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Opinion
of the Scientific Committee on Food
on pulegone and menthofuran

(expressed on 2 July 2002)

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Terms of reference

The Committee is asked to advise the Commission on substances used as flavouring substances or present in flavourings or present in other food ingredients with flavouring properties for which existing toxicological data indicate that restrictions of use or presence might be necessary to ensure safety for human health.

In particular, the Committee is asked to advise the Commission on the implications for human health of pulegone and menthofuran in the diet.

Introduction

Menthofuran is a major metabolite of pulegone and the hepatotoxicity of pulegone is due, at least in part, to this metabolite. The Committee therefore decided that it was appropriate to evaluate pulegone and menthofuran together. It should be noted that this opinion deals with the (R)-(+)-isomers of pulegone and menthofuran since the toxicological database largely relates to these isomers.

Previous evaluations

Council of Europe: Pulegone and (R)-(+)-menthofuran were withdrawn from Part 1, Blue Book, 4th Edition (Council of Europe, 1992).
In 1997 the Committee of Experts on Flavouring Substances (CEFS) of the Council of Europe evaluated pulegone as follows: "Pulegone is mainly a hepatotoxic compound. Metabolic studies have firmly established the role of bioactivation in the hepatotoxicity of pulegone." CEFS set a TDI of 100 µg/kg bw based on the NOEL of 20 mg/kg bw/d in the 28 days oral toxicity study in rats with a safety factor of 200. CEFS requested neurotoxicity and long term studies and proposed the following provisional limits: 20 mg/kg in foods and beverages with the exception of 100 mg/kg in mint/peppermint flavoured alcoholic beverages, 200-400 mg/kg in liqueurs, 100 mg/kg in mint/peppermint flavoured confectionery, 200 mg/kg in mint/peppermint "extra strong mints" and 350 mg/kg in mint/peppermint flavoured chewing gum (Council of Europe, 1997). In 1999, CEFS evaluated menthofuran and concluded that it "is a hepatoxic compound

and is considered as the proximate hepatoxin of pulegone". CEFS set a group TDI of 100 µg/kg bw for menthofuran and pulegone based on the above-mentioned rat study and a safety factor of 200. CEFS proposed the following limits for menthofuran: 20 mg/kg in foods and beverages with the exception of 100 mg/kg in mint/peppermint flavoured alcoholic beverages, 100 mg/kg in mint/peppermint flavoured confectionery and 1000 mg/kg in mint/peppermint flavoured chewing gum (Council of Europe, 1999).

JECFA (Joint FAO/WHO Expert Committee on Food Additives) (2000): On the basis of estimated current daily per capita intake of 0.034 µg/kg bw/d and a NOEL for (R)-(+)-pulegone of 440 µg/kg bw derived from a 90-day study on peppermint oil, a category "No safety concern" was applied to (R)-(+)-pulegone and structurally related flavouring agents isopulegone, isopulegol, isopulegyl acetate, *p*-menth-1,4(8)-dien-3-one and (R)-(+)-menthofuran. This JECFA evaluation was arrived at using the decision tree procedure for chemically defined flavouring substances only and which does not take into account exposure from other sources e.g. herbs and spices. In this respect, the JECFA evaluation differs from that conducted in this opinion where intakes from essential oils are considered.

Current regulatory status

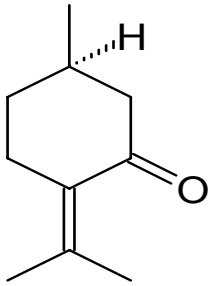
Annex II of Directive 88/388/EEC on flavourings sets the following maximum levels for pulegone in foodstuffs and beverages to which flavourings or other food ingredients with flavouring properties have been added: 25 mg/kg in foodstuffs, 100 mg/kg in beverages, with the exception of 250 mg/kg in peppermint or mint flavoured beverages and 350 mg/kg in mint confectionery. Pulegone may not be added as such to foodstuffs (EEC, 1988).

Menthofuran is listed in the register of chemically defined flavouring substances laid down in Commission Decision 1999/217/EC (EC, 1999), as last amended by Commission 2002/113/EC (EC, 2002).

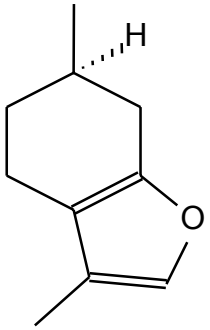
USA: Pulegone and menthofuran have FEMA GRAS status and are listed among the authorised synthetic flavouring substances CFR 21-172.515.

Chemical characterisation

Name:	Pulegone
Synonyms:	d-pulegone; (R)-(+)-pulegone; 1-isopropylidene-4-methyl-2-cyclohexanone; 1-methyl-4-isopropylidene-3-cyclohexanone; 4-(8)-*p*-menthen-3-one; *p*-menth4(8)-en-3-one; *d*-4(8)-*p*-menthen-3-one; 5-methyl-2(1-methylethylidene)-cyclohexanone)
CAS No:	89-82-7
FEMA No:	2963
CoE No:	2050
EINECS:	201-943-2
Structure:	



Name:	(R)-(+)-menthofuran:
Synonyms:	3,9-Epoxy-p-mentha-3,8-diene
Systematic name:	4,5,6,7-Tetrahydro-3,6-dimethylbenzofuran
FL No:	13.035
CAS No:	494-90-6
FEMA No:	3235
CoE No:	2265
EINECS:	207-795-5
Structure:	



Exposure assessment

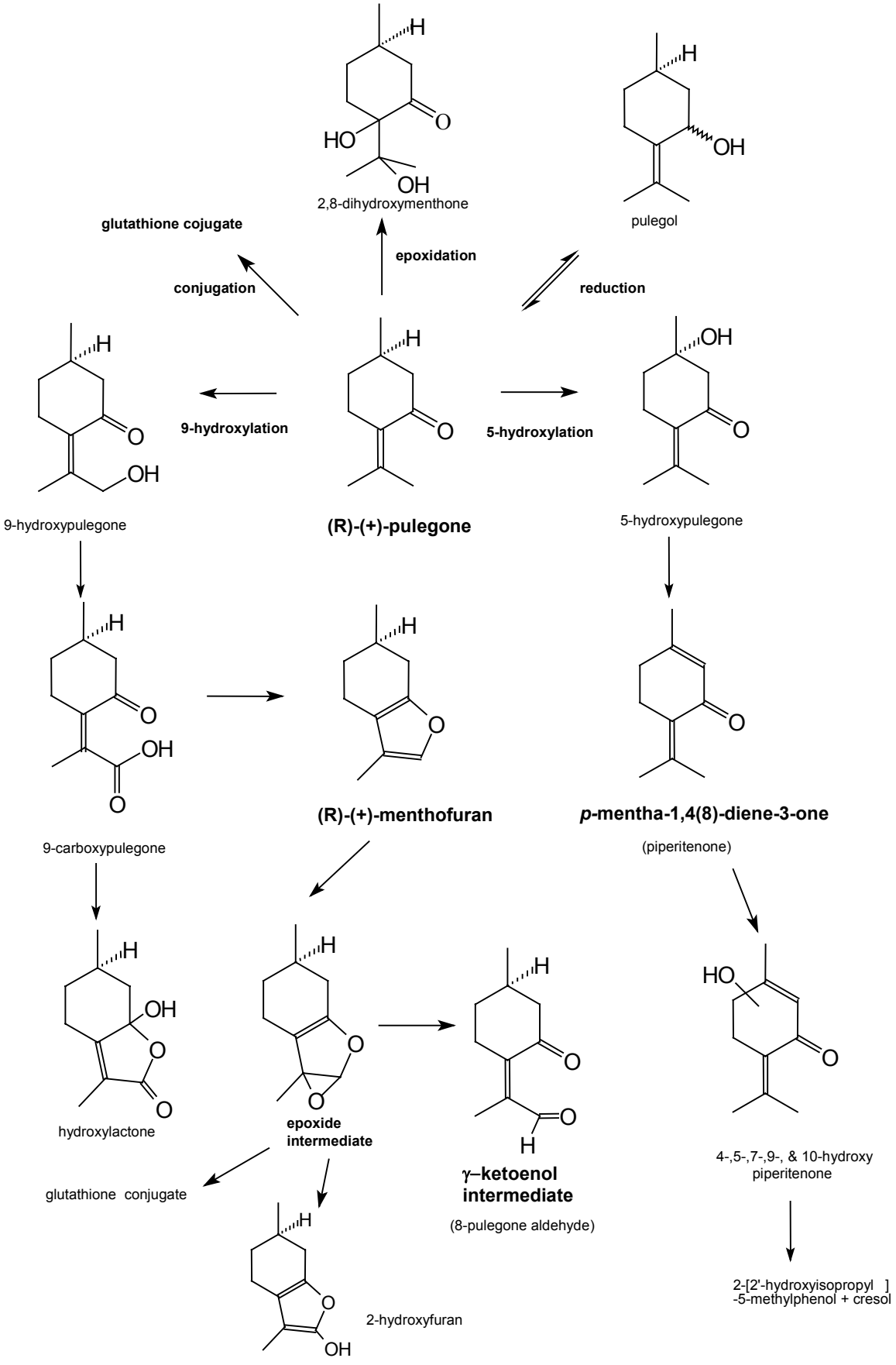
(R)-(+)-pulegone, together with (R)-(+)-menthofuran, is a constituent of peppermint oil and pennyroyal oil and occurs naturally at lower levels in other foods such as oregano, beans and tea (CIVO-TNO, 1996). (S)-(-)-pulegone is contained in Buchu leaf oil, a steam distillate from *Agathosma betulina* which is used as a source for flavourings imparting "cassis" type aromas (Köpke *et al.*, 1994). Estimated mean and 97.5th percentile intakes of (R)-(+)-pulegone in the UK were 0.8 and 3.1 mg/person/day respectively with beverages and chewing gum being the major dietary sources. Estimated intakes of (R)-(+)-menthofuran are similar to those of (R)-(+)-pulegone. Estimates of total intakes for (R)-(+)-pulegone and (R)-(+)-menthofuran in foods and beverages using the National Diet and Nutritional Survey of British Adults and maximum proposed limits for foods and beverages indicates that these are 0.03 and 0.1 mg/kg bw/day for the mean and 97.5th percentile consumers respectively (Council of Europe, 1999)

Estimates of intakes of pulegone and menthofuran have been made in France based on proposed maximum levels of use in various foods, sugar confectionery (including chewing gum), alcoholic and non-alcoholic beverages and on 7-day individual surveys of food consumption. It was assumed that the flavoured foods had a 100% market share. On these bases the mean and 97.5th percentile intakes for pulegone were respectively 43.9 and 72.7 mg/day (0.8 and 1.9 mg/kg bw/day using actual consumer body weights). The corresponding estimated intakes of menthofuran were almost identical. Further estimates based on a 1-year Household Budget Survey indicated that the mean intake of pulegone from sweets, chewing gums and alcoholic beverages was 0.05 mg/day (97.5th percentile 0.5 mg/day) and the corresponding intake of menthofuran was 0.2 mg/day (97.5th percentile 1.7 mg/day).

Information on maximum quantities of menthofuran added as a chemically defined flavouring substance to specific foods and food groups has been provided by the European Flavour & Fragrance Association (EFFA, 2000). If the reported maximum quantities added to alcoholic- and non-alcoholic beverages, candy and chewing gum were used in the intake calculation instead of the maximum use levels proposed by CEFS, the French mean intake estimate, based on the 7-day individual survey, would be 28.7 mg/day (0.6 mg/kg bw/day), whereas the 97.5th percentile intake would be 48.7 mg/day (1.5 mg/kg bw/day). Based on the French Household Budget Survey the corresponding intake estimates would be 0.1 mg/day and 0.6 mg/day, respectively.

It should be noted that the current intake estimates for pulegone and menthofuran are based on accurate food consumption data and on information provided by industry on concentration levels in foods. These levels are not derived from a distribution of analytical results. The resulting intake estimates and particularly the high percentiles of intakes for pulegone and menthofuran should be interpreted as an order of magnitude rather than a precise assessment.

Figure 1: Metabolism of (R)-(+)-pulegone and (R)-(+)-menthofuran



Hazard identification/characterisation

Absorption, metabolism and excretion

The metabolism of (R)-(+)-pulegone has been studied *in vitro* and *in vivo*. The (S)-(-)-stereoisomer has been shown to be metabolised qualitatively in an analogous manner to the (R)-(+)-isomer but there are differences in the relative amounts following the alternative metabolic pathways (Madyastha & Gaikwad, 1998). Further, isopulegone may isomerise to pulegone and also follow similar metabolic pathways which give rise to (R)-(+)-menthofuran and other metabolites.

The metabolism of (R)-(+)-pulegone is shown in Figure 1.

(R)-(+)-pulegone is extensively metabolised by hydroxylation in the 5- and 9-positions to form 5-hydroxypulegone and 9-hydroxypulegone respectively, which then undergo further metabolism. 9-Hydroxylation is the predominant pathway, and the 9-hydroxypulegone undergoes cyclisation to form (R)-(+)-menthofuran. In the second major pathway, the 5-hydroxy metabolite dehydrates to form *p*-mentha-1,4(8)-diene-3-one (piperitenone). In minor pathways, the exocyclic double bond is oxidised to 2,8-dihydroxymenthone, presumably via an epoxide intermediate (Nelson *et al.,*1992b; Madyastha & Raj, 1993; Moorthy *et al.,*1989a). (R)-(+)-pulegone is also reduced to pulegol and may isomerise reversibly to isopulegone, probably through a free radical mechanism (Gordon *et al.,*1987; McClanahan *et al.,*1988).
In addition to the cyclisation reaction to (R)-(+)-menthofuran, 9-hydroxypulegone may undergo detoxication via oxidation to 9-carboxypulegone (i.e. 5-methyl-2-(1-methyl-1-carboxyethylidene)cyclohexanone) which partially cyclises to a hydroxylactone or undergoes further oxidation to polar hydroxyacids which are excreted in urine (Madyastha & Raj, 1993; Moorthy *et al.,*1989a).

Recent studies on the further metabolism of piperitenone in rats have shown this to be extensively hydroxylated in the 4, 5, 7 and 10 positions giving rise to polar urinary metabolites. Hydroxylation also occurs at the 9-position, the position allylic to the ketone. The oxidation product is unstable and not isolated but the cyclisation product, dehydro(R)-(+)-menthofuran has been isolated. This metabolite is thought to contribute to the toxicity, along with (R)-(+)-menthofuran, by formation of a gamma-ketoenal, the ultimate hepatotoxin. The ultimate formation of *p*-cresol is consistent with the formation of a gamma-ketoenal (Madyastha & Gaikwad, 1999). The 4 and 5-hydroxy metabolites are dehydrated to form 2-isopropenyl-5-methylphenol which is then hydroxylated forming the urinary metabolite, 2-[2'-hydroxyisopropyl]-5-methylphenol. All of these metabolites are formed from piperitenone to the extent of 5-12%.
The second major metabolite of (R)-(+)-pulegone is (R)-(+)-menthofuran, which is converted via an epoxide to a reactive gamma-ketoenal, 8-pulegone aldehyde. Ultimately, two stable products, geranic and neric acids are formed by ring cleavage. The epoxide pathway is also consistent with the formation of glutathione conjugates derived from (R)-(+)-menthofuran (Oishi & Nelson, 1993). The possible formation of the ketoenal via a pathway involving 2-

hydroxy-(R)-(+)-menthofuran also is indicated and this compound has been isolated after incubation of (R)-(+)-menthofuran with human cytochrome P450s. Further evidence for this pathway is provided by the isolation both *in vivo* and *in vitro* of (+)-mintlactone and (-)-isomintlactone, which are rearrangement products of 2-hydroxy(R)-(+)-menthofuran (Nelson *et al.,* 1992*a;* Thomassen *et al.,*1992; Khojasteh-Bakht *et al.,*1999).

The reactive intermediate, 8-pulegone aldehyde, has not been detected *in vivo* but has been detected by trapping in semicarbazide after incubating (R)-(+)-pulegone with mouse liver microsomal fractions. In a similar way, it was detected in incubates of (R)-(+)-menthofuran with rat or mouse liver microsomes (McClanahan *et al.,*1989; Thomassen *et al.,*1992; Madyastha & Raj, 1990). The rate of formation of 8-pulegone aldehyde by mouse, rat and human hepatic microsomes is 5-10 times faster from (R)-(+)-menthofuran than from (R)-(+)-pulegone, consistent with the proposed pathway from (R)-(+)-pulegone via (R)-(+)-menthofuran. 8-pulegone aldehyde may be the ultimate toxicant from (R)-(+)-pulegone or (R)-(+)-menthofuran (see below) which suggests that these related compounds should be considered as a group.

Conjugates of (R)-(+)-pulegone, (R)-(+)-menthofuran and other metabolites with glucuronic acid, glutathione or both have been detected in the bile of rats given (R)-(+)-pulegone or (R)-(+)-menthofuran.

Acute toxicity

The oral LD_{50} for (R)-(+)-pulegone in the rat was reported to be 470 mg/kg bw (Moreno, 1975).
Comparison of the i.p. acute toxicity of pulegone in mice indicates that the (S)-(-)-isomer is about one third as toxic as the (R)-(+)-isomer (Gordon *et al.,* 1982).
No standard oral acute toxicity study was found relating to (R)-(+)-menthofuran. On intraperitoneal injection of (R)-(+)-menthofuran in corn oil to mice at doses of 100, 200 or 300 mg/kg bw, the 24-hour mortality was 2/15, 5/15 and 10/16 respectively.

A review of cases of human intoxication with pennyroyal oil (*Mentha pulegium*: pulegone content 62-97%) indicate that ingestion of 10 ml resulted in moderate to severe toxicity and ingestion of greater than 15 ml (ca. 250 mg/kg bw for a 60 kg woman) resulted in death. The clinical pathology is characterised by massive centrilobular necrosis, pulmonary oedema and internal haemorrhage (Anderson *et al.* 1996). These effects are similar to those produced by i.p. administration of both pennyroyal oil and pulegone in mice (Gordon *et al.,*1982).

Investigation of the i.p. acute toxicity of the components of pennyroyal oil (some of which are also mammalian metabolites of pulegone) indicated that isopulegone and *p*-mentha-1,4(8)-diene-3-one (piperitenone) were significantly less toxic than (R)-(+)-pulegone while (R)-(+)-menthofuran was significantly more toxic (Gordon *et al.,*1982). The principal toxic effects observed with pulegone, isopulegone, piperitenone and (R)-(+)-menthofuran were hepatic centrilobular necrosis and, to a lesser degree, bronchiolar necrosis. (R)-(+)-pulegone caused a

depletion of glutathione but (R)-(+)-menthofuran did not. Pretreatment with diethyl maleate to decrease glutathione levels increased the toxicity of (R)-(+)-pulegone, but not of (R)-(+)-menthofuran.

Administration of (R)-(+)-pulegone by gavage to rats at daily doses of 400 mg/kg bw for up to 5 days caused a decrease in liver haem and microsomal cytochrome P450, a decrease in aminopyrine N-demethylase (APDM), glucose-6-phosphatase (G-6-Pase) and an increase in serum alanine aminotransferase (ALAT). At a dose of 100 mg/kg bw the only effect seen was an increase in ALAT. Pretreatment with phenobarbital or diethyl maleate increased the toxicity while pretreatment with 3-methylcholanthrene (3-MC) or piperonyl butoxide resulted in complete protection (Moorthy *et al.*1989b). Similar doses of (R)-(+)-menthofuran by gavage produced a dose-related increase in serum ALAT and a decrease in G-6-Pase and APDM. The effects at 100 mg/kg bw were marginal and pretreatment with phenobarbital increased toxicity while 3-MC had no effect (Madyastha & Raj, 1994).

Sub-acute/subchronic studies

(R)-(+)-pulegone was administered to male and female Wistar rats (10/sex/dose) by gavage in soyabean oil at daily doses of 0, 20, 80 or 160 mg/kg bw for 28 days. The animals showed a dose-dependent atonia after a few days. Water consumption was reduced at the top dose level and body weight gain was significantly reduced at the 80mg/kg and 160 mg/kg dose levels by 10 and 20% respectively. A dose-dependent decrease in plasma creatinine (significant only at the top dose level) was observed after three weeks. At necropsy, rats in the highest dose group showed distended stomachs. At termination, body weights and organ weights were reduced for all dosed animals. Histopathological examination showed vacuolisation of hepatocytes, mainly around the central vein at the 80 and 160 mg/kg dose levels, which were considered by the authors to be an adaptive change. Dose-related changes in the brain, described as "cyst-like spaces" in the white matter, were reported at the two highest dose levels but there was no cellular reaction in the surrounding tissue and there was no demyelinisation. It was stated that the changes resembled hexachloraphene-induced neuropathy (Olsen & Thorup, 1984). The NOEL for this study was considered to be 20 mg/kg bw/day.
Results were reported from the same laboratory in which peppermint oil (1-3% (R)-(+)-pulegone) was given by gavage at dose levels of 0, 10, 40 or 100 mg/kg bw/day to groups of 10 male and 10 female Wistar rats for 28 days. The only significant histopathological change recorded was the appearance of "cyst-like spaces" in the cerebellum at the two highest doses but there were no associated clinical symptoms indicative of encephalopathy.

The study on pulegone was repeated to confirm the earlier reported presence of cyst-like spaces in the cerebellum. Pulegone was given orally by gavage to groups of 28 female Wistar rats at dose levels of 0 or 160 mg/kg bw daily for 28 days. The pulegone-treated animals showed a decreased food consumption and body weight. Clinical biochemical examinations revealed increased plasma glucose, alkaline phosphatase and ALAT and a decreased plasma creatinine in the dosed group. However, there were no significant histopathological changes

in the liver nor the brain, with or without perfusion fixation. The "cyst-like spaces" reported in the cerebellum in the earlier study were thus not confirmed and may have arisen from inadequate tissue fixation procedures (Mølck *et al.,*1998).

Dietary administration of pulegone to male Wistar rats (3-4 animals per dose group) at levels of 0, 0.5 or 1% for 14 days caused a decrease in food intake and body weight gain at the high dose and the triglyceride levels were significantly increased (Imaizumi *et al.,*1985). The dietary level of 0.5% was calculated to correspond to an intake of 250 mg/kg bw/day.

Sub-chronic studies were conducted on peppermint oil (1-2% pulegone) administered by gavage for 5 weeks at 25 or 125 mg/kg bw/day to beagle dogs (3/sex/dose) or at 20, 150 or 500 mg/kg bw/day to male Wistar rats (12/dose group) (Mengs & Stotzem, 1989). In rats, no effects were observed on general health, behaviour, body weight nor on haematological or urinary parameters. Slight, non-significant increases in alkaline phosphatase and urea levels were the only recorded changes in the high dose group of dogs.

In a 90-day study, peppermint oil (1.1% pulegone) was administered by gavage in soybean oil to groups of 14 male and female Wistar rats at doses of 0, 10, 40 and 100 mg/kg bw/day. No differences were recorded in food and water consumption and body weight gain and haematological and clinical biochemical examinations at day 30 or day 86 gave normal values. No effects were seen in either the low or intermediate dose groups but at the high dose nephropathy (hyaline droplets) was reported in males. The authors interpreted these results as an early manifestation of sex- and species-specific nephropathy due to $\alpha 2_u$-globulin (Spindler & Madsen, 1992). "Cyst-like spaces" in the cerebellum were also reported in the high dose animals but there were no other signs of encephalopathy. Based on this study, a NOEL of 40 mg/kg bw/day for peppermint oil was established.

A toxicity screening test in rats was conducted on (R)-(+)-menthofuran at dietary levels corresponding to an intake of 23 mg/kg bw for 14 days. No effects were seen on body weight gain, food consumption, liver or kidney weights, nor on gross and histopathology of the liver and kidney (Van Miller and Weaver 1987).

Genotoxicity

Pulegone was negative in the Ames assay using *Salmonella typhimurium* strains TA1537, TA1535, TA100, TA 98 and TA97 with and without metabolic activation at concentrations of up to 800 µg/plate (Andersen & Jensen, 1984).
Neither (R)-(+)-pulegone nor (R)-(+)-menthofuran were mutagenic in the Ames assay using *S. typhimurium* TA100 and TA98 at concentrations of up to 1000 µg/plate, with and without metabolic activation (Council of Europe, 1999).
In a study of insecticidal and genotoxic activity, concentrations of pulegone in excess of the LD_{50} (0.17 µl) for Drosophila larvae induced a slight increase in "wing mutations" (mosaic spots) (Franzios *et al.,*1997).

Long-term studies for chronic toxicity/carcinogenicity

No data available.

Reproduction and developmental studies

No experimental data available. Anecdotal cases of the use of oil of pennyroyal as an abortifacient are inadequate to evaluate this activity.

Special studies on immunotoxicity

In a screening study for immunotoxicity of (R)-(+)-pulegone in mice, no effects were seen on lymphoid organ weight and cellularity, nor in functional tests of humoral and cell-mediated immunity (Vollmuth *et al.,*1989).

Mechanism of toxicity

(R)-(+)-Pulegone and its metabolite, (R)-(+)-menthofuran, are hepatotoxic and produce similar effects following i.p. injection in mice (Gordon *et al.,*1982). These effects are similar to those reported following human intoxication with pennyroyal oil (Anderson *et al.,*1996).

In rats pulegone (300 mg i.p.) caused dilation of the central veins and distension of sinusoidal spaces within 6 hours and centrilobular necrosis was observable starting at 12 hours. Electron microscopy after 24 hours showed degeneration of endoplasmic reticulum, swelling of mitochondria and nuclear changes (Moorthy *et al.,*1991b). It has been suggested that metabolites of (R)-(+)-pulegone deactivate cytochrome P450s by modifying the prosthetic haem group or the apoprotein (Moorthy *et al.,*1991a; Madyastha *et al.,*1985). In human liver microsomes *in vitro*, (R)-(+)-menthofuran specifically inhibits CYP2A6 and adducts with this enzyme have been isolated. CYP1A2, CYP2D6, CYP2E1 or CYP3A4 were not similarly inactivated (Khojasteh-Bakht *et al.,*1998).

(R)-(+)-pulegone is primarily metabolised to (R)-(+)-menthofuran and *p*-mentha-1,4(8)-dien-3-one. Comparison of the pharmacokinetics of (R)-(+)-pulegone and (R)-(+)-menthofuran after i.p. administration to mice indicated that the hepatotoxicity of (R)-(+)-pulegone could to a large extent be accounted for by the formation of (R)-(+)-menthofuran (Thomassen *et al.,*1988) and (R)-(+)-menthofuran is known to be converted to the reactive (γ-ketoenal, 8-(R)-(+)-pulegone aldehyde. However, the kinetics of (R)-(+)-menthofuran produced endogenously from (R)-(+)-pulegone after i.p. administration differed from that observed after direct administration of (R)-(+)-menthofuran leading the authors to conclude that other processes than activation of (R)-(+)-menthofuran may also be involved in the toxicity of (R)-(+)-pulegone. The metabolite *p*-mentha-1,4(8)-dien-3-one is a doubly α,β-unsaturated ketone

and would also be expected to be biologically active. This metabolite also produces toxicity similar to (R)-(+)-pulegone but with lower potency (Gordon *et al.,*1982).

Incubation of ^{14}C-(R)-(+)-pulegone with rat liver microsomes led to binding to macromolecules (Madyastha & Moorthy, 1989) and binding to mouse liver, lung and kidney proteins has been demonstrated (liver>lung/kidney) (McClanahan *et al.,*1989). The degree of binding to liver protein paralleled the hepatotoxicity *in vivo* (McClanahan *et al.,* 1989). Treatment with semicarbazide decreased the binding, suggesting that 8-pulegone aldehyde is the ultimate toxicant. Covalent binding to mouse liver, lung and kidney protein was also observed with ^{14}C-(R)-(+)-menthofuran (Thomassen *et al.,*1992). The extent of binding to rat, mouse or human microsomes was similar.

Although there is strong evidence that 8-pulegone aldehyde is one ultimate toxicant, there is further evidence to indicate that this metabolite of (R)-(+)-menthofuran does not fully account for the toxicity of (R)-(+)-pulegone. It has been suggested that the formation of p-cresol both from (R)-(+)-menthofuran and from *p*-mentha-1,4(8)-dien-3-one also contribute to the toxicity (Madyastha & Raj, 1991; Thompson *et al.,*1994; Madyastha & Gaekwad, 1999) but this is not consistent with the small amount of p-cresol formed and the fact that it does not demonstrate the same type of toxicity.

The role of cytochrome P450s in metabolic activation of (R)-(+)-pulegone has been demonstrated by the observations that a variety of P450-inhibitors decreased the toxicity while pretreatment with phenobarbital enhanced the toxicity of (R)-(+)-pulegone and (R)-(+)-menthofuran (Mizutani *et al.,*1987; Gordon *et al.,*1987; Moorthy *et al.,*1989b; Madyastha & Raj, 1994). Thus, oxidation appears to enhance the toxicity of (R)-(+)-pulegone and (R)-(+)-menthofuran, which is consistent with the fact that (R)-(+)-pulegone is converted to (R)-(+)-menthofuran via 9-hydroxypulegenone, and the reactive 8-pulegone aldehyde is an ultimate toxicant. Evidence that pulegone is oxidised to 9-hydroxypulegone via a free radical mechanism is provided from the observation that treatment with the free radical scavenger C-phyocyanin decreased the hepatotoxicity of pulegone in rats (Vadiraja *et al.,*1998).

Glutathione plays a role in the detoxication of pulegone. At hepatotoxic doses, pulegone depletes glutathione in the liver and the toxicity of pulegone on i.p. injection in mice is enhanced by treatment with diethyl maleate to decrease glutathione levels. No such increase in toxicity was seen with (R)-(+)-menthofuran (Gordon *et al.,*1982; Thomassen *et al.,*1990). It was suggested that pulegone is analogous to acetaminophen (Nelson, 1995) in that saturation of the glutathione pathway leads to a higher proportion of the dose being metabolised to reactive metabolites (such as 8-pulegone aldehyde). However, in other studies, glutathione has been shown to react with menthofuran epoxide, the precursor to 8-pulegone aldehyde (Oishi & Nelson, 1993). Glutathione conjugation may play a major role in the detoxication of the reactive metabolite produced by cytochrome P450 ((R)-(+)-menthofuran or the gamma-ketoenal) as indicated by the isolation of glutathione conjugates, including a mixed glutathionyl glucuronide, from the bile of rats treated i.p. with pulegone. The evidence

suggests that the metabolic activation of pulegone occurring in animals also occurs in humans, resulting in the formation of (R)-(+)-menthofuran. At high concentrations, (R)-(+)-menthofuran is a proximate hepatoxic product but if the concentration of metabolites of pulegone is not sufficient to deplete hepatocellular concentrations of glutathione, hepatotoxicity may not be observed (Armstrong, 1987).

Conclusion

The metabolism of (R)-(+)-pulegone occurs mainly, though not exclusively, through pathways involving (R)-(+)-menthofuran and these two flavour substances show similar toxicity qualitatively. This suggests that the evaluation of (R)-(+)-pulegone and (R)-(+)-menthofuran cannot be considered independently and that a group evaluation of (R)-(+)-pulegone and (R)-(+)-menthofuran is appropriate. Since pulegyl alcohol and its esters also are converted to pulegone by hydrolysis and oxidation, these flavours should also be considered in the group evaluation. The NOEL for (R)-(+)-pulegone in a short-term rat study was established to be 20 mg/kg bw but this study was of limited duration (28 days). A longer study of 90 days duration in rats was conducted on peppermint oil containing 1.1% pulegone in which a NOEL of 40 mg/kg bw was demonstrated. The Committee considered that studies with (R)-(+)-pulegone were more relevant for risk assessment than studies on complex mixtures in which the significance of other components is unknown. However, the Committee noted that this study was of short duration.

The Committee noted that only a limited database was available on (R)-(+)-pulegone and (R)-(+)-menthofuran and considered that these data were inadequate for the derivation of an ADI. The Committee requires at least further studies to establish a NOEL for (R)-(+)-pulegone and (R)-(+)-menthofuran in 90 day studies together with further studies on genotoxicity at the gene and chromosomal level in line with the general Guideline for Food Additives (SCF, 2001). Dependent on refined intake estimates it might also require studies of reproductive and developmental toxicity. In order to clarify to what extent there is a common mechanism of toxicity and to determine whether (R)-(+)-menthofuran might be included in an overall evaluation on the basis of "(R)-(+)-pulegone equivalents" comparative toxicological data on (R)-(+)-menthofuran and (R)-(+)-pulegone after oral administration are also needed.

The Committee noted the small margins between "worst case" estimated intakes of (R)-(+)-pulegone and (R)-(+)-menthofuran and the dose levels eliciting toxicity in the 28-day study on (R)-(+)-pulegone and in the 90-day study on oil of peppermint. However, the intake estimates are not precise and represent only an order of magnitude. The Committee therefore recommends that, in addition to the toxicological data, industry should provide better usage levels and analytical data on concentrations in relevant products in order to refine the intake estimates to be used in risk assessment.

References

Andersen, P.H. & Jensen, N.J., 1984. Mutagenic investigation of peppermint oil in the Salmonella/mammalian microsome test. *Mutat. Res.*, **138,** 17-20.

Anderson, I.B., Mullen, W.H., Meeker, J.E., Khojasteh-Bakht, S.C., Oishi, S., Nelson, S.D. & Blanc, P.D., 1996. Pennyroyal toxicity: measurement of toxic metabolite levels in two cases and a review of the literature. *Ann. Intern. Med.*, **124,** 726-734.

Armstrong, R.N., 1987. Enzyme catalyzed detoxication reactions: Mechanisms and stereochemistry. *CRC Crit. Rev. Biochem.,* **22,** 39-88

Council of Europe, 1992. Flavouring Substances and Natural Source of Flavourings 4th Edition.

Council of Europe, 1997. Committee of Experts on Flavouring Substances 41st meeting – RD 4.2/8-41. Datasheet on pulegone.

Council of Europe, 1999. Committee of Experts on Flavouring Substances 41st meeting – RD 4.12/1-45. Datasheet on (R)-(+)-menthofuran.

CIVO-TNO, 1996. *Volatile Components in Food – Qualitative and Quantitative Data*. Centraal Instituut Voor Voedingsonderzoek TNO, Zeist, The Netheerlands

EC (European Commission), 1999. Commission Decision 1999/217/EC adopting a register of flavouring substances used in or on foodstuffs drawn up in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council of 28 October 1996. *Official Journal of the European Communities,* 27.3.1999, L84/1-137.

EC (European Commission), 2002. Commission Decision 2002/113/EC amending Decision 1999/217/EC as regards the register of flavouring substances used in or on foodstuffs, *Official Journal of the European Communities,* 20.02.2002, L 49/1.

EEC, 1988. Council Directive 88/388/EEC of 21 June 1988 on the approximation of the laws of the Member States relating to flavourings for use in foodstuffs and to source materials for their production, *Official Journal of the European Communities*, 15.7.1988, L184/61-67.

EFFA (European Flavour & Fragrance Association), 2000. Datasheet on menthofuran submitted to the experts of the Co-ordinating institute of SCOOP Task 1.1. European Flavour & Fragrance Association, Brussels.

Franzios, G., Mirotsou, M., Hatziapostolou, E., Kral, J., Scouras, Z.G. & Mavragani-Tsipidou, P., 1997. Insecticidal and genotoxic activities of mint essential oils. *J. Agr. Food Chem.,* **45**, 2690-2694.

Gordon, W.P., Forte, A.J., McMurty, R.J., Gal, H. & Nelson, S.D., 1982. Hepatotoxicity and pulmonary toxicity of pennyroyal oil and its constituent terpenes in the mouse. *Toxicol. Appl. Pharmacol.* **65**, 413-424

Gordon, W.P., Huitric, A.C., Seth, C.L. McClanahan, R.H. & Nelson, S.D., 1987. The metabolism of the abortifacient terpene, (R)-(+)-pulegone, to a proximate toxin, (R)-(+)-menthofuran. *Drug Metab. Dispos.,* **15**, 589-594.

Imaizumi, K., Hanada, K., Mawartari, K. & Sugano, M., 1985. Effect of essential oils on the concentration of serum lipids and apolipoproteins in rats. *J. Agr. Biol. Chem.* **49**, 2795-2796.

Khojasteh-Bakht, S.G., Koenigs, L.L., Peter, R.M., Trager, W.F. & Nelson, S.D., 1998. (R)-(+)-menthofuran is a potent, mechanism-based inactivator of human liver cytochrome P450 2A6. *Drug Metab. Dispos.,* **26**, 701-704.

Khojasteh-Bakht, S.G., Chen, W., Koenigs, L.L., Peter, R.M. & Nelson, S.D., 1999. Metabolism of (R)-(+)-Pulegone and (R)-(+)-(R)-(+)-menthofuran by human liver cytochrome P450s: Evidence for the formation of a furan epoxide. *Drug Metab. Dispos.,* **27**, 574-580.

Köpke, T., Dietrich, A. & Bosandl A. 1994. Chiral compounds of essential oils XIV: Simultaneous stereoanalysis of Buchu leaf oils compounds. *Phytochem. Anal.* **5**, 61-67

Madyastha, P., Moorthy, B., Vaidyanathan & Madyastha, K.M., 1985. *In vivo* and *in vitro* destruction of rat liver cytochrome P450 by a monoterpene ketone, pulegone. *Biochem. Biophys. Res. Comm.,* **128**, 921-927.

Madyastha, P. & Moorthy, B., 1989. Pulegone-mediated hepatotoxicity: Evidence for covalent binding of (R)-(+)-^{14}C-pulegone to microsomal proteins *in vitro*. *Chem.-Biol. Interact.*, **72**, 325-333.

Madyastha, P. & Raj, C.P., 1990. Biotransformation of R-(+)-pulegone and (R)-(+)-menthofuran *in vitro:* Chemical basis for toxicity. *Biochem. Biophys. Res. Comm.,* **173**, 1086-1092.

Madyastha, P. & Raj, C.P., 1991. Evidence for the formation of a known toxin, *p*-cresol, from (R)-(+)-menthofuran. *Biochem. Biophys .Res. Comm.,* **177**, 440-446.

Madyastha, P. & Raj, C.P., 1992. Metabolic fate of (R)-(+)-menthofuran in rats: novel oxidative pathways. *Drug Metab. Dispos.,* **20,** 295-301.

Madyastha, P. & Raj, C.P., 1993. Studies on the metabolism of a monoterpene ketone, R-(+)-pulegone – a hepatotoxin in rat: Isolation and characterization of new metabolites. *Xenobiotica,* **23**, 509-518.

Madyastha, P. & Raj, C.P., 1994. Effects of (R)-(+)-menthofuran, a monoterpene furan, on rat liver microsomal enzymes *in vivo. Toxicol.,* **89**, 119-125.

Madyastha, P. & Gaikwad, N.W., 1998. Metabolic fate of S-(-)-pulegone in the rat. *Xenobiotica,* **28**, 723-734.

Madyastha, P. & Gaikwad, N.W., 1999. Metabolic disposition of a monterpene ketone, piperitone, in rats: Evidence for the formation of a known toxin, *p*-cresol. *Drug Metab. Dispos.,* **27,** 74-80.

McClanahan, R.H., Huitric, A.C., Pearson, P.G., Desper, J.C. & Nelson, S.D., 1988. Evidence for a cytochrome P-450 catalysed allylic rearrangement with double-bond topomerization. *J. Am. Chem. Soc.,* **110,** 1979-1981.

McClanahan, R.H., Thomassen, D., Slattery, J.T. & Nelson, S.D., 1989. Metabolic activation of (R)-(+)-pulegone to a reactive enonal that covalently binds to mouse liver proteins. *Chem. Res. Toxicol.,* **2**, 349-355.

Mengs, U. & Stotzem, C.D., 1989. Toxicological evaluation of peppermint oil in rodents and dogs. *Med. Sci. Res.* **17**, 499-500.

Mizutani, T., Nomura, H., Nakanishi, K. & Fujita, S., 1987. Effects of drug metabolism modifiers on pulegone-induced hepatotoxicity in mice. *Res.Comm. in Chem.Path. Pharmacol.* **58,** 75-83.

Mølck, A-M., Poulsen, M., Tingard-Lauridsen, S. & Olsen, P., 1998. Lack of cerebellar changes in Wistar rats given pulegone for 28 days. Comparison of immersion and perfusion fixation. *Toxicol. Lett.,* **95**, 117-122.

Moorthy, B., Madyastha, P. & Madyastha, K.M., 1989a. Metabolism of a monoterpene ketone, R-(+)-pulegone, a hepatotoxin in rat. *Xenobiotica,* **19,** 217-224.

Moorthy, B., Madyastha, P. & Madyastha, K.M., 1989b. Hepatotoxicity of pulegone in rats: its effects on microsomal enzymes *in vivo. Toxicol.,* **55**, 327-337

Moorthy, B., Madyastha, P. & Madyastha, K.M., 1991a. Destruction of rat liver microsomal cytochrome P450 *in vitro* by a monoterpene ketone, pulegone – a hepatotoxin. *Indian J. Chem.,* **30,** 138-146

Moorthy, B., Vijayasarathi, S.K., Basu, A. & Madyastha, K.M., 1991b. Biochemical, histopathological and ultrastructural changes in rat liver induced by (R)-(+)-pulegone, a monoterpene ketone. *Toxicol. Environ. Chem.,* **33,** 121-131.

Moreno, O.M., 1975. Acute oral toxicity in rats and dermal toxicity in rabbits. Unpublished report to RIFM. MB research Laboratories Inc. Spinnerstown, PA.

Nelson, S.D. 1995. Mechanisms of the formation and disposition of reactive metabolites that can cause acute liver injury. *Drug Metab. Rev.,* **27**, 147-177

Nelson, S.D., McClanahan, R.H., Thomassen, D. Gordon, W.P. & Knebel, N., 1992a. Investigations of mechanisms of reactive metabolite formation from (R)-(+)-pulegone. *Xenobiotica,* **22,** 1157-1164.

Nelson, S.D., McClanahan, R.H., Knebel, N., Thomassen, D. Gordon, W.P. & Oishi, S., 1992b. The metabolism of (R)-(+)-pulegone, a toxic monoterpene. *Environ. Sci. Res.,* **44**, 287-296.

Oishi, S. & Nelson, S.D., 1993. Metabolic disposition of (R)-(+)-menthofuran in rat: identification of urinary metabolites. ISSX 5th International Meeting, **4**, 167.

Olsen, P & Thorup, I., 1984. Neurotoxicity in rats dosed with peppermint oil and pulegone. *Arch. Toxicol. suppl.,* **7**, 408-409.

SCF (Scientific Committee on Food) (2001). Guidance on submissions for food additive evaluations by the Scientific Committee on Food. Opinion expressed on 11 July 2001. http://europa.eu.int/comm/food/fs/sc/scf/out98_en.pdf

Spindler, P. & Madsen, C., 1992. Subchronic toxicity study of peppermint oil in rats. *Toxicol. Lett.,* **62,** 215-220.

Thomassen, D., Slattery,J.T. & Nelson, S.D., 1988. Contribution of (R)-(+)-menthofuran to the hepatotoxicity of pulegone: assessment based on matched area under the curve and on matched time course. *J. Pharm. Exp. Ther.,* **224,** 825.

Thomassen, D., Slattery,J.T. & Nelson, S.D., 1990. (R)-(+)-menthofuran-dependent and independent aspects of pulegone hepatotoxicity: roles of glutathione. *J. Pharm. Exp. Ther.,* **253,** 567.

Thomassen, D., Knebel, N., Slattery,J.T. McClanahan, R.H.& Nelson, S.D., 1992. Reactive intermediates in the oxidation of (R)-(+)-menthofuran by cytochrome P-450. *Chem. Res. Toxicol.,* **5**, 123-130.

Thompson, D.C., Perera, K., Fisher, R. & Brendel, K., 1994. Cresol isomers: comparison of toxic potency in rat liver slices. *Toxicol. Appl. Pharmacol.* **125,** 51-58.

Vadiraja, B.B., Gaikwad, N.W. & Madyastha, K.M., 1998. Hepatoprotective effect of C-phycocyanin: protection for carbon tetrachloride and (R)-(+)-pulegone-mediated hepatotoxicity in rats**.** *Biochem. Biophys. Res. Comm.,* **249,** 428-431.

Van Miller, J.P. & Weaver, E.V., 1987. Fourteen-day dietary minimum toxicity screen (MTS) in albino rats. Unpublished report to FEMA. Bushy Run Research Center, Export, PA.

Vollmuth, T.A., Keck, J.D., Ratajczak, H.V. & Thomas, P.T., 1989. Immunotoxicity assessment of flavoring ingredients using a rapid and economical screen. *The Toxicologist,* **9**, 206.

WHO, 2001. Evaluation of certain food additives and contaminants. Fifty-fifth report of the Joint FAO/WHO Expert Committee on Food Additives WHO Technical Report Series No 901, pp. 56-61, World Health Organisation, Geneva.