EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions
C2 - Management of scientific committees; scientific co-operation and networks

Scientific Committee on Food

SCF/CS/FLAV/FLAVOUR/30 Final
9 April 2003

Opinion of the Scientific Committee on Food on Isosafrole

(expressed on 4 April 2003)

B-1049 Bruxelles/Brussel - Belgium
Telephone:, switchboard 299.11.11. Fax: (+32-2) 299.4891
Telex: COMEU B 21877. Telegraphic address: COMEUR Brussels
http://europa.eu.int/comm/food/fs/sc/scf/index_en.html

Opinion of the Scientific Committee on Food on Isosafrole

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 implication for human health of isosafrole in the diet.

Introduction

Previous evaluations

In its opinion of 21 September 1979 the Scientific Committee for Food proposed limits for isosafrole in food. For total safrole and isosafrole, limits of 1 mg/kg for foods and for beverages were proposed with the following exceptions: 2 mg/kg in alcoholic beverages with less than 25% alcohol, 5 mg/kg in alcoholic beverages with more than 25% alcohol and 15 mg/kg in foods containing mace and nutmeg (SCF, 1979).

Isosafrole was evaluated by the International Agency for Research on Cancer (IARC) in 1975. It was concluded that isosafrole is carcinogenic in mice and rats, producing liver tumours following its oral administration (IARC, 1976). Based on this monograph, IARC in 1987 concluded for isosafrole that there were no adequate human carcinogenicity data but limited evidence of animal carcinogenicity, and isosafrole was classified in group 3, “not classifiable as to its carcinogenicity for humans” (IARC, 1987).

In 1981 the Joint FAO/WHO Expert Committee on Food Additives (JECFA) also concluded that isosafrole is carcinogenic in rats and mice and no ADI was allocated (WHO, 1981).

In 1999, the Council of Europe Committee of Experts on Flavouring Substances (CEFS) was informed by the International Organisation of Flavour Industry that isosafrole does not occur in any natural source material for flavourings used by flavour industry and therefore the CEFS deleted isosafrole from the list of active principles (Council of Europe, 1999). In 2002 CEFS was informed

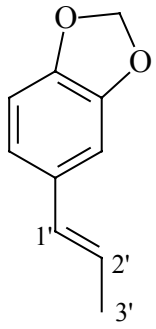
that isosafrole does occur in relevant source materials for flavourings and CEFS decided to reconsider isosafrole at its next meeting (Council of Europe, 2002).

Current regulatory status

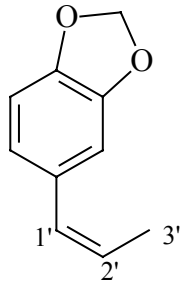
Isosafrole is listed in the Directive 88/388/EEC on flavourings in Annex II with maximum limits for isosafrole (and safrole) of 1 mg/kg in foodstuffs and beverages with exceptions of 2 mg/kg and 5 mg/kg in alcoholic beverages with not more than and with more than 25% volume of alcohol, respectively, and 15 mg/kg in foodstuffs containing mace and nutmeg (EEC, 1988).

Chemical characterisation

Isosafrole is a propenylbenzene derivative.
Name: Isosafrole
Synonyms: 5-(1'-Propenyl)-1,3-benzodioxole; 1,2-(methylenedioxy)-4-(1'-propenyl) benzene
CAS no.: 120-58-1
Structure:



(*E*) - Isosafrole



(*Z*) - Isosafrole

Isosafrole exists as a trans-(E-)isomer (beta-isosafrole) (CAS no:4043-71-4) and as a cis-(Z-)isomer (alfa-isosafrole), (CAS no:17627-76-8).

Exposure assessment

According to CEFS (1999) natural source materials for flavourings used by the flavour industry do not contain isosafrole. Former information on occurrence of isosafrole in ylang-ylang oil and products of sassafras could not be confirmed (MAFF, 1996; Carlson and Thompson, 1997). On the other hand, small amounts of isosafrole were found in some samples of nutmeg oils (range < 0.1 - 3.4%) and oleoresins (range < 0.1 - 2.7%) (MAFF, 1996). In contrast to this report, Lawrence

(1990) did not find isosafrole in nutmeg oil. In a study by Adam and Postel (1992) isosafrole was found in trace amounts (0.01 and 0.03 mg/kg) in two out of 24 alcoholic beverages.

According to available data isosafrole only occurs sporadically and then together with safrole but at much lower concentrations than safrole, roughly an order of magnitude lower (MAFF, 1996). It has therefore been speculated, whether isosafrole occurs as an artefact, formed due to heating during the analytical process and/or during the preparation of source material extracts, essential oils, processed foods and others, containing safrole. This supposition is backed up by the fact that isosafrole can be produced by isomerisation of safrole under hot alkaline conditions (Bert, 1941; Naves and Ardizio, 1957) and hence the possibility that traces of isosafrole may be formed even under neutral conditions (Council of Europe, 1999).
A rough intake estimate for isosafrole could be based on the estimated average safrole intake (for consumers only) (SCF, 2001), assuming that the intake of isosafrole is one tenth of the safrole intake. Using this assumption, the estimated average *per capita* intake of isosafrole amounts to 0.03 mg/day and the 97.5% percentile to 0.05 mg/day.

Hazard identification / characterisation

Absorption, distribution, metabolism and excretion

In an *in vitro* study with epithelial cells from adult rat liver, the major metabolite of isosafrole was 1', 2'-dihydro- 1', 2'-dihydroxyisosafrole with lesser amounts of 1', 2'-epoxyisosafrole and 1'-hydroxysafrole. The metabolites were identified by gas chromatography-mass spectrometry, but no quantitative data are given (Janiaud *et al*., 1976).

At 1 mmol (162 mg) isosafrole ("Cis-trans mixture")/kg bw given to three male albino rats (Wistar strain derived) by stomach tube, metabolite excretion accounted for 89 % of the dose in urine, in 72 hours. Demethylenation leading mainly to 1,2-dihydroxy-4-(1'-propenyl)benzene was the most prominent reaction (92% of the urinary metabolites were demethylenated) but also allylic hydroxylation and epoxide-diol pathway took place. Allylic hydroxylation took place at the 3'-position, but this was a minor pathway in the present rat study. Only 1.3% of the dose was recovered as 3'-hydroxyisosafrole and, contrary to the above *in vitro* study, no 1'-hydroxysafrole was detected. However, the authors did not exclude that some formation of this may occur when very large doses of isosafrole are administered (Klungsøyr and Scheline, 1982). This is supported by the study by Peele and Oswald (1978) who did in fact demonstrate excretion of traces of 1'-hydroxysafrole in the urine of rats to which they had administered 3'-hydroxyisosafrole. It has also been shown that 1'-hydroxysafrole may undergo chemical rearrangement to 3'-hydroxyisosafrole (Borchert *et al*., 1973b) and that this equilibrium between the two isomers strongly favours 3'-hydroxyisosafrole (Peele and Oswald, 1978).

Acute toxicity

LD_{50} oral (mice): 2.47 g/kg bw. LD_{50} oral (rats): 1.34 g/kg bw (Jenner *et al*., 1964; Hagan *et al*., 1965).

Subacute/subchronic toxicity

Rat

Rats (Osborne-Mendel strain, 10 males and 10 females) given 10 000 mg isosafrole/kg in diet showed growth retardation in both sexes. No rats survived beyond 11 weeks of treatment. The livers were enlarged and microscopically slight hepatic cell hypertrophy, which was usually focal and resulted in the formation of nodules, was shown (Hagan *et al*., 1965; Hagan *et al*., 1967).

Daily doses of 460 mg isosafrole/kg bw given by stomach tube for four days to rats (3 males and 3 females) produced severe liver lesions consisting of discolouration, enlargement, and loss of normal texture. No histopathology was performed. Two of the rats died during the test period. Apparently, no control group was included in the study (Taylor *et al*., 1964). Oral intubation to young Osborne-Mendel rats of both sexes of 500 mg isosafrole/kg bw/day for 41 days resulted in mortality ratios of 8/10 rats and of 250 mg isosafrole/kg bw/day for 34 days of 2/10 rats. All ten rats of the control group survived. The following effects were observed: liver hypertrophy and slight focal necrosis and fibrosis, slight degree of focal fatty metamorphosis and bile duct proliferation (Hagan *et al*., 1965).

Chronic toxicity/Carcinogenicity

Mouse

In two strains of mice (C57BL/6 x C3H/Anf)F_1 and (C57BL/6 x AKR) F_1 (group size 18 males and 18 females) isosafrole (vehicle: water) was given by stomach tube from 7 days of age until 28 days of age at 215 mg/kg bw, then subsequently fed *ad libitum* at 517 mg/kg diet for up to 82 weeks. The study included control groups of up to 18 males and up to 18 females per strain. Liver cell tumours occurred in 5/18 males and 1/16 females and in 2/17 males and 0/16 females, pulmonary tumours in 3/18 males and 1/16 females and in 0/17 males and 0/16 females, and lymphomas in 1/18 males and 0/16 females, and in 1/17 males and 0/16 females; of the two strains, respectively. The difference from controls was only statistically significant for the liver tumours (P=0.05) in (C57BL/6 x C3H/Anf)F_1 mice (males and females combined) (Innes *et al*., 1969).

No hepatocarcinogenic activity was found in male B6C3F1 mice given a single i.p. injection (solvent: trioctanoin) of isosafrole (52 % cis-/48 % trans-isomer) to a group of 29 animals or trans-isosafrole (90 % trans-/10 % cis-isomer) to a group of 32 animals, at 12 days of age (dose: 0.75 mmol/kg bw equal to 122 mg/kg bw) and killed at 10 months. Thirty-two animals only given the solvent, served as a control group (Wiseman *et al*., 1987).

Rat

Isosafrole was given in the diet for two years to Osborne-Mendel rats at 0, 1,000, 2,500 or 5,000 mg isosafrole/kg (control group : 35 males and 35 females). At the two lowest dose levels (10 males and 10 females per group) there was slight growth retardation in females. Microscopically, slight hepatic cell hypertrophy but no primary hepatic tumours were reported. At 2,500 mg/kg in the diet there was also slight hyperplasia in the thyroid. At the highest dose, 5,000 mg/kg in the diet (25 males and 25 females) growth retardation was reported in both sexes. The liver was enlarged and microscopically slight hepatic cell hypertrophy, which was usually focal and resulted in the formation of nodules, was shown. Five rats had primary hepatic tumours (two adenomas and three carcinomas). Slight hyperplasia in the thyroid and an increase in the incidence of chronic nephritis were demonstrated. An increased number of interstitial cell tumours was found in testes. The study is poorly reported, and it does not allow a clear NOEL to be established (Hagan *et al*., 1965; Hagan *et al.,* 1967).

No local tumours were observed in 18 male rats (Charles River CD, random bred) given a total of 20 (twice weekly) s.c. injections, each of 3 mg isosafrole (trioctanoin solution) per rat. The rats were examined after surviving 18 months (Borchert *et al*., 1973a).

Genotoxicity

In vitro

Isosafrole (19,7% cis/78.2% trans isomer) did not induce gene mutations in *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537, TA 1538 or in *Escherichia coli* WP 2 uvr A with or without S9. It was negative in a *Bacillus subtilis* DNA repair test (Rec assay) without S9 (Sekizawa and Shibamoto, 1982).

In contrast to safrole, estragole and methyleugenol, it did not induce UDS in cultured rat hepatocytes (Howes *et al*., 1990).

DNA adduct formation

DNA adduct formation using ^{32}P post labelling analysis was studied in livers from adult female CD1 mice, isolated 24 hours after i.p. administration of different alkenylbenzenes, including isosafrole (2 or 10 mg/mouse). After administration of isosafrole, only low binding to the mouse liver DNA was demonstrated, with the two major DNA adducts formed in the N^2-position of guanine. The low DNA binding could also be expressed by the covalent binding index (CBI) value of about 1 for isosafrole. For comparison the CBI values for safrole, estragole and methyleugenol were all about 30 (Randerath *et al.*, 1984).

Reproduction and developmental toxicity

No data available.

Human data

No data available.

Other studies

Isosafrole is a known inducer of some of the liver enzymes of the cytochrome P-450 group in rodents, especially CYP1A2 (cf. Ryan *et al*., 1980; Waxman *et al*., 1985; Murray and Reidy, 1989; Ishida *et al*., 1998; Allis *et al*., 2002).

Summary of hazard identification / characterization

Isosafrole is an efficient inducer of some of the liver cytochrome P-450' s, and is a weak liver carcinogen in rats and mice. Liver DNA adduct formation (^{32}P post labelling) is low. All genotoxicity tests are negative. Overall, these data provide support for a non-genotoxic mechanism of hepatocarcinogenicity associated with hepatic enzyme induction, but the available data from carcinogenicity studies in mouse and rat do not allow establishing a clear NOEL.
Finally, it cannot be completely excluded that high exposure to isosafrole may give rise to some isomerisation of 3'-hydroxyisosafrole to 1'-hydroxysafrole, the anticipated proximate carcinogen of safrole.

Conclusion

Isosafrole is a weak rodent hepatocarcinogen; the carcinogenicity is probably mediated by a non-genotoxic mechanism. Isosafrole metabolites may give rise to only very low binding to liver DNA in mice. It cannot be excluded that high exposure to isosafrole may give rise to isomerisation of 3'-hydroxy-isosafrole to 1'-hydroxysafrole, the proximate carcinogen metabolite of safrole. However, generally the exposure to isosafrole is estimated to be very low.
A clear NOEL could not be demonstrated for hepatic effects in the long-term studies. Therefore, the Committee could not establish a TDI. The Committee notes that isosafrole occurs together with safrole, but at much lower concentrations. Any measure to restrict exposure to safrole in food would also cover isosafrole.

References

Adam, L. Postel, W. (1992). Bestimmung von alpha- und beta-Thujon, Safrol, Isosafrol, beta-Asaron und Cumarin in weinhaltigen Getränken und Spirituosen. Die Brantweinwirtschaft, **132**, 202-206.

Allis, J.W., Anderson, B.P., Zhao, G., Ross, T.M., Pegram, R.A. (2002). Evidence for the involvement of CYP1A2 in metabolism of bromodichloromethane in rat liver. Toxicology, **176**, 25-37.

Bert, M.L. (1941). Sur une nouvelle méthode générale de synthèse des essences allylique et propionylique. *Compt. Rend*., **213**, 873-874.

Borchert, P., Miller, J.A., Miller, E.C. Shires, T.K. (1973a). 1'-Hydroxysafrole, a proximate carcinogenic metabolite of safrole in the rat and mouse. *Cancer Res*., **35**, 590-600.

Borchert, P., Wislocki, P.G., Miller, J.A., Miller, E.C. (1973b). The metabolism of the naturally occurring hepatocarcinogen safrole to 1'-hydroxysafrole and the electrophilic reactivity of 1'-acetoxysafrole. *Cancer Res*., **33**, 575-589.

Carlson, M. Thompson, R.D. (1997). Liquid chromatographic determination of safrole in sassafras-derived herbal products. *J. AOAC*, **80**, 1023-1028.

Council of Europe (1999). Committee of Experts on Flavouring Substances. 45th Session. Record.

Council of Europe (2002). Committee of Experts on Flavouring Substances. 50th Session. Record.

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.

Hagan E.C., Jenner, P.M., Jones, W.I., Fitzhugh, O.G., Long, E.L., Brouwer, J.G., Webb, W.K. (1965). Toxic properties of compounds related to safrole. *Toxicol. Appl. Pharmacol*., **7**, 18-24.

Hagan, E.C., Hansen, W.H., Fitzhugh, O.G., Jenner, P.M., Jones, W.I., Taylor, J.M., Long, E.L., Nelson, A.A., Brouwer, J.B. (1967). Food flavourings and compounds of related structure. II. Subacute and chronic toxicity. *Fd. Cosmet. Toxicol*., **5**, 141-157.

Howes, A.J., Chan, V.S.W., Caldwell, J. (1990). Structure-specificity of the genotoxicity of some naturally occurring alkenylbenzenes determined by the unscheduled DNA synthesis assay in rat hepatocytes. *Fd. Chem. Toxicol*., **28**, 537-542.

IARC (1976). IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man: Some Naturally Occurring Substances, **10**, 231-244.

IARC (1987). IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs Volumes 1-42, Supplement **7**, 51, 65.

Innes, J.M., Ulland, B.M., Valerio, M.G., Petrucelli, L., Fishbein, L., Hart, E.R., Pallotta, A.J., Bates, R.R., Falk, H.L., Gart, J.J., Klein, M., Mitchell, I., Peters, J. (1969). Bioassay of pesticides and industrial chemicals for tumorigenicity in mice: A preliminary note. *J. Nat. Cancer Inst*., **42**, 1101-1114.

Ishida, T., Tasaki, K., Fukuda, A., Ishii,Y., Oguri, K. (1998). Induction of a cytosolic 54 kDa protein in rat liver that is highly homologous to selenium-binding protein. *Environ. Toxicol. Pharmacol*., **6**, 249-255.

Janiaud, P., Delaforge, M., Levi, P., Maume, B.F.Padieu, P. (1976). Etude comparative en culture cellulaire de foie de rat du métabolisme de différents analogues et métabolites d'un hépatocancérogène naturel: le safrol. *CR Soc. Biol*., **170**, 1035-1041.

Jenner, P.M., Hagan, E.C., Taylor, J.M., Cook, E.L. Fitzhugh, O.G. (1964). Food flavourings and compounds of related structure. I. Acute oral toxicity. *Fd. Cosmet. Toxicol*., **2**. 327-343.

Klungsøyr, J., Scheline, R.R. (1982). Metabolism of isosafrole and dihydrosafrole in the rat. *Biomed. Mass Spectrom*., **9**, 323-329.

Lawrence, B.M. (1990). Progress in essential oils. *Perfumer and Flavorist*, **15**, 63-69.

Min. Agr. Fish. Food (1996), Food Surveillance Paper No. 48, Flavourings in Food, London, HMSO.

Murray, M., Reidy, G.F. (1989). *In vitro* formation of an inhibitory complex between an isosafrole metabolite and rat hepatic cytochrome P-450 *PB-B*. *Drug Metab. Dispos*., **17**, 449-454.

Naves, Y.-R., Ardizio, P. (1957). Études sur les matières végétales volatiles CXLVII(I). Sur les cis et trans-isosafroles. *Bull. Soc. Chim. France*, 1053-1057

Peele, J.D., Oswald E.O. (1978). Metabolism of the proximate carcinogen 1'-hydroxysafrole and the isomer 3'-hydroxyisosafrole. *Bull. Environ. Contam. Toxicol.,* **19**, 396-402.

Randerath, K., Haglund, R.E., Phillips, D.H., Reddy, M.V. (1984). ^{32}P-Post-labelling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. *Carcinogenesis*, **5**, 1613-1622.

Ryan, D.E., Thomas, P.E., Levin, W. (1980). Hepatic microsomal cytochrome P-450 from rats treated with isosafrole. *J. Biol. Chem*., **255**, 7941-7955.

SCF (1979). Report of the Scientific Committee for Food on Flavourings (opinion expressed on 21 September 1979). *Reports of the Scientific Committee for Food (9th Series)*, Office for Official Publications of the European Communities, Luxembourg, October 1979.

SCF (2001). Opinion of the Scientific Committee on Food on the safety of the presence of safrole (1-allyl-3,4- methylene dioxy benzene) in flavourings and other food ingredients with flavouring, expressed 12 December 2001. Available at http://europa.eu.int/comm/food/fs/sc/scf/index_en.html.

Sekizawa, J. Shibamoto, J. (1982). Genotoxicity of safrole-related chemicals in microbial test systems. *Mutat. Res*., **101**, 127-140.

Taylor, J.M., Jenner, P.M., Jones, W.I. (1964). A comparison of the toxicity of some allyl, propenyl, and propyl compounds in the rat. *Toxicol. Appl. Pharmacol*., **6**, 378-387.

Waxman, D.J., Dannan, G.A., Guengerich, F.P. (1985). Regulation of rat hepatic cytochrome P-450: Age-dependent expression, hormonal imprinting, and xenobiotic inducibility of sex-specific isoenzymes. *Biochemistry*, **24**, 4409-4417.

WHO (1981). Evaluation of Certain Food Additives. Twenty-Fifth Report of the Joint FAO/WHO Expert Committee on Food Additives. Technical Report Series No. 669. World Health Organization, Geneva.

Wiseman, R.W., Miller, E.C., Miller, J.A., Liem, A. (1987). Structure-activity studies of the hepatocarcinogenicities of alkenylbenzene derivatives related to estragole and safrole on administration to preweanling male C57BL/6J x C3H/HeJ F_1 mice. *Cancer Res*., **47**, 2275-2283.