Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Foods on a request from the Commission on Pulegone and Menthofuran in flavourings and other food ingredients with flavouring properties

Question number EFSA-Q-2003-119

Adopted on 7 December 2005

SUMMARY

The Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food is asked to evaluate substances used as flavouring substances or present in flavourings or present in other food ingredients with flavouring properties. In particular, the Panel is asked to advise the Commission on the implications for human health of pulegone and menthofuran in the diet.

The Panel noted that the Scientific Committee on Food (SCF) had expressed an opinion on pulegone and menthofuran in July, 2002 and took cognizance of the documentation used in arriving at that opinion. The Panel also considered additional data on the metabolism and toxicological properties of pulegone and on \textit{in vivo} extractability of menthofuran present in chewing gum not available to the SCF.

The Panel noted that the metabolism of (R)-(+)-pulegone in mice and rats occurs mainly, though not exclusively, through pathways involving (R)-(+) -menthofuran and these two substances show a qualitatively similar hepatotoxicity. The Panel therefore agreed with the SCF opinion that the evaluation of (R)-(+)-pulegone and (R)-(+) -menthofuran should be considered together.

The new 95-day toxicological data on the (R)-(+) -pulegone enabled the establishment of No Observed Adverse Effect Levels (NOAELs) in rats and mice, meeting one of the requirements of the SCF opinion. However, the data as a whole are not yet sufficient for the Panel to establish a TDI for pulegone.

The Panel noted that refined estimates of pulegone intake from sweets were not available. It also noted the new estimates of intakes from chewing gum are considerably higher than some of the total estimates of mean intake from sweets, chewing gums & alcoholic beverages combined in the previous SCF opinion.

The Panel observed that the current maximum permitted level of pulegone in food may lead to high intakes; particularly in subjects who consume regularly mint flavoured beverages or confectionery. For example it was noted that 500 ml/day of mint flavoured beverage and 100 g/day of mint confectionery could lead to intakes of respectively 4.2 mg/kg bw and 1.2 mg/kg bw for a 30 kg child.
The Panel found the database on (R)-(+)‐menthofuran inadequate to establish an ADI for this flavouring substance. Because (R)-(+)‐menthofuran may be formed during metabolism of (R)-(+)‐pulegone and exhibits qualitatively similar toxicity. The Panel wishes the following studies to be provided within 2 years of the publication of this opinion.

1. Studies to establish a NOEL for (R)-(+)‐menthofuran in 90‐day oral toxicity study in rats;
2. Further genotoxicity studies on (R)-(+)‐menthofuran and (R)-(+)‐pulegone augmenting the database to comply with the SCF General Guideline for Food Additives (studies on mammalian cell gene mutation and chromosome aberration);
3. Further refinement of intake estimates from all dietary sources including actual usage levels and analytical data on concentrations in relevant products;

The Panel considered that dose‐response studies on (R)-(+)‐menthofuran would be helpful in elucidating the toxicological significance of the different dose‐dependent metabolic pathways.

The evaluation of pulegone should also be reviewed when the ongoing NTP two‐year carcinogenicity is completed.

**KEYWORDS**

(R)-(+)‐pulegone, (R)-(+)‐menthofuran, flavourings, CAS No. 89‐82‐7, CAS No. 494‐90‐6.

**BACKGROUND**

**Previous Scientific evaluations**

The Scientific Committee on Food (SCF) issued an opinion on menthofuran and pulegone (expressed on 2 July 2002) (annexed in total to this opinion). The Committee decided that it was appropriate to evaluate pulegone and menthofuran together, as menthofuran is a major metabolite of pulegone and the hepatotoxicity of pulegone is due at least in part, to this metabolite.

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 stated that they require 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 (Scientific Committee on Food, Guidance on submissions for food additive evaluations by the Scientific Committee on Food, expressed on 11 July 2001). It was also considered that depending on refined intake estimates, studies of reproductive and developmental toxicity might be required.

The Committee noted that the intake estimates were not precise and represented only an order of magnitude. The Committee therefore recommended 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.
Industry has now submitted further studies for evaluation, as requested by the Scientific Committee on Food.

**Current EU 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).


As the Commission is currently drafting an updated list of flavouring substances which should be restricted in food, the Commission would need to consider the latest risk assessment on pulegone/menthofuran.

**TERMS OF REFERENCE**

The Commission requests EFSA to issue an opinion on the safety of pulegone and menthofuran.
ASSESSMENT

Chemistry

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-menth-4(8)-en-3-one; d-4(8)-p-menthen-3-one; 5-methyl-2-(1-methylethylidene)cyclohexanone
CAS Name: Cyclohexanone, 5-methyl-2-(1-methylethylidene)-(R)-
CAS No: 89-82-7
CoE No: 2050
EINECS: 201-943-2
Structure:

\[ \text{Structure of Pulegone} \]

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:

\[ \text{Structure of (R)-(+)-menthofuran} \]
Exposure

(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).

The data previously reviewed by the SCF indicated that intakes of (R)-(+-)pulegone and (R)-(+-)menthofuran were similar. Estimated mean and 97.5th percentile intakes of (R)-(+-)pulegone in the UK were 0.8 mg/day and 3.1 mg/day respectively with beverages and chewing gum being the major dietary sources. Estimated intakes of pulegone in France, based on proposed maximum use levels in various foods and beverages and 7-day individual surveys, assuming that flavoured foods had a 100% market share, were 43.9 mg/day and 72.7 mg/day for the mean and 97.5th percentile; 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).

Based on the maximum quantities of menthofuran added as a chemically defined flavour to specific foods and food groups (EFFA, 2000) 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 97.5th percentile intake would be 0.6 mg/day.

The SCF pointed out that the above estimates should be interpreted only as an order of magnitude rather than precise measurements.

New data were provided to the Panel on the exposure resulting from the occurrence of (R)-(+-)menthofuran in peppermint oil used to flavour chewing gum and its extractability from the gum on chewing (EACGI, 2003; Johnson et al., 2003). The range of (R)-(+-)menthofuran concentrations in peppermint oil used to flavour chewing gum was stated to be 1 to 5% and the mean level of the oil in chewing gum is typically 1.5%. In experimental studies, the amount of (R)-(+-)menthofuran extracted after 60 minutes chewing, was 15 to 40%. Based on the assumptions that chewing gum contains 1.5% peppermint oil that contains 3% menthofuran, that 50% is extracted on chewing and that consumption of chewing gum is approx. 1g/day, the estimated intake of (R)-(+-)menthofuran from chewing gum was 0.225 mg/day. However, the Panel notes that the production volume provided in 2003 actually related to 1992 (0.75 g chewing gum/per capita/day) and more recent data would be preferable. The Panel considers it reasonable to assume that the intakes by consumers-only may be 10 times this figure i.e. 7.5 g/day (about 2.5 pieces of gum weighing 3g). Using similar assumptions to those above, intakes of (R)-(+-)menthofuran deriving from regular consumption of chewing gum flavoured with peppermint oil could be about 1.7 mg/day.

Based on data from a Norwegian dietary survey of 1009 (4-day record) 13 year-old children conducted in 2000, the high consumption of chewing gum (95th percentile) was 9.5 g/day (Overby & Andersen, 2000). Using similar assumptions on concentrations of (R)-(+-)menthofuran in peppermint oil and usage
levels to those above leads to an estimated high daily intake of (R)-(+-) -menthofuran from chewing gum of 2.1 mg. If a person has a preference to a brand containing 5% peppermint oil, a consumption of 9.5 g/day would provide an estimated high intake of 3.6 mg/day.

The levels of (R)-(+-)-menthofuran in peppermint oil were stated to be typically 2-3 times the level of (R)-(+-)-pulegone (EACGI, 2003), leading to an estimated high intake of (R)-(+-)-pulegone from chewing gum that may vary between 0.7 mg/day up to 1.8 mg/day.

The Panel noted that refined estimates of pulegone intake from sweets were not available. It also noted the new estimates of intakes from chewing gum are considerably higher than some of the total estimates of mean intake from sweets, chewing gums & alcoholic beverages combined in the previous SCF opinion.

A report from the Netherlands was provided to the Panel describing a dietary intake calculation of pulegone and menthofuran which was based on a probabilistic model (Kruizinga et al., 2004). The food consumption data were from the third Dutch national food consumption survey (1997-98), whereas the concentration data for pulegone and menthofuran were based on the proposal of the European Parliament and the Council on flavourings (2004). The intake from alcoholic beverages was not taken into consideration since the consumption of liqueurs based on peppermint or cassis is negligible in the Netherlands. Market shares were provided by a market research institute and taken into consideration in the model. The estimated mean intakes of pulegone and menthofuran were 0.5 mg/day and 0.4 mg/day, respectively. Estimated high intakes (97.5th percentile) of pulegone and menthofuran among adults were at the most 2.1 mg/day and 3.4 mg/day, respectively (varied between men and women). The group comprising girls aged 1 to 9 years had the highest 97.5th percentile estimated intake of pulegone and menthofuran with 10.3 mg/day and 15.5 mg/day, respectively. However, the author of the report considered the latter figure as an over-estimate, since it was assumed in the model that small children also consume very strong mints. In reality, this is supposed not to be the case.

The Panel noted that the current maximum permitted level of pulegone in food may lead to high intakes, particularly in subjects who consume regularly mint flavoured beverages or confectionery. For example, it was noted that 500 ml/day of mint flavoured beverage and 100 g/day of mint confectionery could lead to intakes of respectively 4.2 mg/kg bw and 1.2 mg/kg bw for a 30 kg child.

Absorption, distribution, metabolism and excretion

The Panel noted that the data previously reviewed by the SCF indicated that (R)-(+-)-pulegone undergoes multiple pathways of metabolism, including one leading to the formation of (R)-(+-)-menthofuran, a major metabolite (See Figure 1 of Appendix 1). Since the SCF evaluation in July 2002, new data on the metabolism of R-(+-)-pulegone have been provided to EFSA and these are summarised below.
A further metabolic study on pulegone was conducted in Fischer 344 rats given a dose of 0.8 mg/kg bw intravenously or doses of 8.0 or 80 mg/kg bw by gavage (Chen et al., 2001). These studies supplemented the earlier studies carried out at high doses. Irrespective of dose, rats excreted a similar proportion of the radioactive dose in urine (ca 45% in males; 54-61% in females in 24 h). A further 14% or 5% of the radioactivity was excreted in urine by males and females respectively in the period 24-72 hours. Three principal pathways of metabolism were identified. The first pathway involves reduction of pulegone to menthone or isomenthone followed by hydroxylation in ring or side chain and subsequent conjugation with glucuronic acid. The second pathway involves conjugation with glutathione by Michael addition leading to mercapturic acid conjugates. The third pathway involves direct hydroxylation in ring or side chain followed by further metabolism by pathways that may yield menthofuran and its metabolite, mint lactone. Analysis of bile of treated rats showed the presence of a glutathione conjugate of menthofuran. This study confirms that menthofuran is an in vivo metabolite of pulegone and that further metabolites of menthofuran are formed, including the hepatotoxic compound 2-Z-(2’-keto-4’-methylcyclohexylidene)propanal (the γ-ketenal, 8-pulegone aldehyde). By comparison of the levels of menthofuran and its metabolites present in urine at higher doses, the authors concluded that the extent of metabolism via this pathway is dose dependent and is greater at higher dose levels. At lower doses than 80 mg/kg bw urinary phase 2 conjugates of hydroxylated metabolites predominate.

In a follow-up comparative study in rats and mice (Chen et al., 2003a), metabolites involving conjugation with glutathione were only observed in mouse bile and not urine whereas in rats these metabolites were detected both in urine and bile. Tissue levels of pulegone-derived radioactivity were highest in the liver of both species and both sexes except that high levels also were found in male rat kidney. It was speculated that urinary metabolites might have been binding to α_u2-globulin in these animals. In mice, clearance was virtually complete in 24 h whereas in rats only 59-81% of the dose was excreted in this time. The proposed metabolic pathways derived from this study are shown in Figure 1.
A study aimed at identifying the biliary metabolites of pulegone has been conducted (Thomassen et al., 1991). Although not a recent study, this was not available to the SCF at its previous evaluation. Pulegone was administered intraperitoneally to biliary duct-cannulated rats at a dose of 250 mg/kg bw. Biliary conjugates characterized were glucuronide and glutathione conjugates, accounting for about 3% of the dose. The conjugates were mainly of hydroxylated pulegone or hydroxylated reduced pulegone. One metabolite was identified tentatively as the glutathione conjugate of menthofuran.

It should be noted that the above studies in rodents were conducted at repeated high oral doses of 8–250 mg/kg bw. A recent study on both (S)-(-) and (R)-(+-)pulegone has been conducted in human volunteers in which the single dose administered was more similar to dietary exposure i.e. ~ 500 µg/kg bw. (Engel, 2003). The major urinary metabolites identified were 8-hydroxymenthone, 1-hydroxymenthone, menthol, and 10-hydroxypulegone; minor metabolites included piperitone and 3-p-menthene-8-ol. In this study, menthofuran was not a major urinary metabolite and further experiments indicated that this might have been an artifact formed from 10-hydroxypulegone during extraction and hydrolysis. The metabolic pathways identified in humans are shown in Figure 2. The reduction of the exocyclic double bond was stereoselective and less favoured for (R)-(+)pulegone than (S)-(+-)pulegone. The author suggested that this might account for the differences in toxicity between the two stereoisomers.

Direct comparisons between the metabolic studies on pulegone in humans and those in laboratory rodents are difficult because (a) there were large differences in dose, (b) repeat doses were given to rodents and single doses to humans, (c) it was not possible to study biliary metabolites in humans, and (d) as radiolabel was not used in the human study, determination of recoveries of the dose was not possible and minor and conjugated metabolites were not identified as such. However, at human doses corresponding to dietary exposure, the significance of metabolism to menthofuran is questionable.

Figure 2. Metabolites of pulegone identified in humans (Engel, 2003)
A study of the metabolism of (R)-(−)-menthofuran in rats was conducted to determine those urinary metabolites of (R)-(−)-pulegone that are derived from the menthofuran pathway (Chen et al., 2003b). The rats received a single gavage dose of 6 or 60 mg menthofuran/kg bw. Three sulphonic acid metabolites were identified in urine, namely hexahydro-3,6-dimethyl-1-(2-sulphoethyl)-2H-indol-2-one, hexahydro-3,6-dimethyl-7α-sulpho-2-(3H)-benzofuranone and 2-sulphomenthofuran. Other urinary metabolites identified could be attributed to further metabolism of mint lactones followed by hydroxylation and conjugation to form glucuronides. Four of the metabolites identified were identical to pulegone metabolites identified in earlier studies. The proposed pathways of metabolism of menthofuran are shown in Figure 3.

Toxicity in laboratory animals

The following short-term studies were completed and the results made available after the previous SCF evaluation.
**Sub-chronic (14 week) studies**

**Mouse**
Groups of male and female B6C3F1 mice (10 of each sex per dose) were given by gavage daily doses of R-(+)-pulegone of 0, 9.375, 18.75, 37.5, 75 or 150 mg/kg bw for 14 weeks (excluding week-ends and holidays). Body weights and clinical observations were recorded weekly. At termination, clinical chemistry and haematological examinations were conducted on blood and liver samples were analysed for reduced and oxidized glutathione levels. At termination, organ weights were measured and comprehensive histological examinations were conducted.

The only significant effects reported were an increase in absolute and relative liver weights at the 150 mg/kg bw/day dose level. Glutathione levels (both reduced and oxidized) were increased in top three dose groups of females and the top two groups of males. Oxidized glutathione was significantly increased in the lowest two dose groups of females and the 37.5 mg/kg bw/day dose group of males.

Based on the significant increase in absolute and relative liver weight, the authors identified a NOAEL of 75 mg/kg bw/day in mice (NTP, 2002a).

**Rat**
Groups of male and female F344N rats (10 of each sex per dose) were given by gavage daily doses of R-(+)-pulegone of 0, 9.375, 18.75, 37.5, 75 or 150 mg/kg bw for 14 weeks (excluding week-ends and holidays). Body weights and clinical observations were recorded weekly. At termination, clinical chemistry and haematological examinations were conducted on blood and liver samples were analysed for reduced and oxidized glutathione levels. At termination, organ weights were measured and comprehensive histological examinations were conducted.

There was one death in the female top dose group prior to termination. At termination, decreased body weights were recorded in the 75 and 150 mg/kg bw/day dose groups of males and the 150 mg/kg bw/day dose group females.

At termination, there were statistically significant increases in relative liver weight, thymus weight and kidney weight in males of the 75 and 150 mg/kg bw/day dose group. Relative and absolute kidney weights were also increased in the 18.75 and 37.5 mg/kg bw/day groups. In females, there were statistically significant increases in absolute and relative weights of liver, thymus and kidney at the 75 and 150 mg/kg bw/day dose levels. Absolute and relative kidney weights were also increased in the 18.75 and 37.5 mg/kg bw/day dose groups. Clinical chemistry showed an increase in alkaline phosphatase and bile acids/salts in both sexes at the 37.5 mg/kg bw/day level and above; at the top dose level, gamma glutamyltranspeptidase and alanine aminotransferase were also elevated.

Haematology at termination showed an increase in reticulocytes, decreased red blood cells, haematocrit and haemoglobin levels in females of the 37.5 mg/kg bw/d dose group with more marked changes in these parameters and increased platelets in higher dose groups of both sexes.

Histological examination showed liver alterations (hepatocyte hypertrophy and bile duct hyperplasia) and nephropathy (nature not specified) in males of the 75 mg/kg bw group. In the top dose group males there was chronic hepatic inflammation, hepatocellular necrosis, oval cell hyperplasia and hepatic
periportal fibrosis. Females exhibited similar hepatic histopathology at the top dose. Both males and females in the top two dose groups showed a significant increase in bone marrow hyperplasia.

A NOAEL of 9.375 mg/kg bw/day was identified from this study based on organ weight changes at the 18.75 mg/kg bw/day dose level and more extensive changes at higher dose levels (NTP, 2002b).

In an earlier 28-day study not previously seen by the SCF, a NOAEL for pulegone in the rat was established as 20 mg/kg bw/day. At higher doses (80 and 160 mg/kg bw/day) pulegone was reported to cause atonia, reduced blood creatinine, histological changes in the liver and reduced terminal body weight (Thorup et al., 1983).

**Genotoxicity**

The results of the following short term studies were not available for evaluation by the SCF in its opinion.

**Menthofuran**

Menthofuran was negative in the Ames assay using *Salmonella typhimurium* strains TA100 and TA1535 at concentrations of 100 to 10000 μg/plate with or without metabolic activation by Aroclor1254-induced Syrian hamster or rat liver S9 preparations. It was also negative against strains TA97, TA98, TA100 or TA1535 at concentrations up to and 667 μg/plate (inclusive) in the presence of hamster or rat liver preparations (NTP, 2002c).

**Pulegone**

Pulegone was negative in the Ames assay using *Salmonella typhimurium* strains TA100 and TA1535 at concentrations of 100 to 10000 μg/plate with or without metabolic activation by Aroclor1254-induced Syrian hamster or rat liver S9 preparations (NTP, 2002d).

**Other toxicological data**

In addition to the above studies, 21 papers were submitted to EFSA relating to studies on the mechanism of α₂u-globulin-mediated renal toxicity in the male rat and involving experimental studies on substances other than (R)-(+)—pulegone and (R)-(+)—menthofuran. In characterizing the risk, the Panel concurred with the SCF evaluation that the critical effect of (R)-(+)—pulegone and (R)-(+)—menthofuran was hepatotoxicity, and therefore these studies are not directly relevant. However, in the new 14 week studies, nephrotoxicity was reported in male rats only and probably arose via this mechanism, which is species and sex specific.

A further 7 papers were submitted dealing with aspects of the toxicity of oil of peppermint or pennyroyal. In its previous opinion the SCF 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 so these studies do not specifically address the deficiencies in the database previously identified by the SCF.
DISCUSSION

In addition to the new studies submitted to EFSA, the Panel gave consideration to the SCF opinion on (R)-(+) -pulegone and (R)-(+) -menthofuran expressed in July 2002. That opinion drew attention to the limited toxicological database on these substances and required at least further studies to establish a NOAEL for (R)-(+) -pulegone and (R)-(+) -menthofuran in 90-day studies together with further studies on genotoxicity at the gene and chromosome level. The SCF also indicated that, dependent on refined intake estimates, it might also require studies of reproductive and developmental toxicity.

The new metabolism studies in rodents indicate that the extent of metabolism of (R)-(+) -pulegone to conjugated (phase 2) metabolites is greater at lower doses and that metabolism to hepatotoxic metabolites is dose-dependent. Nevertheless, they indicate that one pathway of metabolism of pulegone proceeds via menthofuran and that it might be appropriate to consider the toxicity of (R)-(+) -pulegone and (R)-(+) -menthofuran together. However, the extent to which the hepatotoxicity of (R)-(+) -pulegone is dependent on metabolism to (R)-(+) -menthofuran remains unclear and recent metabolic studies in humans suggest that this is not a significant pathway at dietary levels of exposure. The SCF opinion pointed out that comparative oral toxicity studies on the two substances would be required to clarify this and determine whether (R)-(+) -menthofuran might be included in an overall evaluation on the basis of “(R)-(+) -pulegone equivalents”. No such comparative studies were available to the Panel in the current review. Furthermore, the dose-dependence of the competing metabolic pathways is likely to give rise to non-linearity in dose-response characteristics which complicates prediction of relative potencies.

The NTP 95-day studies in rats and mice provide a NOAEL for (R)-(+) -pulegone in both species. This meets one of the requirements of the previous SCF opinion. The NOAELs are 75 mg/kg bw/day in male and female mice based on increased liver weight; no gross or histological changes were observed at the higher dose level of 150 mg/kg bw/day. However, as indicated above, changes in hepatic levels of glutathione (oxidized and/or reduced) were reported in satellite groups at lower dose levels than these. The NOELs in rats were lower at 9.4 mg/kg bw/day in male and female rats based on increased organ weights and at 18.75 and 37.5 mg/kg bw/day based on hepatotoxicity, nephropathy and bone marrow hyperplasia at higher doses.

The genotoxicity studies confirm earlier data but do not meet the requirements for data of the earlier SCF opinion.

There are few studies relating to menthofuran apart from metabolism and genotoxicity.

The new data on extraction of (R)-(+) -menthofuran from chewing gum enable more refined estimates to be made of the contribution to dietary intakes from this source.
CONCLUSIONS AND RECOMMENDATIONS

The new data augment those available at the previous review by the Scientific Committee on Food but do not fully meet the stated requirements with regard to the need for additional genotoxicity studies. The 95-day studies meet the earlier Committee’s requirements to establish a NOAEL for (R)-(+-)pulegone in sub-chronic studies. However, the data as a whole are not yet sufficient for the Panel to establish a TDI for pulegone. Furthermore, there are no comparable data on (R)-(+-)menthofuran.

The Panel noted that refined estimates of pulegone intake from sweets were not available. It also noted the new estimates of intakes from chewing gum are considerably higher than some of the estimates of mean total intake from sweets, chewing gums and alcoholic beverages combined in the previous SCF opinion.

The Panel concluded that the current maximum permitted level of pulegone in food may lead to high intakes, particularly in subjects who consume regularly mint flavoured beverages or confectionery. For example, it was noted that 500 ml/day of mint flavoured beverage and 100 g/day of mint confectionery could lead to intakes of respectively 4.2 mg/kg bw and 1.2 mg/kg bw for a 30 kg child.

The toxicological database for (R)-(+-)menthofuran is extremely limited and does not enable an ADI to be established for (R)-(+-)menthofuran as a chemically defined flavouring. Furthermore, although at high doses (R)-(+-)menthofuran may be formed as a metabolite of (R)-(+-)pulegone and exhibits qualitatively similar toxicity in laboratory rodents, a recent human metabolism study on (R)-(+-)pulegone at sub-toxic doses did not detect menthofuran as a significant metabolite. Nevertheless, the formation of menthofuran as an intermediate leading to other reactive compounds, such as the \( \gamma \)-ketoenal, 8-pulegone aldehyde, cannot be excluded.

The Panel wishes the following studies to be provided within 2 years of the publication of this opinion.

1. Studies to establish a NOEL for (R)-(+-)menthofuran in 90-day oral toxicity study in rats;
2. Further genotoxicity studies on (R)-(+-)menthofuran and (R)-(+-)pulegone augmenting the database to comply with the SCF Guidelines for Food Additives (studies on mammalian cell gene mutation and chromosome aberration); (http://europa.eu.int/comm/food/fs/sc/scf/out98_pdf)
3. Further refinement of intake estimates from all dietary sources including actual usage levels and analytical data on concentrations in relevant products;

The Panel considered that dose-response studies on (R)-(+-)menthofuran would be helpful in elucidating the toxicological significance of the different dose-dependent metabolic pathways.

The evaluation of pulegone should also be reviewed when the ongoing NTP two-year carcinogenicity is completed.
REFERENCES


NTP, 2002a (National Toxicology Program). Toxicity studies of pulegone in B6C3F1 mice (Gavage studies). Batelle Research Laboratories. Study No. G004164-X. Private communication to FEMA; unpublished report
NTP, 2002b (National Toxicology Program). Toxicity studies of pulegone in F344/N rats (Gavage studies). Batelle Research Laboratories. Study No. G004164-W. Private communication to FEMA; unpublished report.

NTP, 2002c (National Toxicology Program). Cellular and Genetic Toxicology Branch, Salmonella Testing Results No. A36119. Private communication to FEMA; unpublished report.

NTP, 2002d (National Toxicology Program). Cellular and Genetic Toxicology Branch, Salmonella Testing Results No. 687373. Private communication to FEMA; unpublished report.


SCIENTIFIC PANEL MEMBERS


ACKNOWLEDGEMENTS

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food wishes to thank Ron Walker for his contribution to the draft opinion.
Appendix 1. Opinion of the SCF, July 2002

The text below is from the Secretariat archives and the published version of the opinion can be found at http://europa.eu.int/comm/food/fs/sc/scf/out133_en.pdf

Scientific Committee on Food

Opinion
of the Scientific Committee on Food
on pulegone and menthofuran

(expressed on 2 July 2002)
Opinion of the Scientific Committee on Food on pulegone and menthofuran

(expressed on 2 July 2002)

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).


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

- 2,8-dihydroymenthone
- pulegol
- 9-hydroxypulegone
- (R)-(±)-pulegone
- 5-hydroxypulegone
- (R)-(±)-menthofuran
- p-mentha-1,4(8)-diene-3-one (piperitenone)
- 9-carboxypulegone
- Hydroxylactone
- Glutathione conjugate
- γ-Ketoenol intermediate (8-pulegone aldehyde)
- Epoxide intermediate
- 2-[2'-Hydroxyisopropyl]-5-methylphenol + cresol
- 4-, 5-, 7-, 9-, & 10-hydroxy piperitenone
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 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 \( \text{p-mentha-1,4(8)-diene-3-one} \) \( (\text{piperitenone}) \). In minor pathways, the exocyclic double bond is oxidised to 2,8-dihydroxymethone, 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 \( (\text{i.e. 5-methyl-2-(1-methyl-1-carboxyethylidene)cyclohexanone}) \) 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., 1992a; 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<sub>50</sub> 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 demyelination. 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 α2u-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 LD50 (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 14C-(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-hydroxypulegonone, 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


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


