mammals, these enzymes occur in most tissues (Heymann, 1980; Anders, 1989), but predominate in the hepatocytes (Heymann, 1980). Acetals also are known to hydrolyse to their corresponding aldehydes and alcohols under acidic conditions, even in the absence of enzyme catalysis (Morgareidge, 1962).

Methods using simulated gastric juice and intestinal fluid have been developed to study ester hydrolysis in vitro. Aromatic esters such as phenylethyl acetate, methyl phenylacetate, ethyl phenylacetate, isopropyl phenylacetate, isoamyl phenylacetate, and citronellyl phenylacetate rapidly hydrolyse in simulated gastric juice and intestinal fluid (Longland et al., 1977). Rapid hydrolysis ($t_{1/2} \leq 1.4 \pm 0.1$ min) occurred when a series of structurally related 3-phenylpropenyl and 3-phenylpropionic acid esters (cinnamyl propionate, propyl cinnamate, butyl cinnamate, 3-phenylpropyl cinnamate and cinnamyl cinnamate) were incubated with artificial intestinal fluid containing pancreatin (Buck & Renwick, 2000).

Aromatic acetals also rapidly hydrolyse in acidic simulated gastric juice. Incubation of the aromatic acetal, 2-phenylpropionaldehyde dimethyl acetal (No. 1468) at a concentration of 1 mmol/l in the presence of simulated gastric juice at 37 °C in vitro resulted in 97% hydrolysis within 1 h, but incubation with simulated intestinal fluid for 5 h resulted in only 5.1% hydrolysis (Morgareidge, 1962).

Analogously, phenyl-substituted esters and acetals are anticipated to be hydrolysed to the corresponding alcohols, aldehydes, and carboxylic acids, before being metabolized further and excreted.

(b) Absorption, distribution and excretion

2-Phenylpropanol derivatives (Nos 1159, 1463, 1467–1474, and 1476) in this group are expected to be rapidly absorbed in the gastrointestinal tract and excreted primarily in the urine.

Five chinchilla rabbits were given a single dose of β-methylphenethyl alcohol (No. 1459) at 500 mg/kg bw suspended in water by oral gavage. Sixty to eighty per cent of the administered dose was excreted as glucuronic acid conjugates in the urine at 24 h (Robinson et al., 1955). In a study by Gruneberg & Ladgecker (1957), a group of seven rabbits was given β-methylphenethyl alcohol orally at a dose of 100, 250, 500, or 1000 mg/kg bw, while a group of four dogs received β-methylphenethyl alcohol orally at doses ranging from 50 to 200 mg/kg bw. Maximum urinary excretion of glucuronic acid conjugates was reached 3 h after dosing in both species. Pooled samples of urine of the rabbits or dogs at 24 h revealed that 50 and 58%, respectively, of the administered dose was excreted as unconjugated or conjugated (glucuronic acid) metabolites. A human given β-methylphenethyl alcohol orally at a dose of 100 mg/kg bw excreted 18.7% of the administered dose in the urine, mainly as glucuronic acid conjugates, within 24 h after dosing (Gruneberg & Ladgecker, 1957).

Three chinchilla rabbits received 2-phenylpropionaldehyde (No. 1467) as a single dose at 300 mg/kg bw by gavage. At 24 h, it was found that 52% of the administered dose was excreted as the glucuronic acid conjugate of the corresponding acid, 2-phenylpropionic acid, in the urine (Robinson et al., 1955).

In two men given 5 mg of (+/–)-[methyl-^{14}C]2-phenylpropionic acid orally, 95% and 100%, respectively, of the administered dose was excreted in the urine as the corresponding glucuronic acid conjugate within 24 h. In two Rhesus monkeys given (+/–)-[methyl-^{14}C]2-phenylpropionic acid at a dose of 81 mg/kg bw by intramuscular injection, 82 and 71% of the administered dose, respectively, was excreted as glucuronic acid conjugates in urine collected for 24 h after dosing. In rabbits given (+/–)-[methyl-^{14}C]2-phenylpropionic acid at a dose of 81 mg/kg bw, 82% of the administered dose was excreted, predominantly as the glucuronic acid conjugate (73%), in the urine within 24 h. Bile-duct cannulated rats were given (+/–)-[methyl-^{14}C]2-phenylpropionic acid at a dose of 5, 50, or 500 mg/kg bw and bile was collected for 3 h after dosing. Independent of dose, approximately 20–30% of the administered dose was excreted in the bile, mainly as the glucuronic acid conjugate (75%) with smaller amounts excreted as the unchanged acid (Dixon et al., 1977).

Male Sprague-Dawley rats given *R*-2-phenylpropionic acid or *S*-2-phenylpropionic acid at a dose of 200 mg/kg bw by intraperitoneal injection excreted the corresponding glucuronic acid conjugates into the urine within 16 h (Li et al., 2002). Male Sprague-Dawley rats given 2-phenylpropionic acid at a dose of 80 mg/kg bw per day for 7 days exhibited increased levels of glucuronic acid conjugates in the urine (Lake et al., 1980). Male Japanese white rabbits given 2-phenylpropionic acid at a dose of 200 mg/kg bw as the sodium salt in aqueous solution by an unspecified route excreted the corresponding glucuronic acid conjugate in the urine within 24 h urine (Nakamura & Yamaguchi, 1987).

In a multispecies study, approximately 50% of a dose of 150 mg/kg bw of *R*,*S*-[methyl-^{14}C]2-phenylpropionic acid administered by intraperitoneal injection was excreted in the urine of male ($n = 4$) and female rats ($n = 4$) **(**57.5 ± 11.6 and 46.6 ± 4.9%, respectively), mice ($n = 4$) (51.3 ± 5.6%), and two rabbits (46.6 and 54.9%, respectively), within 48 h (values quoted are mean ± standard deviation). In both sexes of all species tested, most of the administered dose was eliminated within the first 24 h (Fournel & Caldwell, 1986). Male Wistar albino rats were given *R*-, *S*-, or *R*,*S*-2-phenylpropionic acid at a dose of 150 mg/kg bw by intraperitoneal injection, and samples of blood, urine, and faeces were obtained. The plasma half-lives of the *R*-, *S*-, and *R*,*S*-isomers (3.00, 4.78, and 3.83 h, respectively) and the elimination rates (0.2313, 0.1451, and 0.1811 per hour, respectively) indicate that the *R*-(–) isomer is more rapidly cleared than the *S*-(+) isomer (65.4 versus 43.6 ml/h). In rats, the *S/R* ratio increased from 1 (racemic) to >2 within 8 h of dosing. The stereoselective inversion of *R*- to *S*-has been reported to be more pronounced in female rats than in males. Given the inversion of the *R*- to *S*-isomer, it was not unexpected that the body burden, measured by the area under the curve (AUC), would be greater for the *S*- isomer (AUC_{0-8h} = 214.5, 272.0, and 486.5 μg/ml·h for the *R*-, *S*-, and *R*,*S*-isomers, respectively) (Fournel & Caldwell, 1986).

Once formed, the acyl-glucuronide of *R*- and *S*-2-phenylpropionic acid undergoes internal acyl migration. The rate of conversion of the *R* form is about twofold greater than that for the *S* form. Thus the degradation half-life for the conjugated form of *R*-2-phenylpropionic acid is approximately one-half of that for the *S*-isomer (i.e. 1.8 versus 3.3 h) (Akira et al., 2000). Taken together, these pharmacokinetic data demonstrate that *R*-, *S*-, or *R*,*S*-2-phenylpropionic acid is rapidly distributed and eliminated in laboratory animals (Fournel & Caldwell, 1986; Meffin et al., 1986; Nakamura & Yamaguchi, 1987; Akira et al., 2000). *R*-, *S*-, or *R*,*S*-2-Phenylpropionic acid derived from the metabolism of phenylpropanol and its esters would also be rapidly distributed and eliminated.

The stereoselective disposition of (+/–)-2-phenylpropionic acid was studied in groups of intact, bile-duct cannulated, bile-duct ligated, and both nephrectomized and bile-duct cannulated rats given a single dose at 20 mg/kg bw by intravenous injection. After 5 min, plasma protein binding of the *R*-(–) stereoisomer was slightly higher than that of the *S*-(+) stereoisomer, which corresponded to a slightly increased tissue distribution of the *S*-(+) isomer. After 1 h, the enantiomeric excess of the *S*-(+) isomer in the plasma was 60%, suggesting selective loss of the *R*-(–)-isomer. After 6 h, bile-duct cannulated rats excreted a greater proportion of the administered dose of (+/–)-2-phenylpropionic acid in the bile (51% as the

glucuronic acid conjugate) than in the urine (40%, comprising 8% as free and 32% as conjugated acid). The total amount of *S*-(–) isomer excreted into the bile and urine accounted for 63% of the administered dose, suggesting *R*- to *S*-isomerization. At any time-point, the total elimination (i.e. biliary and urinary excretion) of free and conjugated *R*-(–) isomer was <50%. The combination of a lower rate of excretion and lower plasma concentrations for the *R*-(–) isomer provide further evidence of *R*- to *S*-isomerization (Yamaguchi & Nakamura, 1985).

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, 2-(4-isobutylphenyl)propionic acid, are derivatives of 2-phenylpropionic acid. The pharmacokinetic activity of NSAIDs, especially ibuprofen, has been directly related to chiral inversion and the subsequent yield of the pharmacologically more active stereoisomer (Lee et al., 1985). Therefore, extensive studies, both metabolic and toxicological, in humans have been performed for this chemical class. No attempt has been made to completely review all these studies here. However, the results of the most recent and thorough studies are included to provide relevant data for humans.

Six men and six women were each given 400 mg (a dose of approximately 5.7 mg/kg bw) of (+/–)-ibuprofen and pharmacokinetic parameters were monitored for 10 h after dosing. Maximum serum concentrations (C_{max}) of both enantiomers were achieved 3 h after dosing, with the *S*-(+) isomer showing a slightly higher value than the *R*-(–) isomer. A significantly higher AUC for the *S*-(+) isomer corresponded to a more rapid elimination half-life for the *R*-(–) isomer. Stereoselective protein binding showed that the fraction of the unbound *S*-(+) isomer is significantly greater than the unbound *R*-(–) isomer (*S*/*R* = 2/1). Based on the stereochemical composition of (+/–)-ibuprofen and its metabolites in the urine, approximately 68% of the *R*-(–) isomer undergoes chiral inversion, with a significantly higher clearance rate via inversion than by other routes (Tan et al., 2002).

In a similar study, six healthy human volunteers were given 400 mg of ibuprofen in the form of two tablets on two separate occasions and pharmacokinetic parameters were monitored for 6 h after administration. Maximum serum concentrations were achieved within the first 2 h and, as in the above study, the *S*-isomer demonstrated a significantly higher serum concentration than the *R*-isomer. Both the AUC and the serum half-life were significantly higher for the *S*-isomer than the *R*-isomer. The rate of elimination measured for the *R*-isomer was significantly greater than that of the *S*-isomer (Suri et al., 1997).

In a 1-month study, three groups of human volunteers, young (one male, seven females), elderly (eight males, six females), and elderly with renal impairment (four males, nine females), were given 800 mg of racemic ibuprofen three times per day (2400 mg/day). *S*-D_4-Ibuprofen (10 mg) was administered with the first and last doses of the study. The half-life of *S*-D_4-Ibuprofen was significantly longer ($p < 0.05$) in the elderly volunteers with renal impairment. The inversion of *R*-ibuprofen to *S*-ibuprofen was comparable in all three groups. After the first dose, the *S*/*R* ratio for all groups was 2.1 and after the last dose the *S*/*R* ratios were 2.8, 2.4, and 2.4 for young, elderly, and elderly with renal impairment, respectively. The group of young people showed significantly ($p < 0.05$) lower levels of unbound *S*-ibuprofen than the groups of elderly people and elderly people with renal impair-

ment at the first and last doses tested. As in the above study, in comparison with the *S*-enantiomer, greater proportions of the *R*-enantiomer were found bound to serum protein. After the first and last dose tested, the body burden for the *S*-enantiomer was significantly ($p < 0.0001$) greater than that of the *R*-enantiomer, which was associated with a more rapid clearance of the *R*-enantiomer. Repeated dosing increased the rate of inversion of *R*- to *S*-, but had no effect on the fraction inverted (Rudy et al., 1995). On the basis of these data, the pharmacokinetic behaviour of 2-phenylpropionic acid derivatives are similar in rats and humans.

The four 3-phenylpropyl (Nos 1465, 1466, 1475, and 1478) derivatives in this group are anticipated to show pharmacokinetic behaviour similar to that of other, previously evaluated, 3-phenylpropyl derivatives (Annex 1, reference *149*). Likewise, the six 4-phenylbutyl derivatives (Nos 1458, 1460–1462, 1464, and 1477) are anticipated to show a pattern of absorption, distribution, and elimination similar to that of 2-phenylethyl derivatives (Annex 1, reference *160*).

(c) Metabolism

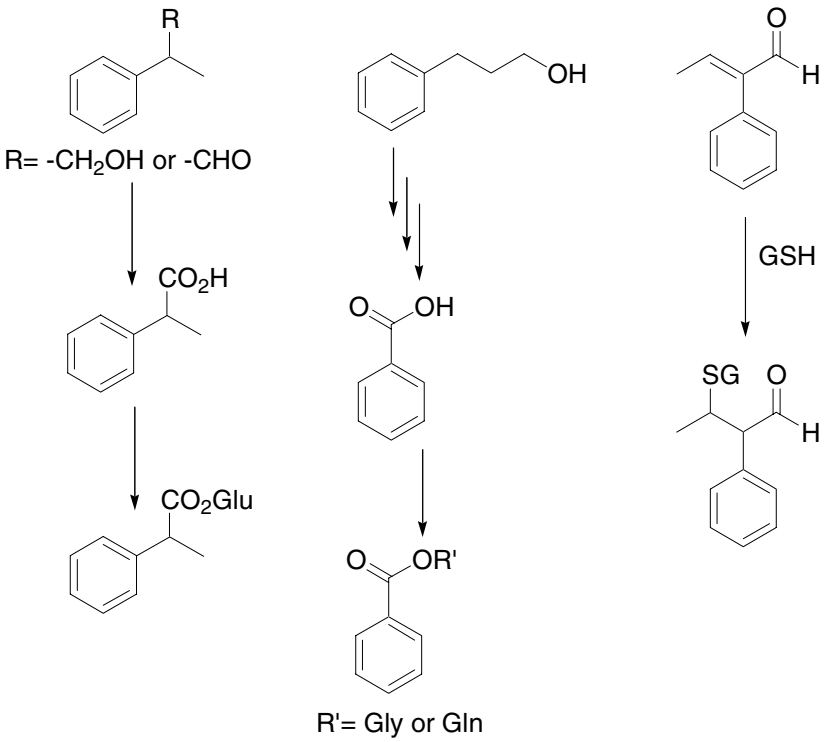
After hydrolysis, the resulting alcohols and aldehydes in this group of flavouring agents are oxidized to yield the corresponding carboxylic acids. In the case of 2-phenylpropyl derivatives, the carboxylic acid is subsequently conjugated with glucuronic acid and excreted primarily in the urine. Derivatives of 3-phenylpropionic acid and 4-phenylbutyric acid may also undergo β-oxidation and cleavage to yield the corresponding derivatives of benzoic acid (Annex 1, reference *149*) and phenylacetic acid (Annex 1, reference *160*). These acids are conjugated predominantly with glycine or glutamine, respectively, and excreted in the urine (see Figure 1).

Groups of three rabbits were given β-methylphenethyl alcohol (No. 1459) at a dose of 500 mg/kg bw, 2-phenylpropionaldehyde (No. 1467) at a dose of 300 mg/kg bw, or (+/–)-, (+)-, or (–)-2-phenylpropionic acid at a dose of 300 mg/kg bw by oral administration, and urine was collected for 24 h. In all cases, the principal metabolite (>52%) was identified as the glucuronic acid conjugate of 2-phenylpropionic acid (Robinson et al., 1955). After administration of *p*-methylphenethyl alcohol at a dose of 544 mg/kg bw, approximately 70% of the administered dose was excreted in the urine as the glucuronic acid conjugate of the acid metabolite. A smaller quantity (12%) of the administered dose was excreted as the glucuronic acid conjugate of the parent alcohol (Williams, 1959).

A comprehensive study has been performed on the effect of species, dose, and mode of administration on the metabolism of (+/–)-2-phenylpropionic acid (Dixon et al., 1977). For doses in the range of 5 to 500 mg/kg bw, the principal metabolite in man (5 mg/kg bw, oral), Rhesus monkeys (81 mg/kg bw, administered by intramuscular injection), rabbits (81 mg/kg bw, administered orally), cats (81 mg/kg bw, administered by intraperitoneal injection), and rats (5, 50, 500 mg/kg bw, administered by intraperitoneal injection) was the glucuronic acid conjugate of the 2-phenylpropionic acid administered. Only in the cat were appreciable quantities of the glycine (9%) and taurine (13%) metabolite form identified.

In a follow-up study with the different stereochemical forms (*R*, *S*, and *R,S*) of 2-phenylpropionic acid (Fournel & Caldwell, 1986), it was shown that chiral inver-

Figure 1. Metabolism of phenyl-substituted aliphatic alcohols and related aldehydes and esters



Gln, glutamine; Gly, glycine; Glu, glutamic acid; GSH, glutathione

sion occurs during the metabolism of 2-phenylpropionic acid in animals (see Figure 2). This process is independent of glucuronidation of the acid. Groups of rats (four of each sex), mice (four males), and rabbits (two females) were given *R,S*-[methyl-^{14}C]2-phenylpropionic acid at a dose of 150 mg/kg bw intraperitoneally, and urine was collected for 24 h. The female rats excreted slightly more of the free (7.4%) and conjugated (37.4%) 2-phenylpropionic acid than the male rats (3 and 30.1%, respectively). The rabbits showed a metabolic profile similar to that of the rats, while the mice excreted more of the unconjugated acid (13.4% free versus 29.1% conjugated). Measurement of the amounts of urinary *R*-(–) and *S*-(+) stereoisomers showed that male and female rats and rabbits exhibited stereoselective inversion of the *R*-(–) to the *S*-(+), with greater stereoselectivity occurring during the 24–48 h after dosing. Although, chiral inversion was reported not to occur in mice during metabolism, mice demonstrated a slight preference for glucuronidation of the *S*-(+) isomer (Fournel & Caldwell, 1986). The results obtained in rats were confirmed in another study in which rats were given (+/–)-2-phenylpropionic acid

at a dose of 20 mg/kg bw by intravenous injection. The enantiomeric enrichment of the *S*-(+) isomer appearing in the bile and urine within 6 h was approximately 63% (Yamaguchi & Nakamura, 1985).

In the same study (Fournel & Caldwell, 1986), the influence of dose and route of administration on the stereochemical course of metabolism was examined. When groups of four male rats were given *R*-(–)-2-phenylpropionic acid at a dose of 30, 150, or 300 mg/kg bw, the stereoisomer ratio measured in the urine at 24 h was approximately *R/S* = 7/3 at the two higher doses, but only 11/9 at the lowest dose. In all cases, inversion to the *S*-(+) form was greater on day 2 than on day 1 of dosing. *S*-(+)-Phenylpropionic acid, administered at the same doses, showed a much slower rate of conversion to the *R*-(–) form. Finally, the isomeric composition in the urine of rats given the racemic form (*R*,*S*), either orally or by intraperitoneal injection, was independent of the route of administration. The authors noted that both enantiomers undergo glucuronidation and that there is some stereoselectivity for glucuronidation of the *S*-(+) isomer in the rat and the mouse. For ibuprofen, a derivative of 2-phenylpropionic acid, glucuronidation of the *S*-form was favoured in humans (Lee et al., 1985).

In a study using deuterium labelled [$^2H_5$](+/–)-4-isobutyl-2-phenylpropionic acid[1] (i.e. ibuprofen) in rats it was shown that chiral inversion involves the loss of the hydrogen at the chiral position. However, hydrogen loss was not identified as a rate limiting step. The authors proposed that chiral inversion occurs via enzyme-catalysed stereoselective formation of a coenzyme A ester of the acid and subsequent formation of a planar enolate tautomer (Sanins et al., 1991).

In the pharmacokinetic study discussed above (section 2.2.1(b); Tan et al., 2002), metabolites in the urine in humans were analysed 24 h after administration of (+/–)-ibuprofen as an oral dose at approximately 5.7 mg/kg bw. Of the administered dose, 74% was excreted as the *S*-(+) isomer (unchanged acid, 11%; oxidized positions on the isobutyl side chain, 63%). A different stereochemical preference for the *S*-(+) isomer occurred for each metabolite. The *S*/*R* ratio of 7/1 was recorded for the glucuronic acid conjugate of (+/–)-ibuprofen, 4.8/1 for the 2′-hydroxylated metabolite, and 3.4/1 for the 4′-carboxy metabolite. No significant difference in metabolism was observed between males and females.

In conclusion, esters of β-methylphenethyl alcohol are hydrolysed to the component alcohol, and then subsequently oxidized to the corresponding aldehyde and carboxylic acid. The resulting 2-phenylpropanionic acid is then conjugated with glucuronic acid and excreted primarily in the urine. It has been shown that stereoselective chiral inversion (*R*- to *S*-) occurs during the metabolism of 2-phenylpropionic acid in vivo.

If the alkyl side-chain contains α,β-unsaturation as in 2-phenylalkenyl aldehyde derivatives (Nos 1472–1474), the electrophilic α,β-unsaturated alkene is suscep-

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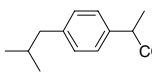
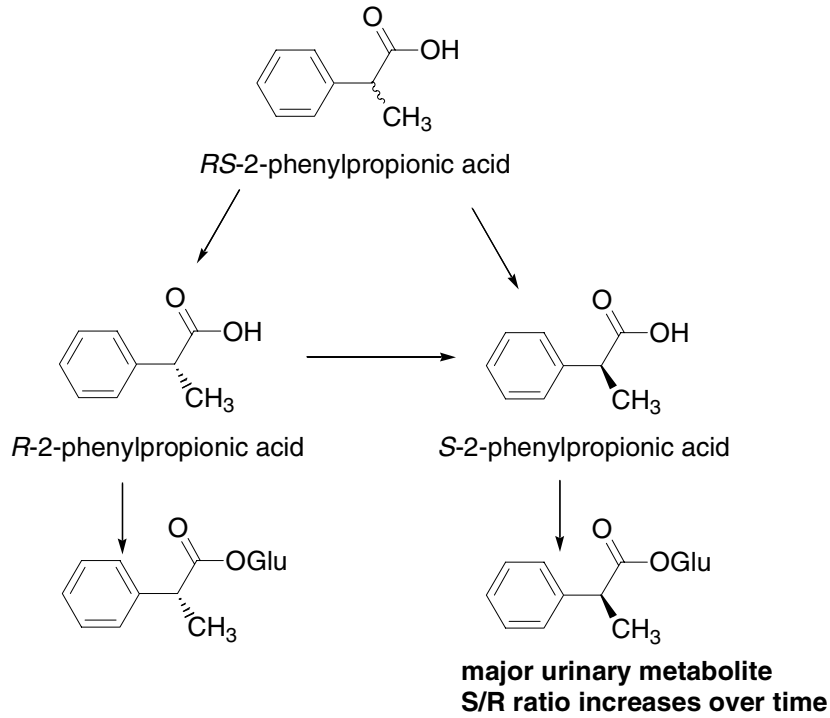
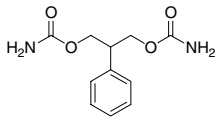
1  (+/–)-4-isobutyl-2-phenylpropionic acid

tible to nucleophilic attack by glutathione or other endogenous thiols. For example, 2-phenylpropenal (No. 1467), a metabolite of the anti-epileptic drug felbamate, has been shown to undergo glutathione conjugation to yield mercapturic acid conjugates in rodents and humans (Thompson et al., 1997). When six adult male Sprague-Dawley rats were given felbamate[2] at a dose of 800 mg/kg bw by gavage, 2-phenylpropenal mercapturic acid conjugate was identified in the urine at 18 h. In the same study, in one healthy male human volunteer given a tablet containing 600 mg of felbamate (a dose of 9 mg/kg bw determined by study authors), the urine

Figure 2. Metabolism of RS-2-phenylpropionic acid in mammals



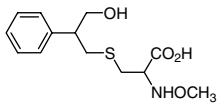
2  felbamate

at 8 h contained reduced[3] and oxidized[4] mercapturic acid conjugates of 2-phenylpropenal (Thompson et al., 1997). Similarly, the α,β-unsaturated derivatives of 2-phenylalkene aldehyde (Nos 1472–1474) may conjugate with glutathione, before being readily converted into mercapturic acid conjugates and eliminated.

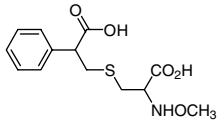
Experiments in vitro provide additional evidence for glutathione conjugation. 2-Phenylpropenal readily forms glutathione conjugates when incubated in vitro with free glutathione at pH 8.0 (Thompson et al., 1996). The half-life for 2-phenylpropenal is approximately 1 min (Thompson et al., 1996). The half-life of formation of the glutathione conjugate of 2-phenylpropenal was determined individually in the presence of three isoforms of human glutathione transferase (GST), GSTM1-1, GSTP1-1, or GSTA1-1, and in the absence of GST. The formation of the glutathione conjugate was more efficiently catalysed by GSTM1-1 than GSTP1-1, followed by GSTA1-1 (half-lives of formation ($t_{1/2}$) were 11.3 ± 1.2, 17.7 ± 0.4, and 26.4 ± 2.4, respectively and k_{cat}/K_m values were 0.275 ± 0.035, 0.164 ± 0.005, and 0.042 ± 0.005 μmol/l^{-1}s^{-1}, respectively) (Dieckhaus et al., 2001). In vitro, 2-phenylpropenal inhibited the catalytic activity of GSTM1-1 and GSTP1-1, with the inhibition of the latter being reversible in the presence of excess glutathione (Diekhaus et al., 2001).

On the basis of these data, two metabolic options were anticipated to exist for the metabolic detoxication of 2-phenylalkenyl aldehydes (e.g. Nos 1472, 1473, and 1476). In one pathway, the aldehyde is oxidized to the corresponding carboxylic acid, and then subsequently conjugated with glucuronic acid and excreted. In the alternative pathway, the alkene function may react with glutathione to yield mercapturic acid conjugates that are readily excreted primarily in the urine.

Derivatives of 3-phenylpropanal (Nos 1465 and 1466) and a related ester (No. 1475) undergo β-oxidation and cleavage to yield benzoic acid and are subsequently converted to hippuric acid and excreted in the urine. Metabolic data related to the evaluation of 3-phenylpropyl derivatives was reviewed previously by the Committee (Annex 1, reference *149*). Dogs given the sodium salt of 2-methyl-3-phenylpropionic acid at a dose of approximately 500 mg/kg bw excreted 77% of the administered dose as hippuric acid and benzoic acid in the urine (Kay & Raper, 1924). Sterically-hindered acids, 2-ethyl- and 2-propyl-3-phenylpropionic acids, were shown not undergo β-oxidation in dogs given an unspecified oral dose, but instead were excreted unchanged in the urine (Carter, 1941).



3 reduced mercapturic acid conjugate of 2-phenylpropenal



4 oxidized mercapturic acid conjugate of 2-phenylpropenal

The 3-phenylpropanoic acid derivatives, 2-oxo-3-phenylpropionic acid (Nos 1478 and 1479), are endogenous in humans, being formed by the oxidative deamination of phenylalanine (Nelson & Cox, 2000). Extensive scientific studies have been performed on this substance as it is a major catabolite of phenylalanine in patients with phenylketonuria. Excess phenylalanine undergoes transamination with pyruvate to form alanine and phenylpyruvate, which can accumulate in the blood, tissues, and urine in patients with phenylketonuria (Nelson & Cox, 2000). At low levels, endogenous 2-oxo-3-phenylpropionic acid was found to be primarily decarboxylated to yield phenylacetate, and readily excreted in the urine. A minor metabolite, the reduction product phenylacetate, was also excreted in the urine (Nelson & Cox, 2000).

After hydrolysis of 4-phenylbutyrate esters (Nos 1458 and 1464), and oxidation of 2-methyl-4-phenylbutyraldehyde (No. 1462), the resulting carboxylic acids undergo β-oxidation and cleavage to yield phenylacetic acid, before being excreted primarily as the glutamine conjugate in humans and a glycine conjugate in rodents (Annex 1, reference *160*). Of the doses of the sodium salt of 2-methyl-4-phenylbutyric acid of 480 and 600 mg/kg bw administered hypodermically to two cats, approximately 42% was excreted in the urine as the glycine conjugate of phenylacetic acid (Kay & Raper, 1924). In dogs, 4-phenylbutyric acid has been shown to undergo β-oxidation, resulting in urinary excretion of phenylacetic acid and its glycine conjugate (Raper & Wayne, 1928).

The remaining three 4-phenylbutyl derivatives (Nos 1460, 1461, and 1477) are tertiary alcohols and do not undergo β-oxidation, but rather, conjugate directly with glucuronic acid and are excreted in the urine. 2-Phenylpropan-2-ol, a structurally related tertiary alcohol at a dose of 500 mg/kg bw given orally to three rabbits resulted in almost complete (60–98%) excretion of the glucuronic acid conjugate of the unchanged alcohol within 24 h (Robinson et al., 1955).

In conclusion, the 2-phenylpropyl or propenyl derivatives in this group are readily hydrolysed and oxidized to the corresponding carboxylic acids, before being subsequently conjugated with glucuronic acid and excreted. α,β-Unsaturated aldehyde derivatives may be conjugated with glutathione and excreted as mercapturic acids, while 3-phenylpropyl derivatives, are expected to oxidize to the corresponding 3-phenylpropionic derivatives. Subsequently, the 3-phenylpropionic derivatives are subjected to β-oxidation and cleavage, to yield benzoic acid. The resulting benzoic acid is conjugated with glycine and excreted as hippuric acid. Finally, 4-phenylbutyl derivatives undergo hydrolysis followed by functional group oxidation (β-oxidation) and cleavage to phenylacetic acid, which is conjugated primarily with glutamine and excreted in the urine. Conversely, 4-phenylbutyl derivatives occurring as tertiary alcohols are instead conjugated with glucuronic acid, before elimination in the urine.

2.2.2 Toxicological studies

(a) Acute toxicity

Oral median lethal dose (LD_{50}) values have been reported for 9 of the 21 substances in this group and are summarized in Table 3. Eight of the phenyl-

Table 3. Studies of the acute toxicity of phenyl-substituted aliphatic alcohols and related aldehydes and esters administered orally

No.	Flavouring agent	Species	Sex	LD_{50} (mg/kg bw)	Reference
1459	β-Methylphenethyl alcohol	Rat	NR	2300	McGee (1974)
1460	2-Methyl-4-phenyl-2-butyl acetate	Rat	NR	4800	Levenstein (1975)
1465	2-Methyl-3-(p-isopropylphenyl)propionaldehyde	Rat	M, F	3810	Jenner et al. (1964)
1466	2-Methyl-3-tolylpropionaldehyde	Rat	NR	4100	Moreno (1977)
1467	2-Phenylpropionaldehyde	Rat	M, F	2800	Jenner et al. (1964)
1467	2-Phenylpropionaldehyde	Rat	M, F	3650	Wong & Weir (1971)
1467	2-Phenylpropionaldehyde	Rat	NR	2800	Bär & Griepentrog (1967)
1468	2-Phenylpropionaldehyde dimethyl acetal	Rat	NR	1850	Moreno (1975)
1471	2-(*p*-Tolyl)propionaldehyde	Rat	NR	3500	Moreno (1973)
1475	Ethyl 2-ethyl-3-phenylpropanoate	Mouse	NR	>41000	Pellmont (1969)
1477	2-Methyl-4-phenyl-2-butanol	Rat	M, F	<5000	Shellanski & Moldovan (1973)
1477	2-Methyl-4-phenyl-2-butanol	Rat	M, F	2112[a]	Shellanski & Moldovan (1973)

F, female; M, male; NR, not reported.

[a] Calculated based on density of 0.96 ml/kg (reported as 2.2 ml/kg).

substituted alcohols and related aldehydes and esters used as flavouring agents (Nos 1459, 1460, 1465–1468, 1471, and 1477) have oral LD_{50} values in the range of 1850 to 5000 mg/kg bw in rats (Jenner et al., 1964; Bär & Griepentrog, 1967; Wong & Weir, 1971; Moreno, 1973, 1975, 1977; Shellanski & Moldovan, 1973; McGee, 1974; Levenstein, 1975). In mice, oral LD_{50} values of >41 000 mg/kg bw were reported for ethyl 2-ethyl-3-phenylpropanoate (No. 1475) (Pellmont, 1969), demonstrating that the oral acute toxicities of phenyl-substituted alcohols and related aldehydes and esters in mice are also low.

(b) Short-term studies of toxicity

The results of short-term studies of toxicity with phenyl-substituted aliphatic alcohols and related aldehydes and esters used as flavouring agents are summarized in Table 4.

(i) β-Methylphenethyl alcohol (No. 1459)

Rats

Groups of 15 male and 15 female weanling Wistar rats were fed diets designed to provide β-methylphenethyl alcohol at a dose of 0 (control), 10, 40, or 160 mg/kg bw per day, for 13 weeks. Animals were observed throughout the study for signs of toxicity and overall condition, and weight was measured at the beginning of the study and twice per week thereafter. Samples of blood, for evaluation of haematological and clinical chemistry parameters, and urine were collected at week 6 and at study termination. At the end of the treatment period, all animals were necropsied and organs were examined grossly for any abnormalities. Samples of organ tissue for microscopic examination were obtained from the control group and the group receiving the highest dose. In comparison with the controls, body weights of males were unaffected by treatment, while treated females showed statistically significant, but non-dose-dependent reductions at week 3, which persisted for the remainder of the period. In males, food intake was slightly decreased at every dose compared with that in the control group; however, the decrease was statistically significant ($p < 0.01$) only at 40 mg/kg bw per day. Food intake in females was significantly decreased at 160 mg/kg bw per day, which was initially reported at week 4 and at every week thereafter. With the exception of significant decreases observed at the lowest dose, water intake in treated males was comparable to that of the controls, while in females at the lowest and highest doses, non-dose-related, statistically significant increases ($p < 0.01$) were reported. Haematological analysis performed at study termination revealed a statistically significant decrease ($p < 0.05$) in erythrocyte counts in males at the highest dose. Urine analysis conducted at 13 weeks revealed an increased incidence of trace proteinuria in females at the lowest dose compared with the control group. Significant clinical chemistry results consisted of elevated concentrations of protein and reduced concentrations of albumin, identified in males at the lowest and intermediate doses and in females at the lowest dose, respectively. Males at the highest dose showed significantly increased absolute and relative weights of the liver. A similar increase was reported in the relative weights of the liver in females at the

Table 4. Results of short-term studies of toxicity with phenyl-substituted aliphatic alcohols and related aldehydes and esters used as flavouring agents

No.	Flavouring agent	Species; sex	No. of test groups[a]/ no. per group[b]	Route	Duration (days)	NOEL (mg/kg bw per day)	Reference
1459	β-Methylphenethyl alcohol	Rat; M, F	3/30	Diet	91	10	Gaunt et al. (1982)
1462	2-Methyl-4-phenylbutyraldehyde	Rat; M, F	1/20-32	Diet	90	0.563[c] (M) 0.656[c] (F)	Posternak et al. (1969)
1466	2-Methyl-3-tolylpropionaldehyde	Rat; M, F	1/20-32	Diet	90	1.17[c] (M) 1.38[c] (F)	Posternak et al. (1969)
1467	2-Phenylpropionaldehyde	Rat; M, F	3/30	Gavage	105	10	Pelling et al. (1976)

F, female; M, male.

[a] Total number of test groups does not include control animals.

[b] Total number per test group includes both male and female animals.

[c] The study was performed with either a single dose or multiple doses that produced no adverse effect. The value is therefore not a true NOEL, but is the highest dose tested that produced no adverse effects. The actual NOEL may be higher.

highest dose. In males, dose-dependent increases were reported in relative weights of the kidney, reaching statistical significance in the groups receiving the intermediate and highest doses, while absolute weights of the kidney were significantly increased only at the highest dose. Additionally, a non-dose-related increase in relative weight of the brain was reported in all treated females. Relative weights of the full and empty caecum and of the adrenals were significantly increased in comparison to those of controls in females at the highest and lowest doses, respectively. Significantly elevated relative weights of the heart and pituitary were reported in males at the intermediate dose. Overall, variations in the relative weights of the brain, heart, pituitary, caecum, and adrenals were not associated with corresponding changes in absolute weights, and, with the exception of a single occurrence of a renal mesenchymal tumour reported in a female at the highest dose, none of the changes in organ weights were identified in the presence of any histopathological findings. Additional renal variations observed in the rat exhibiting the tumour included severe nephrosis, chronic inflammatory cell infiltration of the kidney, and hyperplasia of the renal pelvis and bladder. A higher incidence of alveolar thickening and peribronchial cuffing were reported in treated males and females, respectively, compared with controls. However, no dose–response relationship was presented. On the basis of the renal effects observed in males at the two higher intakes, the no-observed-effect-level (NOEL) was 10 mg/kg bw per day (Gaunt et al., 1982).

(ii) 2-Phenylpropionaldehyde (No. 1467)

Rats

Groups of 15 male and 15 female CFE rats were given 2-phenylpropionaldehyde at a dose of 0 (control), 10, 50, or 500 mg /kg bw in corn oil by gavage, 7 days per week for 15 weeks. Additional groups of 10 (five of each sex) and 15 (ten males, five females) rats were given 2-phenylproionaldehyde at the same doses for 6 and 2 weeks, respectively. Body weight and water and feed consumption were measured each week for 14 weeks. Urine analyses were conducted on samples taken during the last week of treatment. At study termination, blood samples were taken for haematological and serum analyses, and rats were killed and necropsied. Tissue samples were obtained for microscopic examinations. All animals were in good condition and were reported to survive until the end of the study period. In one male, a subcutaneous swelling in the neck was identified at week 6, which was determined by examination to be a cyst. Although the mean body weights of male rats after 14 weeks tended to be lower at the two higher doses, the difference was not statistically significant when compared with the controls. No differences in mean body weights were observed between the controls and any treated females. In both sexes at the highest dose, a non-statistically significant increase in mean feed consumption, and a significant elevation in water intake were reported. Haematological evaluations showed a decrease of 5–7% in the concentration of haemoglobin in males at the highest dose at 6 weeks, as well as at 15 weeks in both sexes at the highest dose and in females at the intermediate dose. Slight polycythaemia was noted in males at the intermediate dose at week 15. The variation, however, was not confirmed in males at the highest dose

or in any treated females. Significantly increased reticulocyte counts were reported in females at the highest dose at week 15. Results obtained from serum and urine analyses were similar in treated and control animals. At necropsy, pulmonary leukocyte infiltration was reported, suggestive of a mild respiratory infection (affected groups not specified). Statistically significant relative organ weight changes ($p < 0.05$), particularly in the liver, occurred mainly in animals at the highest dose. At 2 weeks, relative weights of the liver were increased in males at the intermediate and highest doses and in females at the highest dose. At 6 weeks, relative weights of the liver were increased for males at the highest dose and females at the intermediate and highest doses. At 15 weeks, relative weights of the liver were increased at every dose in males and at the highest dose in females. However, the authors questioned the relevance of the increased weights of the liver at the lowest and intermediate doses, suggesting that they were likely to reflect the slightly lower body weights observed in the two groups relative to those of the controls. At 2 weeks, increased weights of the kidney in males at the intermediate dose and increased weight of the pituitary in females at the highest dose were reported. At 6 weeks, males at the lowest and highest doses exhibited significantly increased relative weights of the heart. Additionally, males at the highest dose had significantly increased relative weights of the kidney, stomach, small intestine and pituitary, and females at the intermediate and highest doses had significantly increased relative weights of the brain and stomach, respectively. At the highest dose, significant increases in relative weights of the kidney and stomach were observed in both sexes, whereas increases in relative weights of the pituitary and heart were limited to males and females, respectively. With the exception of a small granuloma reported in the liver of one male at the highest dose at 15 weeks, the increased organ weights were not accompanied by any histological changes related to treatment. On the basis of these results, the NOEL for 2-phenylpropionaldehyde was 10 mg/kg bw per day in rats, but because of the marginal changes in liver weights the authors stated that the NOEL may be closer to 50 mg/kg bw per day (Pelling et al., 1976).

(iii) 2-Methyl-3-tolylpropionaldehyde (No. 1466)

Rats

Groups of 10–16 male and 10–16 female Charles River CD rats were fed a diet designed to provide 2-methyl-3-tolylpropionaldehyde at a dose of 1.17 (males) or 1.38 (females) mg/kg bw per day for 90 days. Body weight and feed consumption were recorded weekly and efficiency of feed utilization was calculated. Haematological evaluations were conducted on half the animals at week 7 and on all the animals at study termination. At necropsy, kidney and liver weights were measured and gross and histological examinations were conducted on a variety of organs. Compared with controls, no variations in body weight, feed consumption, haematology or clinical chemistry values, organ weights, or pathology were observed (Posternak et al., 1969).

(iv) 2-Methyl-4-phenylbutyraldehyde (No. 1462)

Rats

Groups of 10–16 male and 10–16 female Charles River CD rats were fed a diet designed to provide 2-methy-4-phenylbutyraldehyde at a dose of 0.563 (males) or 0.656 (females) mg/kg bw per day for 90 days. Body weight and feed consumption were recorded weekly and efficiency of feed utilization was calculated. Haematological evaluations were conducted on half the animals at week 7 and all the animals at study termination. At necropsy, weights of the kidney and liver were measured, and gross and histological examinations were conducted on a variety of organs. No effects on body weight, feed consumption, haematology, clinical chemistry, organ weight or pathology were reported. Slightly depressed concentrations of blood urea were observed in males and females, but the authors did not consider this to be of any toxicological significance (Posternak et al., 1969).

(c) Long-term studies of toxicity and carcinogenicity

No information was available

(d) Genotoxicity

(i) In vitro

Testing for genotoxicity in vitro has been performed for four (Nos 1459, 1467, 1468, and 1470) representative members of this group of phenyl-substituted aliphatic alcohols and related aldehydes and esters used as flavouring agents (see Table 5).

In standard assays for mutagenicity in *Salmonella typhimurium*, β-methylphenethyl alcohol (No. 1459), 2-phenylpropionaldehyde (No. 1467), 2-phenylpropionaldehyde dimethyl acetal (No. 1468) and 2-phenylpropyl isobutyrate (No. 1470) were not mutagenic in *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538 when tested at concentrations of up to 3600 μg/plate, with and without metabolic activation (Wild et al., 1983).

Derivatives of cinnamyl[5] are structurally related α,β-unsaturated phenyl-substituted alcohols, aldehydes and carboxylic acids. The Committee previously evaluated a group of cinnamyl alcohol and related flavouring agents and found them to be of no safety concern (Annex 1, reference *150*). Cinnamaldehyde (*trans* and unspecified stereochemistry), cinnamyl alcohol (*trans* and unspecified

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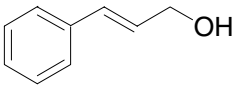
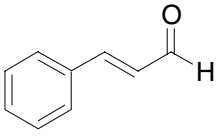
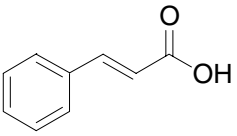
[5] 

cinnamyl alcohol



cinnamaldehyde



cinnamic acid

Table 5. Results of studies of genotoxicity with phenyl-substituted aliphatic alcohols and related aldehydes and esters used as flavouring agents

No.	Flavouring agent	End-point	Test system	Concentration or dose	Result	Reference
In vitro						
1459	β-Methylphenethyl alcohol	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98, and TA100	≤3600 μg/plate	Negative[a]	Wild et al. (1983)
1467	2-Phenylpropionaldehyde	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98, and TA100	≤3600 μg/plate	Negative[a]	Wild et al. (1983)
1468	2-Phenylpropionaldehyde dimethyl acetal	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98, and TA100	≤3600 μg/plate	Negative[a]	Wild et al. (1983)
1470	2-Phenylpropyl isobutyrate	Reverse mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA98, and TA100	≤3600 μg/plate	Negative[a]	Wild et al. (1983)
In vivo						
1467	2-Phenylpropionaldehyde	Sex-linked recessive lethal mutation (Basc test)	*D. melanogaster*	10 mmol/l (1341 μg/m)[b]	Negative	Wild et al. (1983)
1467	2-Phenylpropionaldehyde	Micronucleus formation	NMRI mice	134, 402, or 670 mg/kg bw	Negative	Wild et al. (1983)
1468	2-Phenylpropionaldehyde dimethyl acetal	Sex-linked recessive lethal mutation (Basc test)	*D. melanogaster*	5 mmol/l (901 μg/ml)[c]	Negative	Wild et al. (1983)
1468	2-Phenylpropionaldehyde dimethyl acetal	Micronucleus formation	NMRI mice	360, 630, or 900 mg/kg bw	Negative	Wild et al. (1983)

[a] With and without metabolic activation.
[b] Calculated using relative molecular mass of 2-phenylpropionaldehyde = 134.18.
[c] Calculated using relative molecular mass of 2-phenylpropionaldehyde dimethyl acetal = 180.25.

stereochemistry), cinnamic acid, α-methylcinnamaldehyde, cinnamyl acetate, benzyl cinnamate, cyclohexyl cinnamate, α-amylcinnamaldehyde, α-hexylcinnamaldehyde, and *p*-methoxy-α-methylcinnamaldehyde predominantly were inactive in *S. typhimurium*, including strains TA92, TA94, TA97, TA98, TA100, TA102, TA104, TA1535, TA1537, TA1538, and TA2637. The assays were performed using concentrations ranging up to the level of cytotoxicity, both in the absence and presence of metabolic activation (S9 fraction) obtained from the livers of Aroclor 1254 or methylcholanthrene-induced Sprague-Dawley rats or Syrian hamsters (Dunkel & Simon, 1980; Eder et al., 1980; Florin et al., 1980; Lijinsky & Andrews, 1980; Lutz et al., 1980; Eder et al., 1982a, 1982b; Kasamaki et al., 1982; Lutz et al., 1982; Prival et al., 1982; Sekizawa & Shibamoto, 1982; Neudecker et al., 1983; Wild et al., 1983; Ishidate et al., 1984; Huang et al., 1985; Marnett et al., 1985; Mortelmans et al., 1986; Fujita & Sasaki, 1987; Tennant et al., 1987; Kato et al., 1989; Eder et al., 1991; Dillon et al., 1992; Azizan & Blevins, 1995). The reader is referred to the evaluation made by the Committee at its fifty-fifth meeting (Annex 1, reference *149*) of other reported studies of genotoxicity with cinnamyl compounds and used to support the evaluation of the α,β-unsaturated phenyl-substituted alcohols, aldehydes, and carboxylic acids.

(ii) In vivo

The potential of 2-phenylpropionaldehyde (No. 1467) and 2-phenylpropionaldehyde dimethyl acetal (No. 1468) to induce sex-linked recessive lethal mutations in adult *Drosophila melanogaster* were studied in the Basc test. The frequency of mutation was unaffected when solutions of 2-phenylpropionaldehyde and 2-phenylpropionaldehyde dimethyl acetal at a concentration of 10 and 5 mmol/l, respectively (1341 and 901 μg/ml, respectively) were fed to the flies for 3 days (Wild et al., 1983).

In a test for micronucleus formation, groups of four male and four female NMRI mice given 2-phenylpropionaldehyde as a single intraperitoneal dose at 134, 402, or 670 mg/kg bw or 2-phenylpropionaldehyde dimethyl acetal as a single intraperitoneal dose at 360, 630, or 900 mg/kg bw, demonstrated no increase in micronucleated erythrocytes in samples of bone marrow obtained 30 h after administration (Wild et al., 1983).

Data on genotoxicity after administration of cinnamaldehyde in vivo support the conclusions for 2-phenylpropionaldehyde as well as for the three α,β-unsaturated aldehydes (Nos 1472–1274). An increase in the frequency of sex-linked recessive lethal mutations was reported when *D. melanogaster* was injected with cinnamaldehyde at a concentration of 20 000 mg/l. However, no increase in the frequency of mutations occurred when *D. melanogaster* were fed cinnamaldehyde at a concentration of 800 mg/kg of diet for 3 days. Reciprocal translocations were not observed in either assay (Woodruff et al., 1985). In mammalian test systems, there was no evidence for an increase in unscheduled DNA synthesis in hepatocytes in rats or mice given cinnamaldehyde at a dose of 1000 mg/kg bw by oral gavage (Mirsalis et al., 1989). In the assay for micronucleus formation in rodents, the frequency of formation of micronuclei was not increased when rats or mice were given cinnamaldehyde at a dose of 1700 mg/kg bw or 1100 mg/kg bw, respectively, by oral

gavage (Mereto et al., 1994), or when mice were given cinnamaldehyde at a dose of 500 mg/kg bw by intraperitoneal injection (Hayashi et al., 1984, 1988).

In one study (Mereto et al., 1994) an increase in micronucleated cells was reported in rat and mouse hepatocytes, and in rat (but not in mouse) forestomach cells after oral gavage dosing with cinnamaldehyde at a dose of up to 1100 (rats) or 1700 (mice) mg/kg bw. No increase in the formation of micronuclei in the forestomach were observed in rats or mice at doses of ≤850 mg/kg bw. The increased frequency of micronucleus formation was absent in hepatocytes from rats given a dose of 500 mg/kg bw, but present in hepatocytes from mice given a dose of 850 mg/kg bw. No DNA fragmentation was observed in the rat hepatocytes or gastric mucosa cells. An increase in the incidence and size of GST-positive foci in hepatocytes of rats pre-treated with *N*-nitrosodiethylamine and then given cinnamaldehyde at a dose of 500 mg/kg bw per day by oral gavage for 14 days (Mereto et al., 1994).

The positive findings with cinnamaldehyde in the rat forestomach and in the liver of both rats and mice in vivo are inconsistent with negative results observed in the standard assay in bone marrow and are observed at doses that far exceed those resulting from intake of cinnamaldehyde in food. It has been reported that cinnamaldehyde given at oral doses of ≥500 mg/kg bw results in the depletion of hepatocellular glutathione (Swales & Caldwell, 1991, 1992, 1993). Therefore, increases in micronucleus formation were reported at doses (1100 and 1700 mg/kg bw) that appear to affect cellular defence mechanisms (i.e. glutathione depletion). On the basis of the fact the micronucleus formation is dose-dependent, it appears that induction of micronuclei is a threshold phenomenon which occurs at intakes that are orders of magnitude greater than intake of cinnamaldehyde as a flavouring substance. Also, it is likely that the bolus doses resulting from gavage administration produce much greater exposures of both the forestomach and liver than does administration by dietary admixture. The author (Mereto et al., 1994) acknowledged these facts and concluded that the data did not justify the conclusion that cinnamaldehyde was clastogenic. As a result of the apparent threshold for induction of micronuclei and the lack of activity in the remainder of the studies in vivo, the results obtained with bolus, highest-dose exposures occurring in the liver and forestomach are not considered to be relevant to the safety of cinnamaldehyde used as a flavouring agent.

(iii) Conclusion

The testing of these representative 2-phenylpropanol derivatives in bacterial test systems in vitro (Ames assay) and in mammalian systems (test for micronucleus formation) in vivo showed no evidence of genotoxic potential. These results are further supported by the lack of positive findings in the Basc test. By analogy to structurally related cinnamyl derivatives, the α,β-unsaturated phenyl-substituted aliphatic alcohols and related aldehydes and esters (Nos 1472–1474, and 1476) present no evidence of genotoxicity in bacterial test systems (Ames assay) in vitro and in mammalian systems in vivo (tests for unscheduled DNA synthesis and for micronucleus formation).

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GLYCYRRHIZINIC ACID

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1. EXPLANATION

The Committee was asked to comment on the safety of glycyrrhizinic acid and its monoammonium salt as a natural constituent of liquorice (USA, 'licorice') and in its use as a flavouring substance in various food products. In the call for data, the term 'glycyrrhizic acid' was used rather than the alternative term 'glycyrrhizinic

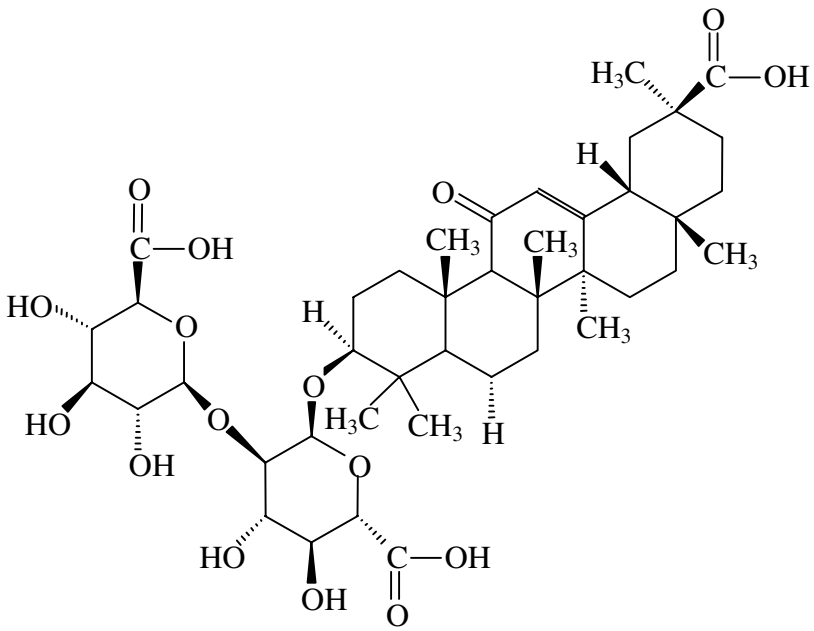
acid'. The Committee agreed to use the latter term. Glycyrrhizinic acid and its monoammonium salt have not been evaluated previously by the Committee.

Glycyrrhizinic acid is a naturally occurring triterpenoid saponin found in the extracts of roots and rhizomes from *Glycyrrhiza glabra*, the liquorice plant. Dried extracts of the roots of the liquorice plant, which may contain between 4% and 25% glycyrrhizinic acid, are present in liquorice confectionery, liquorice herbal teas and in some health products. Glycyrrhizinic acid and the monoammonium salt are both used as flavouring agents. It should be noted that in the literature some authors have used the term 'glycyrrhizin' interchangeably with 'glycyrrhizinic acid'; however, this is not technically correct. Glycyrrhizin is the term historically used to describe the crude acid extract of the liquorice plant.

Glycyrrhizinic acid has been determined to be 'generally recognized as safe' (GRAS) in the United States of America (USA) (Federal Register, 1985) and has been evaluated by the Nordic Council of Ministers (Størmer et al., 1993a) and by the Scientific Committee on Food (Scientific Committee on Food, 1991, 2003).

This monograph reviews the safety of glycyrrhizinic acid (and its ammonium salt) from all dietary sources with a view to establishing an acceptable daily intake (ADI). Data on glycyrrhizin (a crude liquorice extract containing glycyrrhizinic acid), various salts of glycyrrhizinic acid (exists in both α and β stereoisomers), and on the hydrolysis product of glycyrrhizinic acid, glycyrrhetic acid (which exists as both α and β stereoisomers, with the β-isomer being the metabolite of glycyrrhizinic acid), were evaluated.

Figure 1. Structural formula of glycyrrhizinic acid



2. BIOLOGICAL DATA

2.1 Biochemical aspects

It is important to clarify the term 'glycyrrhizin'. In the literature, some authors have used this term interchangeably with 'glycyrrhizinic acid'; however, this is not technically correct. Glycyrrhizin is the term used historically to describe the crude acid extract of liquorice isolated by the Houseman (1922) gravimetric method (described in Bell, 1980). Glycyrrhizinic acid (shown in Figure 1) is a component of glycyrrhizin and is a glycoside that occurs naturally in the liquorice root as the calcium, potassium, or ammonium salt (Mitchell, 1956; Morris & Muller, 1965; Wang et al., 2000; European Flavour and Fragrance Association, 2001). Moreover, the terms 'glycyrrhizinic acid' and '18β-glycyrrhizinic acid' are interchangeable. Similarly, the term 'glycyrrhetic acid' is synonymous with '18β-glycyrrhetic acid'. Current analytical techniques determine the glycyrrhizinic acid content of liquorice and its products; past analytical methods (i.e. Houseman gravimetric method) determined glycyrrhizin content. Glycyrrhizinic acid is composed of glycyrrhetic acid and two molecules of glucuronic acid. Glycyrrhetic acid, also commonly referred to as glycyrrhetinic acid, can be released upon cleavage with glucuronidase.

2.1.1. Absorption, distribution, and excretion

(a) Absorption

Glycyrrhizinic acid, whether in the free form or as the ammonium salt, is poorly absorbed from the gastrointestinal tract. In the gastrointestinal tract, glycyrrhizinic acid is hydrolysed, mainly by the activity of intestinal microflora, to glycyrrhetic acid (the free aglycone of glycyrrhizinic acid), a substance that is readily absorbed.

The results of studies in humans (Carlat et al., 1959; Guillaume et al., 1999) and rats (Parke et al., 1963; Iveson et al., 1971; Ichikawa et al., 1986; Takeda et al., 1996) indicated that glycyrrhizin and monoammonium glycyrrhizinate are hydrolysed in the gastrointestinal tract to glycyrrhetic acid, which is almost completely absorbed into the body. At low doses (i.e. <10 mg/kg bw), uptake of glycyrrhetic acid by the liver after absorption results in low whole-body exposures and rapid elimination via the bile (Terwasawa et al., 1986; Yamamura et al., 1992; Ishida et al., 1994; Krähenbühl et al., 1994).

The times at which maximum plasma concentrations of glycyrrhetic acid are achieved after oral ingestion of glycyrrhizinic acid are reported to be in the range of 12–16 and 8–12 h in rats and humans, respectively. Doses in excess of 25 mg/kg bw may saturate the capacity of intestinal microflora to hydrolyse glycyrrhizinic acid to glycyrrhetic acid, thereby limiting C_{max} values and producing a high-dose absorption plateau. In humans, absorption of glycyrrhetic acid from the gut is virtually complete, regardless of whether it is formed from the hydrolysis of glycyrrhizinic acid or is initially present as either the glycoside or the aglycone in a food matrix (e.g. liquorice) (Størmer et al., 1993b; Mensinga et al., 1998).

Six healthy volunteers were given capsules containing 0.5, 1.0, or 1.5 g of 18β-glycyrrhetic acid (corresponding to a dose of approximately 8.3, 16.7, or

25 mg/kg bw, respectively) (Krähenbühl et al., 1994). Blood samples were taken immediately before administration, every 30 min until 8 h after dosing, and at 9, 10, 12, 14, 24, and 48 h after administration. Analytical results showed that the bioavailability of 18β-glycyrrhetic acid was constant over the range of doses tested. Maximum blood concentrations reached after administration of 0.5, 1.0, and 1.5 g were 4.5, 7.0, and 9.0 mg/l, respectively. At the lowest dose, the elimination of 18β-glycyrrhetic acid from the blood fitted a single compartment model, but was biphasic at doses of 1.0 and 1.5 g. The elimination half-life for the first phase was approximately 2 h. The half-life for the second phase was 11.5 and 38.7 h at 1.0 and 1.5 g, respectively. On the basis of the slow second elimination phase and high lipid solubility, the authors suggested that extensive tissue distribution of 18β-glycyrrhetic acid occurred at the higher doses.

The pharmacokinetic profile of glycyrrhizin was examined in three healthy volunteers (Yamamura et al., 1992). After oral administration of 100 mg of glycyrrhizin (corresponding to a dose of approximately 1.67 mg/kg bw), glycyrrhetic acid was detected in plasma at a maximum concentration of 200 μg/ml; no glycyrrhizin was found in plasma. After intravenous injection, however, only glycyrrhizin was detected in plasma, suggesting that it is not hydrolysed to glycyrrhetic acid in the blood. Elimination of glycyrrhizin from plasma was reported to be biphasic and occurred with a half-life of between 2.7 and 4.8 h. It was also reported in this study that glycyrrhizin was not hydrolysed in stomach juices. The authors concluded that the appearance of the major metabolite of glycyrrhizin (i.e. glycyrrhetic acid) in the plasma after oral administration of glycyrrhizin was due to hydrolysis by intestinal bacteria and that little hydrolysis occurred after absorption.

In 15 healthy volunteers fed liquorice candy (amount not specified) for 2–4 weeks, peak plasma concentrations of glycyrrhetic acid were reported to range from 0 to 480 μg/l (Hughes & Cowles, 1977). Additionally, the authors reported that maximum plasma concentrations of glycyrrhetic acid reached 84 μg/ml in four healthy volunteers who ingested liquorice candy (amount not specified) for 2 days.

Serum concentrations of glycyrrhetic acid were measured in five healthy male volunteers given a single oral dose of liquorice containing 133 mg of glycyrrhizin (corresponding to a dose of glycyrrhetic acid of approximately 2.22 mg/kg bw) (Terwasawa et al., 1986). The maximum serum concentration was reported to be 30 μg/l, which was reached approximately 24 h after administration. The peak serum concentration of glycyrrhetic glycosides was reportedly reached within 4 h.

The rate of absorption of glycyrrhetic acid in human volunteers who consumed an extract of the *Glycyrrhiza* plant was reported to be approximately 75% of that associated with the consumption of pure glycyrrhizinic acid (Cantelli-Forti et al., 1994).

In an investigation of the bioavailability of glycyrrhetic acid after ingestion of liquorice, six healthy volunteers (five females and one male) were given a liquorice confection containing approximately 200 mg of glycyrrhizinic acid (approximately 3.3 mg/kg bw) (Gunnarsdóttir & Jóhannesson, 1997). Mean plasma concentrations

of glycyrrhetic acid rose to a peak of approximately 570 μg/ml at 10 h after ingestion. The area under the curve of concentration–time (AUC) was approximately 7569 μg × h/l, while the mean C_{max} and T_{max} were approximately 794 μg/l and 13 h, respectively. The authors suggested that the biotransformation of glycyrrhizinic acid to glycyrrhetic acid occurs in the large intestine in humans.

In another study of the bioavailability of glycyrrhetic acid in humans (Mensinga et al., 1998), eight men and eight women were given either: (a) 130 mg of glycyrrhetic acid in aqueous suspension; (b) 225 mg of glycyrrhizinic acid in aqueous solution; (c) 225 mg of glycyrrhizinic acid in 150 g of sweet liquorice; or (d) 225 mg of glycyrrhizinic acid in salted liquorice. Samples of plasma were collected for up to 56 h after consumption. Plasma concentrations of glycyrrhetic acid peaked after 3 and 8–10 h for treatments (a) and (b–d), respectively. Intra-individual differences between the treatments were reported to be small, while large inter-individual of differences of four- to fivefold occurred with respect to C_{max} and AUC values, e.g. for treatment (c), C_{max} and AUC values were reported to range from 0.4 to 1.6 mg/l and from 4.8 to 23.1 mg/l × h, respectively.

Egashira et al. (2003) investigated the pharmacokinetics of 'glycyrrhizin' (actually glycyrrhizinic acid) and glycyrrhetic acid in male Wistar rats given single and multiple doses by various routes of exposure. Groups of three rats were given monoammonium glycyrrhizinate (a salt of glycyrrhizinic acid) by intravenous injection, intraperitoneal injection or by oral intubation in saline as a single bolus dose of 10 mg/kg bw. Another group of rats received 20 daily doses (either intravenously or orally) of 2 mg/kg bw. In each case, samples of plasma and bile were collected at various time-points and analysed for the presence of 'glycyrrhizin' (actually glycyrrhizinic acid) and glycyrrhetic acid. Administration of single doses by intravenous or intraperitoneal injection resulted in curves for the plasma concentration of glycyrrhizinic acid that closely resembled a two-compartment model. Administration of single oral doses resulted in much more gradual absorption of glycyrrhizinic acid, with peak plasma concentrations of 70 μg/l being achieved within 10–12 h of administration. Approximately 60% of the oral dose administered was absorbed within 24 h. This value appears to include the absorption of glycyrrhetic acid in addition to any glycyrrhizinic acid. After administration of single does by intravenous, intraperitoneal, or oral routes, the AUC values at 24 h for glycyrrhetic acid were reported to be 1.155, 1.167, and 1.004 μg × h/ml, respectively. The appearance and concentrations of glycyrrhetic acid were similar for all these routes of exposure. Biliary concentrations of glycyrrhizinic acid and glycyrrhetic acid peaked within 2 h of dosing (200–250 μg/ml) and declined rapidly within 8 h of dosing. This finding essentially was the reverse of the observations in plasma.

After dosing at 2 mg/kg bw per day for 20 days, plasma concentrations of glycyrrhizinic acid were 2–10 μg/ml and 0.2–0.7 μg/ml for the intravenous and oral routes of exposure, respectively. Concentrations of glycyrrhetic acid after multiple intravenous and oral doses ranged from 6 to 50 μg/l and from 5 to 60 μg/l, respectively. Equilibrium concentrations of glycyrrhizinic acid and glycyrrhetic acid were achieved within 2 days, with no accumulation of either compound over the 20-day course of treatment (Egashira et al., 2003).

Wistar rats were given radiolabelled glycyrrhetic acid at a dose of 20 mg/kg bw by two different routes (Iveson et al., 1971). After oral gavage, absorption from the stomach was reported to range from 27 to 47% within 6 h, while after intraduodenal infusion, absorption was reported to range from 83 to 95%. When bile collected from the rats was re-administered by intraduodenal infusion to rats that had not been dosed, 31% of the radiolabel given to the first rat was reabsorbed and excreted in the bile.

Takeda et al. (1996) reported that in male Wistar and Sprague-Dawley rats treaed orally with glycyrrhizin (i.e. glycyrrhizinic acid), glycyrrhizin was hydrolysed by intestinal bacteria to glycyrrhetic acid, which was completely absorbed from the gastrointestinal tract. In the absence of intestinal bacteria, as tested using germ-free rats, it was reported that glycyrrhizin was not absorbed and, therefore, did not appear in the plasma. Absorption of glycyrrhetic acid from the intestine of the rats that were not germ-free was considered by the authors to have been complete, because comparable cumulative plasma concentrations of glycyrrhetic acid were obtained both after intravenous injection of glycyrrhetic acid at 5.7 mg/kg bw and oral administration of glycyrrhizin at 10 mg/kg bw (an amount equimolar to 5.7 mg of glycyrrhetic acid).

Wang et al. (1995) investigated the gastrointestinal absorption of pure glycyrrhizin and glycyrrhizin as a constituent of *Glycyrrhiza* extract. Normal male Wistar rats and rats with bile fistula were given glycyrrhizin at a dose of 200 mg/kg bw or an equivalent dose of glycyrrhiza extract in saline (corresponding to a dose of glycyrrhizin of 200 mg/kg bw). The absorption of glycyrrhizin in the *Glycyrrhiza* extract was reported to be reduced when compared with that of pure glycyrrhizin. The excretion of glycyrrhizin in the bile after administration of the *Glycyrrhiza* extract was reported to be approximately 1.1% of the administered dose after 30 h, slightly less than that of pure glycyrrhizin.

Ichikawa et al. (1986) tested the intestinal absorption of glycyrrhizinic acid by injecting bile containing glycyrrhizinic acid into the duodenum of portal vein-cannulated rats. Blood samples were collected for up to 2 h after administration. Absorption from the gut was reported to be apparent as revealed by the steady increase in concentrations of glycyrrhizinic acid in the samples of portal plasma over time. The authors estimated that approximately 80% of the glycyrrhizin excreted in the bile was reabsorbed from the gastrointestinal tract.

(b) Distribution

The results of studies in rats, and inferences that can be drawn from the results of studies in humans, indicate that both glycyrrhizinic acid and its hydrolysis product glycyrrhetic acid are largely confined to the plasma and are not taken up in bodily tissues to a significant extent. In plasma, glycyrrhizinic acid and glycyrrhetic acid are bound to serum albumin. Some hepatic uptake of glycyrrhizin in the blood has been reported to occur, possibly through the actions of organic anion-transporting polypeptides in both rats and humans (Ismair et al., 2003).

In a study in rats given a single intravenous dose of glycyrrhetic acid at 2, 5, or 12 mg/kg bw, analysis of the brain, lung, heart, liver, kidney, stomach, small

intestine, pancreas, spleen, muscle, skin, adipose tissue, plasma, and lymph fluid showed that distribution was poor (Ishida et al., 1989). The authors also reported poor uptake of glycyrrhetic acid into erythrocytes. Ishida et al. (1989) concluded that the elimination of glycyrrhetic acid in the plasma was generally biexponential in nature and dose-dependent.

Ishida et al. (1988) reported that glycyrrhetic acid binds to plasma albumin in vitro at >96% in rats and >99.9% in humans, at concentrations of <0.5 mg/ml and <5 μg/ml, respectively. Ishida et al. (1994) later reported that glycyrrhizin was actively taken up by the isolated rat liver at a rate that exceeded its excretion into the bile.

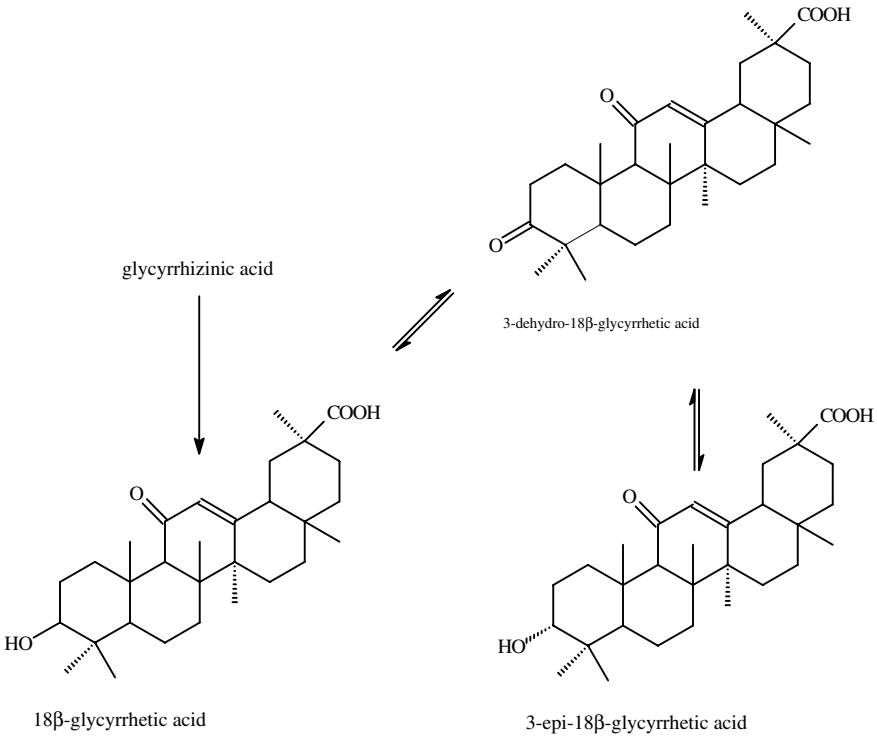
(c) Metabolism

Glycyrrhizin was reported not to be hydrolysed to a significant degree by human stomach juices in vitro, nor was it hydrolysed in human plasma in vivo; however, glycyrrhizin appears in the blood as glycyrrhetic acid after oral administration in humans (Yamamura et al., 1992). The appearance of glycyrrhetic acid in the blood after administration of glycyrrhizin (i.e. glycyrrhizinic acid) is explained by the fact that the intestinal microorganisms in several mammalian species, including humans, are capable of hydrolysing glycyrrhizin (glycyrrhizinic acid) to glycyrrhetic acid (Hattori et al., 1983; Hattori et al., 1985; Akao et al., 1987; Akao, 1997a; Shibata et al., 2000; Akao, 2001).

Hattori et al. (1983) studied the metabolism of glycyrrhizinic acid by human intestinal bacteria isolated from faeces. An intestinal bacterial mixture was prepared and incubated with either monoammonium glycyrrhizinate (glycyrrhizinic acid) or glycyrrhetic acid for 24 and 48 h, respectively. Three metabolites were reported and identified from glycyrrhizinic acid: glycyrrhetic acid, 3-epi-glycyrrhetic acid, and 3-dehydro-glycyrrhetic acid. One metabolite, 3-epi-glycyrrhetic acid, was identified from glycyrrhetic acid and the parent compound was also recovered. 3-Dehydro-glycyrrhetic acid was recovered from the culture medium. This metabolite was incubated with the bacterial mixture and became transformed to glycyrrhetic acid and 3-epi-glycyrrhetic acid, indicating that it may be an intermediate in the formation of 3-epi-glycyrrhetic from either glycyrrhetic acid or glycyrrhizinic acid. Figure 2 illustrates the metabolic pathway of glycyrrhizinic acid in the presence of human intestinal flora.

In a later study, Hattori et al. (1985) examined which microbes of the human intestinal flora were responsible for the metabolism of glycyrrhizinic acid. Their results indicated that *Peptostreptococcus intermedius* and *Clostridium perfringens* together hydrolysed glycyrrhizinic acid to glycyrrhetic acid. *Ruminococcus* sp. and *Clostridium innocuum* were isolated from fresh human faeces and identified as active bacteria in the conversion of 3-dehydro-glycyrrhizinic acid to either glycyrrhizinic acid or 3-epi-glycyrrhizinic acid. The authors reported that a fair number of bacteria studied from the human intestinal flora showed β-glucuronidase activity and, thus, had the ability to metabolize glycyrrhizinic acid to glycyrrhetic acid. However, only a moderate number of bacteria showed the activity required to reduce 3-dehydro-glycyrrhizinic acid to either glycyrrhizinic acid or 3-epi-glycyrrhizinic acid.

Figure 2. Metabolic pathway of glycyrrhizinic acid



From Hattori et al. (1983)

In a more recent study by Akao (1997b), the β-glucuronidase reported to be responsible for the hydrolysis of glycyrrhetyl mono-glucuronide, an intermediate in the hydrolysis of glycyrrhizin to glycyrrhetic acid, was reported to have been isolated from human intestinal bacteria (*Eubacterium* sp.).

Imai et al. (1999) reported that it is the hydrolysis of dipotassium glycyrrhizinate to glycyrrhetinyl monoglucuronide that is the rate-limiting step in the metabolic pathway, and is followed by the rapid hydrolysis of glycyrrhetinyl monoglucuronide to glycyrrhetic acid.

It was reported that glycyrrhizin is metabolized by human intestinal bacteria to 18β-glycyrrhetic acid through two possible pathways (Kim et al., 1999; Kim et al., 2000). The main pathway, which is catalysed by *Eubacterium* L-8 β-glucuronidase, proceeds to the formation of the major product, 18β-glycyrrhetic acid. The second pathway, catalysed by *Streptococcus* LJ-22 β-glucuronidase, results in the production of the minor product, 18β-glycyrrhetic acid-3-*O*-β-D-glucuronide (GAMG), which is subsequently hydrolysed to 18β-glycyrrhetic acid by *Eubacterium* L-8 β-glucuronidase. According to the authors, GAMG and 18β-glycyrrhetic acid have

potent anti-platelet aggregation activity in vitro. Conversely, 18β-glycyrrhetic acid was reported to have the most potent cytotoxic activity against tumour cell lines and *N*-nitrosamines (Malagoli et al., 1998; Kim et al., 2000; Martinez et al., 2000), as well as inhibitory activity against growth of *Helicobacter pylori* and infection with rotavirus (Kim et al., 2000).

Okamura et al. (2003) reported that glycyrrhizin, incubated aerobically or anaerobically for 48 h with fresh faeces from male Wistar rats, was metabolized mainly to glycyrrhetic acid. Under anaerobic conditions, trace amounts of 3-α-hydroxyglygyhetic acid and 3-dehydroglycyrrhetic acid were formed. Under aerobic conditions, the amount of 3-dehydroglycyrrhetic acid was found to increase constantly throughout the incubation period, so that after 48 h concentrations of this metabolite were equal to those of glycyrrhetic acid itself (i.e. about 35 μmol/l).

Akao et al. (1990a, 1990b) reported on a reversible conversion of 18β-glycyrrhetic acid to 3-oxo-18β-glycyrrhetic acid. Akao et al. (1990b) studied the hydroxylation of 18β-glycyrrhetic acid, 3-oxo-18β-glycyrrhetic acid, and 3-epi-18β-glycyrrhetic acid by rat liver microsomes. The results showed that in the presence of nicotinamide adenine dinucleotide phosphate (oxidized form) ($NADP^+$), 18β-glycyrrhetic acid was converted to 3-oxo-18β-glycyrrhetic acid; however, in the presence of nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) the same compound was converted to 22α-hydroxy-18β-glycyrrhetic acid and 24-hydroxy-18β-glycyrrhetic acid. Similarly, 3-oxo-18β-glycyrrhetic acid and 3-epi-18β-glycyrrhetic acid were converted to 22α- and 24-hydroxy derivatives.

Kawakami et al. (1993) reported that glycyrrhetyl-3-*O*-glucuronide, glycyrrhetic acid-3-*O*-hydrogen sulfate, and a glucuronide conjugate of glycyrrhetic acid were biliary metabolites of glycyrrhetic acid in rats.

Human subjects given 2.28 or 2.50 g of glycyrrhetic acid orally failed to show any of the unchanged compound in the urine, although an unidentified metabolite was present (Van Katwijk & Huis in't Veld, 1954).

(d) Excretion

In both humans and rats, glycyrrhizinic acid and glycyrrhetic acid formed by microbe-mediated hydrolysis are excreted mainly in the faeces. In addition, a significant proportion of glycyrrhetic acid absorbed after hydrolysis is subjected to enterohepatic circulation and excretion into the bile.

In a previously described study (Krähenbühl et al., 1994) in which six healthy volunteers were given capsules containing 0.5, 1.0, or 1.5 g of 18β-glycyrrhetic acid (a dose of approximately 8.3, 16.7, and 25 mg/kg bw, respectively), elimination of glycyrrhetic acid from the blood fitted a single compartment model at the lowest dose, but was biphasic at 1.0 and 1.5 g. The elimination half-life was approximately 2 h for the first phase. The half-life of the second phase was 11.5 and 38.7 h at 1.0 and 1.5 g, respectively. On the basis of the slow second elimination phase and high lipid solubility, the authors suggested that extensive tissue distribution of glycyrrhetic acid occurred at the higher dose.

After oral administration of 100 mg of glycyrrhizin (a dose of approximately 1.67 mg/kg bw), elimination of glycyrrhetic acid from plasma was reported to be biphasic and occurred with a half-life of 2.7–4.8 h. It also was reported in this study that glycyrrhizinic acid was not hydrolysed in stomach juices. The authors concluded that the appearance of the major metabolite of glycyrrhizinic acid in the plasma after oral administration was caused by hydrolysis by intestinal bacteria and that little hydrolysis occurred after absorption (Yamamura et al., 1992).

Carlat et al. (1959) reported that orally administered radiolabelled monoammonium glycyrrhizinate was hydrolysed to form glycyrrhetic acid and excreted unchanged in the faeces in humans.

The urinary excretion of 18β-glycyrrhetic acid was examined in two volunteers (age and sex not reported) who received Dutch confectionery liquorice at a dose of 500 g (consumed within 4 h), or at a daily dose of 40 g for a period of 5 days (Guillaume et al., 1999). All urine was collected from the subjects during the days of liquorice consumption, as well as arbitrarily on days 6, 8, and 10 of the study. It was reported that 18β-glycyrrhetic acid was first detected in the urine at 8 and 9 h after the first oral administration of 500 and 40 g of liquorice, respectively. The authors attributed this delay to the hydrolysis of glycyrrhizin to 18β-glycyrrhetic acid by intestinal bacteria, and its subsequent absorption from the gut. Additionally, it was reported that urinary 18β-glycyrrhetic acid could be detected at up to 53 h after consumption of 500 g of liquorice, and up to 72 h after cessation of consumption of liquorice at 40 g/day for 5 days. The authors attributed the extended excretion to the phenomenon of enterohepatic cycling.

To study biliary excretion and enterohepatic cycling of glycyrrhizin (i.e. glycyrrhizinic acid), fasted rats were intravenously injected with glycyrrhizin at a dose of 100 mg/kg bw (Ichikawa et al., 1986). Biliary fistulas and urinary bladder cannulas were placed in some of the rats. Blood samples were taken between 0.5 and 360 min after the administration of glycyrrhizin. In fistulated and cannulated animals, bile and urine samples were drawn for up to 60 h after administration. Samples of faeces were also taken. Approximately 70% of the administered dose was excreted in the bile within 12 h, and the compound was reported not to affect bile flow. Glycyrrhizinic acid was reportedly not detected in the bile or urine of rats after 48 h. In rats without fistulas, the total cumulative excretion in the faeces was 5.5% of the injected dose. It was reported that there was no detectable excretion of glycyrrhizinic acid in the faeces of fistulated rats. The authors concluded that high concentrations of the administered dose of glycyrrhizin (glycyrrhizinic acid) remained in the liver, and were slowly excreted into the bile; however, this transport process was considered to be saturable. Approximately 80% of the glycyrrhizinic acid in the bile was reported to be reabsorbed from the gastrointestinal tract via enterohepatic recycling.

Ishida et al. (1992) gave monoammonium glycyrrhizinate (glycyrrhizinic acid) in 5% glucose solution as a single intravenous injection at a dose of 5, 10, 20, or 50 mg/kg bw to male Wistar rats (number not specified). Blood samples were collected at 1, 5, 10, 30, 60, and 120 min after dosing of all animals. Additional blood samples were collected at 3 h after dosing for the group receiving 10 mg/kg bw, 3 and 5 h after dosing for the group receiving 20 mg/kg bw and 3, 5, 8, and

12 h after dosing for the group receiving 50 mg/kg bw. The elimination of glycyrrhizinate from the plasma was reported to be biexponential for all doses, while the volume of distribution was significantly greater for the two highest doses (20 and 50 mg/kg bw) compared with the other doses; however, there was no evidence of bioaccumulation even at the highest dose. The authors reported that the plasma distribution of glycyrrhizinate was dose-dependent. In another study, similar results were obtained when male Sprague-Dawley rats were injected with a single bolus intravenous dose of glycyrrhizin at 20, 50, or 100 mg/kg bw (Tsai et al., 1992). In this study, blood samples collected for up to 24 h after dosing were reported to show a biphasic, dose-dependent decrease in plasma concentrations of glycyrrhizin at all doses. In another bioavailability study, it also was reported that a similar biphasic response occurred in the plasma in three strains of rats after intravenous administration of glycyrrhetic acid at 10 mg/kg bw (Takeda et al., 1996).

To determine the pharmacokinetics of glycyrrhizinic acid and glycyrrhetic acid in bile, groups of six fasted male Sprague-Dawley rats were given distilled water (vehicle), glycyrrhizinic acid at 480 mg/kg bw, or liquorice extract at 6278 mg/kg bw by oral gavage (Cantelli-Forti et al., 1997). Additional groups of rats were intravenously injected with glycyrrhizinic acid at 2.5 mg/kg bw or with liquorice extract at 32.7 mg/kg bw to avoid gastrointestinal absorption of the test substances. In both experiments, after administration of liquorice extract a significant reduction was reported in the bioavailability (7.4 times less) and clearance rate (20% slower) of glycyrrhizinic acid compared with pure glycyrrhizinic acid.

In male Wistar rats given monoammonium glycyrrhizinate in a 5% glucose solution as a single intravenous dose at 5, 10, 20, or 50 mg/kg bw (Ishida et al. (1992), the primary route of excretion was reported to be via the bile. The rate of total and renal clearance in animals was significantly decreased at 20 and 50 mg/kg bw, indicating that the elimination of monoammonium glycyrrhizinate is dose-dependent; however, there was no evidence of bioaccumulation. This was also the case in male Sprague-Dawley rats given a single intravenous dose of glycyrrhizin at 20, 50, or 100 mg/kg bw (Tsai et al., 1992). Blood samples were collected for up to 24 h after dosing. The authors reported dose-dependent decreases in total clearance and volume of distribution of glycyrrhizin in the rats.

Albino rats were given radiolabelled β-glycyrrhetic acid in arachis oil orally at a dose of 25 mg/kg bw (Parke et al., 1963). After 2–3 days, 70% of the administered dose was reported to be recovered in the bile, 13–20% in the faeces, and 2–3% in the urine. Additionally, intraperitoneal injection of the same dose of β-glycyrrhetic acid was reported to result in virtually 100% of the dose being excreted in the bile within 12 h, with only trace amounts appearing in the urine.

In Wistar rats, 18β-glycyrrhetic acid, administered either by oral gavage or intraperitoneal injection, at a dose of 20 mg/kg bw, was reported to be excreted largely in the faeces (approximately 95%) with a small proportion (approximately 1.7%) appearing in the urine (Iveson et al., 1971). Biliary excretion was reported to account for 75% of the administered dose within 7 h after intraperitoneal injection. Biliary metabolites were identified as the glycyrrhetic acid conjugates glycyrrhetyl-3-*O*-glucuronide, glycyrrhetic acid 3-*O*-hydrogen sulfate, and glycyrrhetyl-3-*O*-glucuronide; however, only glycyrrhetic acid was reported to be recov-

ered from the faeces after oral administration, suggesting that hydrolysis of the biliary metabolites by intestinal microflora occurred.

Metabolites excreted in the bile of rats given glycyrrhetic acid by intravenous administration have been identified as glycyrrhetyl-3-*O*-glucuronide, glycyrrhetic acid 3-*O*-hydrogen sulfate, and a glucuronide conjugate of glycyrrhetic acid (Kawakami et al., 1993). These are similar to metabolites identified after oral and intraperitoneal administration (Iveson et al., 1971). The first appearance of metabolites in the bile was reported to occur after 3 h and, upon immediate intraduodenal infusion to a second rat, appeared in the plasma of the recipient rat after 4 h. The total percentage of glycyrrhetic acid or metabolites excreted in the bile was approximately 17% in the first rat and <2% in the second rat (Kawakami et al., 1993). The authors estimated that the contribution of enterohepatic circulation to the plasma concentrations of glycyrrhetic acid was <3.5%. In contrast, Ploeger et al. (2000a) reported that enterohepatic recycling was responsible for the slow terminal clearance of glycyrrhetic acid. Kawakami et al. (1993) compared the plasma concentration time course for glycyrrhetic acid in bile duct-cannulated and control rats after intravenous administration of glycyrrhetic acid and reported no significant differences; however, the duration of this study was only 3 h and Ploeger et al. (2000a) reported that the influence of the enterohepatic recycling does not become evident until 5 h after dosing. In another study, Ploeger et al. (2000b) reported that enterohepatic recycling of metabolites of glycyrrhetic acid has a significant effect on the kinetics of glycyrrhetic acid in humans and rats.

2.1.2. Pharmacodynamics (mechanism of action)

Glycyrrhizinic acid (glycyrrhizin), liquorice, and glycyrrhetic acid have been reported to have anti-inflammatory, anti-arthritic, antioxidant, and hypolipidaemic properties (Elmadjian et al., 1956; Finney et al., 1958; Evdokimova & Kamilov, 1967; Whitehouse et al., 1967; Mezenova, 1984; Sitohy et al., 1991; Størmer et al., 1993b; Fuhrman et al., 1997; Mauricio et al., 1997), as well as hepatoprotective and immunoprotective effects in humans and animals (Kiso et al., 1984; Yamamura et al., 1997; Okamoto & Kanda, 1999; Paolini et al., 1999; Shiga et al., 1999; Wen-Yu et al., 1999; Al-Qarawi et al., 2001; Dai et al., 2001; Horigome et al., 2001; Okamoto et al., 2001; Utsunomiya et al., 2001; Jeong & Kim, 2002).

Absorbed glycyrrhetic acid (aglycone of glycyrrhizinic acid) has also been reported to produce effects similar to those of the adrenal steroid aldosterone (Groen et al., 1951; Groen et al., 1952; Kraus, 1957; Epstein et al., 1977a; Chandler, 1985; Størmer et al., 1993b; Bijlsma et al., 1996; Ploeger et al., 2000a, 2000b). The precise mechanism of action of glycyrrhetic acid involves the inhibition of 11β-hydroxysteroid dehydrogenase (11β-HSD), which is an enzyme that converts cortisol to cortisone (Epstein et al., 1978; Stewart et al., 1987, Stewart et al., 1988; Monder et al., 1989; MacKenzie et al., 1990a; Kageyama, 1992; Kageyama et al., 1992a; Edwards et al., 1993; Morris, 1993).

The authors of several studies on 11β-HSD have reported that this microsomal enzyme catalyses both the conversion of cortisol to cortisone and the reverse reaction, and consists of two enzymes, 11β-HSD1 (11-oxo-reductase) and 11β-HSD2 (Monder et al., 1989; Stewart et al., 1990; Stewart et al., 1994; Stewart &

Mason, 1995). It was reported that 11β-HSD2 catalyses the oxidation of cortisol to cortisone and shows relatively high activity in the kidney, while 11β-HSD1 catalyses the conversion of cortisone to cortisol and is relatively inactive in the kidney but is active in the liver (Monder et al., 1989; Latif et al., 1990; Stewart et al., 1990; Edwards et al., 1993). Monder et al. (1989) reported that glycyrrhetic acid inhibited 11β-HSD2 but not 11β-HSD1. Inhibition of 11β-HSD2 by glycyrrhetic acid without inhibition of 11β-HSD1 contributes further to the accumulation of cortisol in vivo (Monder et al., 1989; Mantero & Boscaro, 1992; Edwards et al., 1993).

Cortisol has mineralocorticoid activity reportedly equivalent to that of aldosterone, but normally does not express this activity in the tissues where aldosterone acts (i.e. the kidney) because it is converted to inactive cortisone by 11β-HSD2. The inhibition of 11β-HSD2 by glycyrrhetic acid leads to high tissue concentrations of cortisol, resulting in inappropriate mineralocorticoid activity (i.e. sodium and water retention and loss of potassium), as has been reported to occur in humans and animals (Hassan et al., 1954; Revers, 1946; Macabies et al., 1963a, 1963b; Cotterill & Cunliffe, 1973; Epstein et al., 1978; Valentino et al., 1987; Morris, 1993, Bernardi et al., 1994; Funder, 1995; Stewart & Mason, 1995; Armanini et al., 1996; Rossi et al., 1999; Wu et al., 1999; Horigome et al., 2001). In addition, the results of two studies in rats (Hanafusa et al., 2002; Tanahashi et al., 2002) indicate that glycyrrhizinic acid, in addition to inhibiting 11β-HSD after hydrolysis to glycyrrhetic acid, suppresses expression of 11β-HSD mRNA and protein through indirect effects involving a glucocorticoid feedback mechanism. Typically, the effect described above occurs in the absence of any gross or histological abnormalities (Fujimura, 1974).

Investigation into a physiological mechanism for certain effects reported to be caused by liquorice was initiated following a study by Revers (1946), in which liquorice extract had been successfully used in the treatment of 45 patients with gastric ulcer. In this study, it was reported that liquorice extract at 10 g/day, administered orally in three sub-doses, reduced intestinal tonus and mobility, and diminished or cured gastric ulcers; however, liquorice extract was also reported to cause oedema of the face and legs in approximately 20% of the patients. Reduction of the liquorice dose to 3 g/day was reported to result in a decrease in the number of cases and severity of oedema in the patients.

In subsequent investigations, it has been reported that consumption of glycyrrhizinic acid and liquorice or liquorice extracts can induce sodium and water retention, and potassium excretion in healthy individuals (Molhuysen et al., 1950; Card et al., 1953; Chandler, 1985; Heikens et al., 1995). These effects have been compared with those of both aldosterone and deoxycorticosterone, which are mineralocorticoid hormones secreted by the adrenal cortex (Groen et al., 1951; Groen et al., 1952; Kraus, 1957). The principal biological action of aldosterone and, to a lesser extent, deoxycorticosterone is the regulation of electrolyte and water balance in the body by promoting the retention of sodium and water, and excretion of potassium by the kidney. The regulation of concentrations of electrolytes is critical in maintaining normal blood pressure, muscle action, and pH of body fluids (Fox, 1984; Gornall, 1986).

In patients with a defective mechanism for the release of aldosterone (e.g. Addison disease), or in those who have had their adrenals removed because of

disease, the administration of glycyrrhizinic acid (glycyrrhizin) or the ingestion of liquorice has been reported to lessen the symptoms associated with electrolyte imbalance, despite the inability of these patients to secrete aldosterone (Groen et al., 1951; Groen et al., 1952; Card et al., 1953; Pelser et al., 1953; Cotterill & Cunliffe, 1973). For this reason, liquorice and liquorice extracts have been reported to have mineralocorticoid-like activity (Epstein et al., 1978; Morris, 1993; Størmer et al., 1993b; Funder, 1995; Heikens et al., 1995; Sweetman, 2002). In healthy individuals who consume large quantities of products containing glycyrrhizinic acid, such as liquorice, these compounds have been reported to result in sodium and water retention, and potassium loss, leading to hypertension, compensatory reductions in plasma concentrations of aldosterone and renin, and other effects related to electrolyte imbalance (Epstein et al., 1977a, 1977b; Beretta-Piccoli et al., 1985; MacKenzie et al., 1990a; Farese et al., 1991; Morris, 1993; Størmer et al., 1993b; Bernardi et al. 1994; Kerlan et al., 1994; Funder, 1995; Olukoga & Donaldson, 2000; Brouwers & van der Meulen, 2001). The term 'apparent mineralocorticoid excess' (AME) has been used to describe the electrolyte abnormalities involved in a rare monogenic form of early onset hypertension (Edwards et al., 1993; Stewart & Mason, 1995) and has been applied to the effects on electrolyte balance caused by consumption of glycyrrhizinic acid (glycyrrhizin) (Morris, 1993; Olukoga & Donaldson, 2000).

In individuals who consume liquorice and who show evidence of electrolyte imbalance, it has been reported that concentrations of aldosterone are decreased, suggesting that glycyrrhizin or glycyrrhetic acid may have direct mineralocorticoid activity; however, data from studies examining the binding of glycyrrhizin or glycyrrhetic acid to mineralocorticoid receptors (MR) in vitro demonstrate that the affinity of these compounds for MR is approximately 10 000 times less than that of aldosterone (Molhuysen et al., 1950; Ulmann et al., 1975; Armanini et al., 1983, Armanini et al., 1985; Tamaya et al., 1986; Takeda et al., 1987; Baker & Fanestil, 1991). Data from studies in vivo indicate that the concentrations of glycyrrhetic acid in the blood available for binding to MR, in combination with the lower affinity for MR of glycyrrhetic acid compared with that of aldosterone, are not sufficient to explain AME attributable to ingestion of liquorice (Monder et al., 1989; Edwards, 1991); however, several investigators have reported that urinary concentrations of cortisol and the ratio of serum cortisol to cortisone are increased, while plasma and urinary concentrations of cortisone are decreased by consumption of liquorice containing glycyrrhizinic acid (Epstein et al., 1978; MacKenzie et al., 1990a; Kageyama, 1992; Kageyama et al., 1992a; Krähenbühl et al., 1994). Cortisol has been reported to have mineralocorticoid activity and an affinity for MR that is equivalent to that of aldosterone, while cortisone has no mineralocorticoid activity and does not bind to MR (Edwards & Stewart, 1990; Størmer et al., 1993b). On the basis of these observations, several investigators have suggested that induced AME occurs in response to increased renal concentrations of cortisol and, more specifically, as a result of inhibition of the enzyme 11β-HSD2, which converts active cortisol to inactive cortisone (Epstein et al., 1978; Stewart et al., 1987; Funder et al., 1988; Monder et al., 1989; MacKenzie et al., 1990a; Kageyama, 1992; Kageyama et al., 1992a; Edwards et al., 1993; Morris, 1993; Funder, 1995; Heikens et al., 1995; Sweetman, 2002).

Under normal physiological conditions, the concentration of cortisol in the blood is 100–1000 times higher than that of aldosterone, while the affinity of cortisol for MR is equivalent to that of aldosterone (Edwards & Stewart, 1990; Størmer et al., 1993b); however, cortisol is prevented from binding to MR, particularly in tissues with high mineralocorticoid activity (e.g. kidney), because it is converted into inactive cortisone by 11β-HSD2 (Edwards & Stewart, 1990; Morris, 1993). Glycyrrhetic acid inhibits 11β-HSD2, resulting in elevated renal concentrations of cortisol and inappropriate mineralocorticoid effects (Edwards et al., 1993; Morris, 1993; Størmer et al., 1993b). Evidence to support this mechanism comes from several sources. Data from studies of metabolism in vitro using kidney microsomal preparations have confirmed that glycyrrhetic acid, but not glycyrrhizinic acid (glycyrrhizin), inhibits human renal 11β-HSD2 (Monder et al., 1989; Stewart et al., 1994). In addition, glycyrrhizin appears in the blood as glycyrrhetic acid after oral administration and, in rats, oral glycyrrhizin also has been reported to produce inhibition of 11β-HSD2 (Valentino et al., 1987; Wu et al., 1999). Glycyrrhizin and glycyrrhetic acid have also been reported to decrease the levels of mRNA that code for 11β-HSD2 in several tissues (Whorwood et al., 1993; Wu et al., 1999). Furthermore, the effects of glycyrrhizin and, therefore, glycyrrhetic acid do not occur in the absence of cortisol (i.e. following adrenalectomy) (Kageyama et al., 1992b). Finally, the effects of liquorice have been reported to be the same as those seen in individuals known to have a deficiency of 11β-HSD2 (Stewart et al., 1987; Stewart et al., 1988; Monder et al., 1989; Kageyama, 1992; Kageyama et al., 1992a; Morris, 1993).

The time frame for changes in concentrations in cortisol and cortisone in humans after oral administration of 18β-glycyrrhetic acid was studied by Krähenbühl et al. (1994). In this study, single oral doses of 0.5, 1.0, or 1.5 g of 18β-glycyrrhetic acid were provided to six healthy males (approximately 8.3, 16.7, or 25 mg/kg bw, respectively). No adverse effects occurred in any subject and no changes in blood pressure or pulse were reported to occur. Plasma concentrations of cortisol were reportedly not affected at any dose. Plasma concentrations of cortisone was reported to be decreased at each dose compared with those of a control group. The decreases in plasma concentrations of cortisone were reported to be significantly different at 0.5 and 1.0 g at 2.5–7.5 h after administration, and at 1.5 g at 2.5–10 h after administration. As a result of the decrease in concentrations of cortisone, the ratio of plasma cortisol to plasma cortisone was increased, indicating competitive inhibition of 11β-HSD2. Based on the pharmacokinetic parameters of 18β-glycyrrhetic acid also examined in this study, the authors concluded that the development of hypertension and hypokalaemia in humans would require continuous intake of substantial amounts of glycyrrhizinic acid; however, significant variation in the ability of liquorice to induce AME has been reported (Størmer et al., 1993b).

Teelucksingh et al. (1990) investigated how effectively glucocorticoid receptors in human skin could be targeted upon inhibition of cortisol metabolism. Twenty-three healthy volunteers with no previous exposure to exogenous corticosteroids were exposed dermally to a combination of glycyrrhetic acid (20 mg/ml) and hydrocortisone (0.1, 0.3, 1, 3, or 10 mg/ml), which was administered in a volume of 10 µl to 7 × 7 mm sites. When hydrocortisone was administered at concentrations of 1,

3, or 10 mg/ml, a significant potentiation of the cutaneous vasoconstrictor effect was reported. In a companion experiment in vitro, the skin of nude mice was homogenized and incubated with glycyrrhetic acid (0.01, 1, or 100 μmol/l). The activity of 11β-HSD in this skin was reported to be significantly inhibited in a dose-dependent manner at all concentrations of glycyrrhetic acid.

In addition to the inhibitory effect of liquorice components on 11β-HSD2, their effects on other enzymes also contribute to AME in some individuals (Kumagai et al., 1966; Tamura, 1975; Sakamoto & Wakabayashi, 1988; Latif et al., 1990; Morris et al., 1990; Morris, 1993). Glycyrrhetic acid has been reported to inhibit 5β-reductase and 3β-hydroxysteroid dehydrogenase (13β-HSD) enzymes in the liver, resulting in an accumulation of aldosterone and 5α-dihydroaldosterone, both of which are potent mineralocorticoids (Tamura, 1975; Latif et al., 1990). In rats, both glycyrrhizin and glycyrrhetic acid, administered orally at a dose of 45 mg/kg bw per day for 20 days, were reported to inhibit enzymes in Leydig cells in vivo and also inhibited enzymes in ovarian microsomal preparations (Sakamoto & Wakabayashi, 1988). The authors of this study suggested that the resulting accumulation of steroids could provide the necessary precursors for conversion to other corticosteroids (i.e. cortisol).

Beyond effects on enzyme and biochemical systems, it has recently been reported that male Sprague-Dawley rats given glycyrrhizin at a dose of 200 mg/kg bw per day for 0.5–2 weeks showed upregulation of the aquaporin-2 and -3 water channels in the renal inner and outer medulla, as analysed by Western blot (Kang et al., 2003). Creatinine clearance was reported to be unchanged. Upregulation of aquaporin-2 and -3 results in decreased flow of urine and excretion of sodium. Intraperitoneal treatment of the rats with spironolactone (a mineralocorticoid receptor blocker) at a dose of 20 mg/kg bw per day was found to reverse the effects of glycyrrhizin. The authors suggested that their results may indicate that water and salt retention associated with high rates of glycyrrhizin consumption may in part be related to upregulation of aquaporins through the action of cortisol on MRs.

Morris et al. (1990) have suggested that inhibition of 5β-steroid reductase may occur in users of chewing tobacco. This was based on the inhibition of 5β-steroid reductase in cytosolic preparations from rat liver by saliva taken from individuals who had chewed 1 g of tobacco for 5 min. The inhibitory effect was equivalent to that reported to occur in vitro after exposure to 1 μmol of glycyrrhetic acid.

Paolini et al. (1999) reported that oral intake of liquorice root extract (3138 or 6276 mg/kg bw) or its natural constituent, glycyrrhizin, (240 or 480 mg/kg bw) in Sprague-Dawley rats affected their levels of liver monooxygenases. Although a single dose was reported not to have an effect, daily doses given on four consecutive days reportedly induced cytochrome P450 (CYP) CYP3A, CYP1A2 and, to varying extents, CYP2B1-linked enzymes. Also, a lesser increase in activity was reported for testosterone 6β-(CYP3A1/2, CYP1A1/2), 7α-(CYP1A1/2, CYP2A1), 16α- (CYP2B1, CYP2C11), 2α-(CYP2C11) and 2β-(CYP3A1, CYP1A1)-dependent oxidases and androst-4-ene-3,17-dione-(CYP3A1/2)-supported monooxygenases. The authors stated that the highest reported consumption of liquorice root extract in humans is about four times greater than the highest dose (6276 mg/kg bw) used in this study.

Radix *Glycyrrhiza uralensis* Fisch (GRZ), also known as Chinese liquorice, is a component of *Glycyrrhizae* radix that has been traditionally used as an anti-inflammatory, anti-ulcer, detoxification, and liver-protective agent (Wen-Yu et al., 1999; WHO, 1999). Groups of ten male Kunming mice were given GRZ at a dose of 10 g/kg bw per day or glycyrrhetic acid at a dose of 50 mg/kg bw per day orally for 7 days (Wen-Yu et al., 1999). The control animals were given an equivalent volume of normal saline. Mice treated with GRZ or glycyrrhetic acid were reported to have significantly increased levels of the hepatic enzymes CYP450, aminopyrine *N*-demethylase, 7-ethoxycumarin *O*-deethylase, and aryl hydrocarbon hydroxylase compared with controls. The authors attributed the hepatoprotective effect of GRZ to its CYP450-inducing effect, which renders xenobiotics less harmful via metabolism and detoxification.

2.2 Toxicological studies

2.2.1. Acute toxicity

The median lethal dose (LD_{50}) values for glycyrrhizinic acid and related compounds administered by various routes in a variety of animal species are presented in Table 1. Notably, no verifiable LD_{50} value was identified for glycyrrhizinic acid itself.

The oral LD_{50} values for the ammonium, diammonium, potassium, monopotassium, and dipotassium salts of glycyrrhizinic acid were reported to be in the range of 1220–12 700 mg/kg bw in mice (Klosa, 1957; Fujimura, 1974; Fujimura & Okamoto, 1974). Additionally, no deaths were reported in mice given glycyrrhetic

Table 1. Acute toxicity of compounds related to glycyrrhizinic acid

Compound	Species	Route	LD_{50} (mg/kg bw)	Reference
Glycyrrhizin	Mouse	Oral	>2 000	Sasaki et al. (2002)
	Guinea-pig	Subcutaneous	1 000	Tocco (1924)
	Dog	Intravenous	500	Tocco (1924)
Glycyrrhetic acid	Mouse	Intraperitoneal	308	Finney et al. (1958)
Ammonium	Mouse	Oral	12 700	Fujimura (1974)
glycyrrhizinate	Mouse	Intraperitoneal	1 050	Fujimura (1974)
Monoammonium glycyrrhizinate	Mouse	Intraperitoneal	1 070	Fujimura (1974)
Diammonium	Mouse	Oral	9 600	Fujimura & Okamoto (1974)
glycyrrhizinate	Mouse	Intraperitoneal	1 250	Fujimura & Okamoto (1974)
Potassium	Mouse	Oral	2 400	Fujimura (1974)
glycyrrhizinate	Mouse	Intraperitoneal	1 260	Fujimura (1974)
Monopotassium	Mouse	Oral	1 220	Klosa (1957)
glycyrrhizinate	Mouse	Intravenous	412	Klosa (1957)
	Mouse	Intramuscular	695	Klosa (1957)
	Mouse	Subcutaneous	697	Klosa (1957)
Dipotassium	Mouse	Oral	8 100	Fujimura & Okamoto (1974)
glycyrrhizinate	Mouse	Intraperitoneal	1 400	Fujimura & Okamoto (1974)

acid at a maximum dose of 610 mg/kg bw by oral administration (Finney et al., 1958). No effects were reported on the nervous system, cardiovascular system, respiratory system, or the gastrointestinal tract of cats given a single dose of glycyrrhetic acid at 125 mg/kg bw by intraperitoneal administration (Finney et al., 1958).

In groups of four adult female Sprague-Dawley rats, intraperitoneal administration of single doses of glycyrrhizin, 18α- or 18β-glycyrrhetic acid at 0.5, 1, or 1.5 g/kg bw was reported to be well tolerated and to produce no significant lesions upon examination by electrocardiogram (ECG). Conversely, the administration of a single intraperitoneal dose of 18α-glycyrrhetic acid at 2 g/kg bw, or two doses of 18α-glycyrrhetic acid at 1 g/kg bw within 1 h, was reported to result in the death of all four rats, which the authors attributed to an atrio-ventricular block. Upon histopathological examination, these rats were reported to exhibit oedema of the brain, cerebellum and lung, and haematic stasis of the kidney, adrenal glands, spleen and liver. Focal changes in the papillary muscles of the heart also were reported, including oedema, myolisis, and cell distortion (Rossi et al., 1999).

2.2.2. Short-term studies of toxicity

Several short-term studies have reported no toxicologically significant adverse effects in mice and rats treated orally with glycyrrhizinic acid at ≤ 442 mg/kg bw per day (Quaschning et al., 2001), with glycyrrhizin at 533 mg/kg bw per day (Macabies et al., 1963a), or with glycyrrhetic acid at 1500 mg/kg bw per day (Linko & Vasama, 1958; Horigome et al., 2001).

After repeated administration (30 times) of ammonium glycyrrhizinate at a maximum daily dose of 7 or 28 mg/kg bw by gavage into the stomachs of mice and rats (study duration unknown), there were no signs of intoxication, essential changes in the haematological and integral parameters, shifts in the activity of serum enzymes, or morphological changes in cell structures of the internal organs. A second dose of ammonium glycyrrhizinate at 28 mg/kg bw given during the course of treatment, reportedly led to changes in the activity of some enzymes in the brain, and the development of parenchymatous dystrophy of the liver, which was reported to develop to acidophilic necrosis, attended with signs of regeneration (Antov et al., 1997).

Thirty-eight flavouring agents, including liquorice extract, were evaluated for possible immunotoxicity by studying their effect on humoral and cell-mediated immunity. In 135 female CD-1 or B6C3F_1 mice given liquorice extract at a dose of 0, 1250, 2500, or 5000 mg/kg bw per day by gavage for 5 days, liquorice extract was reported to produce a change in spleen weight and spleen : body weight ratio, but these effects were considered by the authors to be incidental changes, as the organ weight difference was not dose-dependent and no other immunomodulation was reported (Vollmuth et al., 1989).

The possible use of glycyrrhetic acid in the treatment of autoimmune diseases was investigated in groups of seven to eight male MRL/Fas 1pr mice given drinking-water containing glycyrrhetic acid at a dose of 5 mg/kg bw per day for 21 or 170 days. The control group received the vehicle (0.3% tragacanth gum) only.

After 21 days of treatment, two mice per group were killed and their organs extracted for examination. No significant differences in the weights of the liver, kidney, thymus, and spleen were reported between animals in the groups treated with glycyrrhetic acid and the control group. Body weights of mice treated with glycyrrhetic acid and mice in the control group were also reported not to be significantly different at the end of the 170-day study. A gradual increase in urinary protein concentrations ($\leq$ 150 μg/ml) was reported in the control mice from days 22–170 of the study. In contrast, urinary protein concentrations in mice treated with glycyrrhetic acid were reported to reach 150 μg/ml after more than 90 days of monitoring. From days 18–50 of the study, urinary protein concentrations in mice treated with glycyrrhetic acid were reported to be significantly lower than those of the controls. Treatment with glycyrrhetic acid was reported to significantly decrease serum concentrations of IgG in the mice after 21 days of treatment, but not serum concentrations of anti-rheumatoid factor and anti-cardiolipin antibodies. Although no significant changes in the serum concentrations of corticosterone were reported in mice treated with glycyrrhetic acid, the significant decrease in serum concentrations of dehydrocorticosterone resulted in a significant suppression of the mean ration of dehydrocorticosterone to corticosterone, which the authors attributed to systemic 11β-HSD2 activity. Concentrations of corticosterone and dehydrocorticosterone in the liver of mice treated with glycyrrhetic acid were reported to be significantly higher than those of controls; however, there were no changes in the ration of dehydrocorticosterone to corticosterone in the liver. No significant changes in the concentrations of these steroids were reported in the kidneys, thymus, and spleen of mice treated with glycyrrhetic acid or of mice in the control group. 11β-HSD activity was reported to be significantly inhibited in the liver and kidneys of mice treated with glycyrrhetic acid compared with controls; however, no significant difference in 11β-HSD activity in the thymus and spleen was reported between mice treated with glycyrrhetic acid and mice in the control groups. According to the authors, glycyrrhetic acid inhibits 11β-HSD activity and subsequently modulates corticosteroid metabolism in 1pr mice, resulting in the suppression of urinary protein excretion and serum concentrations of IgG. Therefore, the authors concluded that continuous treatment with glycyrrhetic acid would retard the development of autoimmune diseases in 1pr mice (Horigome et al., 2001).

Groups of male Wistar-Kyoto rats were given drinking-water containing glycyrrhizinic acid at a concentration of 3000 mg/l for more than 21 days (approximately 442 mg/kg bw per day). Additonal groups were given placebo, spironolactone (5.8 mg/kg bw per day), or eplerenone (182 mg/kg bw per day) by gavage on days 8 to 21. No significant differences in body weight and heart rate were reported for any of the treatment groups. However, significantly increased systolic blood pressure, endothelin-1 (ET-1) protein concentrations, and ET-1-induced vascular contractions were reported for the group receiving placebo + glycyrrhizinic acid compared with the other groups. Conversely, concentrations of nitrate in aortic tissue and nitrous oxide-mediated endothelial function were reported to be significantly reduced in the group receiving placebo + glycyrrhizinic acid compared with the other groups. The authors reported that all the effects attributed to glycyrrhizinic acid (i.e. observed in the group receiving placebo + glycyrrhizinic acid) were restored or normalized by treatment with either one of the two aldosterone receptor antagonists (i.e. spironolactone and eplerenone). The authors attributed the

observed glycyrrhizinic acid-induced hypertension in rats to decreased bioavailability of nitrous oxide and activation of the ET-1 vascular system, and suggested the possible use of aldosterone receptor antagonists in the treatment of cardiovascular diseases associated with reduced 11β-HSD activity (Quaschning et al., 2001).

The possible correlation between high intake of glycyrrhizin and myolysis of the papillary muscles has been studied in groups of 40 adult female Sprague-Dawley rats given distilled water (control), 18α- or 18β-glycyrrhetic acid at 15 mg/kg bw per day, or glycyrrhizin at 30 mg/kg bw per day by oral administration for 30 days. Ten rats per group were killed at days 0, 15, and 30 of the study in order to monitor the possible biochemical or histological effects of the treatment. Mild myolysis of the heart papillary muscles was reported in rats treated with glycyrrhizin or 18α-glycyrrhetic acid at day 30 of the study, and no regression of effects was reported 30 days after cessation of treatment (i.e. day 60). Treatment with 18α-glycyrrhetic acid was also reported to produce tubular calculi (associated with significantly enhanced excretion of calcium ions) and a slight expansion of the bronchus-associated lymphoid tissue of the lungs at day 15. Similar increases in excretion of calcium ions were also reported in rats given glycyrrhizin or 18β-glycyrrhetic acid, but such increases were reported to reach statistical significance only on day 30 of the study. Increased excretion of calcium ions was reported to persist in the rats even after cessation of treatment, particularly in rats treated with 18α-glycyrrhetic acid. According to the authors, the biochemical and histological effects observed are not, in themselves, considered to be serious side-effects; however, they recommended that consumption of products containing liquorice (i.e. liquorice as a confectionery or drug) should be avoided by individuals with hyperaldosteronism, heart disease, or those being treated with diuretics (Rossi et al., 1999).

In a 50-day study, rats fed liquorice extract at a dose of 10 g/kg bw per day or ammoniated glycyrrhizin at a dose of 1 g/kg bw per day were reported to show a progressive increase in blood pressure and growth depression. Relative weight increases and severe lesions were reported in the kidneys, adrenal glands, and heart. The survival rate, after 50 days, was reported to be 36% for rats receiving liquorice extract and 77% for those receiving ammoniated glycyrrhizinate, compared with 100% for controls (Girerd et al., 1958).

In a 165-day study, rats were given glycyrrhizin orally at a dose of 160 mg/day (approximately 533 mg/kg bw per day) at certain intervals. A 25% increase in blood pressure was reported, which returned to normal values when treatment was discontinued (Macabies et al., 1963a). The possible hypertensive action of several glycyrrhizin-related substances was also examined and similar results reported (Macabies et al., 1963b).

Gordon (1974) studied the effect of ammonium glycyrrhizinate on blood pressure, electrolytes, and corticosterone. Within 2–3 weeks, increases in blood pressure were reported in Sprague-Dawley rats fed ammonium glycyrrhizinate at a dose of 1 or 2 g/kg bw per day. These effects were reported not to occur in Osborne-Mendel rats during a 20-week study; however, further details of this study were not available.

In a 90-day study, rats were fed diets containing diammonium and dipotassium glycyrrhizinate at 0.1 and 0.5% (approximately 50 and 250 mg/kg bw per day, respectively). Male rats given the highest dose (250 mg/kg bw per day) were reported to exhibit a slower rate of body-weight gain, but no gross or histological abnormalities were reported in the organs (Fujimura & Okamoto, 1974).

A possible neurobehavioural influence was studied in male Sprague-Dawley rats given diets containing ammoniated glycyrrhizin at 0, 2, 3, or 4% (approximately 0, 1.23, 1.87, and 2.55 g/kg bw per day, respectively). The study consisted of three experimental groups of 40 rats. Within each experimental group, four groups of 10 rats were fed the respective diets. The first experimental group, which was fed the test diet for 4 months, was used to assess growth, physiological state, motor function and exploratory behaviour. The second group, also fed the test diet for 4 months, was used to assess basic learning skills, in addition to growth and physiological parameters. The last experimental group, fed the test diet for 6 months, was used to assess shock-induced behaviour and operant conditioning behaviour. During the exposure period, growth was reported to be significantly reduced in the group receiving the highest dose (2.55 g/kg bw per day) in the first experimental group; however, growth was not reduced in either of the other experimental groups receiving this diet (containing 4% ammoniated glycyrrhizin). A similar pattern was seen for food consumption, which was reported to be significantly reduced at 4% in the second experimental group two, but not in either the first or third experimental groups. Blood pressure and heart rate measurements in the first and second experimental groups were reported to be significantly different from control values at various times during the study and showed an overall hypertensive and bradycardic effect. No significant differences in blood pressure or heart rate were reported in rats receiving diets containing 2% ammoniated glycyrrhizin. At terminal necropsy, significant trends towards dose-related increases in heart weight were reported in the first and second experimental groups, in kidney weights in all three experimental groups, and increased brain and decreased adrenal weights in the second experimental group; however, differences in organ weights for the individual exposure groups within experimental groups were not reported. Tests for motor function, exploratory behaviour, and operant performance were reported not to be significantly different from control values in any group treated with ammoniated glycyrrhizin. Overall, avoidance behaviour was reported to surpass control levels, while no effects were reported on response inhibition learning, retention, or shock sensitivity. The authors suggested that the effects of ammoniated glycyrrhizin on the pituitary–adrenal axis, which are manifested physiologically as effects on heart rate and blood pressure, may have been related to the positive effects on avoidance behaviour (Sobotka et al., 1981).

Linko & Vasama (1958) reported an increase in body weight and excretion of potassium in rats given diets containing glycyrrhetic acid at a dose of ≤ 1500 mg/kg bw per day for 8, 10, or 14 days.

2.2.3. Long-term studies of toxicity and carcinogenicity

A study of carcinogenicity with the disodium salt of glycyrrhizinic acid has been conducted in B6C3F_1 mice. Groups of ≥ 50 male mice were given drinking-water

containing 0.04, 0.08, or 0.15% disodium glycyrrhizinate (approximately 71, 166, and 229 mg/kg bw per day, respectively) for up to 96 weeks. Groups of ≥ 50 or more female mice were given given drinking-water containing 0.08, 0.15, or 0.3% disodium glycyrrhizinate (approximately 117, 217, and 407 mg/kg bw per day, respectively) for up to 96 weeks. The highest dose for each sex was the maximum tolerated dose (MTD) determined in a previous study of subacute toxicity. After the treatment period, mice were maintained on a basal diet with no disodium glycyrrhizinate in the drinking water for an additional 14 weeks. There was a dose-related reduction in the amount of water consumed by the treated animals compared with that in the control animals (significance not stated); however, no dose-related increase was reported in the incidence of tumours or in the specific distribution of benign and malignant neoplasms in mice treated with disodium glycyrrhizinate compared with controls. The most common neoplasms reported were liver cell tumours and lymphoid leukaemia in male and female mice, respectively. No other observations (e.g. haematology) were reported (Kobuke et al., 1985).

In female A/J mice treated orally, a water extract of liquorice was reported to significantly inhibit the formation of lung and forestomach tumours induced by benzo[*a*]pyrene and *N*-nitrosodiethylamine (Wang & Mukhtar, 1992; Wang et al., 1992). The liquorice constituent, glycyrrhizin, also was reported to protect against the initiation of skin tumours by 7,12-dimethylbenz[*a*]anthracene (DMBA) in female Sencar mice (Wang & Mukhtar, 1992).

Glycyrrhizinic acid (glycyrrhizin) has also been reported to inhibit the occurrence of hepatocellular carcinoma induced by diethylnitrosamine (DEN). Groups of 50 mice received DEN at a dose of 75–100 mg/kg bw per week by intraperitoneal injection for 6 weeks plus either 2 mg of glycyrrhizin administered three times per week intramuscularly, or the same volume of saline. Additional control groups of 30 mice received glycyrrhizin or saline without DEN. Mice were killed every 5 weeks after the last injection of DEN, up to 25 weeks. The average number of tumours (adenomas and hepatocellular carcinomas) at 25 weeks was reported to be 0.71 and 1.64 in groups treated with glycyrrhizin + DEN and DEN only, respectively. In the group treated with glycyrrhizin + DEN, the incidence of hepatocellular carcinoma was reported to be lower and the number of apoptotic cells was reported to be higher than that in the group treated with DEN only (Shiota et al., 1997).

In an investigation of the reported chemopreventative effects of liquorice, groups of 16 male Fischer rats were given diets containing 0.38, 1.5, or 3% liquorice root extract (corresponding to a dose of liquorice root of approximately 190, 750, or 1500 mg/kg bw per day, respectively) for 3 months. Half of the animals in each group were killed after 1 month of treatment. A control group of 20 rats received only the basal diet for the same treatment periods. Analyses of liver enzymes at 1 and 3 months were reported to show increased activity of glutathione transferase, catalase, and protein kinase C. The increases were not statistically significant in all cases, but were reported to show a trend towards increase across the treated groups. Levels of ornithine decarboxylase, which indicate promotional activity, were reported to be decreased. No significant changes in food consumption, body weight, haematological, clinical chemistry, organ weight, or histological parameters were reported at any dose. The authors suggested that the increases in protective enzymes without subsequent increases in so-called risk enzymes may

contribute directly or indirectly to the reported chemoprotective activity of liquorice (Webb et al., 1992).

2.2.4. Genotoxicity

(a) In vitro

Several glycyrrhizinate salts and liquorice extracts and/or various components of liquorice containing glycyrrhizinic acid have been tested in a number of short-term tests of mutagenicity/genotoxicity. The results of these tests are summarized in Table 2.

In mammalian cells, chromosome aberrations were reported in Chinese hamster lung fibroblasts treated with glycyrrhizin or sodium glycyrrhizinate, but not in human embryonic lung cells treated with ammoniated glycyrrhizin (Litton Bionetics, 1972; Ishidate et al., 1984; Ishidate, 1988). Glycyrrhizinic acid trisodium salt was reported to produce negative results in tests for sister chromatid exchange and micronucleus formation in Chinese hamster cell cultures and human fibroblastic cell lines (Sasaki et al., 1980). Liquorice extracts were reported to produce negative results in the assay for unscheduled DNA synthesis assay in rat hepatocytes, but reportedly produced positive results in mouse lymphoma cells (Heck et al., 1989).

(b) In vivo

The results of tests on glycyrrhizinic acid salts in vivo are presented in Table 3. Ammoniated glycyrrhizinate was reported to give negative results in the assay for chromosome aberration in rat bone marrow (Litton Bionetics, 1972), and negative results in assays for dominant lethal mutation in mice (Sheu et al., 1986) and host-mediated assays in which *S. typhimurium* bacterial strains were isolated from the organs of host animals and evaluated for mutagenic effects (Litton Bionetics, 1972); however, mixed results were reported for assay for dominant lethal mutation in rats (Litton Bionetics, 1972; Jorgenson et al., 1977; Sheu et al., 1986). The single positive result reported was based on a statistically significant increase in the number of dead implants, which was reportedly due to a high frequency of dead implants in five out of 33 pregnant females mated in the first week after treatment. No effect on the frequency of dead implants was reported in females mated during the second week after treatment.

In a study in male ddY mice (Sasaki et al., 2002), the comet (alkaline single cell gel electrophoresis) assay was used to investigate DNA damage in eight organs (i.e. stomach, colon, liver, kidney, urinary bladder, lung, brain, and bone marrow) after treatment with a single oral (gavage) dose of glycyrrhizin at 2000 mg/kg bw. The authors reported negative results (i.e. no DNA damage at the level of the single cell) for each of the organs studied.

In addition to the studies with ammonium glycyrrhizinate, both disodium and trisodium glycyrrhizinate were reported to give negative results in a test for micronucleus formation in male ddY mice when administered by intraperitoneal injection at doses of ≤140 mg/kg bw (Hayashi et al., 1988).

Table 2. Summary of studies of genotoxicity in vitro with glycyrrhizinic acid salts, liquorice and related compounds

End-point	Test system	Concentration	Result	Reference
Glycyrrhizin				
Chromosomal aberrations	Chinese hamster lung fibroblasts	4–8 mg/ml, −S9	Positive	Ishidate (1988)
Ammoniated glycyrrhizin				
Mutation	*S. typhimurium* TA98, TA100, TA97	0.01–0.5 mg/plate, ±S9	Negative	Cooper & Berry (1988)
Mutation	*S. typhimurium* TA1535, TA1537, TA1538, TA100	0.033–10 mg/plate, ±S9	Negative	SRI (1979)
Mutation	*E. coli* WP2 uvrA	0.033–10 mg/plate ±S9	Negative	SRI (1979)
Chromosomal aberrations	Human embryonic lung cells	≤1 mg/ml	Negative	Litton Bionetics (1972)
Monoammonium glycyrrhizin				
Mutation	*S. typhimurium* TA98, TA100, TA97	0.01–0.5 mg/plate, ±S9	Negative	Cooper & Berry (1988)
Glycyrrhizinate salts				
Mutation	*S. typhimurium* TA92, TA1535, TA100, TA1537, TA94, TA98	≤10 mg/plate, ±S9	Negative	Ishidate et al. (1984)
Chromosomal aberrations	Chinese hamster lung fibroblasts	≤4 mg/ml for disodium glycyrrhizinate, −S9	Positive	Ishidate et al. (1984); Ishidate (1988)
		≤16 mg/ml for trisodium glycyrrhizinate, ±S9	Positive	

Sister chromatid exchange	Chinese hamster cell cultures Don-6	0.89 and 1.78 mg/ml, −S9	Negative	Sasaki et al. (1980)
Sister chromatid exchange	Human fibroblastic cell line HE2144	0.89 and 1.78 mg/ml, −S9	Negative	Sasaki et al. (1980)
Micronucleus formation	Chinese hamster cell cultures Don-6	0.89 and 1.78 mg/ml	Negative	Sasaki et al. (1980)
Micronucleus formation	Human fibroblastic cell cultures HE2144	0.89 and 1.78 mg/ml, ±S9	Negative	Sasaki et al. (1980)
Liquorice powder				
Mutation	*S. typhimurium* TA98, TA100, TA97	0.01–0.5 mg/plate, ±S9	Negative	Cooper & Berry (1988)
Liquorice extracts				
Mutation	*S. typhimurium* TA1537, TA98, TA100, TA1535, TA1538	≤1 mg/plate, ±S9	Negative	Heck et al. (1989)
Mutation	*S. typhimurium* TA98, TA100	Not reported, ±S9	Negative	Yamamoto et al. (1982)
Unscheduled DNA synthesis	Rat hepatocytes	≤669 μg/ml, ±S9	Negative	Heck et al. (1989)
	Mouse lymphoma cells	≤2.8 mg/ml, ±S9	Positive	Heck et al. (1989)

S9, exogenous metabolic activation system consisting of 9000 × *g* supernatant from rodent liver.

Table 3. Summary of studies of genotoxicity in vivo with glycyrrhizinic acid salts, liquorice, and related compounds

End-point	Test system	Dose	Result	Reference
Glycyrrhizin				
Comet assay	Male ddY mice	≤2000 mg/kg bw	Negative	Sasaki et al. (2002)
Ammoniated glycyrrhizin				
Chromosomal abberation	Rat bone marrow	≤5 g/kg bw	Negative	Litton Bionetics (1972)
Dominant lethal mutation	Rat	≤5 g/kg bw	Negative	Litton Bionetics (1972)
Host-mediated assay	Mice	5 g/kg bw	Negative	Litton Bionetics (1972)
Dominant lethal mutation	Sprague-Dawley rat	0.4–4% of diet	Positive[a]	Jorgenson et al. (1977); Sheu et al. (1986)
Dominant lethal mutation	(101 × C3H)F_1 mice	2.25% of diet	Negative	Sheu et al. (1986)
Heritable translocation	(101 × C3H)F_1 mice	2.25% of diet	Negative	Sheu et al. (1986)
Disodium glycyrrhizinate				
Micronucleus formation	Male ddY mouse bone-marrow cells	≤140 mg/kg bw	Negative	Hayashi et al. (1988)
Trisodium glycyrrhizinate				
Micronucleus formation	Male ddY mouse bone-marrow cells	≤140 mg/kg bw	Negative	Hayashi et al. (1988)

[a] Weak but statistically significant increase in the percentage of dead implants in females mated to males receiving diets containing 4% ammoniated glycyrrhizin in the first weeks after exposure.

Overall, although some findings were positive, the available data from studies conducted in vitro and in vivo indicate that glycyrrhizinic acid and its related salts are not genotoxic.

2.2.5. Reproductive toxicity

No birth defects or effects on maternal or fetal survival were reported in mice, rats, hamsters, or rabbits given ammonium glycyrrhizinate at a dose of ≤1 g/kg bw per day by gavage (Food and Drug Research Laboratories, 1972; Mantovani et al., 1988). Another salt, disodium glycyrrhizinate, was also reported to be non-teratogenic in rats given a dose of 60–1480 mg/kg bw per day during the first 20 days of gestation (Itami et al., 1985).

In albino CD-1 mice or Wistar albino rats, monoammonium glycyrrhizinate at a dose of 0, 27, 90, 300, or 1000 mg/kg bw administered by oral intubation on days 6–15 of gestation was reported not to produce teratogenic effects or result in decreased maternal or fetal survival. Administration of monoammonium glycyrrhizinate at the same doses to Syrian golden hamsters on days 6–10 of gestation or to Dutch-belted rabbits on days 6–18 of gestation was also reported not to produce teratogenic effects or result in decreased maternal or fetal survival. The examinations in this study consisted of a caesarean section to determine the number of implantation sites, resorption sites, and live and dead fetuses. The urogenital tract of each dam was examined and the fetuses were also examined for any abnormalities (Food and Drug Research Laboratories, 1972).

A dose-related increase in embryotoxicity and minor anomalies has been reported in rats given ammonium glycyrrhizinate by oral administration. Groups of 16–20 pregnant rats were given drinking-water containing ammonium glycyrrhizinate at a dose of 0, 21, 239, or 680 mg/kg bw per day on days 7–17 of gestation. The authors reported that the effects in the fetuses were not significantly dose-related, with the exception of the number of variants of the sternum. Sternal variations were reported to show a similar increase in the groups receiving the intermediate dose (239 mg/kg bw per day) and the highest dose (680 mg/kg bw per day). The incidence of haemorrhages, haematomas, and ectopic (i.e. displaced) or hypoplastic (i.e. incomplete development) kidneys was reported to show a significant increase in the group receiving the lowest dose (21 mg/kg bw per day) and the highest dose compared with that in the control group, while the incidence in group receiving the intermediate dose was reported to show no significant change. A significant increase ($p < 0.01$) in renal variants was reported in the group receiving the lowest dose; a significant increase ($p < 0.05$) in internal haemorrhages was reported only in the group receiving the intermediate dose. No abnormalities were reported in the dams except for a significant increase in water intake in the groups receiving the intermediate and highest doses. The concentrations of potassium in the dams were reported to remain normal at all doses. The authors also reported a significant increase in resorptions in the groups treated with ammonium glycyrrhizinate, but this occurred at a more sensitive level ($p < 0.03$) than that normally used in this type of study ($p < 0.05$). The sternal variations are considered to represent fetotoxicity and consequently the study showed no dose-related teratogenicity (Mantovani et al., 1988).

Pregnant Wistar rats were given diets containing 0, 0.08, 0.4, or 2.0% disodium glycyrrhizinate (approximately 0, 60, 290, and 1480 mg/kg bw per day, respectively) during the first 20 days of gestation. Fetuses were examined on day 20 of gestation for external, skeletal, or internal malformations, body weight, placental weight, mortality, and sex ratio. No significant differences between animals treated with disodium glycyrrhizinate and control animals were reported. Postnatal development of the offspring treated with disodium glycyrrhizinate was no different to that of control animals. The only significant difference between animals treated with disodium glycyrrhizinate and control animals reported by the authors was in the post-partum body-weight gain of the dams receiving the highest dose (2.0% disodium glycyrrhizinate). At the two higher doses (0.4 and 2.0% disodium glycyrrhizinate), a lower body-weight gain was reported up to 3 weeks after delivery. No explanation was given for this observation. The authors concluded that disodium glycyrrhizinate was not teratogenic to rats fed at the levels indicated (Itami et al., 1985).

2.2.6. Special studies

In addition to conducting studies to evaluate the metabolism and pharmacokinetics of glycyrrhizinic acid and glycyrrhetic acid in rats (Ploeger et al., 2000a, 2000b), Ploeger (2000) and co-workers (2000a, 2000b) have developed a physiologically-based pharmacokinetic-pharmacodynamic model to characterize the probability of humans developing pseudohyperaldosteronism as a result of the consumption of glycyrrhizinic acid, particularly from liquorice and products containing liquorice. The model was calibrated on the basis of data from the literature on plasma concentrations of glycyrrhizinic and glycyrrhetic acid after oral intake of glycyrrhizinic acid/glycyrrhizin, occurrence of pseudohyperaldosteronism at various plasma concentrations of glycyrrhizinic and glycyrrhetic acid, and variables such as the rate of bowel movements, stomach emptying, rate of hydrolysis of glycyrrhizinic acid to glycyrrhetic acid, and enterohepatic circulation of glycyrrhetic acid were also included. With these variables, the model was able to provide estimates of the effects of intake of glycyrrhizinic acid, with hydrolysis to glycyrrhetic acid, on the activity of 11β-HSD2 through the prediction of the ratio of cortisol/cortisone in urine samples at 24 h (Heilmann et al., 1999). Much of these data were also validated against data obtained from independently conducted clinical trials in volunteers receiving multiple oral doses of glycyrrhizinic or glycyrrhetic acid.

From analysis of their model, Ploeger (2000) and Ploeger et al. (2001a, 2001b) determined that the response (likelihood of developing pseudohyperaldosteronism) to intake of glycyrrhizinic acid was mainly a factor of the motility of the gastrointestinal tract (slower motility resulted in greater systemic exposures to both glycyrrhizinic and glycyrrhetic acids), variances in the absorption rate constant, variability in the plasma concentrations of glycyrrhetic acid that produced 50% inhibition of 11β-HSD2, and variability in the initial or baseline activity of the 11β-HSD2 enzyme in any given individual (Mune et al., 1995; Ferrari et al., 2001). By studying the effects of these variables in a Monte Carlo simulation, Ploeger (2000) calculated an estimate of the likelihood of development of disturbances of the ratio of cortisol to corticosterone, and of biochemical and clinical manifestations of 'pseudohyperaldosteronism' with increasing intake of glycyrrhizinic acid. The phar-

macodynamic portion of the model was initialized with the following assumptions obtained from the scientific literature: (a) at the no-observed-effect level (NOEL) for glycyrrhizin of 2.0 mg/kg bw per day reported in a randomized double-blind study in which groups of 39 healthy female volunteers were treated with glycyrrhizinic acid for up to 8 weeks, that the plasma concentration of glycyrrhetic acid never exceeded 800 μg/l (Bijlsma et al., 1996); and (b) the range of potential responses to glycyrrhizinic acid was assumed to have been captured in the studies by Bijlsma et al. (1996) and van Gelderen et al. (2000).

Ploeger (2000) calculated that at an intake of glycyrrhizinic acid of 100 mg/day, about 2 mg/kg bw per day or the NOEL value reported from the Bijlsma et al. (1996) and van Gelderen et al. (2000) 8-week clinical study, approximately 18% of the exposed population would have glycyrrhizinic acid at concentrations of greater than 800 μg/l. Disturbances of the ration of cortisol to corticosterone and of the appearance of clinical manifestations of 'pseudohyperaldosteronism' were predicted to occur in 26% and in 0.04% of the exposed population, respectively. These estimates were considered applicable to the consumption of glycyrrhizinic acid from all dietary sources combined. Overall, the physiologically-based pharmacokinetic-pharmacodynamic model developed by Ploeger (2000) appeared to agree well with the available clinical study data as well as with the case reports of effects of glycyrrhizin at intakes of <100 mg/day in sensitive members of the population.

2.3 Observations in humans

2.3.1. Case reports

In the literature, there have been many case reports of reported intoxications related to excessive consumption of liquorice (Revers, 1946; Molhuysen et al., 1950; Groen et al., 1951; Groen et al., 1952; Pelser et al., 1953; Louis & Conn, 1956; Mollaret et al., 1960; Jenny et al., 1961; Salassa et al., 1962; Chodkiewicz et al., 1963; Gross et al., 1966; Conn et al., 1968; Koster & David, 1968; LeFebvre & Marc-Aurele, 1968; Pelner, 1969; Chamberlain, 1970; Tourtellotte & Hirst, 1970; Robinson et al., 1971; Cotterill & Cunliffe, 1973; Wash & Bernard, 1975; Epstein et al., 1976; Bannister et al., 1977; Cumming, 1977; Epstein et al., 1977a, 1977b; Epstein et al., 1978; Mourad et al., 1979; Takeda et al., 1979; Werner et al., 1979; Blachley & Knochel, 1980; Cumming et al., 1980; Lai et al., 1980; Ibsen, 1981; Sundaram & Swaminathan, 1981; Simpson & Currie, 1982; Yokoyama et al., 1982; Corsi et al., 1983; Cugini et al., 1983; Heidemann & Kreuzfelder, 1983; Joseph & Kelemen, 1984; Nielsen & Pedersen, 1984; Piette et al., 1984; Beretta-Piccoli et al., 1985; Mori et al., 1985; Commeau et al., 1986; Stewart et al., 1987; Achar et al., 1989; Scali et al., 1990; Böcker & Breithardt, 1991; Farese et al., 1991; Chubachi et al., 1992; Hayashi et al., 1992, Shintani et al., 1992; Rosseel & Schoors, 1993; Bernardi et al., 1994; Brayley & Jones, 1994; Kerlan et al., 1994; Hayashi et al., 1995; Heikens et al., 1995; Barrella et al., 1997; Chamberlain & Abolnik, 1997; DeKlerk et al., 1997; Kageyama et al., 1997; Blakey, 1998; Dellow et al., 1998; Doeker & Andler, 1999; Eriksson et al., 1999; Famularo et al., 1999; Ishikawa et al., 1999; Nishioka & Seguchi, 1999; Dobbins & Saul, 2000; Negro et al., 2000; Olukoga & Donaldson, 2000; Russo et al., 2000; Brouwers & van der Meulen,

2001). Effects reported in these cases included retention of serum sodium, depletion of serum potassium, oedema, hypertension, and myopathy. These effects were reported to be intensified in individuals taking medication, especially diuretics.

In Western Europe, people who consumed 1–3 l of alcoholic beverages containing liquorice (levels not specified) daily were reported to develop muscular weakness, paralysis, tetany, and hypokalaemia (Mollaret et al., 1960; Jenny et al., 1961; Chodkiewicz et al., 1963). According to the authors, the symptoms suggested primary aldosteronism (i.e. electrolyte imbalance due to excessive secretion of aldosterone), but such symptoms have also been reported to be the result of the ingestion of large quantities of glycyrrhizin (Jenny et al., 1961).

The case reports document that exposures to glycyrrhizinic acid at <100 mg/day may be associated with the development of effects characteristic of pseudohyperaldosteronism, including increased blood pressure. In these cases, there are often other factors, either genetic or lifestyle, that increase the sensitivity of the individuals to the effects of glycyrrhetic acid on 11β-HSD2. For example, Russo et al. (2000) reported two cases of hypertension encephalopathy associated with high blood pressure induced by prolonged daily ingestion of low doses of liquorice (length of exposure not specified). One case was reported to involve a man aged 42 years who consumed 50 g of liquorice per day (equated by the authors to an intake of glycyrrhizinic acid of 100 mg/day), while the other case involved a man aged 46 years who ingested 40 g of liquorice per day (equated by the authors to an intake of glycyrrhizinic acid of 80 mg/day). Both patients were reported to present with classic symptoms of hypermineralocorticoidism (i.e. hypertension, hypokalaemia and suppressed renin-aldosterone system) in addition to hypertension encephalopathy. The patients were advised to discontinue their intake of liquorice, whereupon rapid improvements in their symptoms were reported. The authors suggested that individual variations in susceptibility to glycyrrhizinic acid exist, such that those who have lower enzyme activity could develop severe induced hypermineralocorticoidism on ingestion of low doses of glycyrrhizinic acid. Similarly, Rosseel & Schoors (1993) reported on the case of a man aged 55 years who developed high blood pressure, low plasma renin activity, and hypokalaemia, despite normal concentrations of plasma aldosterone, after consumption of chewing gum containing glycyrrhizinic acid at approximately 50 mg/day. The symptoms resolved after discontinuation of consumption of chewing gum.

2.3.2 Single-dose studies

It has been reported that single oral doses of 18β-glycyrrhetic acid at 0.5, 1.0, or 1.5 g (approximately 8.3, 16.67, and 25.0 mg/kg bw, respectively) given to six healthy males did not to result in any adverse effects or changes in blood pressure or pulse (Krähenbühl et al., 1994). Plasma concentrations of cortisol were not affected at any dose. Plasma concentrations of cortisone were reported to be decreased at each dose compared with those of a control group. The decreases in plasma concentrations of cortisone were statistically significant in the groups receiving 0.5 and 1.0 g between 2.5 and 7.5 h after administration, and in the group receiving 1.5 g between 2.5 and 10 h after administration.

A man aged 33 years diagnosed with viral meningitis was admitted to the hospital and treated with a preparation called Stronger Neo-Minophagen C (SNMC), a drug consisting of glycine, cysteine and glycyrrhizin, whereupon the patient was reported to develop a drug-induced allergic hepatitis (Akashi et al., 1988). On the basis of the results of an SNMC patch test, the authors concluded that the allergic reaction was induced by the SNMC treatment; however, they were not able to determine which ingredient of SNMC may have potentiated the condition.

An National Cancer Institute-sponsored phase-I clinical trial with glycyrrhetic acid in patients with a prior history of breast cancer was conducted by Vogel et al. (1992). Ten healthy female volunteers who had been previously diagnosed with breast cancer received glycyrrhetic acid as a single oral dose at 100 to 500 mg/m^2 (approximately 2.70 to 13.51 mg/kg bw) and were reported to show no evidence of significant toxicity. No changes (compared with baseline) were reported in systolic or diastolic blood pressure, serum sodium, or plasma renin concentrations. None of the subjects reported any adverse effects and glycyrrhetic acid was reported to be well tolerated up to a dose of 500 mg/m^2.

2.3.3 Multiple-dose studies

In a randomized double-blind treatment study conducted in the Netherlands, 39 healthy female volunteers (aged 18–40 years; body weights, 50–90 kg) were given capsules containing glycyrrhizinic acid at a dose of 0, 1, 2, or 4 mg/kg bw per day administered orally for 8 weeks (Bijlsma et al., 1996; van Gelderen et al., 2000). There were 10 women in the control group, 9 in the group receiving 1 mg/kg bw per day, 9 in the group receiving 2 mg/kg bw per day, and 11 in the group receiving 4 mg/kg bw per day. The study used female volunteers because in a pilot study with higher doses women appeared to be more sensitive than men to glycyrrhizinic acid (van Vloten et al., 1989). Women were eliminated from the study if their blood pressure rose above 95 mmHg (12.7 kPa) diastolic or 150 mmHg (20 kPa) systolic, or if they exhibited peripheral oedema or a decrease in the serum concentration of potassium to below 3.0 mmol/l. One person from the group receiving the highest dose (4 mg/kg bw per day) was withdrawn from the study after 6 weeks due to 'concentration difficulties and general discomfort'. Upon examination, the blood pressure of this subject was reported to be slightly elevated (+7 mmHg (+0.9 kPa) systolic and diastolic), as was the body weight (by 3 kg) compared with baseline levels. Once consumption was discontinued, these parameters were reported to have returned to normal values. During the course of the study, there was no change in body weight in any group, indictating no retention of water. Plasma renin activity decreased significantly only at the highest dose. The serum concentration of aldosterone in the group receiving the highest dose was also significantly lower than that of the control group after 2, 4, 6, and 8 weeks of treatment, but not after 10 weeks. There was no differences between the concentrations of aldosterone reported in the groups receiving a dose of 1 or 2 mg/kg bw per day and those reported in the control group. At 4 mg/kg bw per day, but not at 1 or 2 mg/kg bw per day, an apparent increase in the concentration of atrial natriuretic peptide was found, which decreased after cessation of treatment. At the highest dose, blood pressure was unchanged, but was relatively higher than that in the

control group (diastolic, 72.9 mmHg (9.7 kPa) compared with 67.8 mmHg (9.0 kPa) in the control group; and systolic, 125 mmHg (16.7 kPa) compared with 120 mmHg (16.0 kPa) in the control group), owing to a reduction in blood pressure in the control group over the course of the study. During weeks 2–4, a significant decrease in plasma concentrations of potassium in the group receiving a dose of 4 mg/kg bw per day, relative to the control group, was reported; however, concentrations gradually returned to baseline values throughout the remainder of the experiment. The NOEL was 2 mg/kg bw per day, albeit based on evaluation of a small number of individuals.

In an older clinical study with 'pure' glycyrrhizinic acid (Van Vloten et al., 1989), groups of eight male and eight female human volunteers consumed glycyrrhizinic acid at doses of 400 or 800 mg/day for 2–4 weeks. During the study effects related to 'pseudohyperaldosteronism' were reportedly observed, with females showing a greater sensitivity to the effect.

Revers (1946) reported an improvement in the healing of gastric ulcers in 45 cases in which patients were treated with liquorice extract; however, oedema was reported in 20% of the patients who were given one teaspoon of a preparation of 100 g of extract in 50 g of water three times per day (equivalent to a total of 10 g liquorice extract per day or approximately 850 mg of glycyrrhizinic acid, assuming that liquorice extract contains 8.5% glycyrrhizinic acid). These effects were reported to include initial swelling of the face, followed by enlarged legs and hands. Often, the oedema was reported to be accompanied by violent headache, dizziness, pain in the upper abdomen, tightness of the chest, a marked increase in weight, and slight hypertension. Haematology and cardiac effects were not studied. The total dose, when reduced to 3 g liquorice extract per day (approximately 255 mg of glycyrrhizinic acid, assuming liquorice extract contains 8.5% glycyrrhizinic acid), was reported not to produce the oedematous effects; however, the author commented that, on occasion, oedema also occurred at these lower doses. In addition to this reported incidence of peripheral and facial oedema, a rare case of pulmonary oedema has been reported, for which excessive consumption of liquorice (approximately 3.6 g of glycyrrhizinic acid) was the only reported associated event (structural abnormalities of the heart were ruled out) (Chamberlain & Abolnik, 1997).

In a series of clinical trials, Smorenberg-Schoorl & Vree (1963) evaluated the effects of the consumption of 'succus liquoritiae', reportedly containing 26% glycyrrhizinic acid, on body-weight gain, water retention, oedema and blood pressure in 17 individuals over an unspecified period of time. The individuals reportedly consumed 1560 mg of glycyrrhizinic acid per day. In six of the subjects, intake of 780 mg/day was reported to be associated with less pronounced body-weight increases, and in four of these six, with increased blood pressure. One of the 17 subjects appeared to be especially sensitive to glycyrrhizinic acid since an increase in blood pressure was reported at an intake of 130 mg/day over an unspecified period of time.

Epstein et al. (1977a) measured the levels of electrolyte and the renin-angiotensin-aldosterone axis in four women (aged 38–55 years) withdrawn from consumption of liquorice. The women were hospitalized for chronic toxicity caused by

liquorice after 6 months to 5 years of ingesting 25–200 g of liquorice candy per day. Serum potassium, serum aldosterone, and urinary aldosterone were reported to be below normal concentrations. It was reported that the renin-angiotensin-aldosterone axis was suppressed and plasma renin activity was decreased in all four subjects. All levels returned to the normal range after electrolyte treatment and abstinence from liquorice ingestion.

In a clinical study, 14 healthy individuals ingested 100 or 200 g of liquorice candy (equated by the authors to approximately 700 or 1400 mg of glycyrrhizinic acid, respectively) for 1–4 weeks (Epstein et al., 1977b). Consumption of liquorice was stopped prematurely in six women owing to the development of hypokalaemia and/or oedema of the extremities (the dose received by the subjects was not specified). Other reported side-effects included headaches, lethargy, and weight gain. It was reported that plasma concentrations of potassium dropped, the renin-angiotensin axis was suppressed, plasma renin activity decreased, and urinary and plasma concentrations of aldosterone decreased in subjects continuing the treatment. These effects were reversible upon cessation of liquorice ingestion. No clear distinction was made between the effects seen at the high and low doses; however, the authors did note that the reported effects had a more rapid onset at the higher dose. One male subject was reported to adapt to the levels of liquorice given and his clinical symptoms (oedema and weight gain) returned to normal throughout continued ingestion of liquorice. The authors suggested that this may be due to an adaptive alteration of absorption or degradation of the liquorice.

The possible effect of liquorice ingestion on the pituitary-adrenal function of 13 healthy subjects (aged 18–46 years) was determined by Epstein et al. (1978). Eight subjects (five men, three women) ingested 100 g of liquorice daily and five subjects (all women) ingested 200 g of liquorice daily for 1–4 weeks. The authors determined the dosage to be equivalent to 700–1400 mg of glycyrrhetic acid per day; however, in a previous paper by these authors (Epstein et al., 1977b), the intake of liquorice was determined to be equivalent to 700–1400 mg of glycyrrhizinic acid per day. Glycyrrhizinic acid is comprised of one molecule of glycyrrhetic acid and two molecules of glucuronic acid. It was assumed, therefore, that the authors were actually referring to glycyrrhizinic acid and not glycyrrhetic acid. This assumption was confirmed by personal communication with one of the co-authors, Dr E.A. Espiner. The daily intake of glycyrrhizinic acid was estimated to be 11.7–23.3 mg/kg bw per day for a 60 kg individual. The same authors also measured excretion of cortisol in four women with hypokalaemic hypertension attributed to either long-term ingestion of liquorice or aldosterone-secreting adenomas. Normal subjects were reported to show a significant increase in mean urinary concentration of cortisol during the ingestion period and into the subsequent 1-week observation period. Plasma cortisol and adrenocorticotropic hormone (ACTH) values were not changed. The women reportedly suffering from hypokalaemic (i.e. reduced serum potassium) hypertension due to toxicity caused by liquorice also showed a substantial elevation in urinary cortisol excretion above the normal. This effect was not seen in the women with primary aldosteronism (i.e. alteration of electrolyte metabolism due to excessive secretion of aldosterone).

In a 4-week clinical study, groups of three male and three female volunteers were given a dried aqueous liquorice extract containing glycyrrhizin at 108, 217,

380 or 814 mg/day (approximately equivalent to a dose of glycyrrhizin of 1.8, 3.6, 6.2, or 13 mg/kg bw per day, respectively) (Bernardi et al., 1994). Anthropometric measurements, renal function, serum electrolyte (sodium and potassium concentrations), blood glucose, renin-aldosterone axis and erythrocyte volume fraction were all assessed as part of this study. No effects were reported at the two lowest doses of glycyrrhizin (1.8 and 3.6 mg/kg bw per day), while at the two higher doses (6.2 and 13 mg/kg bw per day), it was reported that three subjects withdrew from the study owing to side-effects including headaches, oedema, and hypertension. Two of the subjects were female and their complaints, headaches and oedema, were reported to be directly associated with premenstrual symptoms. Of these two subjects, one female receiving glycyrrhizinic acid at 13 mg/kg bw per day, who withdrew from the study due to symptoms of borderline hypertension, hypokalaemia, and peripheral oedema, was also reported to be taking an estroprogestinic drug (i.e. an oral contraceptive) at the same time. The third subject, a male, had a history of hypertension that was aggravated by the study protocol. None of the other participants exhibited any significant symptoms.

In six healthy male volunteers, ingestion of 7 g of liquorice (containing 0.5 g of glycyrrhizinic acid; a dose of glycyrrhizinic acid of approximately 8.33 mg/kg bw per day) daily for 7 days was reported to result in a significant decrease in serum concentration of testosterone and a significant increase in 17β-hydroxyprogesterone, while serum androstenedione did not change (Armanini et al., 1999). On the basis of these results, the authors concluded that the enzyme 17,20-lyase, which is responsible for the conversion of 17-hydroxyprogesterone to androstenedione, was inhibited by glycyrrhizinic acid.

Hayashi et al. (1995) reported two cases of hypokalaemic myopathy in patients with a history of ingestion of glycyrrhizin or liquorice. The first patient, a man aged 62 years, was reported to have consumed 273 mg of glycyrrhizin per day for the treatment of liver dysfunction for a period of 2 months (glycyrrhizin at a dose of approximately 4.6 mg/kg bw per day). The second patient in this study, a woman aged 27 years, was reported to have taken a variety of Chinese herbs for weight reduction and constipation for 3 years; however, the amount of liquorice or glycyrrhizin consumed was not reported. In addition to myopathy, both patients were reported to experience severe weakness in their upper and lower extremities. They also were reported to have serum and urine chemistry values indicative of mineralocorticoid excess, which the authors attributed to the intake of glycyrrhizin. Muscle biopsies performed in the patients were reported to show varying fibre diameters, necrosis, phagocytosis, and occasional vacuolated fibres in the male patient, with no vacuolations, but small myofibrillar degeneration in some muscle fibres in the female patient. The authors concluded that the muscle changes observed in the first patient reflected a severely affected stage, and those in the second patient were indicative of a moderately affected stage. Upon discontinuation of consumption of glycyrrhizin and oral treatment with potassium chloride, it was reported that both patients were discharged from the hospital, having made a full recovery of their muscle strengths.

In a study on the potential interaction of liquorice and CYP3A-substrate drugs, 10 healthy men were given 1 g of liquorice or placebo by oral administration twice

daily (approximately equivalent to 33 mg/kg bw per day) for 7 days plus 7.5 mg of midazolam on day 8. Pre-treatment with placebo or liquorice was reported not to result in significantly different effects on the pharmacokinetic and pharmacodynamic parameters of midazolam. On the basis of the results obtained for midazolam, which is a known substrate of CYP3A enzymes, the authors suggested that liquorice does not induce CYP3A activities in humans, and that therefore liquorice would not be expected to interact with CYP3A-substrate drugs in humans (Shon et al., 2001).

Healthy volunteers were given either 225 mg of glycyrrhizin per day, or 0.1 mg of 9α-fluorocortisol per day, or 225 mg of glycyrrhizin plus 1.5 mg of dexamethasone per day, for 7 days. The glycyrrhizinic acid content of the glycyrrhizin was not given. The authors reported that treatment with glycyrrhizin only significantly suppressed plasma renin activity, urinary aldosterone and serum potassium concentrations, while serum sodium, urinary potassium, urinary cortisol excretion and plasma and urinary cortisone concentrations were significantly increased. Similar results were also reported after treatment with 9α-fluorocortisol. The mineralocorticoid effects of glycyrrhizin were reported to be significantly decreased after co-treatment with glycyrrhizin plus dexamethasone (Kageyama et al., 1992a).

In a similar study, five male and five female healthy volunteers were given glycyrrhetic acid at a dose of 500 mg/day (equivalent to approximately 200 g of confectionery per day); however, the glycyrrhizinic acid equivalent of the glycyrrhetic acid was not given. Concentrations of urinary cortisol, urinary potassium, plasma sodium were reported to be significantly elevated with treatment, while plasma and urinary cortisone, plasma potassium, plasma renin activity and aldosterone were reported to be significantly decreased with treatment. Plasma concentrations of cortisol were reported to have decreased, but not significantly. Also, oral administration of 250 mg of glycyrrhetic acid on the first day of treatment was reported to decrease plasma cortisone and increase the ration of plasma cortisol to cortisone within 40 min (MacKenzie et al., 1990b).

Blood pressure and endocrine functions were studied in 83 male users of chewing tobacco and 22 age-matched controls. Users of chewing tobacco were reported to have significantly higher mean blood pressure (133/78 mmHg (17.7/10.4 kPa) compared with 128/73 mmHg (17.1/9.7 kPa), lower plasma renin activity, and lower ratios of spot urinary tetrahydrocortisone to tetrahydrocortisol. Additionally, in five normal volunteers given glycyrrhizinic acid at a dose of 200 mg/day (reportedly equivalent to two pouches of liquorice-containing chewing tobacco per day) for 1 week, significant reductions were reported in plasma renin activity, ratios of urinary tetrahydrocortisone to tetrahydrocortisol, and urinary excretion of aldosterone. The authors concluded that the use of one to two pouches of chewing tobacco may produce mineralocorticoid effects. This study did not provide details of the methodology or the levels of use of chewing tobacco. In addition, individual and statistical data were not reported. Furthermore, no adverse health effects were reported in any of the volunteers (Guthrie, 1992).

The dose– and time–response relationships between liquorice consumption and rise in blood pressure, as well as the variability in response to glycyrrhetic

acid between individuals were investigated in healthy, Caucasian volunteers from Iceland and Sweden. In the first study, 10 volunteers (one male, nine females) received 200 g of sweet liquorice daily for 2 weeks (equated to an intake of glycyrrhetic acid of 540 mg/day by the authors, which corresponds to approximately 9 mg/kg bw per day in a 60 kg individual). In the second study, 30 volunteers (11 males, 19 females) were given 100 g of sweet liquorice daily for 4 weeks (equated to an intake of glycyrrhetic acid of 270 mg/day by the authors, which corresponds to approximately 4.5 mg/kg bw per day in a 60 kg individual). A third group of volunteers (12 males, 12 females) was given 50 g of sweet liquorice daily for 4 weeks (equated to an intake of glycyrrhetic acid of 75 mg/day by the authors, which corresponds to approximately 0.83 mg/kg bw per day in a 60 kg individual). Blood pressure was measured using a standard mercury sphygmomanometer in all of the subjects, twice during each visit, as well as before and after the period of liquorice consumption. All the volunteers were given physical examinations at the beginning of the study, at the end of the period of liquorice consumption, and at the end of the study. In the first study, mean values for systolic and diastolic blood pressures were reported to be significantly higher than baseline after 2 weeks of consumption. Plasma concentrations of potassium were also reported to be significantly decreased in these subjects after 2 weeks, while no significant change in heart rate was reported. In the second study, it was reported that systolic blood pressure was significantly increased after 2 and 4 weeks of liquorice consumption. Significantly decreased plasma concentrations of potassium were also reported in this group after 2 weeks of liquorice consumption, but no significant changes in diastolic blood pressure and heart rate were reported. Although a significantly increased systolic blood pressure was reported at 2 weeks in the third study, no significant changes in systolic blood pressure at 4 weeks, diastolic blood pressure, heart rate and serum concentrations of potassium were reported in any of the volunteers. Additionally, it was reported that the individual response to liquorice followed the normal distribution curve, such that there was a very small interindividual variance in the rise of blood pressure with consumption of liquorice. The authors concluded that the physiological effects of liquorice are dependent on the dose, but not on the length of exposure to liquorice, such that prolongation of consumption from 2 to 4 weeks does not influence the response, as the maximum blood pressure level is reached after the first 2 weeks of consumption (Sigurjónsdóttir et al., 2001).

In summary, the available clinical studies have reported mild clinical effects consisting of hypokalaemia, reduced plasma renin activity, and reduced urinary concentrations of aldosterone in individuals consuming glycyrrhizinic acid at a dose of approximately 12 mg/kg bw per day in the form of soft liquorice candy. No clinical effects have been reported to be associated with intakes of glycyrrhizinic acid of ≤6.2 mg/kg bw per day (Bernardi et al., 1994).

The clinical effects reported to be produced by glycyrrhizin or glycyrrhizinic acid may be described as physiological in nature, mirroring, for all practical purposes, the effects produced by excessive doses of the endogenous adrenal cortical hormone, aldosterone, which is involved in regulating electrolyte balance. As noted above, these effects consist of hypokalaemia and disturbances in renal function resulting in sodium retention and oedema; however, these effects were reported

to be reversible upon cessation of administration of glycyrrhizinic acid (Revers, 1946; Molhuysen et al., 1950; Conn et al., 1968; Chamberlain, 1970; Epstein et al., 1977a, 1977b; Epstein et al., 1978; Lai et al., 1980; Sundaram & Swaminathan, 1981; Yokoyama et al., 1982; Heidemann & Kreuzfelder, 1983; Joseph & Kelemen, 1984; Beretta-Piccoli et al., 1985; Achar et al., 1989; Mackenzie et al., 1990b; Kageyama et al., 1992a; Morris, 1993; Størmer et al., 1993b; Kerlan et al., 1994; Hayashi et al., 1995; Heikens et al., 1995; Olukoga & Donaldson, 2000; Russo et al., 2000; van Gelderen et al., 2000; Brouwers & van der Meulen, 2001; Sigurjónsdóttir et al., 2001). The reversibility of the effects argues further that the changes reflect a physiological response, rather than a phenomenon of toxicity (WHO, 1987), and is consistent with homeostatic adjustments known to occur following release of aldosterone (Guyton, 1987).

2.3.4 Studies in pregnant women

Three studies were identified that evaluated the effects of glycyrrhizinic acid, derived from consumption of liquorice, on pregnancy outcomes (Colley et al., 1982; Strandberg et al., 2001; Strandberg et al., 2002). One of these studies (Colley et al., 1982), in 313 mothers who consumed liquorice as part of cough medication, did not control for any confounding variables and is therefore uninterpretable with respect to evaluating the effects of glycyrrhizinic acid on pregnancy outcome.

The potential effects of liquorice consumption on birth weight and gestational age were studied in 1049 Finnish women who had completed study questionnaires between March and November of 1998. Intake of glycyrrhizin was calculated based on the quantity and frequency of liquorice consumption reported on the questionnaire, together with the data on glycyrrhizin content of liquorice confectionery obtained from manufacturers and the National Food Administration report of 1993 (Blomberg & Hallikainen, 1993). Maternity records provided data on the type of delivery, birth weight and maternal blood pressure, as well as the basis for estimating gestational age. The calculated mean intake of glycyrrhizin was reported to be 363 mg/week among consumers (a dose of glycyrrhizin of approximately 0.87 mg/kg bw per day), with 46 and 2% of the mothers reporting regular weekly and daily consumption of liquorice, respectively. Average values for gestational age were reported to be 40.1, 40, and 39.7 weeks for low (<250 mg/week), medium (250–499 mg/week) and high (≥500 mg/week) maternal intakes of glycyrrhizin, respectively. There were no significant differences in mean birth weights reported between the groups with a low, intermediate and high intake of glycyrrhizin. Regression analyses performed were reported to result in no significant relationship between birth weight and intake of glycyrrhizin; however, a significant reduction in gestational age (by 2.52 days) was reported to be associated with a maternal intake of glycyrrhizin of ≥500 mg/week (approximately ≥1.2 mg/kg bw per day). The effect of glycyrrhizin consumption on gestational age was reported to remain statistically significant despite adjustments for parity, systolic blood pressure, smoking, coffee consumption, and exclusion of augmented or induced births. Additionally, the cumulative frequency plot of gestational age was reportedly shifted to the left among women with a high intake of glycyrrhizin, compared with the rest of the sample. According to the authors, possible mechanisms for the reduced gestational age may include

inhibition of cortisol or prostaglandin metabolism by glycyrrhizin. However, the authors cautioned that the results obtained in this study may have been confounded by factors associated with liquorice consumption, and they recommended that further studies be undertaken to clarify this matter (Strandberg et al., 2001).

In a subsequent study, Strandberg et al. (2002) evaluated the risk of pre-term birth (gestational age, <37 weeks) and high rates of consumption of liquorice (glycyrrhizin, glycyrrhizinic acid) in a sample of 95 Finnish women. One hundred and seven women who gave birth to infants of normal gestational age at the same hospital served as controls. Intake of glycyrrhizin was evaluated on the basis of questionnaire data and was grouped into three categories: <250 mg/week, 250–499 mg/week, and ≥500 mg/week. The authors compared the risk for pre-term (gestational age, <37 weeks) and early pre-term (gestational age, <34 weeks) delivery in the women with a high consumption of liquorice (i.e. ≥500 mg of glycyrrhizin per week) with that for women with a low (<250 mg/week) and moderate (250–499 mg/week) consumption combined. No separate analyses of the low- and moderate-consumption categories were conducted. Compared with the lower consumption categories combined, intake of glycyrrhizin at rates in excess of 500 mg/week was reported to be associated with an increased risk for both pre-term (age-adjusted odds ratio (OR), 2.28; 95% CI, 1.01–5.14) and early pre-term delivery (OR, 3.07; 95% CI, 1.17–8.05). The association with pre-term delivery was slightly weakened (OR, 2.15; 95% CI, 0.83–4.95) upon the inclusion of confounding variables such as smoking and parity in the analysis. The association with early pre-term delivery was unchanged by the addition of confounding variables in the analysis. The authors noted that one limitation of the study was the retrospective nature of the questionnaire. This weak effect on pre-term birth weight is biologically plausible in light of the known effects of glycyrrhizin (glycyrrhetic acid) on concentrations of cortisol and prostaglandin metabolism.

3. INTAKE

Exposure to glycyrrhizinic acid may occur through the consumption of liquorice confectionery and of health products in which liquorice is used, mainly for its laxative and anti-flatulence properties. It may also occur by sucking/chewing dried *Glycyrrhiza* roots. Moreover, glycyrrhizinic acid is present in beverages, chewing gum, tooth paste, and tobacco in which either the purified acid or its ammonium salt or the dried crude root extract of *Glycyrrhiza glabra* have been incorporated as a flavouring.

3.1 Presence and concentration of glycyrrhizinic acid in foods and beverages

(a) Glycyrrhizinic acid as a natural constituent

Concentrations of glycyrrhizinic acid have been determined in liquorice confectionery products available on the market in the USA, Germany, Belgium and the UK (Størmer et al. 1993b). Similar ranges of concentrations of glycyrrhizinic acid (290–7900 mg/kg) were found in these different markets, with few products con-

taining >3500 mg of glycyrrhizinic acid per kg. The mean content of 17 liquorice products from the UK was 2000 mg/kg (Spinks & Fenwick, 1990). In the Netherlands, the glycyrrhizinic acid content of liquorice distributed on the Dutch market by the four major manufacturers (excluding specialties in small packaging) varied from 27 000 to 115 000 mg/kg in block liquorice and from 700 to 2300 mg/kg in liquorice, with cheap products containing less glycyrrhizinic acid. On the basis of block liquorice purchases and market shares of the different liquorice varieties, the mean content of liquorice products marketed in the Netherlands was assumed to be 900 mg/kg (TNO, 1995). On the other hand, Maas (2000) reported analytical data on glycyrrhizinic acid in 19 samples of liquorice marketed in the Netherlands; values ranged from 300 to 5100 mg/kg, with a mean of 1700 mg/kg.

In most exposure assessments for glycyrrhizic acid, liquorice is typically assumed to contain glycyrrhizic acid at 2000 mg/kg, i.e. the mean content in liquorice in the UK (Spinks & Fenwick, 1990; Størmer et al., 1993b; van Gelderen et al., 2000; Commission of the European Communities 2003).

Certain health products, such as liquorice herbal tea (dried) and throat pearls, may contain glycyrrhizinic acid at much higher concentrations — 20 000 and 47 000 mg/kg, respectively (Spinks & Fenwick, 1990).

In 10 brands of herbal teas for which liquorice plant material was stated as an ingredient, the concentration of glycyrrhizinic acid in the prepared beverage was found to vary from 2 to 450 mg/l (average, 126 mg/l). A similar range of concentrations was found in herbal alcoholic beverages (Maas, 2000).

(b) Glycyrrhizinic acid as a flavouring agent

The upper use levels (95th percentile of reported usage) for ammonium glycyrrhizinate and glycyrrhizinic acid used as chemically defined flavouring agents in foods have been provided to the Scientific Committee for Food by the European Flavour and Fragrance Association for various categories (European Flavour and Fragrance Association, 2001, 2003). upper use levels of glycyrrhizinic acid were 375 mg/kg in dairy products and edible ices, 200 mg/kg in bakery wares, 135–550 mg/kg in alcoholic beverages, 50 mg/kg in non-alcoholic soft beverages, 25 mg/kg in meat and meat products, 20 mg/kg in fish and fish products, and 10 mg/kg in composite foods (including casseroles, meat pies, mincemeat). The upper use levels varied from 400 to 5000 mg/kg in confectionery, fondant, sweets and chewing gum.

Maximum reported use levels of the ammonium salt of glycyrrhizinic acid from the Flavor and Extract Manufacturers Association (1994) were listed in the latest version of Fenaroli's handbook (Burdock, 2002): non-alcoholic beverages, 51 mg/kg; alcoholic beverages, 59 mg/kg; baked goods, 61 mg/kg; gelatin/puddings, 79 mg/kg; frozen dairy products, 91 mg/kg; frosting confectionery, 625 mg/kg; hard candies, 676 mg/kg; soft candies, 1511 mg/kg; and chewing gum, 2278 mg/kg.

Glycyrrhizinic acid is used as a flavouring agent in the USA at the following maximum permitted levels: baked foods, 500 mg/kg; alcoholic beverages, 1000 mg/kg; non-alcoholic beverages, 1500 mg/kg; chewing gum, 11 000 mg/kg; hard candy,

160 000 mg/kg; herbs and seasonings, 1500 mg/kg; plant protein products, 1500 mg/kg; soft candy, 31 000 mg/kg; vitamin or mineral dietary supplements, 5000 mg/kg; all other foods except sugar substitutes, 1000 mg/kg (Code of Federal Regulations, 2003).

3.2 Assessment of intake

Recent cases of intoxication attributable to glycyrrhizinic acid reported in the *Opinion of the Scientific Committee for Food* referred to prolonged consumption of large quantities of either liquorice confectionery (50 g/day), herbal tea (3 l per day) and chewing gums (two 16 g packs per day) (Scientific Committee for Food, 2003). These may therefore be considered important potential sources of glycyrrhizinic acid in some sections of the population.

Refined assessments of exposure to glycyrrhizinic acid have been performed by different authors considering only the consumption of liquorice. In the following calculations, it was assumed that liquorice contains glycyrrhizinic acid at 2000 mg/kg.

An exposure assessment was conducted using food consumption data from the 1995 Australian National Nutrition Survey (Australian Bureau of Statistics, 1999) and 1997 New Zealand National Nutrition Survey (Russell et al., 1999). Liquorice consumption data was assessed though individual food recall for 24 h. Individual body weights of respondents were used to express exposure per kg of body weight.

In the survey from Australia, the estimated consumption of liquorice for all respondents (13 858 subjects) was 0.3 g/day, corresponding to an average dietary exposure to glycyrrhizinic acid equal to 0.6 mg/day (0.01 mg/kg bw per day). Estimated consumption of liquorice for consumers only (108 subjects) was 40.2 mg/day at the mean and 162.8 g/day at the 95th percentile, corresponding to dietary exposure to glycyrrhizinic acid of 80.4 mg/day (1.4 mg/kg bw per day) at the mean and 325.5 mg/day (5.6 mg/kg bw per day) at the 95th percentile.

In the survey from New Zealand, the estimated consumption of liquorice for all respondents (4636 subjects) was 0.25 g/day, corresponding to an average dietary exposure to glycyrrhizinic acid of 0.5 mg/day (0.01 mg/kg bw per day). Estimated mean consumption of liquorice for consumers only (30 subjects) was 38.2 mg/day, corresponding to dietary exposure to glycyrrhizinic acid of 76 mg/day (1.1 mg/kg bw per day). The 95th percentile for consumers only is not reported here due to the small sample size.

Owing to the short duration of the surveys from Australia and New Zealand, levels of exposure in consumers with a high intake are probably overestimated, while the percentage of consumers is underestimated.

In Nordic countries (Denmark, Finland, Iceland, Norway and Sweden) gross consumption of liquorice was estimated at 1 to 2.5 kg/person per year. These figures correspond to about 2.7–6.8 g liquorice/person per day and therefore to a mean intake of glycyrrhizinic acid of 6 to 15 mg/person per day.

The consumption of different types of liquorice was also estimated using the data of the Second Dutch National Food Consumption Survey (TNO, 1995). In order to provide estimates comparable to those of other countries, exposure to glycyrrhizinic acid was assessed considering a mean content of 2000 mg/kg of liquorice, although the use of a different value (900 mg/kg) was proposed in the TNO report. The study referred to 6218 persons aged 1–92 years. Food consumption data were obtained through a 2-day record.

The results showed that daily consumption of liquorice products (in all respondents) was 2 g at the mean and 10 g at the 95th percentile. Highest values were found in children and teenagers due to the high percentage of consumers of liquorice in these age classes (35%). In children aged 7–10 years, mean intake was 5 g and intake at the 95th percentile was 20 g.

Among 'liquorice users only' (15% of the whole sample), the daily consumption of liquorice products was 13 g at the mean and 50 g at the 95th percentile. The highest values for consumption in consumers only were found in women and men aged 16–19 and 19–22 years. In these age groups, mean intake ranged from 17 to 26 g/day and intake at the 95th percentile ranged from 54 to 122 g/day. Intake in children 'liquorice users only' aged 7–10 years was 8 g and 7 g at the mean and 25 g and 16 g at the 95th percentile in males and females respectively.

Based on these data, estimated dietary intake of glycyrrhizinic acid for all respondents was therefore 4 mg/day and 20 mg/day at the average and 95th percentile respectively, i.e. 0.07 and 0.3 mg/kg bw assuming a body weight of 60 kg. In children aged 7–10 years, intake would be 10 mg/day at the mean and 40 mg/day at the 95th percentile.

Among 'liquorice users only', estimated dietary intake was 26 and 100 mg/day (mean and 95th percentile). In women and men aged 16–19 and 19–22 years, mean intake ranged from 34 to 52 mg/day and intake at the 95th percentile ranged from 108 to 244 g/day. In children aged 7–10 years, mean estimated intake ranged from to 14 to 16 mg/day and intake at the 95th percentile ranged from 32 to 50 mg/day.

The consumption of liquorice confectionery and the corresponding intake of glycyrrhizinic acid were also estimated through ad hoc statistical analysis of the food consumption data of the INRAN-RM-2001 food survey conducted in a sample of Italian secondary school students. The study referred to 233 adolescents of mean age 17 ± 1.1 and mean body weight 64.1 ± 13.2 (Leclercq et al., 2004). Food intake was assessed on the basis of 12 24-h dietary records (4 consecutive days in three different periods of the year). In the whole sample the estimated consumption of liquorice was 0.4 ± 1.4 g/day at the mean and 2.5 g/day at the 95th percentile. Estimated consumption of liquorice for consumers only (39 subjects, 17% of the whole sample) was, on average, 2.4 ± 2.8 g/day with a maximum intake of 13.5 g/day. Therefore, in the whole sample the estimated dietary intake of glycyrrhizinic acid was 0.8 mg/day at the mean and 5 mg/day at the 95th percentile. Among consumers only, estimated dietary intake was 4.8 mg/day, with a maximum of 27 mg/day.

In summary, the available data on consumption of liquorice confectionery in Australia, New Zealand and, in some European countries, suggest that mean

intake in the whole population is in the range of 0.01 to 0.1 mg/kg bw per day. Where data were available, the percentage of consumers of liquorice in Europe was reported to be in the range of 15–20%, with higher rates in children and teenagers (≤35%). However, these percentages are probably underestimated owing to the short duration of most surveys, which would not allow identification of occasional consumers.

In these surveys, intake by consumers only was estimated to be in the range of 5–50 mg/day at the mean and to reach 100–300 mg/day at the 95th percentile.

The present assessment of intake was performed considering a mean content of glycyrrizinic acid of 2000 mg/kg of liquorice. Higher levels of exposure (up four times higher) can be expected in consumers with a high intake who are loyal to liquorice products which contain higher concentrations of glycyrrhizinic acid (≤7900 mg/kg of liquorice).

No refined assessment of intake has been provided or published in relation to glycyrrhizinic acid intake via consumption of herbal tea containing liquorice. Herbal teas are available as dried products (tea bags) for home preparation or as ready-for-use products which are packaged into bottles as regular soft drinks and usually consumed cool.

It is claimed that the popularity of herbal teas has increased significantly during the past decades in western countries (Manteiga, 1997). However, no quantitative estimates of consumption were made available to the Committee at its present meeting. High levels of consumption of ready-for-use herbal teas were therefore assumed to be similar to that of other soft drinks. Since the highest consumption (per kg bw) of soft drinks is known to be found in children and teenagers, estimates of intake are provided in this age group in order to provide a conservative estimate. Data retrieved from a food consumption study based on 14-day individual records collected in a total of 948 teenagers from five cities in the European Union (Dublin, Ghent, Helsinki, Potsdam and Rome) were used. Mean age ranged from 13 to 16 years and mean body weight ranged from 53 to 64 kg, according to the city. Mean intake of regular carbonated drinks (excluding diet products) was 136 ml/day in the total population (Lambe et al., 2000). The high level of consumption (97.5th percentile of the total population) varied from 371 ml/day in Rome to 995 ml/day in Ghent (Institute of European Food Studies, 1998). These levels of consumption are referred to average intake in 14 days and therefore may be considered as representative of the long-term intake in consumers with a high intake. Exposure in consumers with a high intake would range from 46 mg to 125 mg assuming a mean concentration of of glycyrrhizinic acid of 126 mg/l in herbal teas. The estimated exposure in consumers loyal to the brands of herbal tea with a higher concentration of glycyrrhizinic acid (≤450 mg/l) could reach 167–448 mg.

4. COMMENTS

The absorption, distribution, biotransformation and excretion of glycyrrhizinic acid and/or its monoammonium salt have been investigated in rats and humans.

In both species, glycyrrhizinic acid, whether in the free form or as the monoammonium salt, is poorly absorbed from the gastrointestinal tract. In the gastrointestinal tract, glycyrrhizinic acid is hydrolysed, mainly by the activity of intestinal microflora, to 18β-glycyrrhetic acid (the aglycone of glycyrrhizinic acid), a substance that is readily absorbed. 18β-Glycyrrhetic acid is subject to enterohepatic circulation and can be further metabolized by intestinal bacteria to 3-dehydro-18β-glycyrrhetic acid and 3-epi-18β-glycyrrhetic acid. The time at which maximum plasma concentrations of glycyrrhetic acid are achieved after oral ingestion of glycyrrhizinic acid is reported to be in the range of 12–16 and 8–12 h in rats and humans, respectively. Doses in excess of 25 mg/kg bw may saturate the capacity of intestinal microflora to hydrolyse glycyrrhizinic acid to glycyrrhetic acid. In humans, absorption of glycyrrhetic acid from the gut is virtually complete, regardless of whether it is formed from the hydrolysis of glycyrrhizinic acid or is initially present as either the glycoside or the aglycone in a food matrix (e.g. liquorice). In humans, at a dose of 0.5 g the half-life was approximately 2 h, while at doses of 1.0 and 2.0 g, a second, slower phase of elimination occurred.

The results of studies in rats, and inferences that can be drawn from the results of studies in humans, indicate that both glycyrrhizinic acid and its hydrolysis product glycyrrhetic acid are largely confined to the plasma. In plasma, glycyrrhizinic acid and glycyrrhetic acid are bound to serum albumin and are not taken up in body tissues to a significant extent.

Absorbed glycyrrhetic acid has been reported to produce effects that are similar to those of the adrenal steroid aldosterone. The mechanism of action of glycyrrhetic acid involves inhibition of the type-2 11β-HSD, an enzyme that converts cortisol to cortisone. As a result, levels of cortisol, which has mineralocorticoid activity reportedly equivalent to that of aldosterone, increase. The high renal concentrations of cortisol cause retention of sodium and excretion of potassium. This electrolyte imbalance has been referred to as 'apparent mineralocorticoid excess' or 'pseudohyperaldosteronism'.

The oral LD_{50} values for glycyrrhizinic acid and various salts in mice, guinea-pigs and dogs were reported to be in the range of 308 to 12 700 mg/kg bw. The toxicity of glycyrrhizinic acid and/or its monoammonium salt has been evaluated in a number of short-term studies in rats and mice. At high doses, effects reported included those related to apparent mineralocorticoid excess or pseudohyperaldosteronism. Mild myolysis of the heart papillary muscles was reported in female Sprague-Dawley rats treated with glycyrrhizin (crude extract) at 30 mg/kg bw per day or with 18α- or 18β-glycyrrhetic acid at 15 mg/kg bw per day for 30 days (note: glycyrrhizinic acid is not metabolized to 18α-glycyrrhetic acid).

In a study of carcinogenicity, $B6C3F_1$ mice were treated for 96 weeks with the disodium salt of glycyrrhizinic acid at a dose of ≤229 mg/kg bw per day in males and 407 mg/kg bw per day and observed for an additional 14 weeks. There was a dose-related reduction in the amount of water consumed by the treated animals when compared with the control animals (statistical significance not stated); however, no dose-related increase was reported in the incidence of tumours or in the specific distribution of benign and malignant neoplasms in treated mice compared with controls.

Oral administration of glycyrrhizin, like glycyrrhizinic acid, has been reported to inhibit the development of chemical-induced neoplasms in several models in mice and rats.

The available data indicated that glycyrrhizinic acid and its salts do not have carcinogenic activity.

Several glycyrrhizinic acid salts and liquorice extracts and/or various components of liquorice containing glycyrrhizinic acid have been investigated in a number of tests for mutagenicity and/or genotoxicity. Overall, although some positive findings were reported, the available data indicated that glycyrrhizinic acid and its related salts are not genotoxic in vitro or in vivo.

Ammonium and disodium salts of glycyrrhizinic acid at doses of ≤1.5 g/kg bw per day have been evaluated in several studies of developmental toxicity in mice, rats, hamsters and rabbits. In one of these studies, embryotoxicity was observed, but overall the data indicated that glycyrrhizinic acid and its salts are not teratogenic.

There have been many case reports of effects related to excessive consumption of liquorice (i.e. equivalent to an intake of glycyrrhizinic acid of >200 mg/day). These included retention of serum sodium, depletion of serum potassium, oedema, hypertension, and myopathy. The case reports also documented that consumption of products containing liquorice at levels that would result in intakes of glycyrrhizinic acid of <100 mg/day could be associated with the development of effects characteristic of pseudohyperaldosteronism, including increased blood pressure. The basis for susceptibility in such cases was not known, although several explanations are possible.

The available clinical studies have been reported to demonstrate mild clinical effects, consisting of hypokalemia, reduced plasma renin activity, and reduced urinary concentrations of aldosterone.

In a randomized double-blind study, 39 healthy female volunteers were given glycyrrhizinic acid at a dose of 0, 1, 2, or 4 mg/kg bw per day for 8 weeks. No adverse effects were observed in the groups receiving a dose of 1 or 2 mg/kg bw per day. In the group receiving a dose of 4 mg/kg bw per day, decreases in plasma renin activity and serum concentrations of aldosterone were found. There was an apparent increase in the concentration of atrial natriuretic peptide, which returned to normal after discontinuation of exposure, but there was no increase in blood pressure. However, mean blood pressure was greater at the highest dose than in the controls, owing to a reduction in the blood pressure of the latter over the course of the study.

A physiologically-based pharmacokinetic-pharmacodynamic model has been developed to characterize the probability of humans developing pseudohyperaldosteronism as a result of the consumption of glycyrrhizinic acid. On the basis of modelling, it was calculated that at an intake of glycyrrhizinic acid of 100 mg/day (about 2 mg/kg bw per day), approximately 18% of the exposed population would have glycyrrhizinic acid at concentrations of >800 µg/l. Also, it was predicted that disturbances of the ratio of cortisol to corticosterone would occur in 26% of the

exposed population, and that clinical manifestations of pseudohyperaldosteronism would appear in 0.04% of exposed persons (95% CI, 0.00046–3.0%).

Intake

Exposure to glycyrrhizinic acid through consumption of liquorice confectionery was assessed on the basis of a number of food surveys lasting 1–14 days. Assuming a mean content of 2000 mg of glycyrrhizinic acid per kg of liquorice confectionery, the exposures for consumers only in these surveys were calculated to be in the range of 5 to 50 mg/day at the mean and reached 100 to 300 mg/day at the 95th percentile.

On the basis of a mean content of 126 mg of glycyrrhizinic acid per litre of herbal tea containing liquorice, high levels of exposure may be expected in regular consumers of these beverages.

5. EVALUATION

The most significant effect of glycyrrhizinic acid, after hydrolysis in the gut to glycyrrhetic acid and subsequent absorption, is inhibition of the type-2 11β-HSD, with a consequent increase in concentrations of cortisol, which leads to increased mineralocorticoid activity with retention of sodium and water and symptoms of apparent mineralocorticoid excess. This physiological action of glycyrrhizinic acid (glycyrrhetic acid) is reversible, but when sustained can lead to elevated blood pressure.

The Committee concluded that the safety evaluation of glycyrrizinic acid should be based on the data from humans. It was observed that there is a sensitive subset of the population who appear to show signs of pseudohyperaldosteronism at lower exposures than those which produce effects in the general population, but the available data did not allow the Committee to adequately characterize this subgroup, and hence the data could not be used to assign an ADI. The available data suggest that an intake of 100 mg/day would be unlikely to cause adverse effects in the majority of adults. The Committee recognized that, in certain highly susceptible individuals, physiological effects could occur at intakes somewhat below this figure. The data indicate that consumers with a high intake of liquorice confectionery or herbal tea containing liquorice may have an intake of glycyrrhizinic acid of >100 mg/day.

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ANNEX 1

REPORTS AND OTHER DOCUMENTS RESULTING FROM PREVIOUS MEETINGS OF THE JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

1. **General principles governing the use of food additives** (First report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 15, 1957; WHO Technical Report Series, No. 129, 1957 (out of print).
2. **Procedures for the testing of intentional food additives to establish their safety for use** (Second report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 17, 1958; WHO Technical Report Series, No. 144, 1958 (out of print).
3. **Specifications for identity and purity of food additives (antimicrobial preservatives and antioxidants)** (Third report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as **Specifications for identity and purity of food additives, Vol. I. Antimicrobial preservatives and antioxidants**, Rome, Food and Agriculture Organization of the United Nations, 1962 (out of print).
4. **Specifications for identity and purity of food additives (food colours)** (Fourth report of the Joint FAO/WHO Expert Committee on Food Additives). These specifications were subsequently revised and published as Specifications for identity and purity of food additives, Vol. II. Food colours, Rome, Food and Agriculture Organization of the United Nations, 1963 (out of print).
5. **Evaluation of the carcinogenic hazards of food additives** (Fifth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 29, 1961; WHO Technical Report Series, No. 220, 1961 (out of print).
6. **Evaluation of the toxicity of a number of antimicrobials and antioxidants** (Sixth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Report Series, No. 31, 1962; WHO Technical Report Series, No. 228, 1962 (out of print).
7. **Specifications for the identity and purity of food additives and their toxicological evaluation: emulsifiers, stabilizers, bleaching and maturing agents** (Seventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 35, 1964; WHO Technical Report Series, No. 281, 1964 (out of print).
8. **Specifications for the identity and purity of food additives and their toxicological evaluation: food colours and some antimicrobials and antioxidants** (Eighth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 38, 1965; WHO Technical Report Series, No. 309, 1965 (out of print).
9. **Specifications for identity and purity and toxicological evaluation of some antimicrobials and antioxidants.** FAO Nutrition Meetings Report Series, No. 38A, 1965; WHO/Food Add/24.65 (out of print).
10. **Specifications for identity and purity and toxicological evaluation of food colours.** FAO Nutrition Meetings Report Series, No. 38B, 1966; WHO/Food Add/66.25.
11. **Specifications for the identity and purity of food additives and their toxicological evaluation: some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases** (Ninth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 40, 1966; WHO Technical Report Series, No. 339, 1966 (out of print).
12. **Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour treatment agents, acids, and bases.** FAO Nutrition Meetings Report Series, No. 40A, B, C; WHO/Food Add/67.29.

13. **Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances** (Tenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 43, 1967; WHO Technical Report Series, No. 373, 1967.
14. **Specifications for the identity and purity of food additives and their toxicological evaluation: some flavouring substances and non nutritive sweetening agents** (Eleventh report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 44, 1968; WHO Technical Report Series, No. 383, 1968.
15. **Toxicological evaluation of some flavouring substances and non nutritive sweetening agents**. FAO Nutrition Meetings Report Series, No. 44A, 1968; WHO/Food Add/68.33.
16. **Specifications and criteria for identity and purity of some flavouring substances and non-nutritive sweetening agents**. FAO Nutrition Meetings Report Series, No. 44B, 1969; WHO/Food Add/69.31.
17. **Specifications for the identity and purity of food additives and their toxicological evaluation: some antibiotics** (Twelfth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 45, 1969; WHO Technical Report Series, No. 430, 1969.
18. **Specifications for the identity and purity of some antibiotics**. FAO Nutrition Meetings Series, No. 45A, 1969; WHO/Food Add/69.34.
19. **Specifications for the identity and purity of food additives and their toxicological evaluation: some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances** (Thirteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 46, 1970; WHO Technical Report Series, No. 445, 1970.
20. **Toxicological evaluation of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other substances**. FAO Nutrition Meetings Report Series, No. 46A, 1970; WHO/Food Add/70.36.
21. **Specifications for the identity and purity of some food colours, emulsifiers, stabilizers, anticaking agents, and certain other food additives**. FAO Nutrition Meetings Report Series, No. 46B, 1970; WHO/Food Add/70.37.
22. **Evaluation of food additives: specifications for the identity and purity of food additives and their toxicological evaluation: some extraction solvents and certain other substances; and a review of the technological efficacy of some antimicrobial agents**. (Fourteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 48, 1971; WHO Technical Report Series, No. 462, 1971.
23. **Toxicological evaluation of some extraction solvents and certain other substances**. FAO Nutrition Meetings Report Series, No. 48A, 1971; WHO/Food Add/70.39.
24. **Specifications for the identity and purity of some extraction solvents and certain other substances**. FAO Nutrition Meetings Report Series, No. 48B, 1971; WHO/Food Add/70.40.
25. **A review of the technological efficacy of some antimicrobial agents**. FAO Nutrition Meetings Report Series, No. 48C, 1971; WHO/Food Add/70.41.
26. **Evaluation of food additives: some enzymes, modified starches, and certain other substances: Toxicological evaluations and specifications and a review of the technological efficacy of some antioxidants** (Fifteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 50, 1972; WHO Technical Report Series, No. 488, 1972.

27. **Toxicological evaluation of some enzymes, modified starches, and certain other substances**. FAO Nutrition Meetings Report Series, No. 50A, 1972; WHO Food Additives Series, No. 1, 1972.
28. **Specifications for the identity and purity of some enzymes and certain other substances**. FAO Nutrition Meetings Report Series, No. 50B, 1972; WHO Food Additives Series, No. 2, 1972.
29. **A review of the technological efficacy of some antioxidants and synergists**. FAO Nutrition Meetings Report Series, No. 50C, 1972; WHO Food Additives Series, No. 3, 1972.
30. **Evaluation of certain food additives and the contaminants mercury, lead, and cadmium** (Sixteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 51, 1972; WHO Technical Report Series, No. 505, 1972, and corrigendum.
31. **Evaluation of mercury, lead, cadmium and the food additives amaranth, diethylpyrocarbamate, and octyl gallate**. FAO Nutrition Meetings Report Series, No. 51A, 1972; WHO Food Additives Series, No. 4, 1972.
32. **Toxicological evaluation of certain food additives with a review of general principles and of specifications** (Seventeenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 53, 1974; WHO Technical Report Series, No. 539, 1974, and corrigendum (out of print).
33. **Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers, and thickening agents**. FAO Nutrition Meetings Report Series, No. 53A, 1974; WHO Food Additives Series, No. 5, 1974.
34. **Specifications for identity and purity of thickening agents, anticaking agents, antimicrobials, antioxidants and emulsifiers**. FAO Food and Nutrition Paper, No. 4, 1978.
35. **Evaluation of certain food additives** (Eighteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 54, 1974; WHO Technical Report Series, No. 557, 1974, and corrigendum.
36. **Toxicological evaluation of some food colours, enzymes, flavour enhancers, thickening agents, and certain other food additives**. FAO Nutrition Meetings Report Series, No. 54A, 1975; WHO Food Additives Series, No. 6, 1975.
37. **Specifications for the identity and purity of some food colours, enhancers, thickening agents, and certain food additives**. FAO Nutrition Meetings Report Series, No. 54B, 1975; WHO Food Additives Series, No. 7, 1975.
38. **Evaluation of certain food additives: some food colours, thickening agents, smoke condensates, and certain other substances**. (Nineteenth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Nutrition Meetings Series, No. 55, 1975; WHO Technical Report Series, No. 576, 1975.
39. **Toxicological evaluation of some food colours, thickening agents, and certain other substances**. FAO Nutrition Meetings Report Series, No. 55A, 1975; WHO Food Additives Series, No. 8, 1975.
40. **Specifications for the identity and purity of certain food additives**. FAO Nutrition Meetings Report Series, No. 55B, 1976; WHO Food Additives Series, No. 9, 1976.
41. **Evaluation of certain food additives** (Twentieth report of the Joint FAO/WHO Expert Committee on Food Additives). FAO Food and Nutrition Meetings Series, No. 1, 1976; WHO Technical Report Series, No. 599, 1976.
42. **Toxicological evaluation of certain food additives**. WHO Food Additives Series, No. 10, 1976.
43. **Specifications for the identity and purity of some food additives**. FAO Food and Nutrition Series, No. 1B, 1977; WHO Food Additives Series, No. 11, 1977.

44. **Evaluation of certain food additives** (Twenty-first report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 617, 1978.
45. **Summary of toxicological data of certain food additives**. WHO Food Additives Series, No. 12, 1977.
46. **Specifications for identity and purity of some food additives, including antioxidant, food colours, thickeners, and others**. FAO Nutrition Meetings Report Series, No. 57, 1977.
47. **Evaluation of certain food additives and contaminants** (Twenty-second report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 631, 1978.
48. **Summary of toxicological data of certain food additives and contaminants.** WHO Food Additives Series, No. 13, 1978.
49. **Specifications for the identity and purity of certain food additives**. FAO Food and Nutrition Paper, No. 7, 1978.
50. **Evaluation of certain food additives** (Twenty-third report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 648, 1980, and corrigenda.
51. **Toxicological evaluation of certain food additives**. WHO Food Additives Series, No. 14, 1980.
52. **Specifications for identity and purity of food colours, flavouring agents, and other food additives**. FAO Food and Nutrition Paper, No. 12, 1979.
53. **Evaluation of certain food additives** (Twenty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 653, 1980.
54. **Toxicological evaluation of certain food additives**. WHO Food Additives Series, No. 15, 1980.
55. **Specifications for identity and purity of food additives (sweetening agents, emulsifying agents, and other food additives).** FAO Food and Nutrition Paper, No. 17, 1980.
56. **Evaluation of certain food additives** (Twenty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 669, 1981.
57. **Toxicological evaluation of certain food additives**. WHO Food Additives Series, No. 16, 1981.
58. **Specifications for identity and purity of food additives (carrier solvents, emulsifiers and stabilizers, enzyme preparations, flavouring agents, food colours, sweetening agents, and other food additives**). FAO Food and Nutrition Paper, No. 19, 1981.
59. **Evaluation of certain food additives and contaminants** (Twenty-sixth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 683, 1982.
60. **Toxicological evaluation of certain food additives**. WHO Food Additives Series, No. 17, 1982.
61. **Specifications for the identity and purity of certain food additives**. FAO Food and Nutrition Paper, No. 25, 1982.
62. **Evaluation of certain food additives and contaminants** (Twenty-seventh report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 696, 1983, and corrigenda.
63. **Toxicological evaluation of certain food additives and contaminants**. WHO Food Additives Series, No. 18, 1983.
64. **Specifications for the identity and purity of certain food additives**. FAO Food and Nutrition Paper, No. 28, 1983.

65. **Guide to specifications, general notices, general methods, identification tests, test solutions, and other reference materials**. FAO Food and Nutrition Paper, No. 5, Rev. 1, 1983.
66. **Evaluation of certain food additives and contaminants** (Twenty-eighth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 710, 1984, and corrigendum.
67. **Toxicological evaluation of certain food additives and contaminants**. WHO Food Additives Series, No. 19, 1984.
68. **Specifications for the identity and purity of food colours**. FAO Food and Nutrition Paper, No. 31/1, 1984.
69. **Specifications for the identity and purity of food additives**. FAO Food and Nutrition Paper, No. 31/2, 1984.
70. **Evaluation of certain food additives and contaminants** (Twenty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 733, 1986, and corrigendum.
71. **Specifications for the identity and purity of certain food additives**. FAO Food and Nutrition Paper, No. 34, 1986.
72. **Toxicological evaluation of certain food additives and contaminants**. WHO Food Additives Series, No. 20. Cambridge University Press, 1987.
73. **Evaluation of certain food additives and contaminants** (Thirtieth report of the Joint FAO/WHO Expert Committee on Food Additives). WHO Technical Report Series, No. 751, 1987.
74. **Toxicological evaluation of certain food additives and contaminants**. WHO Food Additives Series, No. 21. Cambridge University Press, 1987.
75. **Specifications for the identity and purity of certain food additives**. FAO Food and Nutrition Paper, No. 37, 1986.
76. **Principles for the safety assessment of food additives and contaminants in food**. WHO Environmental Health Criteria, No. 70. Geneva, World Health Organization, 1987 (out of print). The full text is available electronically at www.who.int/pcs.
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ANNEX 2

ABBREVIATIONS USED IN THE MONOGRAPHS

ADH	alcohol dehydrogenase
ADI	acceptable daily intake
ALDH	aldehyde dehydrogenase
ATP	adenosine triphosphate
AUC	area under the curve
bw	body weight
cDNA	complementary DNA
C_{max}	maximum plasma concentration
CI	confidence interval
coA	coenzyme A
CYP	cytochrome P450
DEN	diethylnitrosamine
DMBA	7,12-dimethylbenz[*a*]anthracene
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ECG	electrocardiogram
ELISA	enzyme-linked immunosorbent assay
F	female
FAO	Food and Agriculture Organization of the United Nations
GEMS/Food	Global Environment Monitoring System — Food Contamination Monitoring and Assessment Programme
GLP	good laboratory practice
GSH	glutathione
GST	glutathione *S*-transferase
HDL	high-density lipoprotein
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
IDL	intermediate-density lipoprotein
Ig	immunoglobulin
IPCS	International Programme on Chemical Safety
LD_{50}	median lethal dose
LDL	low-density lipoprotein

M	male
MTD	maximum tolerated dose
mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NCE	normochromatic erythrocyte
NOEL	no-observed-effect level
NSAID	nonsteroidal anti-inflammatory drug
PARP	poly-ADP-ribose polymerase
PCE	polychromatic erythrocyte
QA	quality assurance
S9	9000 × *g* microsomal fraction of rat liver
SCE	sister chromatid exchange
$t_{1/2}$	half-life
TOS	total organic solids
TPA	12-*O*-tetradecanoylphorbol-13-acetate
UDPGT	UDP-glucuronosyltransferase
UK	United Kingdom
USA	United States of America
VLDL	very-low-density lipoprotein
V_{max}	maximal velocity
WHO	World Health Organization
w/w	weight for weight

ANNEX 3

JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES

GENEVA, 8–17 JUNE 2004

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ANNEX 4

ACCEPTABLE DAILY INTAKES AND OTHER TOXICOLOGICAL INFORMATION, AND INFORMATION ON SPECIFICATIONS

Food additives and ingredients evaluated toxicologically

Food additive	Specifications[a]	Acceptable daily intake (ADI) in mg/kg bw and other toxicological recommendations
Benzoyl peroxide	R	Treatment of whey with benzoyl peroxide at a maximum concentration of 100 mg/kg does not pose a safety concern
α-Cyclodextrin	—	α-Cyclodextrin does not pose a safety concern at the proposed use levels and resulting predicted consumption as food ingredient and food additive The previously established ADI 'not specified' for use as a carrier and stabilizer for flavours, colours, and sweeteners, as a water-solubilizer for fatty acids and certain vitamins, as a flavour modifier in soya milk, and as an absorbent in confectionery was maintained
Hexose oxidase from *Chondrus crispus* expressed in *Hansenula polymorpha*	N	Not specified[b]
Lutein from *Tagetes erecta* L.	N	0–2 (group ADI for lutein and zeaxanthin)[c]
Peroxyacid antimicrobial solutions containing 1-hydroxyethylidene-1,1-diphosphonic acid (HEDP) *Containing HEDP and three or more of the following components: peroxacetic acid, acetic acid, hydrogen peroxide, octanoic acid and peroxyoctanoic acid*		The peroxy compounds in these solutions (hydrogen peroxide, peroxyacetic acid and peroxyoctanoic acid) would break down into acetic acid and octanoic acid, and small residual quantities of these acids on foods at the time of consumption would not pose a safety concern. HEDP does not pose a safety concern at the levels of residue that are expected to remain on foods at the time consumption.

Food additives and ingredients evaluated toxicologically (Contd)

Food additive	Specifications[a]	Acceptable daily intake (ADI) in mg/kg bw and other toxicological recommendations
Acetic acid	R	
1-Hydroxyethylidene-1,1-diphosphonic acid (HEDP)	N	
Hydrogen peroxide	R	
Octanoic acid (as food additive)	N	
Steviol glycosides	N, T	0–2 (temporary)
D-Tagatose	—	Not specified[b]
Xylanase from *Bacillus subtilis* expressed in *Bacillus subtilis*	N	Not specified[b]
Xylanase (resistant to xylanase inhibitor) from *Bacillus subtilis* containing a modified xylanase gene from *Bacillus subtilis*	N	Not specified[b]
Zeaxanthin	N	0–2 (group ADI for lutein and zeaxanthin)[c]

[a] N, new specifications prepared; R, existing specifications revised; T, tentative specifications.

[b] ADI 'not specified' is used to refer to a food substance of very low toxicity which, on the basis of the available data (chemical, biochemical, toxicological and other) and the total dietary intake of the substance arising from its use at the levels necessary to achieve the desired effects and from its acceptable background levels in food, does not, in the opinion of the Committee, represent a hazard to health. For that reason, and for the reasons stated in the individual evaluations, the establishment of an ADI expressed in numerical form is not deemed necessary. An additive meeting this criterion must be used within the bounds of good manufacturing practice, i.e. it should be technologically efficacious and should be used at the lowest level necessary to achieve this effect, it should not conceal food of inferior quality or adulterated food, and it should not create a nutritional imbalance.

[c] This group ADI does not apply to other xanthophyll-containing extracts with a lutein or zeaxanthin content lower than that cited in the specifications.

Food additives considered for specifications only

Food additive	Specifications[a]
Aluminium lakes of colouring matters — general specifications	R
Aluminium powder	R
Hydroxypropyl cellulose	R
Hydroxypropylmethyl cellulose	R
Iron oxides	R
Magnesium sulfate[b]	N, T
Polyvinyl alcohol	R
Titanium dioxide	R
Zeaxanthin-rich extract from *Tagetes erecta* L.	N, T

[a] R, existing specifications revised; R, existing specifications revised; T, tentative specifications.

[b] Magnesium sulfate was not evaluated at the present meeting because the intended use and use levels were not identified.

Revision of heavy metals limits for food additives

INS		Food additive	Limits, not more than (mg/kg)			
			As	Pb	Cd	Hg
523		Aluminium ammonium sulfate	—	3	—	—
510		Ammonium chloride	—	2	—	—
503	(ii)	Ammonium hydrogen carbonate	—	2	—	—
927	a	Azodicarbonamide	—	2	—	—
901		Bees wax	—	2	—	—
210		Benzoic acid	—	2	—	—
—		Benzyl alcohol	—	2	—	—
—		Butan-1,3-diol	—	2	—	—
—		Butan-1-ol	—	2	—	—
—		Butan-2-ol	—	2	—	—
—		Butyl *p*-hydroxybenzoate	—	2	—	—
263		Calcium acetate	—	2	—	—
213		Calcium benzoate	—	2	—	—
170		Calcium carbonate	3	3	—	—
509		Calcium chloride	—	2	—	—
952		Calcium cyclamate	—	1	—	—
341	(ii)	Calcium hydrogen phosphate	3	4	—	—
516		Calcium sulfate	—	2	—	—
902		Candelilla wax	—	2	—	—
1503		Castor oil	—	2	—	—
925		Chlorine	—	2	—	1
—		Citraxanthin	—	2	—	—
459		Cyclodextrin, β-	—	1	—	—
—		Cyclohexane	—	2	—	—
—		Dammar gum	—	2	—	—
—		Diethyl tartrate	—	2	—	—
—		Diethylene glycol monoethyl ether	—	2	—	—
242		Dimethyl dicarbonate	—	2	—	—

Revision of heavy metals limits for food additives (Contd)

INS		Food additive	Limits, not more than (mg/kg)			
			As	Pb	Cd	Hg
—		Diphenyl	—	2	—	—
—		Edible gelatin	1	1.5	0.5	0.15
—		Ferric ammonium citrate	—	2	—	—
422		Glycerol	—	2	—	—
—		Glycerol diacetate	—	2	—	—
—		Heptanes	—	2	—	—
239		Hexamethylene tetramine	—	2	—	—
—		Isoamyl acetate	—	2	—	—
—		Isobutanol	—	2	—	—
—		Isopropyl acetate	—	2	—	—
270		Lactic acid	—	2	—	—
—		Light petroleum	—	2	—	—
1105		Lysozyme hydrochloride	—	2	—	—
504	(i)	Magnesium carbonate	—	2	—	—
511		Magnesium chloride	—	2	—	—
343	(ii)	Magnesium hydrogen phosphate	3	4	—	—
329		Magnesium lactate	—	2	—	—
—		Methanol	—	2	—	—
905		Mineral oil (high viscosity)	—	1	—	—
—		Monoglyceride citrate	—	2	—	—
234		Nisin	—	1	—	—
—		Norhydroguaiaretic acid	—	2	—	—
451	(ii)	Pentapotassium triphosphate	3	4	—	—
231		Phenyl phenol, *o*-	—	2	—	—
1202		Polyvinylpolypyrrolidone, insoluble	—	2	—	—
1201		Polyvinylpyrrolidone	—	2	—	—
261		Potassium acetate	—	2	—	—
212		Potassium benzoate	—	2	—	—
924	a	Potassium bromate	—	2	—	—
508		Potassium chloride	—	2	—	—
501	(ii)	Potassium dihydrogen phosphate	3	4	—	—
917		Potassium iodate	—	2	—	—
252		Potassium nitrate	—	2	—	—
249		Potassium nitrite	—	2	—	—
337		Potassium sodium L(+) tartrate	—	2	—	—
515	(i)	Potassium sulfate	—	2	—	—
—		Propan-1-ol	—	2	—	—
1520		Propylene glycol	—	2	—	—
211		Sodium benzoate	—	2	—	—
466		Sodium carboxy methyl cellulose	—	2	—	—
952		Sodium cyclamate	—	1	—	—
262	(ii)	Sodium diacetate	—	2	—	—
251		Sodium nitrate	—	2	—	—
250		Sodium nitrite	—	2	—	—
232		Sodium *o*-phenyl phenol	—	2	—	—
—		Sodium percarbonate	—	2	—	—
—		Sodium thiocyanate	—	2	—	—
200		Sorbic acid	—	2	—	—

Revision of heavy metals limits for food additives (Contd)

INS		Food additive	Limits, not more than (mg/kg)			
			As	Pb	Cd	Hg
955		Sucralose	—	1	—	—
181		Tannic acid	—	2	—	—
—		Tartaric acid, DL-	—	2	—	—
—		Toluene	—	2	—	—
1518		Triacetin	—	2	—	—
—		Trichlorotrifluoroethane, 1,1,2-	—	2	—	—
927	b	Urea	—	2	—	—

Flavouring agents evaluated by the Procedure for the Safety Evaluation of Flavouring Agents

A. Pyridine, pyrrole and quinoline derivatives

Flavouring agent	No.	Specifications[a]	Conclusion based on current intake
Indole	1301	N	No safety concern
6-Methylquinoline	1302	N	No safety concern
Isoquinoline	1303	N	No safety concern
Skatole	1304	N	No safety concern
1-Ethyl-2-acetylpyrrole	1305	N	No safety concern
1-Methyl-2-acetylpyrrole	1306	N	No safety concern
Methyl 2-pyrrolyl ketone	1307	N	No safety concern
2-Pyridinemethanethiol	1308	N	No safety concern
2-Acetylpyridine	1309	N	No safety concern
N-Furfurylpyrrole	1310	N	No safety concern
2-(2-Methylpropyl)pyridine	1311	N	No safety concern
3-(2-Methylpropyl)pyridine	1312	N	No safety concern
2-Pentylpyridine	1313	N	No safety concern
Pyrrole	1314	N	No safety concern
3-Ethylpyridine	1315	N	No safety concern
3-Acetylpyridine	1316	N	No safety concern
2,6-Dimethylpyridine	1317	N	No safety concern
5-Ethyl-2-methylpyridine	1318	N	No safety concern
2-Propionylpyrrole	1319	N	No safety concern
Methyl nicotinate	1320	N	No safety concern
2-(3-Phenylpropyl)pyridine	1321	N	No safety concern
2-Propylpyridine	1322	N	No safety concern

[a] N, new specifications prepared.

B. Aliphatic and alicyclic hydrocarbons

Flavouring agent	No.	Specifications[a]	Conclusion based on current intake
Camphene	1323	N	No safety concern
β-Caryophyllene	1324	N	No safety concern
d-Limonene	1326	N, T	ADI not specified[b]
Myrcene	1327	N	No safety concern
α-Phellandrene	1328	N	No safety concern
α-Pinene	1329	N	No safety concern
β-Pinene	1330	N	No safety concern
Terpinolene	1331	N	No safety concern
Bisabolene	1336	N	No safety concern
Valencene	1337	N	No safety concern
3,7-Dimethyl-1,3,6-octatriene	1338	N	No safety concern
p-Mentha-1,3-diene	1339	N	No safety concern
p-Mentha-1,4-diene	1340	N	No safety concern
1,3,5-Undecatriene	1341	N	No safety concern
d-3-Carene	1342	N	No safety concern
Farnesene (α and β)	1343	N	No safety concern
1-Methyl-1,3-cyclohexadiene	1344	N	No safety concern
β-Bourbonene	1345	N	No safety concern
Cadinene (mixture of isomers)	1346	N	No safety concern
Guaiene	1347	N	No safety concern

[a] N, new specifications prepared.
[b] An ADI 'not specified' was established for *d*-limonene by the Committee at its forty-first meeting (Annex 1, reference *107*), which was maintained at the present meeting.

C. Aromatic hydrocarbons

Flavouring agent	No.	Specifications[a]	Conclusion based on current intake
p-Cymene	1325	N	No safety concern
Biphenyl	1332	N	No safety concern
p,α-Dimethylstyrene	1333	N	No safety concern
4-Methylbiphenyl	1334	N	No safety concern
1-Methylnaphthalene	1335	N	No safety concern

[a] N, new specifications prepared.

D. Aliphatic, linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters

Flavouring agent	No.	Specifications[a]	Conclusion based on current intake
Butyl 2-decenoate	1348	N	No safety concern
2-Decenal	1349	N	No safety concern
2-Dodecenal	1350	N	No safety concern
Ethyl acrylate	1351	N	No safety concern
Ethyl 2-nonynoate	1352	N	No safety concern
2-Hexenal	1353	N	No safety concern
2-Hexen-1-ol	1354	N	No safety concern
2-(*E*)Hexen-1-yl acetate	1355	N	No safety concern
Methyl 2-nonynoate	1356	N	No safety concern
Methyl 2-octynoate	1357	N	No safety concern
Methyl 2-undecynoate	1358	N	No safety concern
2-Tridecenal	1359	N	No safety concern
trans-2-Heptenal	1360	N	No safety concern
trans-2-Hexenoic acid	1361	N	No safety concern
2-Nonenal	1362	N	No safety concern
2-Octenal	1363	N	No safety concern
2-Pentenal	1364	N	No safety concern
trans-2-Nonen-1-ol	1365	N	No safety concern
2-Undecenal	1366	N	No safety concern
trans-2-Octen-1-yl acetate	1367	N	No safety concern
trans-2-Octen-1-yl butanoate	1368	N	No safety concern
cis-2-Nonen-1-ol	1369	N	No safety concern
(*E*)-2-Octen-1-ol	1370	N	No safety concern
(*E*)-2-Butenoic acid	1371	N	No safety concern
(*E*)-2-Decenoic acid	1372	N	No safety concern
(*E*)-2-Heptenoic acid	1373	N	No safety concern
(*Z*)-2-Hexen-1-ol	1374	N	No safety concern
trans-2-Hexenyl butyrate	1375	N	No safety concern
(*E*)-2-Hexenyl formate	1376	N	No safety concern
trans-2-Hexenyl isovalerate	1377	N	No safety concern
trans-2-Hexenyl propionate	1378	N	No safety concern
trans-2-Hexenyl pentanoate	1379	N	No safety concern
(*E*)-2-Nonenoic acid	1380	N	No safety concern
(*E*)-2-Hexenyl hexanoate	1381	N	No safety concern
(*Z*)-3- & (E)-2-Hexenyl propionate	1382	N	No safety concern
(*E*)-2-Hexenal diethyl acetal	1383	N	No safety concern
2-Undecen-1-ol	1384	N	No safety concern

[a] N, new specifications prepared

E. Monocyclic and bicyclic secondary alcohols, ketones and related esters

Flavouring agent	No.	Specifications[a]	Conclusion based on current intake
Borneol	1385	N	No safety concern
Isoborneol	1386	N	No safety concern
Bornyl acetate	1387	N	No safety concern
Isobornyl acetate	1388	N	No safety concern
Bornyl formate	1389	N	No safety concern
Isobornyl formate	1390	N	No safety concern
Isobornyl propionate	1391	N	No safety concern
Bornyl valerate	1392	N	No safety concern
Bornyl isovalerate (endo-)	1393	N	No safety concern
Isobornyl isovalerate	1394	N	No safety concern
d-Camphor	1395	N	No safety concern
d-Fenchone	1396	N	No safety concern
Fenchyl alcohol	1397	N	No safety concern
Nootkatone	1398	N	No safety concern
1,3,3-Trimethyl-2-norbornanyl acetate	1399	N	No safety concern
Methyl jasmonate	1400	N	No safety concern
Cycloheptadeca-9-en-1-one	1401	N	No safety concern
3-Methyl-1-cyclopentadecanone	1402	N	No safety concern
2(10)-Pinen-3-ol	1403	N	No safety concern
Verbenol	1404	N	No safety concern
7-Methyl-4,4a,5,6-tetrahydro-2(3*H*)-naphthalenone	1405	N	No safety concern
3-Methyl-2-(*n*-pentanyl)-2-cyclopenten-1-one	1406	N	No safety concern
Dihydronootkatone	1407	N	No safety concern
3-L-Menthoxypropane-1,2-diol	1408	N	No safety concern
β-Ionyl acetate	1409	N	No safety concern
α-Isomethylionyl acetate	1410	N	No safety concern
3-(*l*-Menthoxy)-2-methylpropane-1,2-diol	1411	N	No safety concern
Bornyl butyrate	1412	N	No safety concern
D,L-Menthol(+/–)-propylene glycol carbonate	1413	N	No safety concern
L-Monomenthyl glutarate	1414	N	No safety concern
L-Menthyl methyl ether	1415	N	No safety concern
p-Menthane-3,8-diol	1416	N	No safety concern

[a] N, new specifications prepared

F. Amino acids and related substances

Flavouring agent	No.	Specifications[a]	Conclusion based on current intake
β-Alanine	1418	N	No safety concern
L-Cysteine	1419	N	No safety concern[b]
L-Glutamic acid	1420	N	No safety concern[b,c]
Glycine	1421	N	No safety concern[b]
DL-Isoleucine	1422	N	No safety concern
L-Leucine	1423	N	No safety concern[b]
DL-Methionine	1424	N	No safety concern
L-Proline	1425	N	No safety concern[b]
DL-Valine	1426	N	No safety concern
DL-(3-Amino-3-carboxypropyl) dimethylsufonium chloride	1427	N	No safety concern
L-Phenylalanine	1428	N	No safety concern[b]
L-Aspartic acid	1429	N	No safety concern[b]
L-Glutamine	1430	N	No safety concern[b,c]
L-Histidine	1431	N	No safety concern[b]
DL-Phenylalanine	1432	N	No safety concern
L-Tyrosine	1434	N	No safety concern[b]
Taurine	1435	N	No safety concern
DL-Alanine	1437	N	No safety concern
L-Arginine	1438	N	No safety concern[b]
L-Lysine	1439	N	No safety concern[b]

[a] N, new specifications prepared.

[b] Not evaluated using the Procedure for the Safety Evaluation of Flavouring Agents. The substance is a macronutrient and normal component of protein and, as such, human exposure through food is orders of magnitude higher than the anticipated level of exposure from use as flavouring agent.

[c] The group ADI 'not specified' established by the Committee at its thirty-first meeting for L-glutamic acid and its ammonium, calcium, magnesium, monosodium and potassium salts was maintained.

G. Tetrahydrofuran and furanone derivatives

Flavouring agent	No.	Specifications[a]	Conclusion based on current intake
2-Hexyl-4-acetoxytetrahydrofuran	1440	N	No safety concern
2-(3-Phenylpropyl)tetrahydrofuran	1441	N	No safety concern
Tetrahydrofurfuryl acetate	1442	N	No safety concern
Tetrahydrofurfuryl alcohol	1443	N	No safety concern
Tetrahydrofurfuryl butyrate	1444	N	No safety concern
Tetrahydrofurfuryl propionate	1445	N	No safety concern
4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone	1446	N	No safety concern
Tetrahydrofurfuryl cinnamate	1447	N	No safety concern
2-Methyltetrahydrofuran-3-one	1448	N	No safety concern
2-Ethyl-4-hydroxy-5-methyl-3(2*H*)-furanone	1449	N	No safety concern
4-Hydroxy-5-methyl-3(2*H*)-furanone	1450	N	No safety concern
2,5-Dimethyl-4-methoxy-3(2*H*)-furanone	1451	N	No safety concern
2,2-Dimethyl-5-(1-methylpropen-1-yl)tetrahydrofuran	1452	N	No safety concern
2,5-Diethyltetrahydrofuran	1453	N	No safety concern
cis,*trans*-2-Methyl-2-vinyl-5-(2-hydroxy-2-propyl) tetrahydrofuran (linalool oxide)	1454	N	No safety concern
5-Isopropenyl-2-methyl-2-vinyltetrahydrofuran (*cis* and *trans* mixture)	1455	N	No safety concern
4-Acetoxy-2,5-dimethyl-3(2*H*) furanone	1456	N	No safety concern
(+/–)-2-(5-Methyl-5-vinyl-tetrahydrofuran-2-yl) propionaldehyde	1457	N	No safety concern

[a] N, new specifications prepared

H. Phenyl-substituted aliphatic alcohols and related aldehydes and esters

Flavouring agent	No.	Specifications[a]	Conclusion based on current intake
Ethyl 4-phenylbutyrate	1458	N	No safety concern
β-Methylphenethyl alcohol	1459	N	No safety concern
2-Methyl-4-phenyl-2-butyl acetate	1460	N	No safety concern
2-Methyl-4-phenyl-2-butyl isobutyrate	1461	N	No safety concern
2-Methyl-4-phenylbutyraldehyde	1462	N	No safety concern
3-Methyl-2-phenylbutyraldehyde	1463	N	No safety concern
Methyl 4-Phenylbutyrate	1464	N	No safety concern
2-Methyl-3-(*p*-isopropylphenyl) propionaldehyde	1465	N	No safety concern
2-Methyl-3-tolylpropionaldehyde (mixed *o*-,*m*-, *p*-)	1466	N	No safety concern
2-Phenylpropionaldehyde	1467	N	No safety concern
2-Phenylpropionaldehyde dimethyl acetal	1468	N	No safety concern
2-Phenylpropyl butyrate	1469	N	No safety concern
2-Phenylpropyl isobutyrate	1470	N	No safety concern
2-(*p*-Tolyl)propionaldehyde	1471	N	No safety concern
5-Methyl-2-phenyl-2-hexenal	1472	N	No safety concern
4-Methyl-2-phenyl-2-pentenal	1473	N	No safety concern
2-Phenyl-2-butenal	1474	N	No safety concern
Ethyl 2-ethyl-3-phenylpropanoate	1475	N	No safety concern
2-Phenyl-4-pentenal	1476	N	No safety concern
2-Methyl-4-phenyl-2-butanol	1477	N	No safety concern
2-Oxo-3-phenylpropionic acid	1478	N	No safety concern
Sodium 2-oxo-3-phenylpropionate	1479	N,T	

[a] N, new specifications prepared; T, tentative specifications.

Flavouring agents considered for specifications only

No.	Flavouring agent	Specifications[a]
53	Citronellyl formate	R
55	Neryl formate	R
68	Rhodinyl butyrate	R
399	Methyl-β-ionone	R
471	2,8-Dithianon-4-ene-4-carboxaldehyde	R
504	*S*-Methyl benzothioate	R
557	1-Mercapto-2-propanone	R
570	Propenyl propyl disulfide	R
605	1,3-Nonanediol acetate (mixed esters)	R
615	Butyl ethyl malonate	R
628	Ethyl aconitate (mixed esters)	R
631.2	Sodium salt of 3-methyl-2-oxobutanoic acid	S[b]
632.2	Sodium salt of 3-methyl-2-oxopentanoic acid	S[b]
633.2	Sodium salt of 4-methyl-2-oxopentanoic acid	S[b]
919	Glyceryl monooleate	R
1203	Ammonium isovalerate	R
1218	4-Ethyloctanoic acid	R
1263	Isoeugenyl phenylacetate	R
1273	Ethyl 5-hexenoate	R
1291	3-Mercapto-2-methylpentan-1-ol (racemic)	R
1296	spiro[2,4-Dithia-1-methyl-8-oxabicyclo(3.3.0)octane-3,3'-(1′-oxa-2′-methyl)-cyclopentane]	R

[a] R, existing specifications revised; S, existing specifications were maintained; T, the existing, new, or revised specifications are tentative and new information is required.
[b] Specifications will be withdrawn at the next meeting at which flavouring agents are discussed if no information becomes available by that time.

Evaluation of a natural constituent of food

Constituent	Toxicological recommendations
Glycyrrhizinic acid	Available data suggest that an intake of 100 mg/day would be unlikely to cause adverse effects in the majority of adults. In certain highly susceptible individuals, physiological effects could occur at exposure levels somewhat below this figure. The intake data indicate that consumers with a high intake of liquorice confectionery or herbal tea containing liquorice may be exposed to glycyrrhizinic acid at more than 100 mg/day.

ANNEX 5

SUMMARY OF THE SAFETY EVALUATION OF SECONDARY COMPONENTS FOR FLAVOURING AGENTS WITH MINIMUM ASSAY VALUES OF LESS THAN 95%

Summary of the safety evaluation of secondary components for flavouring agents with minimum assay values of less than 95%

No.	Name	Minimum assay value (%)	Secondary components	Comments on secondary components
B. Aliphatic and alicyclic hydrocarbons				
1323	Camphene	80% of $C_{10}H_{16}$	15–19% $C_{15}H_{24}$ terpene hydrocarbons (e.g. valencene)	The $C_{15}H_{24}$ terpene hydrocarbons most likely to be found as secondary components in camphene include valencene (No. 1337), β-caryophyllene (No. 1324), bisabolene (No. 1336), and farnesene (No. 1343). The Committee evaluated all these agents at its present meeting and concluded that they were of no safety concern at estimated current intakes.
1324	β-Caryophyllene	80%	15–19% $C_{15}H_{24}$ terpene hydrocarbons (e.g. valencene)	The $C_{15}H_{24}$ terpene hydrocarbons most likely to be found as secondary components in β-caryophyllene include valencene (No. 1337), bisabolene (No. 1336), and farnesene (No. 1343). The Committe evaluated all these agents at its present meeting and concluded that they were of no safety concern at estimated current intakes.
1327	Myrcene	90% of $C_{10}H_{16}$	5–6% dihydromyrcene	Dihydromyrcene has not been evaluated previously by the Committee. It is expected to share the same metabolic fate as the structurally related substance myrcene (No. 1327), which was evaluated by the Committee at its present meeting. A LOEL/NOEL of 250 mg/kg bw per day was reported in 13-week studies in mice and rats treated by gavage (*1*, *2*). The Committee concluded that this substance was of no safety concern at estimated current intakes.

Summary (contd)

No.	Name	Minimum assay value (%)	Secondary components	Comments on secondary components
1337	Valencene	94%	2–4% other sesquiterpenes	The $C_{15}H_{24}$ terpene hydrocarbons most likely to be found as secondary components in valencene include β-caryophyllene (No. 1324), bisabolene (No. 1336), and farnesene (No. 1343). The Committe evaluated all these agents at its present meeting and concluded that they were of no safety concern at estimated current intakes.
1338	3,7-Dimethyl-1, 3,6-octatriene	80%	15–19% *cis*-β-Ocimene	*cis*-β-Ocimene has not been previously evaluated by the Committee. It is the *cis*-isomer of the primary compound 3,7-dimethyl-1,3,6-octatriene. *cis*-β-Octimene is expected to share the same metabolic fate as the primary compound and the structurally related acyclic hydrocarbons in this group of flavouring agents, which include myrcene (No. 1327). Myrcene was evaluated by the Committee at its present meeting, when a LOEL/NOEL of 250 mg/kg bw per day was identified in a 13-week study in rats and mice treated by gavage (*1*, *2*). The Committee concluded that myrcene was of no safety concern at estimated current intakes.
1339	*p*-Mentha-1, 3-diene	89% of $C_{10}H_{16}$	6–7% 1,4- and 1,8-Cineole	The Committee evaluated 1,4-cineole (No. 1233) at its sixty-first meeting and concluded that it was not a safety concern at estimated current intakes. The Committee also evaluated 1,8-cineole (eucalyptol, No. 1234) at its sixty-first meeting and concluded that it was not a safety concern at estimated current intakes. In an 80-week study in mice, a NOEL of >32 mg/kg bw per day was reported (*3*).
1341	1,3,5-Undecatriene	94% (sum of isomers)	1–3% 2,4,6-Undecatriene (*Z*,*Z*,*E*)	2,4,6-Undecatriene has not been evaluated previously by the Committee. It is expected to share the same metabolic fate as the primary compound 1,3,5-undecatriene and the other acyclic hydrocarbons in this group of flavouring agents, which are oxidized to oxygenated metabolites and excreted in the urine. The Committee concluded that this substance was of no safety concern at estimated current intakes.

1342	δ-3-Carene	92%	2–3% β-Pinene; 1–2% limonene; 1–2% myrcene; 0–1% *p*-cymene	β-Pinene (No. 1330) was evaluated by the Committee at its present meeting, when it was concluded that this substance was of no safety concern at estimated current intakes. *d*-Limonene (No. 1326) was evaluated by the Committee at its present meeting. Based on the ADI 'not specified' that was established for *d*-limonene at the forty-first meeting of the Committee, the Committee concluded that this substance was of no safety concern at estimated current intakes. Myrcene (No. 1327) was evaluated by the Committee at its present meeting. The LOEL/NOEL for myrcene was 250 mg/kg bw per day in 13-week studies in rats and mice treated by gavage (*1*, *2*). The Committee concluded that this substance was of no safety concern at estimated current intakes. *p*-Cymene (No. 1325) was evaluated by the Committee at its present meeting, when it was concluded that this substance was of no safety concern at estimated current intakes.
1343	Farnesene (α and β)	67% (sum of isomers)	21% bisabolene (sum of isomers); 10% other isomers of farnesene and other $C_{15}H_{24}$ terpene hydrocarbons (e.g. valencene, bourbonene, cadinene, guaiene)	Bisabolene (No. 1336) was evaluated by the Committee at its present meeting, when it was concluded that this substance was of no safety concern at estimated current intakes. Other isomersof farnesene are expected to share the same metabolic fate as the primary compounds α and β-farnesene and the structurally related acylic hydrocarbons in this group of flavouring agents, which include myrcene (No. 1327). Myrcene was evaluated by the Committee at its present meeting. The LOEL/NOEL for myrcene was 250 mg/kg bw per day in 13-week studies in rats and mice treated by gavage (*1*, *2*). The Committee concluded that this substance was of no safety concern at estimated current intakes. The $C_{15}H_{24}$ terpene hydrocarbons found as secondary components of farnesene include valencene (No. 1337), bourbonene (No. 1345), cadinene (No. 1346), and guaiene (No. 1347). The Committe evaluated all these agents at its present meeting and concluded that they were of no safety concern at estimated current intakes.

Summary (contd)

No.	Name	Minimum assay value (%)	Secondary components	Comments on secondary components
D. Aliphatic, linear α,β-unsaturated aldehydes, acids and related alcohols, acetals and esters				
1349	2-Decenal	92% (sum of *E* & *Z* isomers)	3–4% 2-decenoic acid	(*E*)-2-Decenoic (No. 1372) acid is a substrate for the fatty acid cycle and is metabolized and excreted primarily as carbon dioxide and water (*4*). The related material, 2,4-decadienal, which is oxidized to 2,4-decadienoic acid, exhibited NOELs of 100 and 200 mg/kg bw per day for male and female mice, respectively, in a 90-day feeding study (*5*). NOELs of 100 and 33.9 mg/kg bw per day were reported for rats in two separate 90-day studies (*5*, *6*). The Committee concluded that this substance was of no safety concern at estimated current intakes.
1350	2-Dodecenal	93% (sum of *E* & *Z* isomers)	3–4% 2-dodecenoic acid	2-Dodecenoic acid is structurally related to the primary material and is expected to be metabolized in the same way. It is a substrate for the fatty acid cycle, and is metabolized and excreted primarily as carbon dioxide and water (*4*), and thus does not present a safety concern at estimated current intakes.
1353	2-Hexenal	92% (sum of *E* & *Z* isomers)	3–4% 2-hexenoic acid	(*E*)-2-Hexenoic acid (No. 1361) is a substrate for the fatty acid cycle and is metabolized and excreted primarily as carbon dioxide and water (*4*). A 98-day study with the structurally related material 2,4-hexadienal, which oxidizes to 2,4-hexadienoic acid, exhibited NOELs of 15 and 60 mg/kg bw for male and female rats, respectively (*7*). The Committee concluded that this substance was of no safety concern at estimated current intakes.
1359	2-Tridecenal	92% (sum of *E* & *Z* isomers)	3–4% 2-tridecenoic acid	2-Tridecenoic acid is structurally related to the primary material and is expected to be metabolized in the same way. It is a substrate for the fatty acid cycle, and is metabolized and excreted primarily as carbon dioxide and water (*4*), and thus does not present a safety concern at estimated current intakes.

1362	2-Nonenal	92% (sum of *E* & *Z* isomers)	3–4% 2-Nonenoic acid	(*E*)-2-Nonenoic acid (No. 1380) is a substrate for the fatty acid cycle, and is metabolized and excreted primarily as carbon dioxide and water (*4*), and thus does not present a safety concern at estimated current intakes.
1363	2-Octenal	92% (sum of *E* & *Z* isomers)	3–4% 2-octenoic acid and ethyl octanoate	2-Octenoic acid is structurally related to other α,β-unsaturated acids and is expected to be metabolized in the same way. It is a substrate for the fatty acid cycle, and is metabolized and excreted primarily as carbon dioxide and water (*4*), and thus does not present a safety concern at estimated current intakes. Ethyl octanoate (No. 33) has been evaluated by the Committee, which concluded that it was of no safety concern at estimated current intakes.
1374	(*Z*)-2-Hexen-1-ol	92%	3-5% (*E*)-2-hexen-1-ol	Both the *E* and *Z* isomers are oxidized in vivo, first to the corresponding aldehyde and then to the acid (*8–10*). They then enter the fatty acid cycle where they are completely metabolized and excreted (*4*). A NOEL of 120 mg/kg bw per day was identified in a 98-day study in rats given drinking-water containing the structurally related material *cis*-3-hexen-1-ol (*11*). The Committee concluded that these substances were of no safety concern at estimated current intakes.
1379	*trans*-2-Hexenyl pentanoate	93%	1–3% propanoic acid; 1–3% 2-hexenol	Propanoic acid (No. 84) has been evaluated by the Committee, which concluded that it was of no safety concern at estimated current intakes. For 2-hexenol see No. 1374 above.
1381	(E)-2-Hexenyl hexanoate	93%	2–3% hexanoic acid; 2–3% 2-hexenol	Hexanoic acid (No. 93) has been evaluated by the Committee, which concluded that it was of no safety concern at estimated current intakes. For 2-hexenol see No. 1374 above.

Summary (contd)

No.	Name	Minimum assay value (%)	Secondary components	Comments on secondary components
E. Monocyclic and bicyclic secondary alcohols and ketones				
1386	Isoborneol	92%	3–5% borneol	Borneol (No. 1385) was evaluated by the Committe at its present meeting. NOELs of 526 and >1300 mg/kg bw per day were reported in 31- and 90-day studies in dogs, respectively (*12*). In addition, NOELs of 15 and 90 mg/kg bw per day were identified for males and females, respectively, for the related material isobornyl acetate (No. 1388) in a 90-day study in rats (*13*). The Committee thus concluded that borneol does not present a safety concern at estimated current intakes.
1398	Nootkatone	93%	2–3% dihydronootkatone	Dihydronootkatone (No. 1407) is metabolized primarily throught the epoxidation and hydration of the isoprenyl side-chain to form the corresponding 13,14-diol (*14*). To a minor extent, reduction of the ketone to the secondary alcohol followed by conjugation with glucuronic acid may occur as with the aliphatic and monocyclic secondary ketones (*15–17*) (Williams, 1959; Lington & Bevan, 1994; Topping et al., 1994). The Committee thus concluded that dihydronootkatone does not present a safety concern at estimated current intakes.
1407	Dihydronootkatone	90%	5–6% nootkatone	Nootkatone (No. 1398) is metabolized primarily throught the epoxidation and hydration of the isoprenyl side-chain to form the corresponding 13,14-diol (*14*) (Asakawa et al., 1986). To a minor extent, reduction of the ketone to the secondary alcohol followed by conjugation with glucuronic acid may occur as with the aliphatic and monocyclic secondary ketones (*15–17*). The Committee thus concluded that nootkatone does not present a safety concern at estimated current intakes.

1409	β-Ionyl acetate	92%	2–3% acetic acid; 1–2% β-ionol	Acetic acid (No. 81) has been evaluated by the Committee, which concluded that it was of no safety concern at estimated current intakes. β-Ionol (No. 392) has been evaluated by the Committee, which concluded that it was of no safety concern at estimated current intakes.
1413	D,L-Menthol (+/–)-propylene glycol carbonate	87%	12% *d,l*-Menthol 2-propylene glycol carbonate	The secondary component is structurally related to the primary material and is expected to be metabolized in the same way. Both are hydrolysed in the liver, producing menthol and propylene glycol. Menthol (No. 427) and propylene glycol (No. 925) have both been previously evaluated by the Committee and considered not to be a safety concern at estimated current intakes.
1414	L-Monomenthyl glutarate	72%	22–24% dimenthyl glutarate; 1–2% glutaric acid	The metabolism of dimenthyl glutarate is expected to follow the same pathway as that for monomenthyl glutarate. The ester functions are hydrolysed in vivo yielding menthol and glutaric acid (*18*, *19*), and thus does not present a safety concern at estimated current intakes. Menthol (No. 427) has been evaluated by the Committee, which concluded that it was of no safety concern at estimated current intakes. Glutaric acid has not been evaluated previously by the Committee, but is endogenous in humans and is structurally related to valeric acid (No. 90), which has been evaluated previously by the Committee, and thus does not present a safety concern at estimated current intakes.
G. Tetrahydrofuran and furanone derivatives				
1456	4-Acetoxy-2,5-dimethyl-3(2*H*)furanone	85%	9–8% 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (No. 1446) is expected to share the same metabolic fate as the primary material, i.e. conjugation with glucuronic acid and excretion in the urine (*20*). A 2-year study with this material reported a NOEL of 200 mg/kg bw per day in both male and female rats (*21*). The Committee concluded that 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone does not present a safety concern at estimated current intakes.

Summary (contd)

No.	Name	Minimum assay value (%)	Secondary components	Comments on secondary components
1457	(+/–)-2-(5-Methyl-5-vinyl-tetrahydrofuran-2-yl) propionaldehyde	90% (sum of 4 isomers)	5–6% 6-hydroxy-2,6 dimethyl 2,7-octadienal	A related substance, hydroxycitronellal (No. 611) was evaluated by the Committee at its 1999 meeting and was concluded to be of no safety concern at estimated current intakes. A NOEL of 250 mg/kg bw per day was reported for hydroxycitronellal in a 2-year study in rats (*22*). The Committee concluded that 6-hydroxy-2,6-dimethyl-2,7-octadienal does not present a safety concern at estimated current intakes.
H. 2-Phenylpropanol derivatives				
1465	2-Methyl-3-(*p*-isopropylphenyl) propionaldehyde	90%	3–5% 2-methyl-3-(p-isopropylphenyl) propionic acid	The initial step in the metabolic pathway for the primary material is oxidation to its corresponding acid. As the secondary material is the corresponding acid of the primary material, it is expected to share the same metabolic fate of conjugation with glucuronic acid and excretion in the urine (*23*). The Committee concluded that 2-methyl-3-(*p*-isopropylphenyl) propionic acid does not present a safety concern at estimated current intakes.

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