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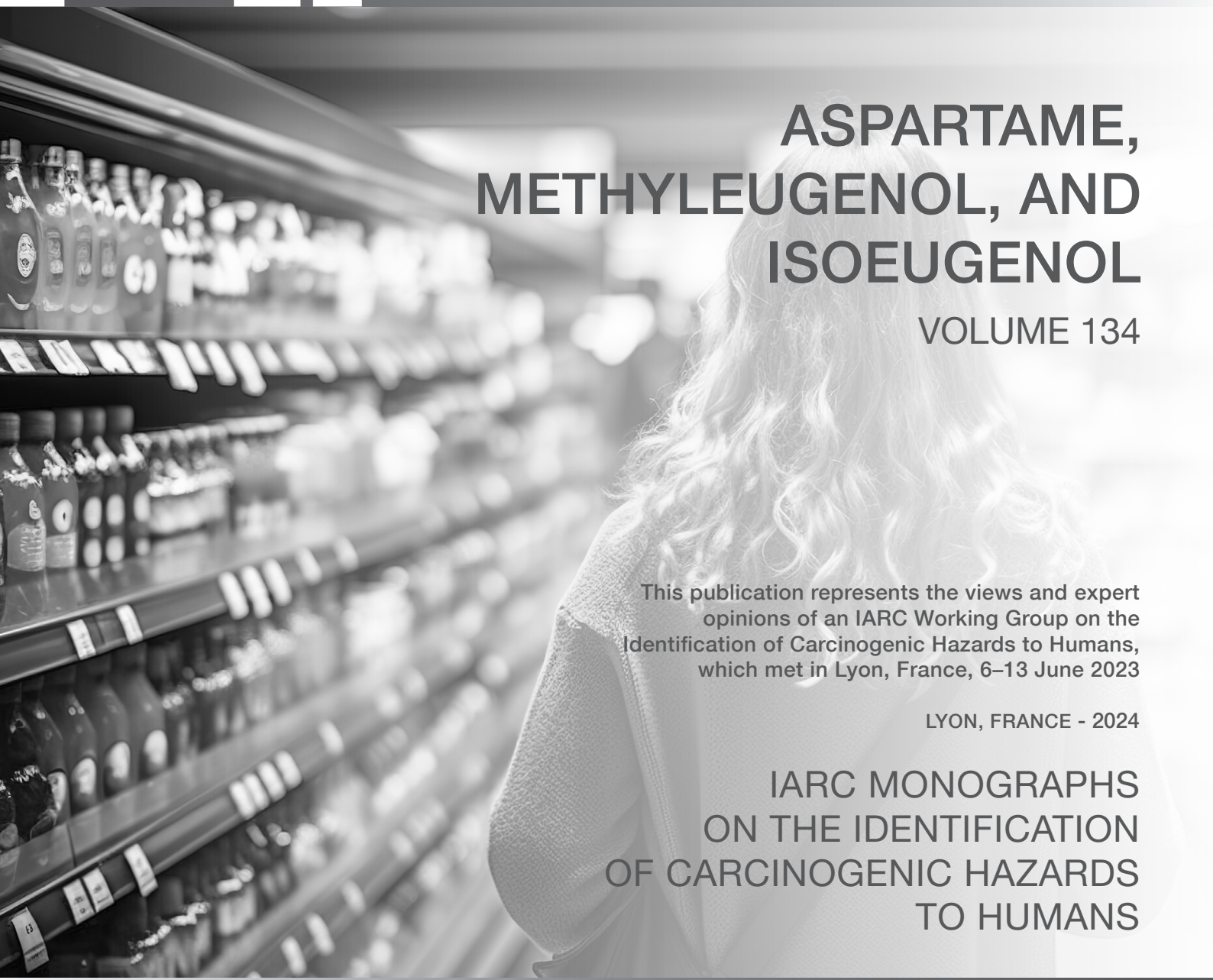

ASPARTAME, METHYLEUGENOL, AND ISOEUGENOL

VOLUME 134

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International Agency for Research on Cancer

World Health Organization

METHYLEUGENOL

1. Exposure Characterization

1.1 Identification of the agent

1.1.1 Nomenclature

Chem. Abstr. Serv. Reg. No.: 93-15-2 (Chemical Abstracts Service, 2022a)

EC/List No.: 202-223-0 (ECHA, 2022)

Chem. Abstr. Serv. name: 1,2-dimethoxy-4-(2-propen-1-yl)-benzene (Chemical Abstracts Service, 2022a)

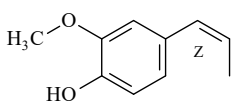
IUPAC systematic name: 1,2-dimethoxy-4-(prop-2-en-1-yl)benzene (ECHA, 2022)

Synonyms: 1-allyl-3,4-dimethoxybenzene; 4-allyl-1,2-dimethyoxybenzene; 4-allyl-veratrole; benzene, 4-allyl-1,2-dimethoxy-; benzene, 1,2-dimethoxy-4-(2-propenyl)-; 1,2-dimethoxy-4-allylbenzene; 3,4-dimethoxyallylbenzene; 1-(3,4-dimethoxyphenyl)-2-propene; 1,3,4-eugenol methyl ether; eugenyl methyl ether; methyl eugenol; *O*-methyleugenol (NCBI, 2022).

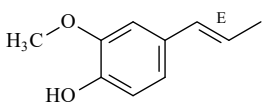
1.1.2 Structural and molecular information

Relative molecular mass: 178.23 (Chemical Abstracts Service, 2022a)

Chemical structure: (Chemical Abstracts Service, 2022a)



Z-(*cis*-) isomer



E-(*trans*-) isomer

Molecular formula: $C_{11}H_{14}O_2$ (Chemical Abstracts Service, 2022a).

1.1.3 Chemical and physical properties

Description: colourless to pale yellow liquid with a clove–carnation odour and a bitter taste (NTP, 2000; Burdock, 2010)

Odour threshold: 820 ppb [µg/L] in water (Ohloff, 1978)

Boiling-point: 254.7 °C (Haynes, 2017)

Melting-point: –2 °C (Haynes, 2017)

Density: 1.0396 g/cm^3 at 20 °C (Haynes, 2017)

Solubility: soluble in ethanol, ethyl ether, chloroform and most other organic solvents; insoluble in water, ethylene glycol, and propylene glycol (NTP, 2000)

Vapour density: > 1.0, relative to air (NCBI, 2022)

Flash-point: 99 °C (NCBI, 2022)

Volatility: evaporates readily at room temperature (NTP, 2000)

Vapour pressure: 0.01 mm Hg (NCBI, 2022)

Auto-ignition temperature: 360 °C at 91 kPa (ECHA, 2022)

Explosion limit: non-explosive (ECHA, 2022)

Octanol/water partition coefficient (P): log K_{ow} = 3.45 (Griffin et al., 1999)

Stability and reactivity: darkens and slowly thickens when exposed to air (NTP, 2000).

1.1.4 Commercial products and impurities

Commercial qualities with purities in the range of 90–94%, 95–98%, and ≥ 99% are available (Chemical Abstracts Service, 2022b). Eugenol is a common impurity with a maximum of 1.0%. (Elan Chemical Co., 2007). β-Caryophyllene (0.68%) and α-humulene (0.08%) were reported as major impurities when eugenol from clove leaf oil was used to produce methyleugenol (Riyanto et al., 2016).

1.2 Production and use

1.2.1 Production process

Methyleugenol is produced by the methylation of eugenol (Burdock, 2010). For example, eugenol may be isolated from leaf oil of *Syzygium aromaticum* (clove), followed by methylation using dimethyl sulfate (Riyanto et al., 2016; Kurniawan et al., 2017). The methylation can be achieved using a one-pot synthesis strategy followed by vacuum fractionation (Agustian et al., 2019). Methyleugenol may also be directly extracted from a variety of plant materials by steam distillation or with organic solvents (Environment Canada, 2010). [The Working Group was unable to find information about which process is currently preferred to produce methyleugenol.]

1.2.2 Production volume

The annual production of methyleugenol in the USA in 1990 was estimated at 11.4 tonnes (NTP, 2000). According to information from 2022, methyleugenol is manufactured in and/or imported to the European Economic Area at a volume of ≥ 1 to < 10 tonnes per annum (ECHA, 2022).

1.2.3 Uses

(a) Direct uses of the isolated compound

Methyleugenol has been used for its delicate clove–carnation odour as a flavouring agent in jellies, baked goods, non-alcoholic beverages, chewing gum, candy, puddings, relishes, and ice cream (Burdock, 2010; IARC, 2013). The presence of methyleugenol in food products may have decreased substantially after the prohibition of its use as such in foods and beverages in the European Union (EU) in 2008 and in the USA in 2018 (see Section 1.5).

Methyleugenol has also been widely used as a fragrance ingredient in various consumer products (NTP, 2000), e.g. in carnation and lilac compositions (Panten and Surburg, 2016). The Consumer Products Information Database (CIPD) lists the use of methyleugenol in six products, including air and fabric fresheners, home pest control products, and automobile cleaners and fresheners (CPID, 2022). According to the Substances in Preparations in Nordic Countries (SPIN) database (SPIN, 2023), four methyleugenol preparations were used in 2000 in the sale, maintenance, and repair of motor vehicles and motorcycles, and in the retail sale of automotive fuel. In 2020, methyleugenol was reported to be present in four cleaning/washing agents (SPIN, 2023).

Methyleugenol is classified as a parapheromone (Australian Government, 2005), and it is used as an insect attractant to male fruit flies in combination with insecticides (NTP, 2000;

NCBI, 2011; Metcalf and Horowitz, 2014a). It is very strongly attractive for the oriental fruit fly, *Bactrocera dorsalis*, and many closely related species (Metcalf and Horowitz, 2014b). In male annihilation programmes for *Bactrocera* species, fibreboard blocks are treated with methyleugenol (the volatile attractant, 15 g/ha) and contact insecticides such as malathion or naled (1 g/ha) (Metcalf and Horowitz, 2014b). Methyleugenol has been used for this purpose in programmes to eradicate the oriental fruit fly in the USA since the 1980s (Turner et al., 1989; US EPA, 2006).

In veterinary medicine, methyleugenol has been used as an anaesthetic agent in rats and mice undergoing surgical procedures (NCBI, 2022).

[The Working Group noted that the literature is often unclear as to whether methyleugenol is used as described above directly or indirectly, due to the use of various plant materials and essential oils that contain it.]

(b) Indirect uses due to natural occurrence in various plant species

Most of the present use of methyleugenol in foods, beverages, or herbal medicinal products may occur inadvertently because of its natural occurrence in essential oils of various herbs and spices (Grosch et al., 2013) (see Section 1.4).

In intensive animal production, laurel leaf oil can be used as a feed additive for fattening piglets, poultry, or fin fish (EFSA, 2023).

1.3 Detection and analysis

Methods for the extraction and quantitation of methyleugenol have been reviewed (Turner and Shuker, 2006; Dang and Quirino, 2021a). Samples are homogenized, and methyleugenol is separated using various extraction techniques, including solvent extraction including liquid–liquid extraction, liquid-phase microextraction, solid-phase extraction, and supercritical fluid extraction. Extraction efficiency can be increased by the use of ultrasound or microwaves. Distillation (steam or hydro-) is commonly used for the extraction of methyleugenol from spices and herbal drugs. Sample preparation techniques, methods of analysis, and the limits of detection (LODs) from a selection of recent studies are presented in Table 1.1.

1.3.1 Air

Gas chromatography-mass spectrometry (GC-MS) has been used to quantify methyleugenol in indoor air (Lamas et al., 2010a) and tobacco smoke (Stanfill and Ashley, 2000) with sample collection using absorbents or filters, respectively.

1.3.2 Water

Methyleugenol in water can be measured using liquid chromatography (LC) (Shaver and Bull, 1980) and in wastewater effluent using liquid chromatography-mass spectrometry (LC-MS) (Clark et al., 1991).

1.3.3 Food

GC-MS (Lopez et al., 2015) or gas chromatography-tandem mass spectrometry (GC-MS/MS) have been used for the quantification of methyleugenol in foods and beverages (Bousova et al., 2011; Ke et al., 2016; Li et al., 2018). A GC-MS method was compared with a nuclear magnetic resonance (NMR)-based method that involves only minimal sample preparation for the measurement of methyleugenol in food. GC-MS proved more sensitive, precise, and suitable for regulatory purposes but was time-consuming and expensive. NMR can be used for a faster screening analysis and appears to be ideal for conducting larger surveys to estimate human exposure (Grosch et al., 2013). [The Working Group noted that NMR would not detect very low levels (LOD, 2.1 mg/L), which in food analysis is

Table 1.1 Analytical methods for the measurement of methyleugenol in various matrices

Sample matrix	Sample preparation	Instrument (LOD)	Comments	Reference
Air				
Indoor air	Vacuum pump; activated magnesium silicate (Florisil) adsorbent in glass tube; ultrasound-assisted solvent extraction	GC-MS (0.027 µg/m^3)		Lamas et al. (2010a)
Tobacco smoke particulate	Smoking machine; glass fibre (Cambridge) filter; hexane/MDAP extraction, SPE	SIM-GC-MS (5.1 ng/cigarette)		Stanfill and Ashley (2000)
Water				
Water	Dichloromethane extraction	GC, HPLC (NR)		Shaver and Bull (1980)
Water	No pre-treatment	EFM-based LFIA (16.5 µg/kg)		Lei et al. (2023)
Wastewater effluent	Liquid–liquid (methylene chloride) extraction	LC-MS and GC-MS (NR)		Clark et al. (1991)
Food				
Allspice	Powder hydrodistillated, essential oil diluted in dichloromethane; head-space SPME	GC-MS (NR)		Bajer et al. (2016)
Cinnamon	Ultrasound extraction in methanol	HPLC-UV (0.10 µg/cm^3)		Gursale et al. (2010)
Pepper	Ultrasound extraction with ethyl acetate (SLE)	GC-HRMS-Q-Orbitrap (10 µg/kg)	Validated for black pepper according to SANTE 11813/2017 guidelines.	Rivera-Pérez et al. (2020); European Commission (2017)
Beverages	Head-space SPME	GC-MS/MS (1 µg/kg)		Bousova et al. (2011)
Beverages	Ethyl acetate extraction with $MgSO_4$ and NaCl	GC-MS (62.5 µg/kg)		Lopez et al. (2015)
Beverages	Ultrasound extraction in ethyl acetate with $MgSO_4$ and NaCl, clean-up with PSA	SIDA-GC-MS/MS (0.4 µg/kg)		Li et al. (2018)
Rose water	Liquid–liquid extraction: ultrasound extraction in dichloromethane; SPE: polystyrene-based sorbent and dichloromethane extraction or silica-based sorbent and methanol extraction	GC-MS (0.780 µg/L)		Canbay (2017)
Flavouring ingredients	Ultrasound extraction in methanol	HPLC-UV (0.03 µg/mL)		Dang and Quirino (2021b)
Foods, solid	Homogenization in blender; head-space SPME	GC-MS/MS (10 µg/kg)		Bousova et al. (2011)

Table 1.1 (continued)

Sample matrix	Sample preparation	Instrument (LOD)	Comments	Reference
Food	Homogenized in 60% ethanol	^{1}H NMR (2.1 mg/L)		Grosch et al. (2013)
Food	Homogenization using liquid nitrogen; ethyl acetate extraction with $MgSO_4$ and NaCl, clean-up with PSA	GC-MS (500 µg/kg)		Lopez et al. (2015)
Fish fillet	Homogenization, ultrasound extraction in hexane, SPE with ethyl acetate	GC-MS/MS (0.2 µg/kg)		Ke et al. (2016)
Fish and meat	Homogenization, ultrasound extraction in ethyl acetate with $MgSO_4$ and NaCl, clean-up with PSA	SIDA-GC-MS/MS (20 µg/kg)		Li et al. (2018)
Fish	Homogenization, extraction by acetonitrile, SPE, polypropylene sorbent	UPLC-MS/MS (0.03 µg/kg[a])		Liu et al. (2019b)
Fish	Homogenization, ultrasound extraction with acetonitrile, air drying at room temperature, dissolved in methanol solution	EFM-based LFIA (16.7 µg/kg)		Lei et al. (2023)
Fish	Ultrasound extraction in acetonitrile and Na_2SO_4, a second extraction with acetonitrile; degreasing by *n*-hexane; concentration by pressure blowing, dissolved in methanol solution	Electrochemical detection. Pt NPs@RL-SiO_2/GCE (0.16 µmol/L)		Shi et al. (2021)
Fish, shrimp	Ultrasound extraction in acetonitrile; DSPE with polystyrene-glycidylmethacrylate microspheres (PS-GMA), clean-up with PSA and C18; DMSO-assisted concentration	HPLC-UV (43 µg/kg)		Shi et al. (2022)
Fish, shrimp	Fluorinated covalent organic polymer adsorbent, SPME with acetonitrile	HPLC-UV-vis (3.3 µg/kg)		Wang et al. (2021a)
Herbal medicines				
Manchurian wildginger (*Asarum* spp)	Powderization, ultrasound extraction in methanol	HPLC-UV-Vis (2.17 µg/g)		Chen et al. (2009)
Mosla soochowensis	Supercritical fluid extraction with carbon dioxide	GC-MS (NR)		Chen and Wu (2005)
Uncaria hook (*Uncaria rhynchophylla*)	Hydrodistillation	GC-MS (NR)		Iwasa et al. (2015)
Aniba canelilla	Hydrodistillation, head-space SPME	GC-FID/MS (0.16 µg/mL)		Kreutz et al. (2018)
Pimenta pseudocaryophyllus	Hydrodistillation	HPLC UV-Vis (NR)		Niculau et al. (2018)
Ocimum gratissimum, Ocium campechianum	Microwave-assisted hydrodistillation	GC-MS (NR)		Pino Benitez et al. (2009)
Tea tree oil	Mixing with *n*-hexane and *n*-tetradecane	GC-MS (150 µg/L)		Raymond et al. (2017)

Table 1.1 (continued)

Sample matrix	Sample preparation	Instrument (LOD)	Comments	Reference
Acori tatarinowii rhizome essential oil	Hydrodistillation	GC-MS (NR)		Yan et al. (2020)
Asarum oils	Distill/steam dried plants to obtain volatile oil; desiccate oil using anhydrous sodium sulfate	GC-MS (NR)		Yang (1986); Xu, (1984, 1986)
Asarum herbs	Hydrodistillation	GC-MS (0.01 µg/mL, LOQ)		Yao et al. (2020)
Kaixin San	Powderization, head-space, no pre-treatment Crushing in grinder, extraction by heating with petroleum ether	GC-IMS (NR) GC-MS (0.02 µg/mL, LOQ)		Yin et al. (2021)
Mahuang Fuzi Xixin	Powderization, microwave extraction in methanol	UPLC-PDA (0.62 µg/mL)		Zhang et al. (2015)
Consumer products				
Cigarette tobacco	SPME	GC-MS (0.0022 µg/g)		Stanfill and Ashley (1999)
Aromatherapy massage oil	Dual dispersive liquid–liquid microextraction	GC-MS (3.0 ng/mL)		Tsai et al. (2015)
Cosmetics				
Creams, lotions	Solid-phase dispersion-pressurized liquid extraction	GC-MS [12 µg/kg]		Lamas et al. (2010b)
Creams	Direct contact sorptive tape extraction	GC-MS (15 µg/kg)		Sgorbini et al. (2010)
Human biological specimens				
Serum	SPE	IDGC-MS (3.1 pg/g)		Barr et al. (2000)
Serum	SPE	GC-HRMS (3.1 pg/g)		Schecter et al. (2004)

DMSO, dimethyl sulfoxide; DSPE, dispersive solid-phase extraction; EFM, europium-fluorescent microspheres; GC-MS, gas chromatography-mass spectrometry; GCE, glassy carbon electrode; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; IDGC, isotope dilution gas chromatography; IMS, ion mobility spectrometry; LFIA, lateral-flow immunoassay; LOD, limit of detection; MDAP, 3′,4′-methylenedioxyacetophenone; MS/MS, tandem mass spectrometry; $MgSO_4$, magnesium sulfate; Na_2SO_4, anhydrous sodium sulfate; ^{1}H NMR, proton nuclear magnetic resonance; NR, not reported; PDA, photometric diode array; PSA, primary secondary amine; PS-GMA, polystyrene-glycidylmethacrylate microspheres; RL-SiO_2, raspberry-like silicon dioxide; SIDA, stable isotope dilution assay; SIM, selected ion monitoring; SLE, solid–liquid extraction; SPE, solid-phase extraction; SPME, solid-phase microextraction; UPLC, ultra-high-performance liquid chromatography; UV, ultraviolet.

[a] The LOD for methyleugenol in fish is 0.10 µg/kg (signal:noise ratio, 10). The authors could detect 0.03 µg/kg (signal:noise ratio, 3) in their samples. This could be interpreted as an LOQ of 0.10 µg/kg and LOD of 0.03 µg/kg.

rarely needed, see Section 1.4.] High-performance liquid chromatography with ultraviolet detection (HPLC-UV) was used to quantify methyleugenol in spices or flavouring ingredients (Gursale et al., 2010; Dang and Quirino, 2021b) and in seafood (Wang et al., 2021a; Shi et al., 2022).

Rivera-Pérez et al. (2020) used ultrasound-assisted solvent extraction (UAE) and GC-MS to measure methyleugenol; the analytical method was validated for black pepper according to SANTE/11813/2017 guidelines (European Commission, 2017).

An immunoassay for the determination of methyleugenol was developed recently. The LOD was 16.5 µg/kg in water and 16.7 µg/kg in fish (Lei et al., 2023).

1.3.4 Medicinal herbs or plants

Both GC-MS and HPLC-photo-diode assay have been used for the quantification of methyleugenol in aromatic plants (e.g. Miele et al., 2001; Kothari et al., 2004; Kreutz et al., 2018; Yao et al., 2020), oils (e.g. Verdian-rizi and Hadjiakhoondi, 2008; Zheljazkov et al., 2008; Pino Benitez et al., 2009; Raymond et al., 2017; Yan et al., 2020) and herbal drugs (e.g. Chen et al., 2009; Zhang et al., 2015; Yin et al., 2021).

1.3.5 Consumer products

GC-MS has been used to quantify methyleugenol in cosmetic creams and lotions (Lamas et al., 2010b; Sgorbini et al., 2010), aromatherapy massage oil (Tsai et al., 2015), and cigarette tobacco (Stanfill and Ashley, 1999).

1.3.6 Biological specimens

Methyleugenol can be measured in human serum by solid-phase extraction followed by isotope dilution gas chromatography-high-resolution mass spectrometry (GC-HRMS), with an LOD of 3.1 pg/g (Barr et al., 2000; Schecter et al., 2004).

1.4 Occurrence and exposure

1.4.1 Environmental occurrence

Methyleugenol is a compound that occurs naturally in the essential oils of more than 450 plant species, including *Artemisia dracunculus* (tarragon), *Syzygium aromaticum* (clove), *Daucus carota* (carrot), *Myristica fragrans* (nutmeg), *Rosmarinus officinalis* (rosemary), *Laurus nobilis* (bay leaf or laurel), *Ocimum basilicum* (basil), and *Thymus serpylloides* (thyme) (De Vincenzi et al., 2000; European Medicines Agency, 2005; Burdock, 2010; Government of Canada, 2010; Tan and Nishida, 2012; Eisenreich et al., 2021). The amount of methyleugenol in the essential oils extracted from these plants is variable and depends on several factors, such as the plant variety, maturity at harvest, harvesting method, storage conditions, and extraction method (Smith et al., 2002; Yahyaa et al., 2019). Several species have essential oils that contain more than 90% methyleugenol, for example, *Croton malambo* (Euphorbiaceae), *Cinnamomum cordatum* (Lauraceae), *Melaleuca bracteata*, *M. ericifolia*, *M. leucadendra*, *M. quinquenervia*, *Pimenta racemosa* (all Myrtaceae), *Piper divaricatum* (Piperaceae), and *Clusena anisata* (Rutaceae). In an additional 68 species, the essential oils of either the whole plant or a part of the plant have a methyleugenol content that ranges from 20% to 90% (Tan and Nishida, 2012). For example, the wood oil of the huon pine (*Lagarostrobos franklinii*) grown in Tasmania, Australia, contains 74% methyleugenol (Brophy et al., 2003).

An overview of concentrations of methyleugenol in the essential oils of common fruits and spices is given in Table 1.2.

Methyleugenol occurs naturally in foods and beverages, can be added to foods as a flavouring agent, and occurs in some herbal medicinal products. Other sources include cosmetics and personal care products, insect repellents, and some tobacco and cannabis products. The

Table 1.2 Relative concentrations of methyleugenol in the essential oils of some common fruits and spices

Common name	Botanical name	Concentration (%)
Allspice berries	*Pimenta dioica*	0.1–68
Allspice leaves	*Pimenta dioica*	2–15.4
Anise seeds	*Pimpinella anisum*	0.1–2
Asafetida	*Ferula assa-fetida*	0.03
Basil leaves	*Ocimum basilicum*	< 0.2–87
Black pepper berries	*Piper nigrum*	0.9
Canadian snake root	*Asarum canadense*	11–45.0
Cardamom	*Elettaria cardamomum*	0.1
Clove	*Syzygium aromaticum*	0.03–0.5
Common sage	*Salvia officinalis*	1.45–19.8
Fennel	*Foeniculum vulgare*	0.18–1
Guava fruit	*Psidium guajava*	0.2
Hyssop	*Hyssopus officinalis*	0.01–43.9
Laurel leaves	*Laurus nobilis*	0.02–8.3
Lemongrass	*Cymbopogon flexuosus*	0.05–82.4
Lovage	*Levisticum officinale*	0.001–1.3
Mugwort	*Artemisia vulgaris*	1–7
Nutmeg kernel	*Myristica fragrans*	0.002–17.9
Parsley	*Petroselinum crispum*	0.01–3.8
Peppermint	*Mentha piperita*	0.1
Rhubarb	*Rheum rhabarbarum*	2–7
Rocket/rucola salad	*Eruca sativa*	0.9
Rose	*Rosa (various species)*	0.04–6.9
Rosemary	*Rosmarinus officinalis*	< 0.01–1.5
Chinese star anise seeds	*Illicium verum*	0.11–0.4
Oregano	*Origanum vulgare*	16.5
Tarragon	*Artemisia dracunculus*	0.03–38
Thyme	*Thymus vulgaris*	0.1–0.2

Compiled by the Working Group using data from Eisenreich et al. (2021); De Vincenzi et al. (2000); Tan and Nishida (2012); Davidsen et al. (2023a); Rosol et al. (2023); Rietjens et al. (2023); Davidsen et al. (2023b); Environment Canada (2010); and Zhao et al. (2021).

production and use of methyleugenol can result in its release to the environment through various waste streams, and its use as an insect attractant is expected to result in its direct release to the environment (NCBI, 2011).

(a) Air

Methyleugenol is expected to exist almost exclusively as a vapour in the ambient atmosphere (NCBI, 2011). Vapour-phase methyleugenol is degraded in the atmosphere by reaction with photochemically generated hydroxyl radicals (Meylan and Howard, 1993). The atmospheric half-life has been estimated at between 5 hours and 1 day (Atkinson and Carter, 1984; Meylan and Howard, 1993). Methyleugenol lacks functional groups susceptible to hydrolysis and is therefore not expected to be hydrolysed in the environment (Lyman, 1990), nor is it expected to be directly photolysed, because of its lack of absorption in the environmental UV spectrum (> 290 nm) (NCBI, 2011).

In the context of the use of methyleugenol in bait stations and lures during invasive pest con-

trol programme activities in California, USA, a screening-level air dispersion model estimated a maximum 1-hour air concentration of 0.052 µg/m³ to which the average resident living near the treated area is probably exposed (OEHHA, 2018). In Los Angeles County, California, USA, the air around insect traps baited with methyleugenol was analysed for the presence of the substance; methyleugenol was found in samples taken from within a distance of 5 m from the traps during the first few days (323–1050 ng/m³ on the day of application) (Turner et al., 1989).

(b) Water

Methyleugenol has been detected at a concentration of 5 ppb [0.005 mg/L] in the effluent of a publicly owned treatment works in New Jersey, USA, located at an industrial site (industrial contribution to the influent was 18%) (Clark et al., 1991), and at concentrations of 0.001–0.002 mg/L in the raw and partially treated effluent of an unbleached kraft paper mill, but not in the final effluent (Keith, 1976). Methyleugenol was found to dissipate rapidly from water. Methyleugenol had a half-life of approximately 6 hours in water at 32 °C and 34 hours in water at 22 °C (Shaver and Bull, 1980). Its potential for bioconcentration in aquatic organisms is low, and biodegradation in water may be an important environmental process (NCBI, 2011).

(c) Soil

On the basis of its physical and chemical properties (see Section 1.1), methyleugenol is not expected to adsorb to suspended solids and sediment and is expected to be highly mobile in soil. However, it was immobile in silty loam, Lufkin fine sandy loam, Houston clay, and Brazos river bottom sand from Texas, USA (Shaver, 1984). Volatilization of methyleugenol from moist soil surfaces is expected to be an important process (NCBI, 2011). Methyleugenol had a half-life of approximately 6 hours in soil at 32 °C and 16 hours in soil at 22 °C (Shaver and Bull, 1980). Methyleugenol is not expected to volatilize from dry soil surfaces on the basis of its vapour pressure (Perry and Green, 1984, cited by NCBI, 2011). Biodegradation may be an important environmental process in soil (NCBI, 2011). [The Working Group noted that limited data were available to support or refute the theoretical assumptions about the fate of the compound in soil. Some of the information available appeared contradictory (e.g. methyleugenol volatilizes from moist but not from dry soil) and in need of experimental validation.]

(d) Consumer products

Some essential oils, including citronella (*Cymbopogon* spp.), basil (*Ocimum* spp.), bay (*Laurus nobilis*), and tea tree (*Melaleuca* spp.), that may contain a high percentage of methyleugenol are used as fragrances in consumer products such as personal care and household cleaning products (Environment Canada, 2010). Citronella oil is an active ingredient in some commercially available personal insect-repellent lotions and sprays that are applied to the skin. It is also used in outdoor candles and torches as an ambient insect repellent (Environment Canada, 2010).

The usual and maximum concentrations of methyleugenol in some cosmetic products were 0.02% and 0.2% in soap, 0.002% and 0.02% in detergents, 0.01% and 0.05% in creams and lotions, and 0.3% and 0.8% in perfumes, respectively (Opdyke, 1975).

In a study of eight commercial brands of cigarettes in the USA, only one brand was found to contain methyleugenol at above the LOD (5.1 ng/cigarette) in the smoke particles of unblocked cigarettes (average of three measurements, 46.5 ng in the particulate matter of one cigarette) (Stanfill and Ashley, 2000). The effect of blocking the ventilation holes in the cigarette filter was investigated in another brand (containing methyleugenol at 81 ng/cigarette). Methyleugenol was not detected in the unblocked

cigarette smoke but was detected in the smoke when the holes were partially or completely blocked (6.4 ng and 10.8 ng in the smoke particulate of one cigarette, respectively).

Bidi cigarettes (small hand-rolled cigarettes produced mainly in India) have been sold in the USA in a wide variety of exotic (e.g. clove and mango) and candy-like flavours (e.g. raspberry, dewberry, and chocolate). Some of these tobacco flavourings contain alkenylbenzenes, including methyleugenol (Stanfill et al., 2003, 2006). Methyleugenol was found in 11 out of 20 bidi cigarette brands purchased in the USA and in Indian bidi cigarettes at levels ranging from 0.49 µg/g to 61 µg/g. Lower levels of methyleugenol, ranging from 0.003 to 0.86 µg/g, were found in US cigarettes (Stanfill et al., 2003).

In a study by Stanfill et al. (2006), compounds were analysed in the combustible parts of the filler and wrapper material consumed during smoking of bidi cigarettes and US cigarettes. Methyleugenol was not detected in the three US cigarettes (< 6.3 µg/cigarette) but was detected in two bidi cigarettes (≤ 36.6 µg/cigarette).

The volatile oil of *Cannabis sativa* may contain approximately 0.1–0.2% methyleugenol (Wanas et al., 2020; Pieracci et al., 2021).

(e) Food

Some of the plant species containing methyleugenol are culinary herbs and spices, e.g. basil, tarragon, lemongrass, bay leaf, nutmeg, allspice, cloves, and mace (Siano et al., 2003; Ávila et al., 2009; WHO, 2009; Environment Canada, 2010; Sharopov et al., 2016; Złotek et al., 2016; Rietjens et al., 2023; Davidsen et al., 2023a). Additionally, some edible fruits, such as grapefruit and bananas also contain methyleugenol (Smith et al., 2002).

Commercially prepared foods could also contain methyleugenol, including ice cream; bakery products such as cookies, pies, pastries, and buns; puddings and other gelatin-based desserts; condiments, soups and sauces, especially pesto; various meat products; candy and chewing gum; and beverages prepared with spices and herbs containing methyleugenol (Environment Canada, 2010). An overview of methyleugenol in foods is given in Table 1.3.

(i) Historical occurrence (before changes in regulations)

Historically, the pure compound has been reported to be added as a flavouring agent to bakery products (27–40 mg/kg), chewing gum (10–45 mg/kg), condiments and relishes (3–7 mg/kg), frozen dairy products (15–17 mg/kg), gelatins and puddings (15–17 mg/kg), hard candy (0.6 mg/kg), non-alcoholic beverages (9–12 mg/kg), and soft candy (19–24 mg/kg) (Burdock, 2010).

Some brands of cookies available in the USA were found to contain methyleugenol at approximately 3.3 mg/kg as an added flavouring, i.e. 18 µg/cookie. Lower concentrations were found (in decreasing order) in other brands of gingersnaps, cinnamon-flavoured oatmeal, vinaigrette salad dressing, cinnamon-flavoured mints, chewing gum, cake doughnuts, and cola beverages. In 20 other brands of gingersnaps and other cookies, doughnuts, colas, and foods flavoured with cinnamon, nutmeg, or ginger, methyleugenol was either not detected or was found at concentrations of < 0.05 mg/kg (Schecter et al., 2004).

[The Working Group noted that these levels are no longer to be expected in foods because of changes in the regulation of use of methyleugenol as a food flavouring (see Section 1.5 for details).]

(ii) Occurrence not affected by regulations and current occurrence (after changes in regulations)

Processed foods can be flavoured with essential oils or extracts of specific plants that contain methyleugenol, such as sassafras (*Sassafras albidum*), tarragon (*Artemisia dracunculus* L.), laurel (*Laurus nobilis* L.), and Ceylon citronella (*Cymbopogon nardus*) (Burdock, 2010).

Table 1.3 Concentrations of methyleugenol in selected food products

Food product	Methyleugenol concentration (mg/kg)
Vienna sausage	0.10–0.14
Tomato sauce with basil	0.01–0.33
Hard candy*	0.6**
Gingersnaps (cookies)*	3.3
Pesto sauce	0.01–5.3
Condiments and relishes*	3–7**
Non-alcoholic beverages*	0.03–12
Frozen dairy products*	15–17**
Gelatins and puddings*	15–17**
Soft candy*	19–24**
Bakery products*	27–40**
Chewing gum*	10–45**
Indonesian instant herbal beverages	3–444***

* Historical use in flavoured products.
** "Usual" and "max." (maximum) values.
*** Range for detected samples. Detection rate was 43%.
Compiled by the Working Group using data from Siano et al. (2003); Schecter et al. (2004); Burdock (2010); Grosch et al. (2013); and Suparmi et al. (2019).

Methyleugenol was measured in food products purchased on the Italian national market and found to be present in tomato sauce with basil (0.01–0.33 mg/kg) and in Vienna sausage (0.10–0.14 mg/kg), probably due to the addition of nutmeg (Siano et al., 2003). In a survey of 120 German food products suspected of containing methyleugenol, the highest levels were found in basil, allspice, and nutmeg (mean, 202 mg/kg for basil, 1351 mg/kg for nutmeg and 4288 mg/kg for allspice), with lower mean levels in tarragon and laurel leaves (56 mg/kg and 26 mg/kg, respectively). Other products such as teas, beverages, and compound foods contained mean levels of around 1 mg/kg and below (Grosch et al., 2013). In a survey of 114 herbal beverages suspected to contain methyleugenol in Indonesia, methyleugenol was detected in 49 samples (2.6–444 mg/kg) (Suparmi et al., 2019).

Whole citrus fruit samples were collected near insect traps baited with methyleugenol in Sacramento County, California, USA, in September 1988. Methyleugenol was detected at concentrations ranging from 70 to 210 ppb [µg/kg] in some fruit from two of the four sites sampled (Turner et al., 1989).

(f) Herbal medicinal products

The WHO monographs on selected medicinal plants describe the use of Folium Ocimi Sancti (fresh or dried leaves of *Ocimum sanctum* L.), which contains methyleugenol as a major constituent (up to 86%) of its essential oil, in traditional systems of medicine and in folk medicine (WHO, 2002). Similarly, Xixin (Asari radix et rhizoma) containing methyleugenol (Asari radix up to 39% in its essential oil), has been used in traditional Chinese medicine as a local anaesthetic and a remedy for toothache, headache, and inflammatory diseases (Wang et al., 1997; Wang et al., 2015). Fennel fruits, possibly containing methyleugenol as a minor constituent (up to 1%) of the essential oil, have been used as a traditional herbal medicine in Europe and China. Fennel tea is administered as a carminative to infants in private homes and in maternity clinics and is highly appreciated for its mild flavour and good tolerance (Iten and Saller, 2004). In several

EU countries, sweet and bitter fennel herbal tea is traditionally used for treating the symptoms of digestive upsets and as a remedy for cough associated with colds (European Medicines Agency, 2008).

Methyleugenol is a component of several essential oils that are sold for use as complementary medicines (Government of Canada, 2010).

1.4.2 Occupational exposure

In the context of use in oriental fruit fly eradication programmes, the United States Environmental Protection Agency (US EPA) states that workers using these insect traps are not expected to have dermal contact with the traps, although dermal contact could occur during preparation of the traps, e.g. preparation of the methyleugenol mixture or filling of the traps with the mixture (US EPA, 2006).

In the USA, the National Institute for Occupational Safety and Health (NIOSH) conducted a National Occupational Exposure Survey (NOES) in 1981–1983. NIOSH randomly selected 4490 fixed-site worksites in 522 different industries employing about 1 800 000 workers in 377 occupational categories. NOES revealed that 12 682 [95% confidence intervals (95% CI), 7402–18 262] employees, including 9413 [95% CI, 4895–13 931] women (74%), were potentially exposed to methyleugenol (NIOSH, 1990a, b, 1994), including 9429 [74% of total] hairdressers or cosmetologists, 967 [8%] machine operators, 827 [7%] packaging and filling machine operators, and 595 [5%] mixing and blending machine operators. [The Working Group noted that these data included all potentially exposed workers and were estimated in the 1980s.] Aromatherapists (professionals who use essential oils for body massages) are liable to be exposed repeatedly to methyleugenol through dermal contact and also through inhalation of vapour (Burfield, 2004).

[The Working Group noted the lack of comprehensive exposure data in an occupational context. Despite lacking exposure data, the Working Group also noted that occupational exposure to methyleugenol by dermal or inhalation routes may occur through the production or use of foods, cosmetics, and insect attractants.]

1.4.3 Exposure of the general population

(a) Exposure data

The average intake of methyleugenol was assessed by the United Kingdom delegation to the Council of Europe and was estimated (for consumers only) to be 13 mg/person per day; the 97.5th percentile was 36 mg/person per day or, expressed on a body-weight basis, 0.19 and 0.53 mg/kg body weight (bw) per day, respectively (Council of Europe, Committee of Experts on Flavouring Substances, 2001; European Commission, 2001). In the USA, Smith et al. (2002) estimated the mean daily per capita intake of methyleugenol from all sources to be approximately 0.8 μg/kg bw per day, with more than 85% resulting from the consumption of basil, allspice, and nutmeg, and their essential oils (Smith et al., 2002). The same authors also highlighted that consumers of some foods containing methyleugenol, such as pesto, could have exposures to methyleugenol that were least 10 times higher, since fresh pesto is prepared from a large quantity of fresh sweet basil (Smith et al., 2002). Burdock (2010) estimated individual exposure to methyleugenol at 0.12 μg/kg bw per day. Miele et al. (2001) estimated intake of methyleugenol from a single serving of pasta with pesto and concluded that it could reach 250 μg/kg [bw] per meal for adults and 500 μg/kg [bw] per meal for children (Miele et al., 2001). Recently, a series of assessments developed under the Flavor and Extract Manufacturers Association of the United States (FEMA) "generally recognized as safe" (GRAS) programme were used to estimate intakes of methyleugenol from the consumption of natural flavouring complexes. Depending on the flavouring ingredients

considered, the intakes were estimated as: for derivatives of basil, nutmeg, parsley, tarragon, and mace, 0.4, 3, 0.01, 0.7, and 0.1 µg/person per day, respectively (Davidsen et al., 2023a); for lemongrass oil, chamomile oils, and citronella oil, 0.0005–0.04 µg/person per day (Rosol et al., 2023); for allspice and anise, fennel-derived, 0.1–0.25 µg/kg bw per day (Rietjens et al., 2023); and for asafetida oil, 0.0007 µg/person per day (Davidsen et al., 2023b).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) also estimated the maximum dietary intake of methyleugenol in the USA, finding an exposure of 424 µg/person per day or about 6–8 µg/kg bw per day for an adult (WHO, 2009).

Levels of exposure to methyleugenol through consumption of instant herbal beverages available on the market in Indonesia were assessed and the estimated daily intake was found to be 0.1–51.2 µg/kg bw per day for adults and 1.1–3.3 µg/kg bw per day for children (Suparmi et al., 2019). Specifically, the consumption of *jamu*, an Indonesian traditional herbal medicine, was estimated to lead to an exposure to methyleugenol equivalents of 0.9–551 µg/kg bw per day (Suparmi et al., 2018). Van den Berg et al. (2011) assessed exposure to methyleugenol from the consumption of different plant food supplements of botanicals and botanical preparations. Possible exposure to methyleugenol was estimated to be in the range of 0.02 to 2960 µg/kg bw per day (Van den Berg et al., 2011). On the basis of an analysis of 120 food products from Germany, exposure was less than 1 µg/kg bw per day for the average population of Germany (Grosch et al., 2013).

Another source of exposure to methyleugenol is the use of personal care products that include essential oils containing methyleugenol. Environment Canada (2010) estimated that daily systemic exposure to methyleugenol in adult women was 1.5 µg/kg bw per day as a result of dermal exposure through the use of body lotion, face moisturizer, skin cleanser, and fragrance including various essential oils containing methyleugenol (Environment Canada, 2010).

On the basis of an assessment by Health Canada (Health Canada, 2004a), exposure to methyleugenol from the use of a personal citronella-based insect repellent has been estimated to be 0.13 µg/kg bw for adults and 0.21 µg/kg bw for children (IARC, 2013).

In conclusion, exposure of the general population to methyleugenol is dominated by the ingestion of food and beverages. In addition to dietary exposure, the use of personal care products containing methyleugenol, as well as insect repellent, represent additional sources of exposure for the general population. [The Working Group noted that the estimations obtained present significant sources of uncertainty, mainly because of the lack of exposure data. Additionally, new regulations concerning the addition of pure methyleugenol to foods might have an impact on the levels reported. For the general population, a daily exposure of low micrograms per kg bw is expected. For some high-level consumers of certain methyleugenol-rich foods, considerably higher levels of exposure of up to milligrams per kg bw are expected. Although exposure through inhalation is expected, no data are presently available.]

(b) Biomonitoring

Few data on biomonitoring in humans are available to determine the exposure of the general population to methyleugenol. The Centers for Disease Control and Prevention in the USA measured methyleugenol levels in serum samples collected during the Third National Health and Nutrition Examination Survey (NHANES III, 1988–1994) in a non-representative subset of 206 adults. The results revealed a mean serum methyleugenol concentration of 24 pg/g serum (whole weight), and a range of < 3.1 to 390 pg/g serum (whole weight) (Barr et al., 2000). Methyleugenol was detected in 98% of the samples analysed,

demonstrating that exposure to methyleugenol is ubiquitous in the USA (Barr et al., 2000). Smokers (geometric mean, GM, 17 pg/g for smokers or living with 1 smoker, or 19 pg/g for smokers or living with ≥ 2 smokers) had higher levels of methyleugenol than did non-smokers (GM, 5.6 pg/g for non-smokers with no smokers in the home) (Barr et al., 2000). [The Working Group noted that the report by Barr et al. (2000) did not clearly describe whether the two smoker populations were partly overlapping.] The highest serum levels of methyleugenol were dependent on factors such as diet, genetics, and body weight. It has been noted that because methyleugenol is found in air, water, food and beverages, and oils, day-to-day variations in exposure could have an effect on serum levels of methyleugenol (Albertini et al., 2006). [The Working Group noted that methyleugenol is rapidly metabolized, thus sampling strategy and analytical methods affect the human biomonitoring results for this substance (for more detail about absorption, distribution, metabolism, and excretion, see Section 4.1) (Albertini et al., 2006). For this reason, information on sampling strategies is crucial for the informativeness of biomonitoring studies.]

1.5 Regulations and guidelines

The available regulations for methyleugenol have evolved, mainly due to re-evaluations of its hazard classification (e.g. the previous evaluation by the *IARC Monographs* programme as *possibly carcinogenic to humans*, Group 2B) (IARC, 2013; US FDA, 2004).

In the USA, methyleugenol was classified as GRAS as a food additive by the United States Food and Drug Administration (US FDA) under 21 CFR §172.515 (US FDA, 2004), and its addition as a synthetic flavouring substance was permitted (US FDA, 2010). However, in 2018, the US FDA withdrew its authorization for the use of methyleugenol as a synthetic flavouring substance in foods as a result of a petition submitted by various health, environmental, and consumer rights organizations (US FDA, 2018a). To allow food companies to reformulate their products to remove methyleugenol content, the US FDA set October 2020 as the deadline after which this change in regulations was enforced (US FDA, 2018b).

In the EU, according to the Scientific Committee on Food of the European Commission, and Annex III of Regulation (EC) No. 1334/2008 (European Commission, 2008b), it is not permitted to add methyleugenol as such to foods for flavouring purposes. This regulation entered into force in January 2009 and has applied since January 2011. Furthermore, maximum levels of methyleugenol, (which occurs naturally in certain foods with flavouring properties) were established for specific food groups, i.e. dairy products, 20 mg/kg; meat preparations and meat products, including poultry and game, 15 mg/kg; fish preparations and fish products, 10 mg/kg; soups and sauces, 60 mg/kg; ready-to-eat savouries, 20 mg/kg; and non-alcoholic beverages, 1 mg/kg. It must be noted that these maximum levels do not apply for compound foods that contain no added flavourings and to which the only food ingredients with flavouring properties that have been added are fresh, dried, or frozen herbs and spices. As an example, pesto made with basil is permitted in food preparations, regardless of its methyleugenol content.

The International Organization for Standardization (ISO) has provided international standards for minimum and maximum percentages of methyleugenol in essential oils from various plant species; these oils are widely used in the food and perfumery industries (ISO, 2023; Table 1.4). [The Working Group noted that ISO values that include minimum and maximum requirements for methyleugenol content are used for product standardization and are not health-based values.]

Table 1.4 International standards regarding methyleugenol content in various plant essential oils

Common name	Botanical name	Methyleugenol content (% in essential oil)		ISO Standard No.
		Minimum	Maximum	
Tarragon leaves	*Artemisia dracunculus* L.	ND	< 1.0	10115:2013
Dehydrated tarragon (leaves and flowering tops)	*Artemisia dracunculus* L.	Main constituent[a]		7926:1991
Basil leaves	*Ocimum basilicum* L.	0.3	2.5	11043:1998
Magnolia flower, China type	*Michelia* × *alba* DC	1.2	4.4	17382:2007
Bay leaves	*Pimenta racemosa* (Mill.) JW Moore	0.1	2.0	3045:2004
Rose flowers	*Rosa* × *damascena* Miller	Not reported[a]		9842:2003

ISO, International Organization for Standardization; ND, not determined.
[a] No quantitative range provided in standard.
All ISO standards from ISO (2023).

Regarding the use of methyleugenol in cosmetics, the European Commission Directive 2002/34/EC included methyleugenol in the list of substances that cosmetic products must not contain (Annex II of Council Directive 76/768/EEC), except for normal content in the natural essences used and provided the concentration does not exceed 0.01% in fine fragrance; 0.004% in eau de toilette; 0.002% in fragrance cream; 0.0002% in other leave-on products and in oral hygiene products; and 0.001% in rinse-off products (European Commission, 2002). The same approach has been taken in Canada (Health Canada, 2010).

For insect repellents containing citronella oil, Health Canada's Pest Management Regulatory Agency (PMRA) proposed a phase-out of citronella-based personal insect repellents, based on the re-evaluation of available information on these products, including the fact that these products typically contain methyleugenol (Health Canada, 2004b). Subsequently, the PMRA re-evaluated the associated risks and established guidelines for the registration of nonconventional pest control products, noting that the level of methyleugenol in the final product must be less than 0.0002% (2 ppm) (Health Canada, 2017).

For the use of the technical product of citronella on non-food crops to control for ragwort, the European Commission has specified that the product must contain no more than 0.1% of the manufacturing impurities methyleugenol and (structurally related) methylisoeugenol (European Commission, 2008a). In Australia and the USA, the use of methyleugenol in insect traps and lure products as an insect attractant is permitted as a measure in eradication programmes (Australian Government, 2005; US EPA, 2010).

According to European Standard EN 17648 regarding liquids for electronic cigarettes (e-liquids), the maximum level of methyleugenol in the finished e-liquid is 1 mg/kg (CEN, 2022).

[The Working Group noted that no threshold has been established for occupational exposure to methyleugenol.]

1.6 Quality of exposure assessment in key mechanistic studies in humans

Three studies quantified two DNA adducts of methyleugenol ($[^{15}N_5]N^6$-MIE-dA and $[^{15}N_5]N^2$-MIE-dG) in human tissues by ultra-performance

liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) (see discussion on DNA adducts findings in Section 4.2.1). The studies by Herrmann et al. (2013) and Tremmel et al. (2017) were conducted on liver tissue samples collected from individuals undergoing liver surgery in Germany. The study by Monien et al. (2015) was conducted on non-tumour pulmonary tissues from patients undergoing surgery in France. The level of exposure to methyleugenol in people from whom liver and lung tissues were collected was not assessed. [The Working Group noted that the presence of methyleugenol–DNA adducts indicates that exposure has occurred, although the source and amount of exposure could not be determined. As the studies did not determine exposure by any other means, no plausible correlation between exposure levels and amounts of DNA adducts could be ascertained. Because of widespread exposure to food and consumer products containing methyleugenol, background exposure of the general population is plausible.]

2. Cancer in Humans

No data were available to the Working Group.

3. Cancer in Experimental Animals

See Table 3.1.

3.1 Mouse

3.1.1 Oral administration (gavage)

In a well-conducted study of chronic toxicity and carcinogenicity that complied with Good Laboratory Practice (GLP), groups of 50 male and 50 female B6C3F$_1$ mice (age, 5–6 weeks) were treated with methyleugenol (purity, 99%) in 0.5% methylcellulose by gavage at a dose of 0 (vehicle control), 37, 75, or 150 mg/kg body weight (bw) on 5 days per week for 105 weeks (NTP, 2000; also reported by Johnson et al., 2000). At study termination, survival was: 38/49, 36/50, 37/50, and 35/50 in males and 31/50, 18/50, 18/50, and 2/50 in females, for the groups at 0 (control), 37, 75, and 150 mg/kg bw, respectively. In males, the probability of survival to study completion for the treated groups was similar to that for the control group. In females, the probability of survival to study completion for the treated groups was significantly lower than that for the control group ($P \leq 0.013$, life-table test). In males, terminal group mean body-weight values decreased by 10%, 16%, and 13% in the groups at 37, 75, and 150 mg/kg bw per day, respectively. In females, terminal group mean body-weight values decreased by 39%, 44%, and 46% in the groups at 37, 75, and 150 mg/kg bw per day, respectively.

In males, there was a significant positive trend in the incidence of hepatocellular adenoma ($P = 0.006$ poly-3 trend test; [$P = 0.024$, Cochran–Armitage trend test]), and the incidence – 26/49 (53%) 43/50 (86%) 38/50 (76%) 39/50 (78%) – was significantly increased in each treated group ($P < 0.001$, poly-3 test, [$P = 0.0003$, Fisher exact test]; $P < 0.001$, poly-3 test, [$P = 0.0144$, Fisher exact test]; $P = 0.003$, poly-3 test, [$P = 0.0079$, Fisher exact test], respectively), and exceeding the upper bound of the range observed in historical controls from this laboratory – 201/514 (39.1%); range, 21–58%. The incidence of hepatocellular carcinoma – 10/49 (20%) 20/50 (40%) 19/50 (38%) 9/50 (18%) – was significantly increased at 37 and 75 mg/kg bw ($P = 0.030$, poly-3 test, [$P = 0.0281$, Fisher exact test]; and $P = 0.044$, poly-3 test, [$P = 0.0439$, Fisher exact test], respectively). The incidence of hepatocellular carcinoma exceeded the upper bound of the range observed in historical controls from this laboratory at the lowest dose – 102/514 (19.8%); range, 8–38%. There was a significant positive trend in the incidence of hepatocellular adenoma or carcinoma (combined) ($P = 0.018$, poly-3 trend

Table 3.1 Studies of carcinogenicity in experimental animals exposed to methyleugenol

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (M) 5–6 wk 105 wk NTP (2000)	Gavage Methyleugenol, 99% 0.5% methylcellulose 0, 37, 75 or 150 mg/kg bw, 5 days/wk for 105 wk 50, 50, 50, 50 38, 36, 37, 35	*Liver* Hepatocellular adenoma 26/49 (53%), 43/50* (86%), 38/50* (76%), 39/50** (78%)	$P = 0.006$ poly-3 trend test; [$P = 0.024$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P = 0.0003$, Fisher exact test] *$P < 0.001$, poly-3 test; [$P = 0.0144$, Fisher exact test] **$P = 0.003$, poly-3 test; [$P = 0.0079$, Fisher exact test]	*Principal strengths*: well-conducted GLP study; adequate number of animals used; randomly allocated in groups; adequate duration; males and females used; multiple doses used. Historical controls: hepatocellular adenoma, 201/514 (39.1%); range, 21–58%; hepatocellular carcinoma, 102/514 (19.8%); range, 8–38%; hepatocellular adenoma or carcinoma, 267/514 (52%); range, 25–72%; hepatoblastoma, 2/514 (0.4%); range, 0–3%; hepatocellular carcinoma or hepatoblastoma, 104/514 (20.2%); range, 8–38%; hepatocellular adenoma, hepatocellular carcinoma or hepatoblastoma, 267/514 (52%); range, 25–72%.
		Hepatocellular carcinoma 10/49 (20%), 20/50* (40%), 19/50** (38%), 9/50 (18%)	*$P = 0.030$, poly-3 test; [$P = 0.0281$, Fisher exact test] **$P = 0.044$ poly-3 test; [$P = 0.0439$, Fisher exact test]	
		Hepatocellular adenoma or carcinoma 31/49 (63%), 47/50** (94%), 46/50** (92%), 40/50* (80%)	$P = 0.018$, poly-3 trend test; **$P < 0.001$, poly-3 test, [$P = 0.0002$, Fisher exact test] **$P < 0.001$, poly-3 test, [$P = 0.0005$, Fisher exact test] *$P = 0.016$, poly-3 test, [$P = 0.0517$, Fisher exact test]	
		Hepatoblastoma 0/49, 0/50, 1/50 (2%), 3/50 (6%)	$P = 0.019$, poly-3 trend test; [$P < 0.01$, Cochran–Armitage trend test]	
		Hepatocellular carcinoma or hepatoblastoma (combined) 10/49 (20%), 20/50* (40%), 20/50* (40%), 11/50 (22%)	$P \leq 0.030$, poly-3 trend test; [$P = 0.0281$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (M) 5–6 wk 105 wk NTP (2000) (cont.)		Hepatocellular adenoma, hepatocellular carcinoma, hepatoblastoma (combined) 31/49 (63%), 47/50** (94%), 46/50**(92%), 41/50*(82%)	$P = 0.012$, poly-3 trend test; **$P < 0.001$, poly-3 test; [$P \leq 0.0002$, Fisher exact test] **$P < 0.001$, poly-3 test; [$P \leq 0.0005$, Fisher exact test] *$P = 0.011$ poly-3 test; [$P = 0.0305$, Fisher exact test]	
		Glandular stomach Malignant neuroendocrine tumour 0/49, 0/48, 0/49, 2/50	[NS]	
		Carcinoma 0/49, 0/48, 0/49, 1/50	[NS]	
Full carcinogenicity Mouse, B6C3F$_1$ (F) 5–6 wk 105 wk NTP (2000)	Gavage Methyleugenol, 99% 0.5% methylcellulose 0, 37, 75 or 150 mg/kg bw, 5 days/wk for 105 wk 50, 50, 50, 50 31, 18, 18, 2	*Liver* Hepatocellular adenoma 20/50 (40%), 48/50* (96%), 46/49* (94%), 41/50* (82%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P \leq 0.001$, poly-3 test, [$P < 0.0001$, Fisher exact test]	*Principal strengths*: well-conducted GLP study; adequate number of animals used; randomly allocated in groups; adequate duration; males and females used; multiple doses used. Historical controls: hepatocellular adenoma, 108/511 (21.1%); range, 6–40%; hepatocellular carcinoma, 37/511 (7.2%); range, 0–22%; hepatocellular adenoma or carcinoma, 138/511 (27%); range, 8–58%; hepatoblastoma, 0/511; hepatocellular carcinoma or hepatoblastoma, 37/511 (7.2%); range, 0–22%; hepatocellular adenoma, hepatocellular carcinoma or hepatoblastoma, 138/511 (27.0%); range, 8–58%; hepatocholangiocarcinoma, 1/511 (0.2%); range, 0–2%.
		Hepatocellular carcinoma 7/50 (14%), 37/50* (74%), 47/49* (96%), 47/50* (94%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Hepatocellular adenoma or carcinoma (combined) 25/50 (50%), 50/50* (100%), 49/49* (100%), 49/50* (98%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (F) 5–6 wk 105 wk NTP (2000) (cont.)		Hepatoblastoma 0/50, 6/50* (12%), 11/49** (22%), 15/50*** (30%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P = 0.009$, poly-3 test; [$P = 0.0133$, Fisher exact test] **$P < 0.001$, poly-3 test; [$P = 0.0002$, Fisher exact test] ***$P < 0.001$, poly-3 test; [$P = 0.0001$, Fisher exact test]	
		Carcinoma or hepatoblastoma (combined) 7/50 (14%), 38/50* (76%), 48/49* (98%), 49/50* (98%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma (combined) 25/50 (50%), 50/50* (100%), 49/49* (100%), 49/50* (98%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Hepatocholangiocarcinoma 0/50, 0/50, 0/49, 2/50 (4%)	[NS]	
Full carcinogenicity Mouse, B6C3F$_1$ (M) 1 day 18 mo Miller et al. (1983)	Intraperitoneal injection Methyleugenol, > 98% Trioctanoin 0 (control), 4.75 µmol on days 1, 8, 15, and 22 of lactation, weaning at 4 wk then purified diet for 18 mo 60, 59 58, 58	*Liver* Hepatoma Tumour incidence: 24/58, 56/58* Tumour multiplicity: 0.5, 3.2	*$P < 0.001$, Fisher exact test	*Principal strengths*: adequate duration and adequate number of animals per group. *Principal limitations*: one sex; only one dose group; limited number of organs examined.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (M) 1 day 18 mo Miller et al. (1983)	Intraperitoneal injection 1′-hydroxymethyleugenol, > 98% Trioctanoin 0 (control), 2.85 µmol on days 1, 8, 15, and 22 of lactation, weaning at 4 wk then purified diet for 18 mo 60, 44 58, 44	*Liver* Hepatoma Tumour incidence: 24/58, 41/44* Tumour multiplicity: 0.5, 3.5	*$P < 0.001$, Fisher exact test	*Principal strengths*: adequate duration and adequate number of animals per group. *Principal limitations*: only one dose group; one sex; limited number of organs examined.
Full carcinogenicity Rat, F344 (M) 5–6 wk 105 wk NTP (2000)	Gavage Methyleugenol, 99% 0.5% methylcellulose 0, 37, 75, 150 mg/kg bw 0, 37, 75, 150 mg/kg bw, 5 days/wk for 105 wk 50, 50, 50, 50 20, 16, 15, 0	*Liver* Hepatocellular adenoma 5/50 (10%), 12/50* (24%), 23/50** (46%), 38/50** (76%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P = 0.042$, poly-3 test; [$P = 0.0542$, Fisher exact test] **$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	*Principal strengths*: well-conducted GLP study; adequate number of animals used; randomly allocated in groups; adequate duration; both males and females used; multiple doses used. Historical controls: hepatocellular adenoma, 12/400 (3.0%); range, 0–8%; hepatocellular carcinoma, 4/400 (1.0%); range, 0–4%; hepatocellular adenoma or carcinoma (combined), 16/400 (4.0%); range, 0–10%; renal tubule adenoma, 3/400 (0.8%); range, 0–2%; renal tubule carcinoma, 2/400 (0.5%); range, 0–4%; renal tubule adenoma or carcinoma (combined), 5/400 (1.3%); range, 0–4%; malignant mesothelioma, 7/402 (1.7%); range, 0–6%; skin fibroma, 17/402 (4.2%); range, 0–12%; skin fibrosarcoma, 3/402 (0.8%); range, 0–2%; skin fibroma or fibrosarcoma (combined), 20/402 (5.0%); range, 0–12%; benign adrenal medulla pheochromocytoma, 94/401 (23.4%); range, 14–34%; malignant adrenal medulla pheochromocytoma, 8/401 (2.0%); range, 0–6%; benign or malignant adrenal medulla pheochromocytoma (combined), gavage, 101/401 (25.20%); range, 16–36%.
		Hepatocellular carcinoma 2/50 (4%), 3/50 (6%), 14/50* (28%), 25/50** (50%)	$P < 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P \leq 0.001$, poly-3 test; [$P = 0.0009$, Fisher exact test] **$P \leq 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Hepatocellular adenoma or carcinoma (combined) 7/50 (14%), 14/50 (28%), 28/50* (56%), 43/50** (86%)	$P \leq 0.001$, poly-3 trend test [$P < 0.001$, Cochran–Armitage trend test] *$P = 0.049$, poly-3 test; [$P < 0.0001$, Fisher exact test] **$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344 (M) 5–6 wk 105 wk NTP (2000) (cont.)		Hepatocholangioma 0/50, 0/50, 0/50, 1/50 (2%) Hepatocholangiocarcinoma 0/50, 0/50, 1/50 (2%), 1/50 (2%) Hepatocholangioma or hepatocholangiocarcinoma (combined) 0/50, 0/50, 1/50 (2%), 2/50 (4%) *Glandular stomach* Benign neuroendocrine tumour 0/50, 0/50, 0/50, 3/50 (6%) Malignant neuroendocrine tumour 0/50, 0/50, 0/50, 4/50* (8%) Benign or malignant neuroendocrine tumour (combined) 0/50, 0/50, 0/50, 7/50* (14%) *Kidney* Renal tubule adenoma (single and step sections) 4/50 (8%), 6/50 (12%), 17/50** (34%), 13/50* (26%)	[NS, Fisher exact test] [NS, Fisher exact test] [NS, Fisher exact test] NS, poly-3 test $P = 0.002$, poly-3 trend test; [$P = 0.001$, Cochran–Armitage trend test] *$P = 0.033$, poly-3 test $P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test] *$P = 0.002$, poly-3 test; [$P = 0.0062$, Fisher exact test] $P < 0.001$, poly-3 trend test; [$P = 0.004$, Cochran–Armitage trend test] **$P < 0.001$, poly-3 test; [$P = 0.0013$, Fisher exact test] *$P = 0.003$, poly-3 test; [$P = 0.0155$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344 (M) 5–6 wk 105 wk NTP (2000) (cont.)		Renal tubule carcinoma (single and step sections) 1/50, 0/50, 0/50, 0/50 *Mammary gland* Fibroadenoma 5/50 (10%), 5/50 (10%), 15/50** (30%), 13/50* (26%) *All organs* Malignant mesothelioma 1/50 (2%), 3/50 (6%), 5/50 (10%), 12/50* (24%) *Skin (subcutaneous tissue)* Fibroma 1/50 (2%), 9/50** (18%), 8/50* (16%), 5/50 (10%) Fibrosarcoma 0/50, 3/50 (6%), 0/50, 3/50 (6%) Fibroma or fibrosarcoma (combined) 1/50 (2%), 12/50** (24%), 8/50* (16%), 8/50* (16%)	[NS, Fisher exact test] $P < 0.001$, poly-3 trend test; [$P = 0.0061$, Cochran–Armitage trend test] **$P = 0.004$, poly-3 test; [$P = 0.0114$, Fisher exact test] *$P = 0.008$, poly-3 test; [$P = 0.0332$, Fisher exact test] $P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P = 0.0009$, Fisher exact test] **$P = 0.006$, poly-3 test; [$P = 0.0078$, Fisher exact test] *$P = 0.011$, poly-3 test; [$P = 0.0154$, Fisher exact test] NS, poly-3 test; [Fisher exact test] $P = 0.037$, poly-3 trend test; **$P < 0.001$, poly-3 test; [$P = 0.0009$, Fisher exact test] *$P \leq 0.011$, poly-3 test; [$P = 0.0154$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344 (F) 5–6 wk 105 wk NTP (2000)	Gavage Methyleugenol, 99% 0.5% methylcellulose 0, 37, 75 or 150 mg/kg bw, 5 days/wk for 105 wk 50, 50, 50, 50 22, 25, 22, 11	*Liver* Hepatocellular adenoma 1/50 (2%), 8/50* (16%), 11/49** (22%), 33/49*** (66%)	$P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test] *$P = 0.017$, poly-3 test; [$P = 0.0154$, Fisher exact test] **$P = 0.002$, poly-3 test; [$P = 0.0017$, Fisher exact test] ***$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	*Principal strengths*: well-conducted GLP study, adequate number of animals used; randomly allocated in groups; adequate duration; both males and females used; multiple doses used. Historical controls: hepatocellular adenoma, 1/401 (0.3%); range, 0–2%; hepatocellular carcinoma, 0/401; hepatocellular adenoma or carcinoma (combined), 1/401 (0.3%); range, 0–2%; forestomach squamous cell papilloma, 2/401 (0.5%); range, 0–2%; forestomach squamous cell carcinoma, 0/401; forestomach squamous cell papilloma or carcinoma (combined), 2/401 (0.5%); range, 0–2%; benign adrenal medulla pheochromocytoma, gavage, 18/401 (4.5%); range, 0–10%; malignant adrenal medulla pheochromocytoma, gavage, 3/400 (0.8%); range, 0–4%; benign or malignant adrenal medulla pheochromocytoma (combined), gavage, 22/400 (5.5%); range, 0–14%.
		Hepatocellular carcinoma 0/50, 0/50, 4/49* (8%), 8/49** (16%)	$P < 0.001$, poly-3 trend test; [$P = 0.001$, Cochran–Armitage trend test] *$P = 0.066$, poly-3 test; [$P = 0.056$, Fisher exact test] **$P = 0.002$, poly-3 test; [$P = 0.026$, Fisher exact test]	
		Hepatocellular adenoma or carcinoma (combined) 1/50 (2%), 8/50* (16%), 14/49** (28%), 34/49*** (68%)	$P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test] *$P = 0.017$, poly-3 test; [$P = 0.0154$, Fisher exact test] **$P < 0.001$, poly-3 test; [$P = 0.0002$, Fisher exact test] ***$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344 (F) 5–6 wk 105 wk NTP (2000) (cont.)		Hepatocholangiocarcinoma		
		0/50, 0/50, 0/49, 3/49 (6%)	$P = 0.010$, poly-3 trend test; [$P = 0.003$, Cochran–Armitage trend test] NS, poly-3 test; [NS, Fisher exact test]	
		Glandular stomach		
		Benign neuroendocrine tumour		
		0/50, 0/50, 13/50** (26%), 9/50* (18%)	$P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test] **$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test] *$P = 0.029$, poly-3 test; [$P = 0.0013$, Fisher exact test]	
		Malignant neuroendocrine tumour		
		0/50, 1/50 (2%), 12/50* (24%), 26/50* (52%)	$P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P \leq 0.0001$, Fisher exact test]	
		Benign or malignant neuroendocrine tumour		
		0/50, 1/50 (2%), 25/50* (50%), 34/50* (68%)	$P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test] *$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		All organs		
		Mononuclear cell leukaemia		
		17/50 (34%), 20/50 (40%), 19/50 (38%), 28/50* (56%)	$P = 0.007$, poly-3 trend test [$P = 0.012$, Cochran–Armitage trend test] *$P = 0.008$, poly-3 test; [$P = 0.0219$; Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344 (M) 5–6 wk 52 wk NTP (2000)	Gavage Methyleugenol, 99% 0.5% methylcellulose 0, 300 mg/kg bw, 5 days/wk for 52 wk followed by vehicle control for 53 wk 50, 50 20, 0	*Liver* Hepatocellular adenoma 5/50 (10%), 32/50* (76%)	*$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	*Principal strengths*: well-conducted GLP study, adequate number of animals used; randomly allocated in groups; adequate duration; both males and females used; multiple doses used. Historical controls: hepatocellular adenoma, 12/400 (3.0%); range, 0–8%; hepatocellular carcinoma, 4/400 (1.0%); range, 0–4%; hepatocellular adenoma or carcinoma (combined), 16/400 (4.0%); range, 0–10%; renal tubule adenoma, 3/400 (0.8%); range, 0–2%; renal tubule carcinoma, 2/400 (0.5%); range, 0–4%; renal tubule adenoma or carcinoma (combined), 5/400 (1.3%); range, 0–4%; malignant mesothelioma, 7/402 (1.7%); range, 0–6%; skin fibroma, 17/402 (4.2%); range, 0–12%; skin fibrosarcoma, 3/402 (0.8%); range, 0–2%; skin fibroma or fibrosarcoma (combined), 20/402 (5.0%); range, 0–12%; benign adrenal medulla pheochromocytoma, 94/401 (23.4%); range, 14–34%; malignant adrenal medulla pheochromocytoma, 8/401 (2.0%); range, 0–6%; benign or malignant adrenal medulla pheochromocytoma (combined), gavage, 101/401 (25.20%); range, 16–36%.
		Hepatocellular carcinoma 2/50 (4%), 36/50* (72%)	*$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Hepatocellular adenoma or carcinoma (combined) 7/50 (14%), 45/50* (90%)	*$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Hepatocholangioma 0/50, 6/50* (12%)	$P = 0.004$, poly-3 test; [$P = 0.0133$, Fisher exact test]	
		Hepatocholangiocarcinoma 0/50, 7/50* (14%)	$P < 0.001$, poly-3 test; [$P = 0.0062$, Fisher exact test]	
		Hepatocholangioma or hepatocholangiocarcinoma (combined) 0/50, 13/50* (26%)	$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Glandular stomach Benign and malignant neuroendocrine tumour (combined) 0/50, 4/50* (8%)	*$P = 0.032$, poly-3 test	
		Kidney Renal tubule adenoma (single and step sections) 4/50 (8%), 20/50* (40%)	*$P < 0.001$, poly-3 test; [$P = 0.0002$, Fisher exact test]	
		Renal tubule adenoma or carcinoma (single and step sections) 5/50 (10%), 20/50* (40%)	$P < 0.001$, poly-3 test; [$P = 0.0005$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344 (M) 5–6 wk 52 wk NTP (2000) (cont.)		*All organs* Malignant mesothelioma 1/50 (2%), 5/50* (10%)	*P = 0.041, poly-3 test	
Full carcinogenicity Rat, F344 (F) 5–6 wk 52 wk NTP (2000)	Gavage Methyleugenol, 99% 0.5% methylcellulose 0, 300 mg/kg bw, 5 days/wk for 52 wk followed by vehicle control for 53 wk 50, 50 22, 16	*Liver* Hepatocellular adenoma 1/50 (2%), 43/50* (86%) Hepatocellular carcinoma 0/50, 22/50* (44%) Hepatocellular adenoma or carcinoma (combined) 1/50 (2%), 43/50* (86%) Hepatocholangioma 0/50, 8/50* (16%) Hepatocholangiocarcinoma 0/50, 9/50* (18%) Hepatocholangioma or hepatocholangiocarcinoma (combined) 0/50, 17/50* (34%) *Glandular stomach* Benign neuroendocrine tumour 0/50, 5/50* (10%)	**P < 0.001, poly-3 test; [P < 0.0001, Fisher exact test] *P < 0.001, poly-3 test; [P < 0.001, Fisher exact test] *P < 0.001, poly-3 test; [P < 0.0001, Fisher exact test] P = 0.003, poly-3 test; P = 0.0029, Fisher exact test] P < 0.001, poly-3 test; P = 0.0013, Fisher exact test] P < 0.001, poly-3 test; P < 0.0001, Fisher exact test] *P = 0.029, poly-3 test; [P = 0.0287, Fisher exact test]	*Principal strengths*: well-conducted GLP study; adequate number of animals used; randomly allocated in groups; adequate duration; both males and females used; multiple doses used. Historical controls: hepatocellular adenoma, 1/401 (0.3%); range, 0–2%; hepatocellular carcinoma, 0/401; hepatocellular adenoma or carcinoma (combined), 1/401 (0.3%); range, 0–2%; forestomach squamous cell papilloma, 2/401 (0.5%); range, 0–2%; forestomach squamous cell carcinoma, 0/401; forestomach squamous cell papilloma or carcinoma (combined), 2/401 (0.5%); range, 0–2%; benign adrenal medulla pheochromocytoma, gavage, 18/401 (4.5%); range, 0–10%; malignant adrenal medulla pheochromocytoma, gavage, 3/400 (0.8%); range, 0–4%; benign or malignant adrenal medulla pheochromocytoma (combined), gavage, 22/400 (5.5%); range, 0–14%.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Agent tested, purity Vehicle, Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344 (F) 5–6 wk 52 wk NTP (2000) (cont.)		Malignant neuroendocrine tumour 0/50, 36/50* (72%)	*$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
		Benign or malignant neuroendocrine tumour 0/50, 41/50* (82%)	*$P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]	
Initiation–promotion (tested as initiator) Rat, F344 (M) 5–6 wk 40 wk Williams et al. (2013)	Gavage Methyleugenol, > 98% Methylcellulose 0, 62, 125, 250 mg/kg bw, by gavage, 3 days/wk, for 16 wk followed by control diet for 24 wk 12, 12, 12, 12 12, 12, 12, 12	*Liver* Hepatocellular adenoma 0/12, 0/12, 1/12, 12/12* Tumour multiplicity: 0, 0.1, 1, 1.1 Hepatocellular carcinoma Tumour incidence: 0/12, 0/12, 0/12, 0/12 Tumour multiplicity: 0, 0, 0, 0	[$P < 0.001$, Cochran–Armitage trend test] *[$P < 0.001$, Fisher exact test]	*Principal strengths*: multiple dose study. *Principal limitations*: limited number of organs examined; limited number of animals; short duration; one sex study.
Initiation–promotion (tested as initiator) Rat, F344 (M) 5–6 wk 40 wk Williams et al. (2013)	Gavage Methyleugenol, > 98% Methylcellulose 0, 62, 125, 250 mg/kg bw gavage, 3 days/wk, for 16 wk followed by 500 ppm phenobarbital in the diet for 24 wk 14, 14, 14, 14 14, 13, 14, 13	*Liver* Hepatocellular adenoma Tumour incidence: 0/14, 0/13, 3/14, 10/13* Tumour multiplicity: 0, 0, < 1, 2.8 Hepatocellular carcinoma Tumour incidence: 0/14, 0/13, 0/14, 0/13 Tumour multiplicity: 0, 0, 0, 0	[$P < 0.001$, Cochran–Armitage trend test] *[$P < 0.001$, Fisher exact test]	*Principal strengths*: multiple dose study. *Principal limitations*: limited number of organs examined; limited number of animals; short duration; one sex.

bw, body weight; F, female; GLP, Good Laboratory Practice; M, male; mo, month(s); NS, not significant; wk, week(s).

test), with the incidence – 31/49 (63%), 47/50 (94%), 46/50 (92%), 40/50 (80%) – being significantly increased in all treated groups ($P < 0.001$, poly-3 test, [$P = 0.0002$, Fisher exact test]; $P < 0.001$, poly-3 test, [$P = 0.0005$, Fisher exact test]; $P = 0.016$, poly-3 test, [$P = 0.0517$, Fisher exact test], respectively), and exceeding the upper bound of the range observed in historical controls – 267/514 (52%); range, 25–72%. There was a significant positive trend in the incidence of hepatoblastoma ($P = 0.019$, poly-3 trend test; [$P < 0.01$, Cochran–Armitage trend test]), with the incidence at 150 mg/kg bw per day (3/50, 6%) exceeding the range observed in historical controls from this laboratory – 2/514 (0.4%); range, 0–3%. There was a significant positive trend in the incidence of hepatocellular carcinoma or hepatoblastoma (combined) ($P \leq 0.030$, poly-3 trend test), with the incidence – 10/49 (20%), 20/50 (40%), 20/50 (40%), 11/50 (22%) – being significant [$P = 0.0281$; Fisher exact test] at 37 and 75 mg/kg bw per day. The incidence at these two doses exceeded the upper bound of the range observed in historical controls from this laboratory – 104/514 (20.2%); range, 8–38%. There was a significant positive trend in the incidence of hepatocellular adenoma, hepatocellular carcinoma, or hepatoblastoma (combined) ($P = 0.012$, poly-3 trend test), with the incidence – 31/49 (63%), 47/50 (94%), 46/50 (92%), 41/50 (82%) – being significantly increased at all doses ($P < 0.001$, poly-3 test, [$P \leq 0.0002$, Fisher exact test]; $P < 0.001$, poly-3 test, [$P = 0.0005$, Fisher exact test]; ($P = 0.011$, poly-3 test, [$P = 0.0305$, Fisher exact test], respectively), and exceeding the upper bound of the range observed in historical controls from this laboratory – 267/514 (52%); range, 25–72%.

In females, there was a significant positive trend in the incidence of hepatocellular adenoma ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 20/50 (40%), 48/50 (96%), 46/49 (94%), 41/50 (82%) – being significantly increased in each treated group ($P < 0.001$, poly-3 test, [$P < 0.0001$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this laboratory – 108/511 (21.1%); range, 6–40%. There was a significant positive trend in the incidence of hepatocellular carcinoma ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 7/50 (14%), 37/50 (74%), 47/49 (96%), 47/50 (94%) – being significantly increased in each treated group ($P < 0.001$, poly-3 test, [$P < 0.0001$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this laboratory – 37/511 (7.2%); range, 0–22%. There was a significant positive trend in the incidence of hepatocellular adenoma or carcinoma (combined) ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 25/50 (50%), 50/50 (100%), 49/49 (100%), 49/50 (98%) – being significantly increased in each treated group ($P < 0.001$, poly-3 test, [$P < 0.0001$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this laboratory – 138/511 (27%); range, 8–58%. There was a significant positive trend in the incidence of hepatoblastoma ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 0/50, 6/50 (12%), 11/49 (22%), 15/50 (30%) – being significantly increased in each treated group ($P = 0.009$, poly-3 test, [$P = 0.0133$, Fisher exact test]; $P < 0.001$, poly-3 test, [$P = 0.0002$, Fisher exact test]; $P < 0.001$, poly-3 test, [$P = 0.0001$, Fisher exact test], respectively), and exceeding the historical controls from this laboratory (0/511). There was a significant positive trend in the incidence of hepatocellular carcinoma or hepatoblastoma (combined) ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 7/50 (14%), 38/50 (76%), 48/49 (98%), 49/50 (98%) – being significantly increased in each treated group ($P < 0.001$, poly-3 test, [$P < 0.0001$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this

laboratory – 37/511 (7.2%); range, 0–22%. There was a significant positive trend in the incidence of hepatocellular adenoma, hepatocellular carcinoma or hepatoblastoma (combined) ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 25/50 (50%), 50/50 (100%), 49/49 (100%), 49/50 (98%) – being significantly increased in each treated group ($P < 0.001$, poly-3 test, [$P < 0.0001$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this laboratory – 138/511 (27%); range, 8–58%. The incidence of hepatocholangiocarcinoma at the highest dose – 2/50 (4%) – exceeded the upper bound of the range observed in historical controls from this laboratory – 1/511 (0.2%); range, 0–2%. [The Working Group noted that hepatoblastoma and hepatocholangiocarcinoma are rare neoplasms of the liver and that neuroendocrine tumours of the glandular stomach are rare neoplasms of the glandular stomach in experimental animals of this strain and age.]

In treated groups, a significantly increased incidence of preneoplastic and non-neoplastic lesions was observed in liver (eosinophilic foci and chronic active inflammation in males; oval cell hyperplasia, hypertrophy, bile duct hyperplasia, haematopoietic cell proliferation, and haemosiderin pigmentation in females), and glandular stomach (atrophy, ectasia, hyperplasia, chronic inflammation in both males and females).

[The Working Group noted that this was a well-conducted study that complied with GLP and used an adequate number of animals (randomly allocated in groups), both males and females, and multiple doses, and with a duration of most of the lifespan.]

3.1.2 Intraperitoneal administration

In a study performed by Miller et al. (1983), groups of 60 male B6C3F$_1$ mice received methyleugenol (purity > 98%; dissolved in trioctanoin) by intraperitoneal injection at a dose of 0 (vehicle controls) or total dose of 4.75 µmol/mouse on days 1, 8, 15, and 22 of lactation. Mice were weaned at 4 weeks and then maintained on a purified diet for 18 months. A group of 60 animals was used as controls.

A statistically significant increase ($P < 0.001$, Fisher exact test) in the incidence of hepatoma was observed in the methyleugenol-treated group (56/58, 96%) compared with controls (24/58, 41%). [The Working Group noted that the distinction between benign and malignant hepatoma had not been clearly defined at the time when the study was conducted.]

Non-neoplastic lesions have not been reported.

[The Working Group noted that this study used an adequate duration of exposure and observation and an adequate number of animals; however, only one sex and one dose group were used, and a limited number of organs was examined (which is appropriate for this particular assay).]

3.1.3 Intraperitoneal administration of the metabolite, 1′-hydroxymethyleugenol

In a study performed by Miller et al. (1983), groups of 44 male B6C3F$_1$ mice received 1′-hydroxymethyleugenol (purity, > 98%; dissolved in trioctanoin) by intraperitoneal injection at a dose of 2.85 µmol/mouse on lactation days 1, 8, 15 and 22. Mice were weaned at 4 weeks and then maintained on a purified diet for 18 months. A group of 60 animals was used as controls.

A statistically significant increase ($P < 0.001$, Fisher exact test) in the incidence of hepatoma was observed in the group treated with 1′-hydroxymethyleugenol (41/44, 93%) compared with the controls (24/58, 41%)). [The Working Group noted that the distinction between benign and malignant hepatoma had not been clearly defined at the time when the study was conducted.]

[The Working Group noted that this study used an adequate duration of exposure and

observation and an adequate number of animals per group; however, it was limited by the use of one sex only, only one dose group, and the limited number of organs examined (which is appropriate for this particular assay).]

3.2 Rat

3.2.1 Oral administration (gavage)

In a well-conducted study of chronic toxicity and carcinogenicity that complied with GLP, groups of 50 male and 50 female Fischer 344 (F344) rats (age, 5–6 weeks) were treated with methyleugenol (purity, 99%; in 0.5% methylcellulose) by gavage at a dose of 0 (vehicle control), 37, 75, or 150 mg/kg bw per day on 5 days per week for 105 weeks. Additional groups of 50 males and 50 females received methyleugenol (in 0.5% methylcellulose) at a dose of 300 mg/kg bw per day by gavage for 52 weeks, followed by the vehicle alone for the remaining 53 weeks of the study (NTP, 2000; also reported by Johnson et al., 2000). At study termination, survival was: 20/50, 16/50, 15/50, 0/50, and 0/50 in males and 22/50, 25/50, 22/50, 11/50, and 16/50 in females, for the groups at 0 (control), 37, 75, 150, and 300 mg/kg bw per day, respectively. In males, the probability of survival to study completion for the groups at 150 and 300 mg/kg bw per day was significantly decreased ($P < 0.001$, life-table test) and all animals from these groups died before the end of the study. In females, the probability of survival to study completion for the group at 150 mg/kg bw per day approached a significant decrease ($P = 0.053$, life-table test). In males, terminal group mean body-weight values decreased by 12%, 23%, and 26% in the groups at 75, 150, and 300 mg/kg bw per day, respectively. In females, terminal group mean body-weight values decreased by 20%, 26%, and 26% in the groups at 75, 150, and 300 mg/kg bw per day, respectively.

In males, there was a significant positive trend in the incidence of hepatocellular adenoma ($P \leq 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 5/50 (10%), 12/50 (24%), 23/50 (46%), and 38/50 (76%), for the groups at 0 (control), 37, 75, or 150 mg/kg bw per day, respectively – being significantly increased at the lowest dose ($P = 0.042$, poly-3 test; [approached significance, $P = 0.0542$, Fisher exact test]), and the two other doses ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this laboratory – 12/400 (3.0%); range, 0–8%. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of hepatocellular adenoma in the treated group – 5/50 (10%) and 32/50 (64%) at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of hepatocellular carcinoma ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 2/50 (4%), 3/50 (6%), 14/50 (28%), and 25/50 (50%) in groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 75 mg/kg bw per day ($P \leq 0.001$, poly-3 test; [$P = 0.0009$, Fisher exact test]) and at 150 mg/kg bw ($P \leq 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]), respectively, and exceeding the upper bound of the range observed in historical controls from this laboratory – 4/400 (1.0%); range, 0–4%. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of hepatocellular carcinoma in the treated group – 2/50 (4%) and 36/50 (72%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of hepatocellular adenoma or carcinoma (combined) ($P \leq 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 7/50 (14%), 14/50 (28%), 28/50 (56%), and 43/50 (86%)

for the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 37 mg/kg bw ($P = 0.049$, poly-3 test; [$P < 0.0001$, Fisher exact test]); and at 75 mg/kg bw and 150 mg/kg bw ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test], for each), and exceeding the upper bound of the range observed in historical controls from this laboratory – 16/400 (4.0%); range, 0–10%. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased the incidence of hepatocholangioma – 0/50 (control), 6/50 (12%) ($P = 0.004$, poly-3 test; [$P = 0.0133$, Fisher exact test]; $P < 0.001$, poly-3 test; [$P = 0.0062$, Fisher exact test]; $P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) – hepatocholangiocarcinoma – 0/50 (control), 7/50 (14%) – and hepatocholangioma or hepatocholangiocarcinoma (combined) – 0/50 (control), 13/50 (26%) – in the treated groups compared with controls. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of hepatocellular adenoma or carcinoma (combined) in the treated group – 7/50 (14%) and 45/50 (90%), for the groups at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of malignant neuroendocrine tumours of the glandular stomach ($P = 0.002$, poly-3 trend test; [$P = 0.001$, Cochran–Armitage trend test]), with the incidence – 0/50, 0/50, 0/50, and 4/50 (8%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at the highest dose ($P = 0.033$, poly-3 test). There was a significant positive trend in the incidence of benign or malignant neuroendocrine tumours of the glandular stomach (combined) ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 0/50, 0/50, 0/50, 7/50 (14%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at the highest dose ($P = 0.002$, poly-3 test; [$P = 0.0062$, Fisher exact test]). Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P = 0.032$, poly-3 test) the incidence of benign or malignant neuroendocrine tumours of the glandular stomach (combined) in the treated group – 0/50, 4/50 (8%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of renal tubule adenoma (single and step sections) ($P < 0.001$, poly-3 trend test; [$P = 0.004$, Cochran–Armitage trend test]), with the incidence – 4/50 (8%), 6/50 (12%), 17/50 (34%), and 13/50 (26%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 75 ($P < 0.001$, poly-3 test; [$P = 0.0013$, Fisher exact test]) and 150 mg/kg bw ($P = 0.003$, poly-3 test; [$P = 0.0155$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this laboratory – 3/400 (0.8%); range, 0–2%. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P = 0.0002$, Fisher exact test]; $P < 0.001$, poly-3 test; [$P = 0.0005$, Fisher exact test]) the incidence of renal tubule adenoma (single and step sections) – 4/50 (8%) (control), 20/50 (40%) ($P < 0.001$, poly-3 test; [$P = 0.0002$, Fisher exact test]) – and renal tubule adenoma or carcinoma (combined) (single and step sections) – 5/50 (10%) (control), 20/50 (40%) ($P < 0.001$, poly-3 test; [$P = 0.0005$, Fisher exact test] – in the treated groups. There was a significant positive trend in the incidence of mammary gland fibroadenoma ($P < 0.001$, poly-3 trend test; [$P = 0.0061$, Cochran–Armitage trend test]), with the incidence – 5/50 (10%), 5/50 (10%), 15/50 (30%), and 13/50 (26%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 75 ($P = 0.004$, poly-3 test; [$P = 0.0114$, Fisher exact test]) and 150 mg/kg bw ($P = 0.008$, poly-3 test; [$P = 0.0332$, Fisher exact test]). There was a significant positive trend in the incidence of malignant mesothelioma (all organs) ($P < 0.001$, poly-3 trend test;

[$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 1/50 (2%), 3/50 (6%), 5/50 (10%), and 12/50 (24%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 150 mg/kg bw per day ($P < 0.001$, poly-3 test; [$P = 0.0009$, Fisher exact test]), and exceeding the upper bound of the range observed in historical controls from this laboratory – 7/402 (1.7%); range, 0–6%. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P = 0.041$, poly-3 test) the incidence of malignant mesothelioma (all organs) in the treated group – 1/50 (2%) and 5/50 (10%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant increase in the incidence – 1/50 (2%), 9/50 (18%), 8/50 (16%), and 5/50 (10%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – of skin (subcutaneous) fibroma at 37 ($P = 0.006$, poly-3 test; [$P = 0.0078$, Fisher exact test]) and 75 mg/kg bw ($P = 0.011$, poly-3 test; [$P = 0.0154$, Fisher exact test]), with the incidence exceeding the upper bound of the range observed in historical controls from this laboratory – 17/402 (4.2%); range, 0–12%. There was a significant positive trend in the incidence of skin (subcutaneous) fibroma or fibrosarcoma (combined) ($P = 0.037$, poly-3 trend test), with the incidence – 1/50 (2%), 12/50 (24%), 8/50 (16%), and 8/50 (16%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased ($P < 0.001$, poly-3 test; [$P = 0.0009$, Fisher exact test]) at 37 mg/kg bw; ($P = 0.011$, poly-3 test; [$P = 0.0154$, Fisher exact test]; ($P = 0.005$, poly-3 test; [$P = 0.0154$, Fisher exact test] at 75 and 150 mg/kg bw, respectively), and exceeding the upper bound of the range observed in historical controls (20/402 (5.0%); range, 0–12%) from this laboratory.

In females, there was a significant positive trend in the incidence of hepatocellular adenoma ($P \leq 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 1/50 (2%), 8/50 (16%), 11/49 (22%), and 33/49 (67%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at all doses ($P = 0.017$, poly-3 test; [$P = 0.0154$, Fisher exact test]; $P = 0.002$, poly-3 test; [$P = 0.0017$, Fisher exact test]; $P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test], respectively), and exceeding the upper bound of the range observed in historical controls from this laboratory – 1/401 (0.3%); range, 0–2%. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of hepatocellular adenoma in the treated group – 1/50 (2%), 43/50 (86%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of hepatocellular carcinoma ($P < 0.001$, poly-3 trend test; [$P = 0.001$, Cochran–Armitage trend test]), with the incidence – 0/50, 0/50, 4/49 (8%), and 8/49 (16%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 150 mg/kg bw per day ($P = 0.002$, poly-3 test; [$P = 0.0026$, Fisher exact test]), and exceeding the incidence observed in historical controls from this laboratory (0/401). Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.001$, Fisher exact test]) the incidence of hepatocellular carcinoma in the treated group – 0/50, 22/50 (44%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of hepatocellular adenoma or carcinoma (combined) ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence (1/50 (2%), 8/50 (16%), 14/49 (29%), 34/49 (69%) for the 0 (control), 37, 75, and 150 mg/kg bw, respectively) being significantly increased at 37 mg/kg bw per day ($P = 0.017$, poly-3 test; [$P = 0.0154$, Fisher exact test]); 75 mg/kg bw per day ($P < 0.001$, poly-3 test; [$P = 0.0002$, Fisher exact test]); and 150 mg/kg bw per ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]); and exceeding the upper bound of the

range observed in historical controls from this laboratory – 1/401 (0.3%); range, 0–2%. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of hepatocellular adenoma or carcinoma (combined) in the treated group – 1/50 (2%), 43/50 (86%), at 0 (control) and 300 mg/kg bw per day, respectively. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P = 0.003$, poly-3 test; [$P = 0.0029$, Fisher exact test]) the incidence of hepatocholangioma in the treated group – 0/50, 8/50 (16%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend ([$P = 0.010$, poly-3 trend test; $P = 0.003$, Cochran–Armitage trend test]) in the incidence of hepatocholangiocarcinoma – 0/50, 0/50, 0/49, and 3/49 (6%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P = 0.0013$, Fisher exact test]) the incidence of hepatocholangiocarcinoma in the treated group – 0/50, 9/50 (18%), at 0 (control) and 300 mg/kg bw per day, respectively. Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of hepatocholangioma or hepatocholangiocarcinoma (combined) in the treated group – 0/50, 17/50 (34%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of benign neuroendocrine tumours of the glandular stomach ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 0/50, 0/50, 13/50 (26%), and 9/50 (18%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 75 mg/kg bw per day ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) and 150 mg/kg bw per day ($P < 0.001$, poly-3 test; [$P = 0.0013$, Fisher exact test]). Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P = 0.0029$, poly-3 test; [$P = 0.0287$, Fisher exact test]) the incidence of benign neuroendocrine tumours of the glandular stomach in the treated group – 0/50, 5/50 (10%), at 0 (control) or 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of malignant neuroendocrine tumours of the glandular stomach ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 0/50, 1/50 (2%), 12/50 (24%), and 26/50 (52%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 75 and 150 mg/kg bw per day ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]). Exposure to methyleugenol at 300 mg/kg bw for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of malignant neuroendocrine tumours of the glandular stomach in the treated group – 0/50, 36/50 (72%), at 0 (control) and 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of benign or malignant neuroendocrine tumours of the glandular stomach (combined) ($P < 0.001$, poly-3 trend test; [$P < 0.001$, Cochran–Armitage trend test]), with the incidence – 0/50, 1/50 (2%), 25/50 (50%), and 34/50 (68%) in the groups at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased at 75 and 150 mg/kg bw ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]). Exposure to methyleugenol at 300 mg/kg bw per day for 52 weeks significantly increased ($P < 0.001$, poly-3 test; [$P < 0.0001$, Fisher exact test]) the incidence of benign or malignant neuroendocrine tumours of the glandular stomach (combined) in the treated group – 0/50, 41/50 (82%), for 0 (control) or 300 mg/kg bw per day, respectively. There was a significant positive trend in the incidence of mononuclear cell leukaemia (all organs) ($P = 0.007$, poly-3 trend test; [$P = 0.012$, Cochran–Armitage trend test]), with the incidence – 17/50 (34%), 20/50 (40%), 19/50 (38%), and 28/50 (56%) in the groups

at 0 (control), 37, 75, and 150 mg/kg bw per day, respectively – being significantly increased (P = 0.008, poly-3 test; [P = 0.0219, Fisher exact test]) at 150 mg/kg bw per day. [The Working Group noted that hepatocholangiocarcinoma is a rare neoplasm of the liver, that mesothelioma is a rare neoplasm, and that neuroendocrine tumours are rare neoplasms of the glandular stomach in experimental animals of this strain and age. The Working Group also noted that in the main and stop-exposure studies, there was consistency in the tumour response for cancers of the liver and the glandular stomach in male and female rats, and for renal tubule tumours in male rats.]

Regarding preneoplastic and non-neoplastic lesions, significant increases in incidence were observed in the liver (bile duct hyperplasia, oval cell hyperplasia, hypertrophy, cystic degeneration, eosinophilic foci, basophilic foci, mixed cell foci), glandular stomach (atrophy, neuroendocrine cell hyperplasia) in treated groups for both sexes. In males, an increased incidence of preneoplastic lesions was observed in the kidney (renal tubule hyperplasia). [The Working Group noted that this was a well-conducted GLP study using an adequate number of animals (randomly allocated in groups), both males and females, and multiple doses, and with a duration of most of the lifespan.]

3.2.2 Initiation–promotion

In a study performed by Williams et al. (2013), groups of 12 male F344 rats (age, 5–6 weeks) were treated with methyleugenol (purity, > 98%; in 0.5% methylcellulose) by gavage at a dose of 0 (vehicle control), 62, 125, or 250 mg/kg bw on 3 days per week for 16 weeks, followed by either control diet or diet containing phenobarbital at 500 ppm for 24 weeks. Three deaths occurred in the treated groups immediately after dosing and were attributed to the gavage procedure. The first death occurred on the second day of dosing, and the other two deaths occurred later; the animals were not replaced. The body weights of rats selected for inclusion in the study did not vary by more than 15% from the mean body weight.

Statistically significant increases in the incidence of hepatocellular adenoma were observed only in the groups treated with methyleugenol alone at 250 mg/kg bw [P < 0.001, Fisher exact test] alone or together with phenobarbital at 500 ppm. No hepatocellular carcinoma was observed in treated or control groups. Statistically significant increases in the incidence of hepatocellular altered foci were observed in the groups treated with methyleugenol at 125 mg/kg bw and 250 mg/kg bw [P < 0.001, Fisher exact test] alone or together with phenobarbital at 500 ppm.

[The Working Group noted that this study used multiple doses. However, it also used a small number of animals, only one sex, and a limited number of organs was examined (which was appropriate for the assay being conducted). The short duration may have precluded the formation of carcinomas.]

3.3 Evidence synthesis for cancer in experimental animals

The carcinogenicity of methyleugenol has been assessed in one well-conducted GLP study in male and female B6C3F$_1$ mice by oral administration (gavage) (NTP, 2000; also reported by Johnson et al., 2000), an intraperitoneal injection study in neonate male B6C3F$_1$ mice Miller et al. (1983), in one well-conducted GLP study in male and female F344 rats by oral administration (gavage) (NTP, 2000; also reported by Johnson et al., 2000), and in an initiation–promotion experiment in male F344 rats (Williams et al., 2013).

In male and female B6C3F$_1$ mice treated by gavage, there were increases in the incidence of hepatocellular adenoma, hepatocellular carcinoma, hepatocellular adenoma or carcinoma

(combined), hepatoblastoma, hepatocellular carcinoma or hepatoblastoma (combined), and hepatocellular adenoma, hepatocellular carcinoma or hepatoblastoma (combined). In male B6C3F$_1$ mice treated as neonates by intraperitoneal injection, there was an increased incidence of liver hepatoma. In male and female F344 rats treated by gavage, there were increases in the incidence of hepatocellular adenoma, hepatocellular carcinoma, hepatocellular adenoma or carcinoma (combined), hepatocholangioma, hepatocholangiocarcinoma, hepatocholangioma or hepatocholangiocarcinoma (combined), malignant neuroendocrine tumours of the glandular stomach, and benign or malignant neuroendocrine tumours of the glandular stomach. In male F344 rats treated by gavage, there were increases in the incidence of renal tubule adenoma, renal tubule adenoma or carcinoma (combined), malignant mesothelioma, mammary gland fibroadenoma, and subcutaneous fibroma or fibrosarcoma (combined). There was a significant increase in the incidence of mononuclear cell leukaemia (all organs) and benign neuroendocrine tumours of the glandular stomach in females. In male F344 rats treated in an initiation–promotion assay, there was a dose-dependent induction of hepatocellular adenoma. In male B6C3F$_1$ mice treated with 1′-hydroxymethyleugenol by intraperitoneal injection, there was a significant increase in the incidence of hepatocellular adenoma.

4. Mechanistic Evidence

4.1 Absorption, distribution, metabolism, and excretion

4.1.1 Absorption, distribution, and excretion

(a) Humans

Only the absorption of methyleugenol has been investigated in a few studies in exposed humans, after oral or dermal exposure.

(i) Oral exposure

Schecter et al. (2004) examined serum concentrations of methyleugenol in nine fasted (overnight) volunteers (five men; four women; body weight, 63–74 kg) who ingested ginger-snap cookies containing approximately 216 µg of methyleugenol. The mean serum concentration of methyleugenol before exposure was 16.2 ± 4.0 pg/g wet weight (LOD, 3.1 ng/pg; range, < LOD to 37 pg/g). After exposure, the mean peak serum concentration in the nine participants rose to 53.9 ± 7.3 pg/g wet weight (range, 25–100 pg/g) within 15 minutes. [The Working Group noted that the mean serum concentration of methyleugenol measured before exposure probably reflects background exposure via dietary sources.] A half-life of approximately 90 minutes was calculated. The maximum concentration measured by Schecter et al. (2004) was within the range of concentrations of methyleugenol (i.e. < 3.1 to 390 pg/g; mean, 24 pg/g) measured in the serum of 206 non-fasting participants in the United States National Health and Nutrition Examination Survey (NHANES III, 1988–1994) (Barr et al., 2000).

(ii) Dermal exposure

Using a sorptive tape (absorbent polydimethylsiloxane tape) extraction technique, Sgorbini et al. (2010) quantified methyleugenol on the skin surface of two volunteers after the application of a skin cream containing

methyleugenol at 50 ppm. One hour after application, the amount of methyleugenol detected on the stratum corneum decreased by approximately 90.1%. On the basis of its reported boiling-point (255 °C), methyleugenol is a volatile/semi-volatile organic compound and a proportion of loss from the skin surface is probably caused by volatilization. In one volunteer, Sgorbini et al. (2010) estimated that methyleugenol had a permeation rate of 14.5%, 30 minutes after exposure.

(b) Experimental systems

The National Toxicology Program (NTP) investigated the toxicokinetics of methyleugenol using ^{14}C-labelled and unlabelled methyleugenol in F344 rats and B6C3F$_1$ mice (NTP, 2000). The doses administered ranged from 11.8 to 300 mg/kg bw, and exposure methods included a single gavage exposure, a single intravenous exposure, and repeated gavage exposures (e.g. 6, 12 and 18 months; 5 days per week). Overall, methyleugenol is absorbed extensively within minutes. For example, the time taken to reach maximum concentration (T_{max}) values of approximately 5 minutes were observed in male and female rats and mice, and > 70% of the orally administered radioactivity was found in the urine of male rats and female mice 72 hours after a single gavage exposure to [^{14}C]methyleugenol at a dose of between 25 and 150 mg/kg bw. Notably, most of the radioactivity in rats and mice exposed orally to [^{14}C]methyleugenol was detected within the first 24 hours after dosing, with very little excretion between 24 and 72 hours. Of the administered radioactivity, < 13% was recovered in the faeces and < 0.1% in the expired air. The absolute bioavailability of the parent compound after oral exposure was low and increased in a dose-dependent manner, ranging from 6% to 20% in rats and from 4% to 19% in mice. [The Working Group noted that the low percentage values for absolute bioavailability probably reflect substantial first-pass metabolism and that the dose-dependent increase in absolute bioavailability suggests saturation of metabolism at higher doses.]

After a single intravenous exposure (11.8 mg/kg bw in male F344 rats) or oral exposure (118 mg/kg bw in male F344 rats and female B6C3F$_1$ mice) to [^{14}C]methyleugenol, methyleugenol was eliminated in the urine within 12 hours, with most of the radioactivity being excreted within the first 24 hours. At 72 hours after dosing, most residual radioactivity was found in the liver; in fact, the tissue-to-blood ratio of radioactivity was 2:3 in the liver, 0.9:1.4 in the kidney, and < 1 in all other tissues (i.e. brain, fat, heart, large intestine, lungs, muscle, skin, small intestine, spleen, stomach, and testes). [The Working Group noted that the urinary bladder was not included in the analysis.]

After repeated oral exposures (at doses ranging from 37 to 300 mg/kg bw per day in F344 rats and from 37 to 150 mg/kg bw per day in B6C3F$_1$ mice), the terminal half-life of methyleugenol was 1–2 hours, and elimination of methyleugenol appeared to be similar in young adult and aged rodents. However, values for the area under the curve (AUC) appeared to decrease in males after repeated exposures and to increase with age in males and females. [The Working Group noted that these observations suggest metabolic induction, but also the attenuation of methyleugenol metabolism with ageing.]

Hong et al. (2013) explored dose-, sex- and species-dependent effects on various toxicokinetic parameters after exposure to methyleugenol by gavage or intravenous administration in male and female rats and mice. In contrast to the studies described in NTP (2000), Hong et al. (2013) used corn oil, not methylcellulose, as the vehicle. Non-fasted F344 rats (21 males and 21 females, age, ~13 weeks) were exposed by gavage to methyleugenol as a single dose at 37, 75, or 150 mg/kg bw. A separate group of 18 male and 18 female non-fasted rats was exposed intravenously to methyleugenol as a single dose at 37 mg/kg bw. Similarly, B6C3F$_1$ mice (42 males and 42 females, age, ~13 weeks) were exposed by

gavage to methyleugenol as a single dose at 37, 75, or 150 mg/kg bw. A separate group of 36 male and 36 female mice was exposed intravenously to methyleugenol as a single dose at 37 mg/kg bw. After exposure by gavage at 37 mg/kg bw, the absolute bioavailability of methyleugenol was ~4% in rats and 7–9% in mice). [The Working Group noted that the volume of distribution (> 10 L/kg) in rats and mice exceeded the total body water volume (~0.7 L/kg), suggesting extensive distribution, high tissue binding, and/or plasma protein binding.] In addition, it was observed that after exposure by gavage, the plasma concentration-versus-time curves in rats and mice showed secondary peaks. [The Working Group considered that the secondary peaks in the plasma concentration-versus-time curves were caused by the vehicle used (e.g. corn oil acted as a reservoir in the gut that retarded systemic absorption).]

In general, rats showed higher AUC_{∞} values than did mice. After intravenous exposure (37 mg/kg bw) or exposure by gavage (at doses ranging from 37 to 150 mg/kg bw), methyleugenol was rapidly cleared from the systemic circulation in rats and mice. After intravenous exposure (37 mg/kg bw), systemic clearance of methyleugenol from the plasma appeared to be biphasic in rats and mice; a rapid initial phase was followed by a slower terminal phase.

[The Working Group noted that the clearance values (> 59 mL/minute per kg in rats and > 182 mL/minute per kg in mice after intravenous exposure; and > 293 mL/minute per kg in rats and > 717 mL/minute per kg in mice after gavage) were greater than the hepatic blood flow rates (~55 mL/minute per kg in rats and 90 mL/minute per kg in mice), especially after gavage, suggesting significant first-pass metabolism.] No sex-specific differences in toxicokinetic parameters were noted in rats and mice exposed by gavage at doses ranging from 37 to 150 mg/kg bw.

Kreutz et al. (2018) quantified methyleugenol in skin samples after the application of *Aniba canelilla* oil (containing 16.2% methyleugenol), using the porcine ear skin and Franz-type diffusion cell system. The permeation of methyleugenol from the oil increased in a dose-dependent manner, and the order of retention of methyleugenol was receptor fluid > dermis > epidermis > stratum corneum, indicating that methyleugenol is capable of deep tissue penetration. Using the Franz-type diffusion cell system and heat-separated human epidermis, Schmitt et al. (2009) determined that the apparent permeability of methyleugenol ranged from 1.08×10^{-5} to 2.06×10^{-5} cm/second.

The kinetics of methyleugenol administered as part of an herbal extract may differ from those of pure methyleugenol. For example, an estimated T_{max} of approximately 7 hours and an elimination half-life of approximately 4 hours were observed in the plasma of male Sprague-Dawley rats (body weight, 240 ± 10 g; n = 6) exposed orally to an *Asarum* extract containing methyleugenol at approximately 766 mg/kg bw (Fan et al., 2021). Conversely, Meng et al. (2013) reported shorter T_{max} (10 minutes) and half-life (~68 minutes) values in the plasma of male Sprague-Dawley rats after oral exposure to 0.2 g/kg of an extract of *Acorus tatarinowii* Schott containing 0.5% methyleugenol (equivalent to a dose of 1 mg/kg bw).

4.1.2 Metabolism

(a) Exposed humans

Tremmel et al. (2017) investigated the role of the sulfotransferase SULT1A1 enzyme in the formation of methyleugenol–DNA adducts and the influence of genetic variance in the SULT1A1 gene on SULT1A1 phenotypes in the human liver. Liver and blood tissue samples (n = 121) collected from 121 Caucasian [White] patients undergoing liver surgery were analysed for: (i) blood sample genotyping, i.e. SULT1A1 copy number

variation; (ii) SULT1A1 mRNA expression levels; (iii) SULT1A1 protein expression levels; and (iv) concentration of methyleugenol–DNA adducts, i.e. N^2-(*trans*-methylisoeugenol-3′-yl)-2′-deoxyguanosine (N^2-MIE-dG). Methyleugenol-specific DNA adducts were detected in all the liver samples, and the levels measured positively correlated with both mRNA and protein expression levels of SULT1A1. Additionally, a positive association was noted between SULT1A1 copy number variations and levels of methyleugenol–DNA adducts (see also Section 4.2.1(a)).

(b) Metabolism in human tissue-derived microsomes and cell lines

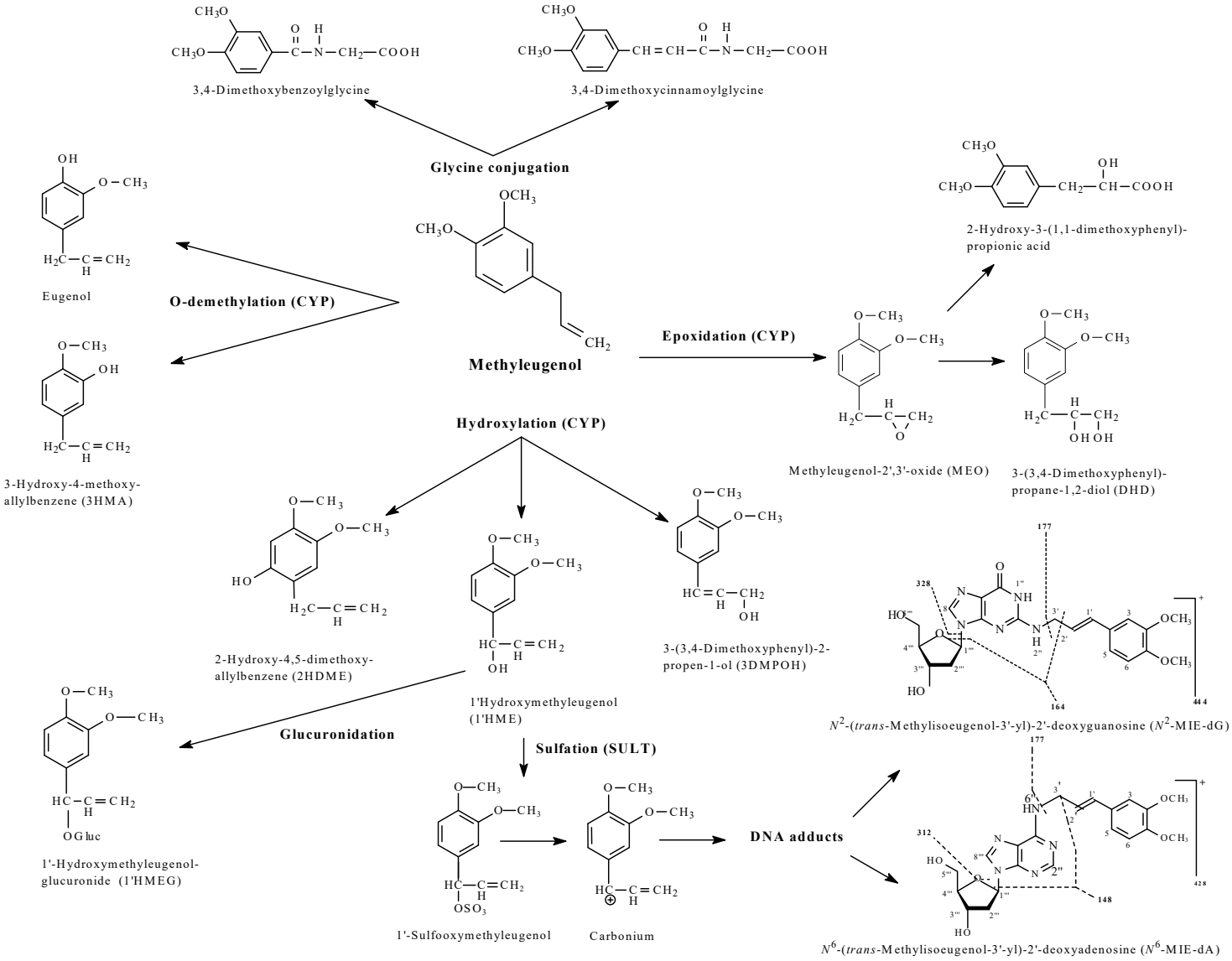
Al-Subeihi et al. (2012) investigated the metabolism of methyleugenol by microsomal preparations from the human liver, lung, kidney, or small intestine. Using pooled microsomal preparations, it was observed that only microsome preparations from the liver were capable of metabolizing methyleugenol and that six metabolites were formed (see Fig. 4.1), of which 1′-hydroxymethyleugenol and its glucuronide conjugate were the most abundant.

When examining human liver microsomes, Jeurissen et al. (2006) reported that the average rate of 1′-hydroxylation of methyleugenol by cytochrome P450 (CYP) in microsomes from 15 individual livers was 2.45 ± 0.34 nmol min^{-1} nmol CYP^{-1} and that activities varied 5-fold (from 0.89 to 4.30 nmol min^{-1} nmol CYP^{-1}). In comparison, Gardner et al. (1997) reported that the mean rate of 1′-hydroxylation of methyleugenol in microsomes from 13 individual human liver samples (~0.50 nmol min^{-1} nmol CYP^{-1}) varied by as much as 27-fold (from 0.05 to 1.33 nmol min^{-1} nmol CYP^{-1}). Al-Subeihi et al. (2015) reported an average rate of oxidation of 1′-hydroxymethyleugenol to 1′-oxomethyleugenol of 5.21 nmol min^{-1} nmol CYP^{-1} in microsomal 9000 × *g* (S9) preparations from 20 individual human liver samples and a difference of approximately 3-fold between the lowest and highest reported activities (from 3 to 8.3 nmol min^{-1} nmol CYP^{-1}). Al-Subeihi et al. (2015) also reported an average rate of sulfonation of 1′-hydroxymethyleugenol of 0.12 nmol min^{-1} nmol CYP^{-1} and an approximate 8-fold difference between the lowest and highest reported activities (0.03–0.25 nmol min^{-1} nmol CYP^{-1}). Gardner et al. (1997) reported that the variation in 1′-hydroxylation activities of the human liver samples was not correlated with CYP content.

[The Working Group noted the substantial variability in the rate of metabolism, i.e. 1′-hydroxylation of methyleugenol, observed in human liver microsomal preparations; however, it was also acknowledged that the results from studies using liver tissue biopsies may have limited relevance to the general population, since the livers of patients at the time of the biopsy probably did not exhibit normal function.]

Al-Subeihi et al. (2013) investigated the effects of nevadensin on the formation of methyleugenol–DNA adducts in human hepatocellular carcinoma cells (HepG2) exposed to 1′-hydroxymethyleugenol. The cells were incubated in the presence of 1′-hydroxymethyleugenol at 100 μM and nevadensin at 0, 0.002, 0.02, 0.2, or 2 μM for approximately 14 hours. Co-incubation with 1′-hydroxymethyleugenol at 100 μM and pentachlorophenol at 12.5 μM was used to model SULT enzyme inhibition. Co-exposure with 1′-hydroxymethyleugenol and nevadensin (from ≥ 0.02 μM nevadensin) resulted in a dose-dependent decrease (up to 88%) in the formation of DNA adducts. Co-exposure with 1′-hydroxymethyleugenol and pentachlorophenol resulted in nearly complete inhibition of the formation of DNA adducts. [The Working Group noted that a previous study identified nevadensin as a potent SULT inhibitor (Alhusainy et al., 2010), which is probably the reason for the observed reduction in methyleugenol–DNA adduct formation with nevadensin at concentrations of ≥ 0.02 μM. Alhusainy et al. (2014) also observed a 24-fold

Fig. 4.1 Proposed metabolic pathways for methyleugenol and its metabolites



CYP, cytochrome P450.
Adapted from Solheim and Scheline (1976); Al-Subeihi et al. (2012); Herrmann et al. (2012).

reduction in DNA adduct formation in male F344 rats after co-administration of nevadensin and methyleugenol (see Section 4.2.1).]

(c) Experimental systems

(i) Non-human mammals in vivo

Solheim and Scheline (1976) exposed male Wistar rats (body weight, 250–300 g) to methyleugenol at a dose of 100, 200, or 400 mg/kg bw by gavage or intraperitoneal injection. Urine and bile were sampled and analysed using GC-MS. No parent molecule was detected in the urine or bile of rats exposed by gavage or by intraperitoneal injection. Twenty-four hours after exposure by gavage, the predominant urinary metabolites were: 2-hydroxy-3-(3,4-dimethoxyphenyl)-propionic acid (20% of the administered dose); 3,4-dimethoxybenzoylglycine (32%); and 3,4-dimethoxycinnamoylglycine (22%). Similar urinary metabolites were observed after intraperitoneal exposure. The major metabolite detected in the bile after oral and intraperitoneal exposure (after treatment of the samples with β-glucuronidase and sulfatase) was 1′-hydroxymethyleugenol. Solheim and Scheline (1976) also noted that 4-dihydroxypropylbenzene is a urinary metabolite of methyleugenol; however, this metabolite was not detected in the urine of rats pre-exposed to neomycin. [The Working Group noted that the absence of 4-dihydroxypropylbenzene in the urine of neomycin-treated rats suggested that rat intestinal microflora might play a role in the metabolism of methyleugenol.]

As previously reported in Section 4.1.1(b), methyleugenol undergoes substantial first-pass metabolism when administered orally. To characterize the urinary metabolites, male F344 rats (n = 3) and female B6C3F$_1$ mice (n = 3) were exposed by gavage to [^{14}C]methyleugenol in corn oil as a single dose at 118 mg/kg bw (NTP, 2000). Three male F344 rats were also exposed by intravenous injection to [^{14}C]methyleugenol in ethanol:Emulphor:saline at a dose of 11.8 mg/kg bw. Urine samples were collected at various intervals up to 72 hours after exposure. Because of the low amounts of radioactivity detected 24 hours after exposure, samples from 24 hours and later were not characterized. Reverse-phase HPLC analysis of pooled urine samples at up to 24 hours revealed that no parent molecule was detected in the urine of male rats exposed intravenously or by gavage or in the urine of female mice after exposure by gavage. The metabolites detected in the urine of both species included hydroxylated, sulfated, and glucuronidated forms.

Feng et al. (2018) developed a method for detecting methyleugenol–DNA adducts (i.e. N^6-(methylisoeugenol-3′-yl)-2′-deoxyadenosine, N^6-MIE-dA) in rat urine. The analytical technique was validated by exposing groups of male Sprague-Dawley rats by gavage to pure methyleugenol (single and repeated doses) and various herbal extracts containing methyleugenol (i.e. *Asari* radix extract, *Acori tatarinowii* rhizoma extract, *Myristicae* semen extract, or Shi San Xiang extract). The doses of methyleugenol administered to each rat ranged from 1 to 25 mg/kg bw for pure methyleugenol and approximately 0.3 to 1.9 mg/kg bw as part of an herbal extract preparation. The amount of N^6-MIE-dA excreted in the urine increased in a dose-responsive manner after the administration of pure methyleugenol. N^6-MIE-dA was also detected in the urine of all rats exposed to the herbal extracts. Another DNA adduct form, N^2-(methylisoeugenol-3′-yl)-2′-deoxyguanosine, N^2-MIE-dG, was also detected in the rat urine; however, only at a dose of 25 mg/kg bw. [The Working Group noted that N^2-(methylisoeugenol-3′-yl)-2′-deoxyguanosine was the type of DNA adduct predominantly detected in the liver tissues of patients studied by Tremmel et al. (2017).] In an earlier study by Yao et al. (2016), several glutathione and related cysteine conjugates were detected in the urine and bile of rats exposed to methyleugenol at 100 mg/kg bw. [The Working Group noted that Yao et al. (2016)

detected three glutathione conjugates in the bile after either gavage or intraperitoneal administration. The conjugates were possibly formed from the α,β-unsaturated aldehyde, carbonium ion, or quinone methide of methyleugenol, e.g. reactive metabolites that may be associated with the putative metabolic pathway for genotoxicity of methyleugenol in rodents.]

(ii) Non-human mammalian cells in vitro

The NTP (2000) investigated the formation of methyleugenol metabolites in rat and mouse hepatocytes and compared it with that in human cells. The cells were incubated with various concentrations of methyleugenol for 18 hours, and the metabolites were characterized by mass spectrometry. Although no definitive structures were assigned, five common metabolites were identified, i.e. a glycine conjugate with a molecular weight of 239 Da; a demethylated and sulfated metabolite (molecular weight, 243 Da); a hydroxylated and sulfated metabolite (molecular weight, 273 Da); a sulfated diol metabolite (molecular weight, 291 Da) in mouse and human cells, but not in rat hepatocytes; and a hydroxylated glucuronide (molecular weight, 369 Da) in rat and mouse but not human hepatocytes.

Al-Subeihi et al. (2011) investigated the metabolism of methyleugenol in microsomal preparations from the liver, lung, and kidney of male Sprague-Dawley rats. The metabolism of methyleugenol in the liver of rats is very similar to that described for humans (Al-Subeihi et al., 2012); 1′-hydroxymethyleugenol is the most abundant metabolite formed. Metabolism in the lung of male rats appeared to be predominantly via O-demethylation; no 1′-hydroxymethyleugenol was formed in incubations with either the lung or the kidney. Only one metabolite was identified after exposure of kidney cells to methyleugenol, i.e. methyleugenol-2′3′-oxide. Male rat liver microsomes were also shown to form glucuronide conjugates, 1′-sulfooxymethyleugenol, and 1′-oxomethyleugenol. Cartus et al. (2012) and Gardner et al. (1997) described similar metabolism in rat liver microsomes.

Gardner et al. (1997) suggested that, at low concentrations, CYP2E1 enzyme but not CYP3A, CYP1A2, CYP2D1, or CYP2C11 catalysed 1′-hydroxylation of methyleugenol in rat hepatocytes in vitro. Additionally, Gardner et al. (1997) showed that pre-treatment of rats with methyleugenol at doses of between 30 and 300 mg/kg bw per day for 5 days increased the 1′-hydroxylation capacity of the harvested hepatocytes in vitro; this suggested that repeated oral exposure to high doses of methyleugenol may induce other enzymes responsible for 1′-hydroxylation (e.g. CYP2B and CYP1A2). Notably, Gardner et al. (1997) did not observe induction of 1′-hydroxylation in rat livers after pre-treatment with methyleugenol at 10 mg/kg bw per day for 5 days. In addition, the increases observed at higher doses (e.g. 30, 100, and 300 mg/kg bw per day) ranged from 38% to 67% higher than control levels. Carlsson et al. (2022) observed significantly higher levels of methyleugenol–DNA adducts (i.e. N^2-MIE-dG and N^6-MIE-dA) in V79 hamster fibroblast cells expressing human SULT1A1 (see also Section 4.2.1).

(iii) Enzymes involved in methyleugenol metabolism in human cells or humanized model systems

Jeurissen et al. (2006) identified human CYP enzymes involved in the metabolism of methyleugenol (i.e. methyleugenol 1′-hydroxylation) using microsomes expressing individual recombinant human CYP genes stably transfected into either insect or human lymphoblastoid cell lines. The activities of CYPs 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C19, 2D6*1, 2E1, and 3A4 in insect and human lymphoblastoid cells were investigated and compared with the activities of pooled microsomes from 15 samples of human liver. The enzyme activities of the human lymphoblastoid cells were roughly similar to those of human liver microsomes, but the activities of the

enzymes from the insect cells were much higher. After 20 minutes of incubation with 200 µM methyleugenol at 37 °C, insect cells expressing CYPs 1A2, 2A6, 2C9, 2C19, and 2D6 as well as human lymphoblastoid cells expressing CYPs 1A2, 2C9, 2C19, and 2D6 were able to 1′-hydroxylate methyleugenol. Co-incubating the human liver microsomes with various enzyme inhibitors showed that the CYP1A2 inhibitor α-naphthoflavone and the CYP2C9 inhibitor sulfaphenazole were most effective at inhibiting methyleugenol 1′-hydroxylation, with inhibitions of approximately 54% and 70% of control activities, respectively. For all other inhibitors investigated, the inhibition of 1′-hydroxylation was < 12%. This indicates that CYP1A2 and CYP2C9 are important enzymes for the 1′-hydroxylation of methyleugenol. However, at physiologically relevant concentrations of methyleugenol, CYP1A2 showed a significantly higher enzyme efficiency (i.e. $k_{cat}/K_m = 169$) than did CYP2C9 ($k_{cat}/K_m = 5$), CYP2C19 ($k_{cat}/K_m = 3$), and CYP2D6 ($k_{cat}/K_m < 3$). Jeurissen et al. (2007) suggested that related alkylbenzenes, such as estragole, may compete for binding sites on the relevant hydroxylation enzymes (e.g. CYP1A2), affecting methyleugenol metabolism. Additionally, lifestyle factors (e.g. smoking and use of barbiturates) or polymorphisms that may induce or inhibit these enzymes may contribute to interindividual differences in metabolism and, potentially, toxicity (Jeurissen et al., 2006, 2007).

Al-Subeihi et al. (2015) also used insect cells expressing human CYPs (i.e. CYPs 1A2, 2A6, 2B6, 2C8, 2C9*1, 2C19, 2D6*1, 2E1, or 3A4), and UDP-glucuronosyltransferase (i.e. UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, or 2B17) to evaluate the metabolism of methyleugenol and the bioactivation of its metabolite (i.e. 1′-hydroxymethyleugenol). The results from the insect cell experiments suggested that: (i) CYP1A2 is the main enzyme involved in 1′-hydroxylation of methyleugenol; (ii) CYP2B6 is predominantly responsible for epoxidation of 1′-hydroxymethyleugenol; and (ii) UGT1A9 and UGT2B7 are predominantly responsible for glucuronidation of 1′-hydroxymethyleugenol.

Herrmann et al. (2014) conducted a study using genetically modified strains of FVB/N mice. Four mouse strains were studied, i.e. (i) wildtype (WT) for mouse Sult1a1; (ii) deficient in mouse Sult1a1 (knockout; KO); (iii) expressing human SULT1A1/2 (transgenic; TG); or (iv) hemizygous for human SULT1A1/2 (KO/TG). Mice were exposed to methyleugenol at doses ranging from 0.28 to 280 µmol/kg bw (from 0.05 to 50 mg/kg bw) via gavage or an equimolar high dose of 1′-hydroxymethyleugenol (280 µmol/kg bw; 54.5 mg/kg bw) via intraperitoneal injection. All animals were killed 6 hours after dosing, according to the optimal interval for detecting DNA adduct formation in the liver. Herrmann et al. (2014) found that, after exposure to methyleugenol, the formation of DNA adducts in the livers of KO mice was reduced by ≥ 97% compared with WT mice and that TG mice showed enhanced DNA adduct formation compared with WT mice. Additionally, there was significantly greater DNA adduct formation in WT, TG, and KO/TG mice after equimolar intraperitoneal dosing with 1′-hydroxymethyleugenol than after dosing with methyleugenol exposure by gavage. These results confirmed that SULT1A is a key enzyme responsible for the bioactivation of methyleugenol. Subsequent analysis of extrahepatic tissue from a similar experiment using these transgenic mice showed that an increase in the formation of DNA adducts was also detected in the extrahepatic tissues in the following order: liver > caecum > kidney > colon > stomach > small intestine > lung > spleen (Herrmann et al., 2016). Closer examination of SULT1A activities in the liver, caecum, kidney, and stomach suggested that the formation of DNA adducts in extrahepatic tissue after methyleugenol exposure is dependent on levels of SULT1A enzymes in the tissue. For example, high expression levels of human SULT1A1/2 and mouse Sult1a1 are observed in

the liver and caecum, but extremely low levels of both enzymes are observed in the stomach (Herrmann et al., 2016) (see also Section 4.2.1).

Herrmann et al. (2012) investigated whether recombinant *S. typhimurium* bacteria strain TA100 expressing human sulfotransferases (i.e. SULT1A1, 1C2, 1A2, 1E1, 1A3, 1C1, 1C3, 2A1, and 2B1b) were capable of activating hydroxylated metabolites of methyleugenol to mutagens in the Ames assay. For this experiment, three hydroxylated forms of methyleugenol (i.e. (+)-1′-hydroxymethyleugenol, (–)-1′-hydroxymethyleugenol, and (*trans*)-3′-hydroxymethylisoeugenol) were synthesized and incubated with *S. typhimurium* expressing various individual human sulfotransferases versus those deficient in sulfotransferases. *S. typhimurium* expressing SULT1A1 (expressed in eukaryotes in many tissues) and SULT1C2 (expressed primarily in fetal tissue) showed higher revertant frequencies with very low concentrations of hydroxylate than did the respective deficient TA100 strain. No activation was observed for strains expressing SULT1A3, SULT1C1, SULT1C3, SULT2A1, or SULT2B1b. Some activation was observed for SULT1A2 and SULT1E1 but at a much lower level than observed for SULT1A2 and SULT1E1. [The Working Group noted that since the level of protein expression in bacteria differs significantly from that in eukaryotes, the relevance of these results to the metabolism of methyleugenol by humans should be considered cautiously.]

Herrmann et al. (2012) also investigated the effectiveness of murine sulfotransferases (Sult1a1 or Sult1d1) to activate hydroxylated metabolites of methyleugenol (i.e. (+)-1′-hydroxymethyleugenol, (–)-1′-hydroxymethyleugenol, and (*trans*)-3′-hydroxymethylisoeugenol) using recombinant bacteria (*S. typhimurium* strain TA100) and the Ames assay. Murine Sult1a1 required higher concentrations of all three hydroxylated methyleugenol metabolites to cause a noticeable, but much lower, increase in revertant frequencies compared with its human orthologue (SULT1A1). Murine Sult1d1 did not activate any of the hydroxylated methyleugenol metabolites. In a similar experiment, Honda et al. (2016) used modified TA100 strains of *S. typhimurium* in the Ames assay to investigate the activities of human SULT1C2. Using optimized in vitro conditions (i.e. protein content, 1.2 mg/plate; pre-incubation time, 2 hours), methyleugenol was found to increase the number of revertants significantly and reproducibly in the modified TA100 strains (Honda et al., 2016) (see also Section 4.2.2).

(iv) Acellular systems

Sipe et al. (2014) investigated the metabolism of methyleugenol using horseradish peroxidase and electron paramagnetic resonance spectroscopy. [The Working Group noted that the study provided evidence (through spectral analysis) that methyleugenol underwent peroxidative metabolism in vitro to form free radicals and that these radicals were not good substrates for catalase (see also Section 4.2.5).]

4.1.3 Physiologically based kinetic modelling

The evaluation of the toxicokinetics of methyleugenol has also been approached through physiologically based kinetic (PBK) modelling. For example, the NTP developed PBK models for methyleugenol in rats and mice on the basis of in vivo toxicokinetic parameters determined in F344 rats and B6C3F$_1$ mice and on information available in the literature (NTP, 2000). More recently, Al-Subeihi et al. developed a series of PBK models for methyleugenol. One PBK model in rats was based on in vitro metabolic parameters, in silico-derived partition coefficients, and information on physiological parameters identified in the literature (Al-Subeihi et al., 2011). A second PBK model accounted for SULT inhibition by nevadensin (Al-Subeihi et al., 2013) A third PBK model was developed specifically for humans and was based on in vitro (i.e. phase I

and II metabolism by human cell microsomes) and in silico-derived parameters (Al-Subeihi et al., 2012). The human PBK model was further refined by including: (i) CYP1A2-catalysed bioactivation of methyleugenol; (ii) CYP2B6-catalysed epoxidation of methyleugenol; (iii) the apparent kinetic constants for oxidation of 1′-hydroxymethyleugenol; and (iv) the apparent kinetic constants for sulfation of 1′-hydroxymethyleugenol (Al-Subeihi et al.,2015).

4.2 Evidence relevant to key characteristics of carcinogens

This section reviews the mechanistic data for the key characteristics of carcinogens (Smith et al., 2016) encompassed by methyleugenol. Evidence was available on whether methyleugenol exhibits the key characteristics "is electrophilic or can be metabolically activated to an electrophile", "is genotoxic", "induces oxidative stress", "induces chronic inflammation", "is immunosuppressive", and "modulates receptor-mediated effects", "causes immortalization", and "alters cell proliferation, cell death, or nutrient supply". The evidence for the key characteristics of carcinogens "alters DNA repair or causes genomic instability" and "induces epigenetic alterations" was sparse. The exposure assessments for the mechanistic studies in humans are reported in Section 1.6.

4.2.1 Is electrophilic or can be metabolically activated to an electrophile

(a) Humans

(i) Exposed humans

See Table 4.1 and Fig. 4.1.

Evidence of electrophilicity in exposed humans was reported in three studies. Herrmann et al. (2013) investigated the formation of DNA adducts in liver tissue samples collected from surgical liver interventions from a group of 30 individuals (18 males and 12 females) at the Campus Virchow, University Medical Center Charité, Berlin, Germany. Of the 30 surgical non-tumour liver samples, 29 were found to contain the major DNA adduct formed by methyleugenol, namely N^2-MIE-dG, at levels in the range of 1.1–36.2 adducts/10^8 nucleosides, detected by isotope dilution UPLC-MS/MS (Herrmann et al., 2013). The analogous adenosine adduct, N^6-MIE-dA, was also detected in most samples, although at a level that was lower by 60-fold. The maximum and median levels of both adducts combined were 37 and 13 adducts/10^8 nucleosides, respectively. In a further study from the same research group, Tremmel et al. (2017) detected specific methyleugenol–DNA adducts in 121 human liver tissue specimens from subjects undergoing surgery in the department of general, visceral, and transplantation surgery at the Charité centre (Campus Virchow, University Medical Center Charité, Humboldt University Berlin, Germany). The levels of adducts varied by 122-fold (Tremmel et al., 2017). These levels correlated significantly with mRNA and protein levels of human sulfotransferase SULT1A1, indicating the role of this isozyme in the metabolic activation of methyleugenol, as also mentioned in Section 4.1.

In another study, Monien et al. detected the most common DNA adduct formed by methyleugenol, N^2-MIE-dG, in the range of 1.7–23.7/10^8 nucleosides, as measured by UPLC-MS/MS in non-tumour pulmonary tissue samples from 4 men and 6 women with lung cancer, provided by Biopredic International, Rennes, France (Monien et al., 2015). The less frequent adduct, N^6-MIE-dA, was detected in the range of 0.31–1.18 adducts/10^8 nucleosides in 5 out of 10 lung tissue samples. The levels of N^6-MIE-dA were on average 27-fold lower than the levels of N^2-MIE-dG. [The Working Group noted that, although these studies did not investigate specific sources of exogenous exposure of methyleugenol (see Section 1.6), the results might

Table 4.1 End-points relevant to electrophilicity in humans exposed to methyleugenol

End-point (assay)	Biological sample type	Location	No. of exposed and controls	Response[a] (No. of individuals with/without DNA adducts)	Covariates controlled	Comments	Reference
DNA adducts (^{32}P-postlabelling)	Surgical, non-tumourous liver	Germany	18 men, 12 women	+ (29/30)	Patients with hepatitis, cirrhosis, or chronic excessive alcohol use	Source of exposure not documented.	Herrmann et al. (2013)
DNA adducts (^{32}P-postlabelling)	Surgical, non-tumourous liver	Germany	121 participants	+ (121/121)		Source of exposure not documented. Inverse relationship between adducts levels and age.	Tremmel et al. (2017)
DNA adducts (^{32}P-postlabelling)	Surgical, non-tumourous lung	France	4 men, 6 women	+ (10/10)		Source of exposure not documented.	Monien et al. (2015)

[a] +, positive.

demonstrate evidence of universal human exposure to methyleugenol.]

(ii) Human cells in vitro

See Table 4.2.

DNA adducts, analysed by ^{32}P-postlabelling, were formed in a dose-dependent manner in metabolically competent human hepatocellular carcinoma HepG2 cells when exposed to methyleugenol at increasing concentrations (50–450 µM) (Zhou et al., 2007).

N^2-MIE-dG and *N*^{6}-MIE-dA adducts were also formed in HepG2 cells treated with 1′-hydroxymethyleugenol (25–150 µM), as the main phase I metabolite of methyleugenol (Carlsson et al., 2022).

(b) Experimental systems

(i) Humanized animals

The role of sulfotransferases (SULTs) in the metabolism of methyleugenol has been demonstrated in genetically modified mice (see also Section 4.1). The formation of DNA adducts in the liver induced by oral exposure to methyleugenol or by intraperitoneal exposure to 1′-hydroxymethyleugenol and 3′-hydroxymethylisoeugenol was greatly reduced in the liver of *Sult1a1* knockout mice, compared with wildtype mice.

In mice transgenic for human *SULT1A1/2*, adduct levels were higher by about 10-fold than in wildtype mice, and were similar whether or not the mouse gene was also present (i.e. the combination of both human and mouse genes) (Herrmann et al., 2014).

SULT1A forms also dominate metabolic activation in several extrahepatic tissues. Using the same knockout and transgenic mouse strains, Herrmann et al. (2016) found that DNA adduct formation by methyleugenol in mouse caecum and kidney was almost entirely dependent on the presence of mouse *Sult1a1* or human *SULT1A1/2*. In the stomach, however, adduct formation was independent of SULT1A status.

(ii) Non-human mammals in vivo

See Table 4.3.

Intraperitoneal exposure of adult female CD-1 mice to methyleugenol at a dose of 2 or 10 mg/mouse in 0.1 mL of trioctanoin induced the formation of DNA adducts in the liver at levels of 150 ± 15 or 646 ± 88 adducts/10^7 nucleotides, respectively, as assessed by ^{32}P-postlabelling (Randerath et al., 1984).

Methyleugenol was the most potent DNA adduct-forming compound out of the seven compounds tested in a study designed to test the carcinogenicity of alkenylbenzenes. Male C57B1 × C3H/He F_1 (B6C3F_1) mice were injected with methyleugenol at a dose of 0.25, 0.5, 1.0, or 3.0 µmol on days 1, 8, 15, and 22 after birth, respectively. Liver DNA was isolated on days 23, 29, and 43 and analysed by ^{32}P-postlabelling. DNA adducts were observed at levels of 72.7 ± 10.7 pmol/mg DNA on day 23, 37.1 ± 9.7 pmol/mg DNA on day 29, and 25.6 ± 6.2 pmol/mg DNA on day 43 (Phillips et al., 1984).

DNA adducts were also detected, but not quantified, by HPLC-^{32}P-postlabelling analysis of the livers of mice treated intraperitoneally with methyleugenol at a dose of 2000 µmol/kg (Levy and Weber, 1988).

Male Sprague-Dawley rats exposed to methyleugenol at a dose of 1, 5, or 25 mg/kg bw by gavage produced the DNA adduct *N*^{6}-MIE-dA, which was detected in urine samples collected at 12-hour intervals. *N*^{6}-MIE-dA was excreted in a time- and dose-dependent manner (Feng et al., 2018).

The potential of methyleugenol to induce DNA-adduct formation was also proven by the co-exposure of male F344 rats to nevadensin (a SULT inhibitor; 120 mg/kg bw per day for 3 days per week) and methyleugenol (250 mg/kg bw per day, three times per week), resulting in a reduction of 24-fold in adduct formation after 8 weeks compared with rats exposed to methyleugenol only (Alhusainy et al., 2014) (see also

Table 4.2 End-points relevant to genotoxicity and related effects in human cells in vitro exposed to methyleugenol

End-point (assay)	Cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
DNA strand breaks (alkaline comet assay)	Human colon carcinoma H29 cells	+	NT	50 µM	Comets also formed by 1′-hydroxymethyleugenol (at 50 µM), 3-oxomethylisoeugenol, and methyleugenol-2′,3′-oxide (at ≥ 25 µM)	Groh et al. (2016)
Micronucleus formation	Human colon carcinoma H29 cells	–	NT	100 µM	Micronuclei also formed by 1′-hydroxymethyleugenol, 3′-oxomethylisoeugenol, and methyleugenol-2′,3′-oxide (at ≥ 10 µM)	Groh et al. (2016)

HIC, highest ineffective concentration; LEC, lowest effective concentration, NT, not tested.

[a] +, positive; –, negative.

Table 4.3 End-points relevant to electrophilicity and genotoxicity in non-human mammals in vivo exposed to methyleugenol

End-point (assay)	Species, strain (sex)	Tissue or cells	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
DNA adducts	Mouse, CD-1 (F)	Liver	+	2 mg/kg	i.p., for 24 h		Randerath et al. (1984)
DNA adducts	Mouse, B6C3F$_1$ (M)	Liver	+	0.25, 0.5, 1.0, and 3.0 µmol on days 1, 8, 15, and 22 after birth	Injected; DNA isolated on days 23, 29, and 43		Phillips et al. (1984)
DNA adducts	Mouse, C57BL/6J	Liver	+	2000 µmol/kg	i.p, for 3 h		Levy and Weber (1988)
DNA adducts	Mouse, wildtype (FVB/N), *Sult1a1*-knockout, transgenic human *SULT1A1/2*	Liver	+	50 mg/kg	By gavage, for 6 h	Adduct levels greatly reduced in *Sult1a1*-knockout mice; 10-fold higher in human *SULT1A1/2*-transgenic mice than in wildtype mice.	Herrmann et al. (2014)
DNA adducts	Mouse, wildtype (FVB/N), *Sult1a1* knockout, transgenic human *SULT1A1/2*	Caecum, kidney	+	50 mg/kg	By gavage, 1.5–24 h	Adduct formation highly dependent on the presence of mouse *Sult1a1* or human *SULT1A*.	Herrmann et al. (2016)
DNA adducts	Rat, F344 (M)		+	250 mg/kg per day	By gavage, 3×/wk for 8 wk	Adduct levels reduced 24-fold by co-administration of nevadensin, a SULT inhibitor.	Alhusainy et al. (2014)
DNA adducts	Rat, Sprague-Dawley (M)	Urine	+	1, 5, or 25 mg/kg	By gavage; urine collected at 12 h intervals	dA adduct of methyleugenol excreted in the urine.	Feng et al. (2017)
DNA strand breaks (alkaline comet assay)	Rat, F344 (M)	Liver, bladder, kidney, lung	–	2000 mg/kg, at 1–8 h	By gavage, 1, 3, 6 and 8 h	Comet formation observed at 6 h and 8 h. when endonuclease III included in the protocol.	Ding et al. (2011)
		Bone marrow	+/–	2000 mg/kg		Bone marrow gave positive results at 8 h only.	
Gene mutation	Rat, *Gpt* delta (M, F)		+	100 mg/kg per day	By gavage, for 13 wk		Jin et al. (2013)

Table 4.3 (continued)

End-point (assay)	Species, strain (sex)	Tissue or cells	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
Gene mutation	Mouse, B6C3F$_1$/N (M, F)	Liver tumour	+	37, 75, or 150 mg/kg bw	By gavage, for 2 years	Analysis of samples from a 2-year cancer study. Exome sequencing revealed a higher mutation burden than found in spontaneous tumours.	Auerbach et al. (2018)
Micronucleus formation	Mouse, B6C3F$_1$/N (M, F)	Peripheral blood erythrocytes	–	1000 mg/kg	By gavage for 14 wk	No increase in percentage of polychromatic erythrocytes.	NTP (2000)

dA, deoxyadenosine; dG, deoxyguanosine; F, female; h, hour(s); HID, highest ineffective dose; i.p., intraperitoneal; LED, lowest effective dose; M, male; NT, not tested; ppm, parts per million; SULT, sulfotransferase; wk, week(s).

[a] +, positive; –, negative; +/–, equivocal (variable response in several experiments within an adequate study).

Section 4.1.2). Pentachlorophenol (11 mg/kg per day), included in the study as a positive control, caused a reduction in DNA-adduct formation that was similar to that caused by methyleugenol.

Methyleugenol has also been shown to covalently modify RNA. Yang et al. (2020) detected three adenosine adducts (formed at the 1-, 3-, and 6-positions of adenine, characterized by LC-MS/MS) plus two guanosine adducts and one cytidine adduct in the hepatic tissues of male Kunming mice exposed intraperitoneally to a mixture of chemically synthesized methyleugenol d_0-Me/d_3-Me (1:1) at a dose of 10, 30, or 50 mg/kg.

Intraperitoneal exposure of male Fisher rats to methyleugenol, either as a single dose at 10 or 100 mg/kg or at doses of 10, 30, 100, or 300 mg/kg per day for five consecutive days, induced the formation of covalent protein adducts in the liver, as assessed by enzyme-linked immunosorbent assay (ELISA) using specific antisera raised in rabbits treated with serum albumin conjugated with 1′-acetoxymethyleugenol (Gardner et al., 1996). Adducts were formed in a dose-dependent manner, with the highest levels found in microsomal fractions and lesser amounts in the nuclear, mitochondrial, and cytosolic fractions. Methyleugenol was also shown to interact with glutathione and proteins (see Section 4.1.2(b)). In fact, biliary glutathione and urinary cysteine conjugates were observed in male Sprague-Dawley rats exposed to methyleugenol at 100 mg/kg (Yao et al., 2016).

(ii) Non-human mammalian cells in vitro

See Table 4.4.

Incubation of primary hepatocytes isolated from male Wistar rats with methyleugenol and some of its metabolites formed by rat, bovine and human liver microsomes, such as 1′-hydroxymethyleugenol, 3′-hydroxymethylisoeugenol, and 3′-oxomethylisoeugenol, induced the formation of the deoxyguanosine adduct, N^2-MIE-dG. The deoxyadenosine adduct N^6-MIE-dA was also detectable in some incubations at levels 50 times as low as the deoxyguanosine adduct. The levels of adducts formed by 1′-hydroxymethyleugenol were higher than those formed by methyleugenol itself, suggesting the importance of this metabolite in the pathway of metabolic activation of the parent compound (Cartus et al., 2012).

Carlsson et al. observed a significant increase in both N^2-MIE-dG and N^6-MIE-dA DNA adducts in V79 hamster fibroblasts expressing the human SULT1A1 enzyme and exposed to methyleugenol, thus proving the role of SULT enzyme in the metabolic activation of 1′-hydroxymethyleugenol (Carlsson et al., 2022).

As reported in studies in vivo, reactive metabolites of methyleugenol have also been shown to modify RNA in vitro. Yang et al. (2020) used LC-MS/MS to detect and characterize three adenosine adducts, formed at the 1-, 3-, and 6-positions of adenine, in RNA from primary mouse hepatocytes incubated with methyleugenol at 100 μM.

(iii) Non-mammalian experimental systems

See Table 4.5.

In studies that investigated the potential effects of methyleugenol in a turkey egg genotoxicity assay, it was observed that the injection of methyleugenol (2–4 mg/egg) into turkey eggs containing 22–24-day-old fetuses induced DNA adducts in the fetal liver, as assessed by ^{32}P-postlabelling analysis (Kobets et al., 2016, 2018). In a follow-up study, Kobets et al. (2019) confirmed the formation of N^2-guanine and N^6-deoxyadenine derivatives by mass spectrometry and also observed DNA adduct formation in fetuses contained in chicken eggs treated with methyleugenol (Kobets et al., 2019).

As mentioned in Section 4.1.2(b), hydroxylated metabolites of methyleugenol induced the formation of DNA adducts, determined by UPLC-MS/MS, in *S. typhimurium* strain TA100 expressing either human or murine sulfotransferases (Herrmann et al., 2012). Specifically, (+)-1′-hydroxymethyleugenol, (–)-1′-

Table 4.4 End-points relevant to electrophilicity and genotoxicity in non-human mammalian cells in vitro exposed to methyleugenol

End-point (assay)	Species, strain, tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
DNA adducts (mass spectrometry)	Rat, Wistar (M), primary hepatocytes	+	NT	250 µM	dG adduct detected, plus dA adduct at 50-times lower level. Adducts also formed by 1′-hydroxymethyleugenol, 3′-hydroxymethylisoeugenol and 3′-oxomethylisoeugenol	Cartus et al. (2012)
DNA strand breaks (comet assay)	Hamster, Chinese, lung fibroblasts, V79	+	NT	25 µM		Groh et al. (2012)
Unscheduled DNA synthesis	Rat, F344 (M), primary hepatocytes	+	NT	1000 µM		Howes et al. (1990); Chan and Caldwell (1992)
Unscheduled DNA synthesis	Rat, F344 (M), primary hepatocytes	+	NT	10 µM	One concentration tested	Burkey et al. (2000)
	Mouse, $B6C3F_1$ (F), primary hepatocytes	+	NT	10 µM		
Micronucleus formation	Hamster, Chinese, lung fibroblasts, V79	–	NT	100 µM	Although methyleugenol gave negative results, several metabolites gave positive results	Groh et al. (2012)
Sister-chromatid exchange	Hamster, Chinese, ovary, CHO cells	–	+	17 µg/mL	Toxic at 233 µg/mL –S9	NTP (2000)
Chromosomal aberrations	Hamster, Chinese, ovary, CHO cells	–	–	233 µg/mL	Toxic at 500 µg/mL	NTP (2000)

CHO, Chinese hamster ovary; dA, deoxyadenosine; dG, deoxyguanosine; HIC, highest ineffective concentration; LEC, lowest effective concentration, NT, not tested; S9, 9000 × *g* supernatant.

[a] +, positive; –, negative.

Table 4.5 End-points relevant to genotoxicity and related effects of methyleugenol and metabolites in non-mammalian experimental systems

Test system (species, strain)	End-point (assay)	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Turkey embryos, age 22–24 days	DNA adducts (^{32}P-postlabelling)	+	NA	3 doses totalling 4 mg/egg	Adducts confirmed by mass spectrometry	Kobets et al. (2016, 2018, 2019)
Chicken embryos, age 9–11 days	DNA adducts (^{32}P-postlabelling)	+	NA	3 doses totalling 2 mg/egg	Adducts confirmed by mass spectrometry	Kobets et al. (2019)
Turkey embryos, age 22–24 days	DNA strand breaks (comet assay)	–	NA	3 doses totalling 4 mg/egg		Kobets et al. (2016, 2018)
Saccharomyces cerevisiae, RS9	Recombination	+	+	0.68–2.04 mM [121.3–363.75 µg/mL]	Intrachromosomal (*HIS*$^+$) and interchromosomal (*ADE*$^+$) recombination in a dose-related manner	Schiestl et al. (1989)
Saccharomyces cerevisiae, RS112	Recombination	+	NA	0.6 mg/mL	DEL recombination; nonlinear dose–response relation	Brennan et al. (1996)
Salmonella typhimurium TA97, TA100, TA1535, TA1537	Mutation	–	–	6 µM/plate	Highest dose is lower than OECD 471 recommendations because of toxicity	Schiestl et al. (1989)
Salmonella typhimurium TA98, TA98, TA100, TA102	Mutation	–	–	333 µg/plate	Highest dose is lower than OECD 471 recommendations due to toxicity	NTP (2000)
Salmonella typhimurium TA100-hSULT1A1, -hSULT1A1, -hSULT1A2, -hSULT1E1	Mutation	+	NA	~3 nmol (+)- and (–)-1′-hydroxymethyleugenol, 3′-hydroxymethylisoeugenol	Metabolites tested	Herrmann et al. (2012)
Salmonella typhimurium TA100-hSULT1A3, -hSULT1C1, -hSULT1C3, -hSULT2A1, -hSULT2B1b	Mutation	–	NA	100 nmol (+)- and (–)-1′-hydroxymethyleugenol, 3′-hydroxymethylisoeugenol	Metabolites tested	Herrmann et al. (2012)
Salmonella typhimurium TA100-hSULT1A1	Mutation	+	NA	31 µM 1′-hydroxymethyleugenol	Metabolite tested	Berg et al. (2016)
Escherichia coli WP2 *uvrA*	Mutation	–	–	300 µg/plate	Highest dose is lower than OECD 471 recommendations due to toxicity	Sekizawa and Shibamoto (1982)

Table 4.5 (continued)

Test system (species, strain)	End-point (assay)	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Salmonella typhimurium strain with hSULT1C2	Mutation	–	+	0.391–62.5 µg/plate	Dose–response relation up to 62.5 µg/plate +S9	Honda et al. (2016)
Bacillus subtilis rec H17 Rec$^+$) and M45 (Rec$^-$) strains	DNA damage (rec assay)	+	NA	1.0 mg/disk		Sekizawa and Shibamoto (1982)

HIC, highest ineffective concentration; LEC, lowest effective concentration; NA, not applicable; NR, not reported; OECD, Organisation for Economic Co-operation and Development; S9, 9000 × *g* supernatant.

[a] +, positive; –, negative.

hydroxymethyleugenol, and (*trans*)-3′-hydroxymethylisoeugenol induced the formation of DNA adducts in bacteria expressing human SULT1A1; low levels of DNA adducts were also detected in bacteria expressing human SULT1A2 and SULT1E1 and murine Sult1a1. No DNA adduct formation was observed in bacteria expressing other human SULT forms (1A3, 1C1, 1C3, 2A1, and 2B1b) or murine Sult1d1, or in the unmodified parent TA100 strain. The main adduct formed was N^2-MIE-dG, with much lower levels of the minor adduct N^6-MIE-dA detectable in some cases.

(iv) Acellular systems

Methyleugenol labelled with ^{14}C was reported to bind to calf thymus DNA both in the presence of Araclor 1254-induced rat and mouse liver S9 and in the presence of uninduced rat, mouse, and human liver S9 (NTP, 2000). Methyleugenol (100 μM) was also reported to modify protein cysteine residues in mouse liver microsomes, forming three types of modification derived from the elctrophilic intermediates, α,-β-unsaturated aldehyde and the 1′-carbonium ion of methyleugenol, as characterized by LC-MS/MS (Feng et al., 2017).

4.2.2 Is genotoxic

(a) Humans

(i) Exposed humans

No data on genotoxicity-associated endpoints in exposed humans were available to the Working Group.

(ii) Human cells in vitro

See Table 4.2.

Methyleugenol and three of its metabolites (1′-hydroxymethyleugenol, methyleugenol-2′,3′-epoxide, and 3′-oxomethylisoeugenol) induced alkali-labile lesions in the DNA of human colon carcinoma H29 cells, as measured by the alkaline comet assay (Groh et al., 2016). The parent compound and 1′-hydroxymethyleugenol gave positive results at 50 μM, the other two metabolites at 25 μM and above. Methyleugenol did not induce micronucleus formation at up to 100 μM, although methyleugenol-2′,3′-epoxide at 100 μM and 3′-oxomethylisoeugenol at ≥ 10 μM gave positive results. Micronuclei were CREST-positive (kinetochore-containing), indicating an aneugenic mode of action (Groh et al., 2016).

A DNA damage response, including induction of γH2AX, was observed in human liver HepG2 cells treated with the methyleugenol metabolite 1′-hydroxymethyleugenol at 25–150 mM, but strand breaks, measured by the comet assay, were not detected (Carlsson et al., 2022).

(b) Experimental systems

(i) Non-human mammals in vivo

See Table 4.3.

DNA damage was assessed by the comet assay conducted on male F344 rats exposed to methyleugenol as a single oral dose at 400 or 1000 mg/kg bw (Ding et al., 2011). There was no evidence of increased DNA damage in the liver, bone marrow, bladder, kidney, and lung at 3 and 24 hours after treatment. After a higher dose of 2000 mg/kg bw, the liver, bone marrow, and bladder did not exhibit DNA damage at 1, 3, 6, and 8 hours after treatment, with the exception of the bone marrow at 8 hours. However, when a modified comet assay (including endonuclease III in the protocol for liver samples) was used (Table 4.6), comet formation was detected in methyleugenol-exposed rats at 6 and 8 hours, suggesting that methyleugenol induced oxidative damage in DNA.

When methyleugenol was investigated in the F344 *gpt* delta transgenic rat model, in male and female rats treated orally at 0, 10, 30, or 100 mg/kg for 13 weeks, a significant increase in *gpt* and Spi^- mutation frequencies was observed in the liver at the highest dose (a carcinogenic dose) (Jin et al., 2013). [The Working Group noted that

Table 4.6 End-points relevant to oxidative stress in experimental systems exposed to methyleugenol

End-point	Assay	Species, strain (sex), cell line, system	Results[a]	Dose or concentration (LEC/LED or HIC/HID)	Comments	Reference
Non-human mammals in vivo						
Oxidative DNA damage	Endo III-comet assay	Rats, F344 (M), liver	+	2000 mg/kg, gavage, 1, 3, 6 and 8 h	Comet formation was observed at 6 h and 8 h when endonuclease III was included in the protocol.	Ding et al. (2011)
ROS	Hydroethidine	Rats, SD (M)	↓	100 mg/kg, i.p.	ROS ↑ after experimentally induced ischaemia (visualized microscopically). The increase was partially reversed by methyleugenol. No control using methyleugenol with non-ischaemic tissue.	Choi et al. (2010)
GSH/GST GSH content GST content SOD CAT NO Lipid peroxidation	GSH content GST content Enzyme activity Enzyme activity Griess reagent TBARS	Intestinal mucosal tissue, homogenized from adult Wistar rats, treated with methyleugenol, hypoxia (in parentheses), or both, respectively	NC, (↓), ↑ NC, (↓), ↑ NC, (↓), ↑ NC, (↓), ↑ NC, (↑), ↓ NC, (↑), ↓	100 mg/kg per day, gavage, 30 days	Methyleugenol treatment of rats with and without ischaemia induced by surgical ligation of intestinal tissue for 30 min followed by perfusion. In most cases, methyleugenol treatment of ischaemic tissue changed the measured parameters to control values.	Saleh and El-Shorbagy (2017)
DSBs Oxidative DNA damage	Fpg-comet assay	V79 Chinese hamster lung fibroblasts	+	25 µM	Enhanced formation of DSBs	Groh et al. (2012)
Non-human mammalian cells in vitro						
ROS	DCFDA	Mixed glial cells cultured from prefrontal cortex of SD rats (M), age 1 day	↓	10 µM	ROS ↑ after hypoxia (damage visualized microscopically and by LDH release) or stimulation with IFNγ/LPS. Partially reversed by methyleugenol exposure. No methyleugenol control experiments without hypoxia or IFNγ/LPS, respectively.	Choi et al. (2010)

Table 4.6 End-points (continued)

End-point	Assay	Species, strain (sex), cell line, system	Results[a]	Dose or concentration (LEC/LED or HIC/HID)	Comments	Reference
MnSOD, CAT	Enzyme activity	Primary cortical neuronal cultures from SD (M) rats, day 17–18 of gestation	↑	10 μM	Increase demonstrated after hypoxia treatment (but methyleugenol concentration unclear). methyleugenol (10 μM) partially reversed increases in NO and iNOS gene expression induced by IFNγ/LPS. No control experiments without hypoxia or IFNγ/LPS treatments, respectively.	Zhou et al. (2017)
ROS	DCF fluorescence	Mouse macrophage cell line RAW 264.7	NC, (↑), ↓	140 μM [25 μg/mL] methyleugenol, tBHP (alone), or both, respectively	Cells were exposed to methyleugenol alone, tBHP, or tBHP with increasing concentrations of methyleugenol. tBHP increased ROS and decreased GSH and SOD activity. Methyleugenol was not cytotoxic (MTT assay) but 2 mM tBPH was. Co-incubation of 2 mM tBHP and increasing methyleugenol concentrations partially reversed tBPH cytotoxicity and impact on ROS generation and SOD activity.	
GSH	Protein content, tBHP	Mouse macrophage cell line RAW 264.7	↑, (↓), ↑			
SOD	Protein content, tBHP	Mouse macrophage cell line RAW 264.7	↑, (↓), ↑			
HO-1 GCLC GCLM NQO1	Protein expression – western blot analysis	Mouse macrophage cell line RAW 264.7	↑ ↑ ↑ ↑	LEC, 70 μM LEC, 35 μM LEC, 35 μM LEC, 70 μM		
Nrf2 Keap1/β-actin	Protein expression – western blot analysis	Mouse macrophage cell line RAW 264.7	↑ ↓	140 μM 140 μM		
ARE response	Luciferase activity	Mouse macrophage cell line RAW 264.7	↑	140 μM		
ROS	DCF fluorescence	Mouse macrophage cell line J774A.1	NC, (↑), ↓	140 μM methyl-eugenol, tBHP (alone), or both, respectively		

Table 4.6 End-points (continued)

End-point	Assay	Species, strain (sex), cell line, system	Results[a]	Dose or concentration (LEC/LED or HIC/HID)	Comments	Reference
Nrf2	Transcription activity (ARE luciferase) and qRT-PCR	NIH 3T3 cell line HEK293 cell line	No transcription ↑ Transcription	25 µg/mL		Ma et al. (2021)
Non-mammalian species						
Lipid peroxidation	TBARS	*Candida albicans*	↑	LEC, 56 µM		Khan et al. (2011)
SOD, CAT, GPX			↑	LEC, 56 µM		
GSH			↓	LEC, 56 µM		
Acellular systems						
GSH activity	In vitro reaction of GSH with 1-chloro-2,4-dinitrobenzene	GST isozymes isolated from male Wistar rat liver			Concentration that inhibited 25% of the reaction for various isozymes: GST 1-1, 0.6 mM GST 1-2, > 2.5 mM GST 3-3, 0.5 mM GST 4-4, 1.3 mM GST 7-7, 1.2 mM	Rompelberg et al. (1996)

ARE, antioxidant response element; CAT, catalase; DCF, dichlorofluorescein; DCFDA, 2′,7′-dichlorofluorescein diacetate; DSB, double-strand break; Endo III, endonuclease type III; ESR, electron spin resonance; Fpg, formamidopyrimidine DNA glycosylase (also known as 8-oxoguanine DNA glycosylase); GCLC, glutamate cysteine ligase catalytic/modifier subunit; GSH, glutathione; GST, glutathione *S*-transferase; h, hour(s); HEK293 human embryonic kidney cell line; HIC/HID, highest ineffective concentration/dose; HO-1, haem oxygenase 1; IFNγ, interferon gamma; iNOS, inducible nitric oxide synthase; i.p., intraperitoneal; LDH, lactate dehydrogenase; LEC/LED, lowest effective concentration/dose; LPS, lipopolysaccharide; M, male; MnSOD; manganese-dependant (mitochondrial) superoxide dismutase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NIH 3T3, transformed mouse fibroblast cell line; NO, nitric oxide; NQO1, quinone oxidoreductase; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species (cytoplasmic unless otherwise specified); SD, Sprague-Dawley; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; tBHP, *tert*-butylhydroperoxide.

[a]↓, decrease; ↑, increase; NC, no change; +, positive.

these results suggested that the hepato-carcinogenic potential of methyleugenol might involve a genotoxic mechanism.]

Exome sequence analysis of DNA extracted from samples of hepatocellular carcinoma induced in B6C3F$_1$/N mice (n = 3) exposed daily by gavage to methyleugenol at 0 (vehicle control), 37, 75, or 150 mg/kg for 2 years revealed a higher mutation burden than was found in spontaneous tumours from untreated controls, and the mutation frequency increased linearly with dose (Auerbach et al., 2018). When compared with the Catalogue of Somatic Mutations in Cancer (COSMIC) 30-signature data set, the trinucleotide motif signature most closely resembled signature 4 (attributed to benzo[*a*]pyrene) and signature 24 (attributed to aflatoxin B$_1$).

However, in male and female B6C3F$_1$ mice exposed to methyleugenol at doses of up to 1000 mg/kg for 14 weeks, no increase in the percentage of micronucleated normachromatic erythrocytes was observed, nor was there an increase in the percentage of polychromatic erythrocytes (NTP, 2000).

(ii) Non-human mammalian cells in vitro

See Table 4.4.

Methyleugenol induced unscheduled DNA synthesis (UDS) in primary cultures of freshly isolated hepatocytes from male F344 rats (Howes et al., 1990). In a subsequent study (Chan and Caldwell, 1992), the inclusion of 1′-hydroxymethyleugenol indicated that this metabolite was more potent in inducing UDS than was its parent compound. In a subsequent study, methyleugenol induced UDS in cultured primary hepatocytes from male F334 rats and female B6C3F$_1$ mice (Burkey et al., 2000).

Groh et al. (2012) investigated methyleugenol-treated V79 Chinese hamster lung fibroblasts for DNA damage using the comet assay, for micronucleus formation, and for mutation at the *hprt* locus. The metabolites 1′-hydroxymethyleugenol, methyleugenol-2′,3′-epoxide, and 3′-oxomethylisoeugenol, were also tested. All four compounds induced comet formation, with 1′-hydroxymethyleugenol and methyleugenol-2′,3′-epoxide causing greater DNA damage than did methyleugenol. However, only methyleugenol-2′,3′-epoxide and 3′-oxomethylisoeugenol increased the frequencies of micronuclei. Only methyleugenol-2′,3′-epoxide showed marginal mutagenicity at the *hprt* locus.

In Chinese hamster ovary (CHO) cells, methyleugenol at a concentration of 17 µg/mL induced sister-chromatid exchange in the presence of S9 (NTP 2000). In the absence of S9, methyleugenol gave negative results at 50 µg/mL and was toxic at 167 µg/mL. Methyleugenol did not induce chromosomal aberrations in CHO cells when tested at up to 233 µg/mL in either the presence or absence of S9 and was toxic at 500 µg/mL (NTP, 2000).

(iii) Non-mammalian experimental systems

See Table 4.5.

In studies that investigated methyleugenol by injecting it into turkey eggs containing fetuses aged 22–24 days, the compound did not induce DNA damage, as detected by the comet assay. These studies are also described in Section 4.2.1 (Kobets et al., 2016, 2018).

In diploid *Saccharomyces cerevisiae* strain RS9, methyleugenol induced intrachromosomal (*HIS*$^+$) and interchromosomal (*ADE*$^+$) recombination in a dose-related manner (Schiestl et al., 1989). In another diploid strain, RS112, methyleugenol induced DEL recombination (Brennan et al., 1996).

Methyleugenol was inactive in inducing mutations in *S. typhimurium* strains TA100, TA1535, TA98, TA1537, and TA1538, and in *Escherichia coli* WP2 *uvrA* (Sekizawa and Shibamoto, 1982), at up to 300 µg/plate. [The Working Group noted that toxicity precluded testing at higher doses.]

Similarly, Schiestl et al. (1989) found methyleugenol to be inactive in *S. typhimurium* strains TA97, TA98, TA100, and TA102 when tested at

up to 6 µM/plate. It was inactive when tested with either hamster or rat S9 in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 at up to 333 µg/mL and was toxic at the next concentration tested, 666 µg/mL (NTP, 2000).

In a modified strain of *S. typhimurium* TA100 expressing human sulfotransferase SULT1C2, methyleugenol induced mutations in the presence of rat liver S9 (Honda et al., 2016). The same investigators had previously demonstrated that 1′-hydroxymethyleugenol was mutagenic in this strain and with human SULT1A1 (Herrmann et al., 2012) (see also Section 4.1.2). The latter was also reported in a study in which 1′-hydroxymethyleugenol in TA100-hSULT1A1 and TA100-hSULT1C2 without rat liver S9 served as a positive control (Berg et al., 2016).

Methyleugenol gave positive results in the absence of S9 in the DNA repair test in *Bacillus subtilis* (Sekizawa and Shibamoto, 1982).

4.2.3 Alters DNA repair or causes genomic instability

(a) Humans

No studies relative to DNA repair or genomic instability in humans or in human cells exposed to methyleugenol were available to the Working Group.

(b) Experimental systems

Yafune et al. (2013) investigated cell cycle effects in the liver of rats treated with methyleugenol for 28 days (1000 mg/kg bw per day, by gavage). Methyleugenol was reported to increase the number of liver cells overexpressing $p21^{Cip1}$, a cyclin-dependent kinase inhibitor playing a key role in the cell cycle checkpoint in G_1 phase. Methyleugenol also increased the number of cells overexpressing Aurora B or Incenp proteins. The authors considered these findings to be an indication of cell population growth, in line also with an increase in the percentage of cells expressing nuclear antigen Ki-67 cell proliferation marker (see also Section 4.2.4(b)).

Methyleugenol reduced the expression of several genes involved in the DNA repair and damage response in the livers of male F344 rats 3 hours after an oral dose of 2000 mg/kg bw (Ding et al., 2011). Among the affected genes were DNA damage-binding genes *Brca1* and *Rad1*; base excision repair genes *Mpg* and *Ogg1* (an oxidative DNA damage repair gene that encodes 8-oxoguanine DNA glycosylase); the double-strand break repair gene *Rad52*; the mismatch repair genes *Pms1*, *Pms2*, and *Pold3*; and the cell cycle check point gene *Rad9*. Expression levels recovered at 6 hours and 8 hours after treatment, except for *Brca1*, *Ogg1*, and *Pold3*.

4.2.4 Induces epigenetic alterations

(a) Humans

(i) Exposed humans

No studies relative to the induction of epigenetic alterations on exposed humans were available to the Working Group.

(ii) Human cells in vitro

Groh et al. (2013) treated human colon carcinoma HT29 cells for 24 hours with methyleugenol and three of its metabolites, 1′-hydroxymethyleugenol, methyleugenol-2′,3′-epoxide, and 3′-oxomethylisoeugenol at 10–100 µM. Methyleugenol and 1′-hydroxymethyleugenol did not inhibit histone deacetylase (HDAC) activity, but the other two metabolites (methyleugenol-2′,3′-epoxide and 3′-oxomethylisoeugenol) did. HDAC inhibition was not accompanied by changes in the levels of HDAC1 protein. [The Working Group noted that growth inhibition was observed after treatment. The growth inhibitory potency of the investigated alkylbenzenes in HT29 cells was ranked as follows: 3′-oxomethylisoeugenol > methyleugenol-2′,3′-epoxide > 1′-hydroxymethyleugenol ≈ methyleugenol.]

(b) Experimental systems

Yafune et al. (2013) observed an increase in the number of cells positive for phosphorylated histone H3 (p-H3) and for heterochromatin protein 1 α (HP1α⁺) in the livers of rats treated with methyleugenol compared with rats in untreated control groups and in groups treated with non-carcinogens. The data suggested a role for histone modification in cell transition through different cell cycle phases, such as M-phase arrest through G_1/S. (see also Sections 4.2.3 and 4.2.8(b)).

4.2.5 Induces oxidative stress

(a) Humans

No studies relative to the induction of oxidative stress in humans or in human cells exposed to methyleugenol were available to the Working Group.

(b) Experimental systems

See Table 4.6.

Methyleugenol alone was tested in few studies. Oxidative stress was not observed in homogenized small intestinal tissue of male Wister rats treated with methyleugenol at 100 mg/kg bw per day by gavage (Saleh and El-Shorbagy, 2017). In adult male Wistar rats treated with methyleugenol at 100 mg/kg per day for 30 days, there was no impact on levels of nitric oxide (NO) or oxidized lipids (thiobarbituric acid reactive substances, TBARS), cellular glutathione (GSH), or the activity of glutathione-*S*-transferase (GST), superoxide dismutase (SOD), or catalase (CAT) in homogenized intestinal tissue. Surgical ligation of the mesenteric arterial blood supply was used to induce ischaemia. The resulting ischaemia increased NO and TBARS and reduced GSH levels and GST, SOD, and CAT activities. Treating the rats with methyleugenol before surgical ligation prevented these changes in the markers of the oxidative stress response, and levels remained similar to those in sham-operated animals.

In rats, prior administration of methyleugenol at 100 mg/kg was partially protective against the induction of reactive oxygen species (ROS) and ischaemic damage in cerebral tissue caused by blocking the cerebral blood supply in adult male Sprague-Dawley rats. It was also protective against the induction of intracellular ROS and cytotoxicity caused by hypoxia in mixed glial cells cultured from the prefrontal cortex of male Sprague-Dawley rats (age, 1 day) (Choi et al., 2010). No experimental animals or cells were exposed to methyleugenol in the absence of hypoxia in this study.

Methyleugenol (50 or 100 µg/mL) increased transcription of nuclear factor erythroid 2-related factor 2 (NRF2) in a human embryonic kidney cell line (HK293) and a mouse embryonic fibroblast cell line (NIH 3T3), but no increase in NRF2 protein was evident by western blot in the human embryonic kidney cell line (Ma et al., 2021). Methyleugenol (25 µg/mL) did not increase intracellular ROS detected by 2′,7′-dichlorodihydrofluorescein diacetate (DCDFA) fluorescence in a murine macrophage cell line but did increase intracellular GSH and SOD activity. Treating the cells with *t*-butyl hydroperoxide depressed intracellular GSH and SOD activity; these changes were partially reversed in a concentration-dependent manner by co-treatment with methyleugenol (Zhou et al., 2017). [The Working Group noted that, in these studies, methyleugenol altered biomarkers of oxidative stress only after the induction of oxidative stress by another treatment, i.e. hypoxia or *t*-butylhydroperoxide.]

Khan et al. (2011) found that growing *Candida albicans* to mid-exponential phase with as little as 10 µg/mL (56 µM) methyleugenol in the growth medium produced evidence of oxidative stress. This was demonstrated by an increase in lipid peroxidation (TBARS) and antioxidant activity (SOD and CAT activity), as well as a decrease in cytosolic GSH. [The Working Group noted that

these results may be of limited direct relevance to humans, because the studies were conducted in non- mammalian species versus mammalian cells.]

(c) Acellular systems

Rompelberg et al. (1996) reported reversible inhibition of each of the several GST isozymes isolated from rat and human liver cytosol. Methyleugenol has been reported to have weak free radical scavenging activity in aqueous solution, on the basis of monitoring its reaction with 1,1-diphenyl-2-picrylhydrazyl (DPPH). The concentration of methyleugenol that caused 50% radical scavenging (IC_{50}) was 80 mol/mol DPPH at 4–400 mM (Nenadis et al., 2021) and 13.7 µM (about 25% as active as ascorbic acid), when tested at concentrations of 0.1–100 µM (Choi et al., 2010).

The electron paramagnetic resonance spectrum of the product of oxidation of methyleugenol by H_2O_2 and horseradish peroxidase is consistent with conversion of the 1-methoxy group to an oxy radical (Sipe et al., 2014). Perhaps more significantly, these authors noted an unexpected electron paramagnetic resonance signal in the methyleugenol source material. The results of additional experiments suggested that the source material lots were contaminated with 10–30 ppm of an unidentified hydroperoxide formed by methyleugenol auto-oxidation. This hydroperoxide was found to be a substrate for horseradish peroxidase (in the absence of H_2O_2) but not for catalase. The authors suggested that this auto-oxidation may have implications for understanding the metabolism of methyleugenol. [The Working Group noted that the presence of this hydroperoxide, particularly if formed in larger amounts, may also have an impact on toxicological test results for synthetic methyleugenol.]

4.2.6 Induces chronic inflammation

(a) Humans

(i) Exposed humans

No studies relative to chronic inflammation in humans exposed to methyleugenol were available to the Working Group.

(ii) Human cells in vitro

Methyleugenol, administered at 50, 100, and 200 µM, caused a non-significant reduction in matrix metallopeptidase 9 (MMP-9) activity in BEAS-2A cells induced with tumour necrosis factor alpha (TNFα) and interleukin 4 (IL-4) (Kim et al., 2014). [The Working Group noted that MMP-9 is a known mediator of inflammation through its involvement in the processing of various chemokines or cytokines.]

(b) Experimental systems

(i) Non-human mammals in vivo

As described in Section 3, evidence of inflammation was reported in the 2-year studies in rodents (NTP, 2000). Inflammation was also reported in subchronic studies in male and female $B6C3F_1$ mice and F344/N rats treated with methyleugenol at doses of 0, 10, 30, 100, 300, or 1000 mg/kg bw in 0.5% aqueous methylcellulose by gavage, 5 days per week, for 14 weeks (NTP, 2000; Abdo et al., 2001).

In the 14-week subchronic study in rats, the incidence of atrophy and chronic inflammation of the mucosa of the glandular stomach was significantly increased (NTP, 2000). Sustained inflammation and atrophy were observed at 300 mg/kg bw (minimal to mild lesions) and 1000 mg/kg bw (mild to moderate lesions) in both male and female rats. Inflammation, although not atrophy, was reported in the glandular stomach at a lower dose of 100 mg/kg bw in female rats only. The inflammation was of mild severity, consisting of fibrosis and diffuse infiltration of the lamina propria by lymphocytes, neutrophils, and macrophages. However, in the 2-year gavage

study, chronic inflammation was not reported in the glandular stomach or the liver of male or female rats. [The Working Group noted that consideration of non-neoplastic inflammatory lesions at the 2-year time point may have limited value in terms of carcinogenesis.]

Atrophy of the glandular stomach (100%, 5/5 exposed versus 0/5 controls), but not residual inflammatory changes, was reported at the 6- or 12-month interim kill of male and female rats at the highest dose (300 mg/kg, stop-exposure study). Of note, the incidence of splenic fibrosis in female rats at 150 and 300 mg/kg was also significantly increased (NTP, 2000). [The Working Group noted that chronic tissue inflammation often leads to tissue fibrosis (Koyama and Brenner, 2017; Tanwar et al., 2020), suggesting that the splenic fibrosis observed after 2 years resulted from chronic inflammation, even though there was no evidence of inflammation at the end of the 2-year study in exposed rats. The diagnosis of inflammation, especially chronic inflammation in the spleen, is notably challenging because of the constituent cell types in splenic parenchyma.]

In mice, chronic inflammation in the liver was not reported in the 14-week study in mice, although subacute inflammation in the liver was observed in male (1000 mg/kg) and female (300 mg/kg) mice (NTP, 2000), and significant increases in the incidence of atrophy, degeneration, and mitotic alterations of the glandular stomach were seen in female mice at 300 mg/kg. In the 2-year gavage study, a dose-related increase in chronic active inflammation was observed in the glandular stomach of 51% and 66% of male mice at 75 and 150 mg/kg, respectively. Similarly, at these two concentrations, non-proliferative chronic active inflammation was observed in the liver of 56% of treated male mice (NTP, 2000).

Of note, oral administration of methyleugenol at doses of 3 and 10 mg/kg bw in male ddY strain mice did not affect the activity of cyclooxygenase-1 (COX-1) or cyclooxygenase-2 (COX-2), which are important mediators of inflammation (Yano et al., 2006).

(ii) Non-human mammalian cells in vitro

One in vitro study was identified in which methyleugenol at non-cytotoxic doses (ranging from 5 to 100 μM) attenuated the immune response elicited by exposure of a rat basophilic leukaemia mast cell line (RBL-2H3) to immunoglobulin E (IgE) and 2,4-dinitrophenol–bovine serum albumin. Methyleugenol markedly suppressed the release of TNFα and IL4 in a dose-dependent manner and inhibited the formation of prostaglandin E_2 (PGE2), prostaglandin D_2 (PGD2), and leukotriene C_4 (LTC4). Similarly, methyleugenol suppressed the formation of leukotriene B_4 (LTB4) (Tang et al., 2015). [The Working Group noted that these results collectively suggest that methyleugenol may induce immunosuppression by inhibiting the release of pro-inflammatory cytokines TNFα and IL4 and the allergic inflammatory response mediated by PGE2, PGD2, LTC4, and LTB4.]

4.2.7 Modulates receptor-mediated effects

(a) Humans

(i) Exposed humans

No studies relative to the modulation of receptor-mediated effects by methyleugenol in exposed humans were available to the Working Group.

(ii) Human cells in vitro

One study reported that methyleugenol selectively activated human transient receptor potential ankyrin 1 (hTRPA1), with a half-maximal effective concentration (EC_{50}) value of 160.2 ± 21.9 in exposed hTRPA1-Flp-In 293 stably transfected cells or in HEK293T cells transiently expressing hTRPA1. These results suggest agonism on TRPA1, which is a mediator of several downstream signalling pathways involved in sensory neural processes (Moon et al., 2015).

(b) Experimental systems

(i) Non-human mammals in vivo

Methyleugenol at dose of 3 or 10 mg/kg significantly inhibited the duration of pain-related behaviours (biting or licking the tail and hind paws) induced by 0.25 nM intrathecal injection of *N*-methyl-D-aspartic acid (NMDA) in mice. Bicuculline (3 mg/kg, via subcutaneous injection), a known antagonist of the gamma-aminobutyric acid type A ($GABA_A$) receptor, was shown to suppress the behaviours reported after oral administration of methyleugenol at 10 mg/kg, suggesting $GABA_A$ agonism (Yano et al., 2006).

(ii) Non-human mammalian systems ex vivo

Three studies conducted in mammalian models ex vivo provided evidence that methyleugenol interacts with GABA receptors. Ding et al. (2014) studied $GABA_A$ receptor agonism and reported that methyleugenol enhanced GABA-induced ionic currents in primary cultures of mouse hippocampal neurons harvested from pregnant C57BL/6J mice. In another study, methyleugenol demonstrated agonist activity in ionotropic $GABA_A$ receptors when infused bilaterally into transverse central lateral brain slices from C57BL/6J mice (Zhu et al., 2018). Similarly, GABAergic inhibitory activity in the central amygdala was significantly increased by methyleugenol infused bilaterally into the central amygdala brain slices, acting on the $GABA_A$ receptor and reducing anxiety in mice (Liu et al., 2019a).

4.2.8 Alters cell proliferation, cell death, or nutrient supply

(a) Humans

(i) Exposed humans

No studies relative to alterations in cell proliferation, cell death, or nutrient supply with methyleugenol in exposed humans were available to the Working Group.

(ii) Human cells in vitro

Few studies in human cells in vitro were available to the Working Group. Of these, most investigated the potential beneficial effects of methyleugenol as a cytostatic, antiproliferative, and antioxidant agent, limiting their relevance to the key characteristic under evaluation.

Kuang et al. (2021) showed that although methyleugenol alone (0–40 µmol/L) did not induce any changes in the viability of human immortalized kidney cells (HK-2), pre-treatment with methyleugenol at 0–40 µmol/L in an in vitro model of hypoxia/reoxygenation-exposed HK-2 cells promoted the expression of NRF2 and haem oxygenase-1 (HO-1) and translocation of NRF2 to the nucleus, and downregulated the expression of NADPH oxidase 4 (NOX4), reducing apoptosis. The effect of methyleugenol was reversed by treatment with the NRF2 inhibitor ML385. [The Working Group considered that the study was of limited informativeness, since no results on NRF2, HO-1, or NOX4 expression levels after treatment with methyleugenol alone were reported.]

Yi et al. (2015) evaluated the potential effects on cell growth and apoptosis of methyleugenol treatment alone or in combination with cisplatin (a known antiproliferation anticancer drug) in human cervical cancer cells (HeLa). Methyleugenol treatment at 10, 20, 40, 60, 80, and 100 µM induced a concentration-dependent increase in the growth inhibition rate, as measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A synergistic effect on growth inhibition was observed when HeLa cells were treated simultaneously with methyleugenol (60 µM) and cisplatin (1 µM) for 48 hours. However, methyleugenol treatment at 60 µM for 48 hours was reported to shift 15% of the cell population to G_0/G_1 phase, compared with 9% observed in untreated cells and to induce apoptosis, as shown by a significant increase in the activity of

apoptotic mediator factor caspase 3 and reduction in mitochondrial membrane potential. [The Working Group considered that the study had some limitations since the duration of methyleugenol treatment was not reported clearly across all the tests performed.]

Yin et al. (2018) investigated the potential antiproliferative effects of methyleugenol in a human retinoblastoma cell line, RB355. Methyleugenol treatment (0–200 μM) for 48 hours induced a significant concentration-dependent decrease in cell viability (IC_{50} = 50 μM) as measured by the MTT assay. The vital staining of cells treated with methyleugenol (50 μM) with the autophagolysosome marker monodansylcadaverine (MDC) showed induction of autophagy, which was confirmed by a concentration-dependent (25, 50, 100 μM) increase in the expression of autophagy protein LC3-II and a decrease in LC3-I and p62, but no change in BECLIN-1 or VSP4. In addition, a concentration-dependent (25, 50, 100 μM) increase in the proportion of cells arrested in G_2/M cell cycle phase was observed; this was accompanied by downregulation of PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha), m-TOR (mechanistic target of rapamycin kinase), AKT (AKT serine/threonine kinase 1) and respective phosphorylated forms, as measured by western blot analysis of levels of the three proteins. [The Working Group noted that the relevance of this study was limited by the lack of detailed statistical analysis of the data.]

Groh and Esselen (2017), while investigating the potential of methyleugenol and its metabolites (1′-hydroxymethyleugenol, 3′-oxomethylisoeugenol, methyleugenol-2′,3′-epoxide) to trigger the DNA damage response in human colon adenocarcinoma HT29 cells, observed that it was mostly the metabolites that induced the accumulation of HT29 cells in G_2 phase. The DNA damage response was characterized by a time-delayed phosphorylation of ATM (ataxia-telangiectasia, mutated)/ATR (ATM- and Rad3-related) kinases and checkpoint kinase 1 after 2 hours of incubation, and the tumour suppressor protein P53 after 24 hours of incubation. The test compounds induced apoptotic cell death, as indicated by cleavage of caspase 3 and poly-(ADP-ribose)-polymerase after 72 hours.

Deng et al. (2021) studied the effects of methyleugenol and one of its metabolites, 2-allyl-4,5-dimethoxyphenol (DMP), on cell proliferation and growth in two human cell lines (breast cancer MDA-MB-231 and fetal lung fibroblast HFL1 cells). Cells were incubated with methyleugenol or DMP at concentrations of 15.625, 31.25, 62.5, 125, 250, 500, or 1000 μg/mL in the culture medium for 24 hours. Methyleugenol and DMP significantly inhibited the cell proliferation rate, as measured by the MTT assay. The IC_{50}s for methyleugenol and DMP were 454.0647 and 62.7670 μg/mL, respectively, in MDA-MB-231 cells and 687.9166 μg/mL and 539.6506 μg/mL, respectively, in fetal lung fibroblast HFL1 cells. [The Working Group considered this study to be of limited relevance because the metabolite DMP investigated is normally formed in non-mammalian species (i.e. Insecta). In addition, methyleugenol was used as the reference compound.]

(b) Experimental systems

(i) Non-human mammals in vivo

In a repeated-dose toxicity study, B6C3F_1 female mice were exposed to methyleugenol in the diet at a dose of 75 mg/kg per day, 5 days per week, for 2 weeks. After treatment, no evidence of liver tissue alterations was observed by histopathology; however, in liver tissue homogenates methyleugenol was shown to induce alterations in the expression of several genes and expressed sequence tags previously identified by the authors as potential early biomarkers of mouse liver carcinogenesis. Specifically, methyleugenol induced upregulation of cell cycle genes encoding cyclin G1 (Ccng1) and p21 (Cdkn1a) proteins, as well as strong upregulation of growth arrest and

DNA-damage-inducible, beta (Gadd45b), upregulation of the transcription factor early growth response 1 (Egr1), the jun B proto-oncogene (Junb), deoxyribonuclease IIa (Dnase2a), and transforming growth factor beta stimulated clone 22 (Tsc22), a transcriptional repressor and putative tumour suppressor gene. As assessed by oligonucleotide array and quantitative reverse transcription-polymerase chain reaction (qRT-PCR), methyleugenol also induced the downregulation of two tumour suppressor genes encoding fragile histidine triad diadenosine triphosphatase (Fhit) and WW domain-containing oxidoreductase (Wwox), which are involved in apoptotic evasion, as well as the gene encoding cytokine inducible SH2-containing protein (Cish), a member of a family of intracellular proteins that regulates the response of immune cells to cytokines (Iida et al., 2005).

Characterization of the events leading to hepatocarcinogenesis in male F344/NSlc rats (age, 5 weeks) after exposure to methyleugenol at 1000 mg/kg per day by gavage for 28 days was also performed in a series of studies carried out by Taniai et al. (2012), Yafune et al. (2013), and Kimura et al. (2016). Taniai et al. (2012) observed that methyleugenol caused diffuse distribution of cytomegalic liver cells with anisokaryosis and scattered centrilobular necrosis, as assessed by histopathology. Immunohistochemical analysis of liver tissue samples showed that methyleugenol, at the dose tested, significantly increased the number [percentage] of cells that stained positive for the cell proliferation marker Ki-67 compared with untreated or non-carcinogen-treated controls [α-naphthyl isothyocianate or acetaminophen] and the number of cells that were positive for minichromosome maintenance complex component 3 (Mcm3) compared with α-naphthyl isothiocyanate but not untreated controls. Methyleugenol also caused a significant increase in the number of cells that stained positive for DNA topoisomerase II alpha (TopoIIα); as well as an increase in the number of cells staining positive for ubiquitin D (Ubd) and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) compared with untreated or non-carcinogen-treated controls.

In the study by Yafune et al. (2013), it was reported that exposure to methyleugenol at 1000 mg/kg bw per day for 2 days significantly decreased body weight but increased liver weight, compared with untreated animals. The authors also investigated several proteins involved in cell proliferation and apoptosis in liver tissues; the corresponding genes had been previously selected by a global gene screen using microarrays as those most upregulated in the liver of rats receiving thioacetamide (a representative cytomegaly-inducing hepatocarcinogen) as repeated oral doses (400 ppm in the diet). Liver tissue samples from methyleugenol-treated animals showed a significant increase in the percentage of immunoreactive cells expressing $p21^{Cip1}$[Cdkn1a], a cycle-dependent kinase CDK inhibitor; nuclear Cdc2 (cell division cycle 2), which drives the G_2/M transition; Aurkb (aurora kinase B); Incenp (inner centromere protein); p-H3; HP1α (heterochromatin protein 1 alpha); Klf6 (Kruppel like transcription factor 6); and p53, compared with the untreated and non-carcinogen controls, thus sustaining cell proliferation and apoptosis in response to G_2/M cycle arrest (see also Section 4.2.4).

In a follow-up study, Kimura et al. (2016) further investigated the onset of carcinogen-specific cell cycle-related alterations during the early time course of repeated carcinogen administration, studying the effects of methyleugenol after 3 and 7 days of treatment compared with 28 days. Time-dependent significant bodyweight decreases and liver-weight increases were observed in treated animals compared with untreated controls. No morphological alterations in the liver were observed after 3 days or 7 days of treatment, although centrilobular liver cell hypertrophy associated with a cytoplasmic ground glass appearance was reported; after

28 days of treatment, the presence of liver cells exhibiting cytomegaly and anisokaryosis was confirmed, as assessed by histopathology. At day 3, the number of cells that were positive for TopoIIα, p-H3, and Mad2 (a spindle checkpoint protein) significantly decreased compared with untreated controls; lower levels were observed for Ubd+ cells, and the numbers of cells expressing $p21^{Cip1}$ and p-Mdm2 (phosphorylated Mdm2) were significantly increased. The number of cells expressing TopoIIα, p-H3, Mad2, Ubd, γH2AX, and Ubd decreased on day 7 compared with the increased levels in untreated controls. Instead, the number of Ki-67-positive cells remained unchanged until day 28 of treatment, and cleaved caspase 3-positive cells increased starting from day 7. The number of TopoIIα+, p-H3+, Mad2+, Ubd+, γH2AX+, $p21^{Cip1}$+ and p-Mdm2+ cells was significantly increased after 28 days of treatment. In addition, modulation of the transcripts of cyclins (Cdkn1a and Cdkn2a), retinoblastoma 1 and 2 (Rb1 and Rb2), and p53 were observed (by RT-PCR) from day 3 to day 28, confirming the involvement of G_1/S checkpoint-related genes starting from earlier time points, and of genes related to spindle checkpoints, M phase, and DNA damage at later time points.

Abdo et al. (2001) reviewed the NTP studies (NTP, 2000) investigating the effects of methyleugenol in groups of 10 female rats at a dose of 0, 37, 75, or 150 mg/kg per day, 5 days per week, for 30 or 90 days, or 300 or 1000 mg/kg per day for 30 days, and in groups of male mice exposed to a dose of 0, 9, 18.5, 37, 75, 150, or 300 mg/kg per day for 30 or 90 days. They observed a significant increase in cell proliferation, indicated by an increase in the percentage of cells labelled with bromodeoxyuridine (BrdU), mainly in the fundic glands of the glandular stomach in male mice at 150 or 300 mg/kg for 30 days or 18.5 37, or 75 mg/kg for 90 days. The treatment induced non-neoplastic lesions in the liver, glandular stomach, and nose of male and female mice. Cytological alterations, necrosis, subacute inflammation (see also Section 4.2.6), and bile duct hyperplasia were observed in male mice at 1000 mg/kg and in female mice at 300 and 1000 mg/kg. Significant increases in the incidence of atrophy, degeneration, and mitotic alterations of the glandular stomach were seen in female mice at 300 mg/kg. Serum gastrin levels were significantly increased in male mice at 150 and 300 mg/kg after 30 days but not after 90 days of exposure. In male and female rats, there were increases in the incidence of cytological alterations, cytomegaly, Kupffer cell pigmentation, and mixed foci of cellular alteration in the liver at 1000 mg/kg. In addition, increases in the incidence of atrophy and chronic inflammation in the glandular stomach were observed in male and female rats at 300 and 1000 mg/kg. There were significant increases in serum gastrin levels in female rats at all doses after 30 days and after higher (150, 33, 1000 mg/kg) exposures at 90 days. The BrdU labelling index showed significant increases in cell proliferation in the fundic glands of the glandular stomach in female rats in almost all dosed groups at both 30 days (37, 150, 300 and 1000 mg/kg) and 90 days (37, 75 and 150 mg/kg). In addition, increased BrdU labelling was also observed in the liver in rats at 150, 300, and 1000 mg/kg at 30 days and 150 mg/kg at 90 days.

Williams et al. (2013) studied the effects of methyleugenol administered at a dose of 62–250 mg/kg bw for 24 weeks in male F344 rats. Methyleugenol produced diffuse hepatocellular hypertrophy and enlargement of the cholangiole plexus in the periportal regions, midzonal macrovesicular phanerosis, periportal infiltrate of mononuclear cells, and multiple foci of hepatocellular necrosis. The hepatocellular replicating fraction values (cells positive for proliferating cell nuclear antigen, PCNA) increased more than twofold.

While investigating genotoxicity in F344 *gpt* delta transgenic rats exposed to methyleugenol at the dose of 10, 30 or 100 mg/kg for 13 weeks, Jin et al. (2013) observed increases in the number and

area of foci positive for the placental form of GST in the liver and increases in cell proliferation, (as measured by the ratio of PCNA-positive hepatocytes) in male and female rats at 100 mg/kg.

In the 2-year cancer study conducted by the NTP (Johnson et al., 2000; NTP, 2000; see also Section 3 of the present monograph), male and female F344/N rats and B6C3F$_1$ mice were treated with methyleugenol at doses of 37, 75, or 150 mg/kg per day by gavage, 5 days per week, for 105 weeks (rats) or 104 weeks (mice). A stop-exposure group of rats received methyleugenol at 300 mg/kg per day by gavage for 53 weeks and then vehicle only for the remaining 52 weeks. Tissues were examined microscopically. In rats, methyleugenol increased the incidence of hepatocyte hypertrophy and oval cell hyperplasia (except for males at 37 mg/kg per day) and also increased the incidence of bile duct hyperplasia in groups of males at 37, 75, and 150 mg/kg per day and females at 150 and 300 mg/kg per day. Increases in the incidence of oval cell hyperplasia, hypertrophy, and mixed cell foci were also noted at the 6- and 12-month interim evaluations in male and female rats at 300 mg/kg per day. In addition, atrophy of the glandular stomach was observed at the 6- and 12-month interim evaluations in male and female rats at 300 mg/kg per day. Atrophy of the glandular stomach was observed in male and female rats in all dose groups. The incidence of neuroendocrine cell hyperplasia in the fundic region of the glandular stomach was increased in groups at 150 and 300 mg/kg per day and was more prevalent and severe in females than in males. In mice, the incidence of bile duct and oval cell hyperplasia increased, starting at 37 mg/kg per day for males and 75 mg/kg per day for females. The incidence of haematopoietic cell proliferation increased, starting at 37 mg/kg per day. Atrophy of the glandular stomach was observed in male and female mice at 75 and 150 mg/kg per day. Hyperplasia of the glandular stomach increased at 75 mg/kg per day in male and female mice.

In a further analysis of the lesions observed in rats from the above study (NTP, 2000; see also Section 3.2), Janardhan et al. (2015) reviewed by microscopy the original haematoxylin and eosin-stained tissue sections of the stomach with a diagnosis of neuroendocrine cell hyperplasia and benign and malignant neuroendocrine tumour. In addition, haematoxylin and eosin-stained slides of metastatic neuroendocrine tumours in other tissues were reviewed. Hyperplasia and non-neoplastic lesions in the neuroendocrine cells were observed mainly in the fundus region of the stomach and showed positive immunoreactivity for chromogranin A and synaptophysin.

(ii) Non-human mammalian cells in vitro

Wang et al. (2021b) investigated the potential protective effects of methyleugenol on liver ischaemia reperfusion injury (LIRI) and whether this effect was regulated by the PI3K/Akt signalling pathway. They used a partially warm LIRI model in which C57BL/6J mice underwent 60 minutes of ischaemia, followed by reperfusion for 6 hours. They also used also used a hypoxia–reoxygenation injury cell model, mouse liver cell line (AML12), which underwent 24 hours of hypoxia, followed by 18 hours of normoxia. The extent of liver injury was assessed by serum transaminase concentrations, haematoxylin and eosin staining, quantitative real-time PCR, myeloperoxidase activity, and TUNEL analysis. Apoptosis was detected using flow cytometry. Protein levels of p-PI3K, PI3K, p-Akt, Akt, p-Bad, Bad, Bcl-2, Bax, and cleaved caspase 3 were detected by western blotting. LY294002 (an inhibitor of PI3K/Akt signalling) was used to elucidate the relationship between methyleugenol and PI3K/Akt signalling. The results showed that methyleugenol alleviated the LIRI-induced liver injury, the inflammatory response, and the apoptosis induced by hypoxia–reoxygenation.

Deng et al. (2021) studied the cytotoxicity of methyleugenol and its metabolite, DMP, in mouse embryonic hepatocytes (BNL-CL.2). The

concentrations studied were 15.625, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL, and cytotoxicity was measured by the MTT assay after 24 hours of exposure. The authors reported IC_{50}s of 114.5169 and 1171.5902 µg/mL, respectively. [The Working Group considered the study to be of limited relevance because the metabolite investigated is mainly formed in non-mammalian species (i.e. Insecta). In addition, methyleugenol was used as the reference compound.]

4.2.9 Data relevant to multiple key characteristics

(a) Results from microarrays and omics

(i) Non-human mammals in vivo

See Table 4.7.

Yafune et al. (2013) (see also Section 4.2.8(b)) aimed to clarify the regulatory molecular mechanisms behind cell cycle aberrations related to the early stages of hepatocarcinogenesis. Gene expression analysis using microarrays and qRT-PCR was performed on RNA samples from the liver of rats exposed to several hepatocarcinogens, including methyleugenol, for 28 days. There were increases in cell proliferation (Ki-67-positive cells) and apoptosis (TUNEL-positive cells) after exposure to methyleugenol and consistent increases in several marker genes and proteins involved in cell proliferation. Increased staining for p53, $p21^{Cip1}$, Cdc2, Aurkb, Incenp, p-H3, and HP1α proteins was observed in the livers of mice exposed to methyleugenol. qRT-PCR showed increased expression of Klf6 but not Ndrg1. [The Working Group noted that the study had some limitations because no description of pathway analyses was provided, or comparisons of gene profiles between the different chemicals examined or between results of microarray and qRT-PCR.]

To understand gene expression patterns produced after exposure to hepatocarcinogens with different mechanisms of action, Iida et al. (2005) used qRT-PCR and oligonucleotide microarray analysis to identify genes that were altered in $B6C3F_1$ mouse liver after treatment with different known carcinogens, including methyleugenol (75 mg/kg per day, in the diet, for 2 weeks). Liver gene expression of 20 842 genes was assessed by oligonucleotide microarray. Several cancer-related genes, including those involved in apoptosis (*Fhit*, *Wwox*) and those involved in the cell cycle (*Tsc22*, *Gadd45b*) were induced or repressed in unique patterns for specific carcinogens but were not altered by the non-carcinogens. The authors noted that even if molecular alterations in the tumours were similar, such as in the case of oxazepam and methyleugenol, early gene expression changes appeared to be carcinogen-specific and involved apoptosis and cell cycle-related genes. [The Working Group noted that pathway analysis was not performed.]

To identify hepatocarcinogens using computational approaches, Auerbach et al. (2010) developed a series of classification prediction models based on gene expression in the male F344 rat liver and generated using full genome microarrays after 2, 14, or 90 days of exposure to a collection of hepatocarcinogens, including methyleugenol. Methyleugenol increased the expression of *Mybl2* and *Adam8* and downregulated *Wwox* and *Fhit* after 90 days of treatment. All four of these genes play mechanistic roles in cell proliferation or apoptosis. [The Working Group noted that pathway analysis was not performed.]

(ii) Human mammalian cells in vitro

Kreuzer et al. (2020) performed experiments to identify genes that would be predictive of DNA damage in vitro. Metabolically active human HepaRG hepatocarcinoma cells were exposed to five food-relevant genotoxic carcinogens, including methyleugenol. Transcriptomic responses were analysed using RNA sequencing technology and validated by real-time RT-PCR. Various biostatistical approaches revealed a characteristic transcript signature of 37 genes

Table 4.7 Microarray and omics results for multiple key characteristics in non-human mammalian systems in vivo and in vitro after exposure to methyleugenol

End-point	Normalization and statistics	Curation	Results	Tissue, cell type, or cell line	Relevant KCs[a]	Exposure concentration or range and duration	Comments	Reference
Transcriptomics Agilent Mouse two-colour oligonucleotide array of ≈ 20 000 genes	Agilent Feature Extraction software: Rosetta Resolver (version 3.2, build 3.2.2.0.33) Expression analysis performed with GeneSpring 6.2	None provided	Microarray: 47 genes altered; cell cycle-related genes Microarray and qRT-PCR: upregulated genes: *Cdkn1a*, *Ccng1*, *Gadd45b*, *Junb*, *Dnase2*; reduced or absent *Fhit* and *Wwox* expression	Liver	KC10 (cell proliferation and apoptosis)	Female $B6C3F_1$ mice (age 6 weeks) dosed with methyleugenol at 75 mg/kg per day	No pathway analysis	Iida et al. (2005)
Transcriptomics Agilent Rat Whole Genome oligonucleotide microarrays in 4 × 44K format	Agilent Feature Extraction software (v9.5); normalized using quantile normalization followed by per chip median centring; significant changes determined using a *t*-test (Benjamini and Hochberg multiple testing correction); GSEA performed using GeneSpring GX 10	NIEHS CEBS [not confirmed]	Increased mitosis at 2 and 14 days; reduced or absent *Fhit* and *Wwox* expression	Liver	KC10 (cell proliferation and apoptosis)	Male F344/N rats dosed with methyleugenol at 150 mg/kg per day by gavage in methylcellulose (training set); 35.6 or 356 mg/kg per day in corn oil (test set); killed on days 3, 15, 91	No pathway analysis on methyleugenol, only on hepatocarcinogens versus non-hepatocarcinogens	Auerbach et al. (2010)

Table 4.7 (continued)

End-point	Normalization and statistics	Curation	Results	Tissue, cell type, or cell line	Relevant KCs[a]	Exposure concentration or range and duration	Comments	Reference
Transcriptomics RNA-Seq	Alignment to the human genome (hg19) performed using STAR (version 2.5.2b); differentially expressed genes (DEGs) identified using DESeq2 A false discovery rate-adjusted *P*-value (*Q* value) < 0.05 (Benjamini-Hochberg correction) was defined as threshold for statistical significance Ingenuity pathway analysis used	GSE14654	Data set overlap using Venn diagrams showed that 37 genes were commonly regulated by all five test chemicals including methyleugenol; several of the 37 genes were functionally linked to cell cycle progression, DNA damage response, and/or cell death, e.g. *FHIT*, *FOSL1*, *GDF15*, *MACROD2*, *NINL*, *PAK7*, *SRC*, and *WWOX*; upstream regulators downregulated	Human hepatoma, HepaRG cells	KC2; KC10 (apoptosis; nutrient supply)	250 µM; 24-hour treatment	Methyleugenol was used as a reference genotoxic agent	Kreuzer et al. (2020)

Table 4.7 (continued)

End-point	Normalization and statistics	Curation	Results	Tissue, cell type, or cell line	Relevant KCs[a]	Exposure concentration or range and duration	Comments	Reference
Transcriptomics RNA-Seq (cont.)			(*HNF4A*, *SCAP*, *SREBF2*, *SREBF1*, *INSR*). Several toxicity functions/ pathways were perturbed (necrosis of liver, apoptosis of hepatocytes, apoptosis of liver cells, focal necrosis of liver, cell death of liver cells); diseases and biofunctions (fatty acid metabolism, transport of molecule, export of molecule, synthesis of lipid, transport of lipid)					

CEBS, Chemical Effects in Biological Systems database; GSEA, gene set enrichment analysis; KC, key characteristic of carcinogens; NIEHS, National Institute of Environmental Health Sciences; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

[a] KC2, "is genotoxic"; KC10, "alters cell proliferation, cell death, or nutrient supply".

that exhibited a similar expression pattern after exposure to the test chemicals. The 37-gene signature could differentiate between genotoxic and nongenotoxic carcinogens. The genes in the signature were shown by pathway analyses to be involved in the DNA damage response and p53 activation. In addition, methyleugenol treatment was predicted by IPA ingenuity pathway analysis to lead to the suppression of several metabolic transcription factors, metabolic diseases, and metabolic biofunctions (Kreuzer et al., 2020). [The Working Group noted that this study was not targeted towards understanding the mechanisms of methyleugenol action but used methyleugenol as a reference agent for DNA damage.]

(iii) Summary

In summary, the four studies demonstrated effects on one or more of the key characteristics. The studies in mice and rats (Iida et al., 2005; Auerbach et al., 2010; Yafune et al., 2013) provided evidence that in the livers of exposed rodents methyleugenol causes increases in hepatocyte proliferation and suppression of hepatocyte apoptosis. In human HepaRG cells, there was evidence for increases in DNA damage and weak evidence for alteration of nutrient supply.

(b) Evaluation of high-throughput in vitro toxicity screening data

The analysis of the in vitro bioactivity of the agents reviewed in *IARC Monographs* Volume 134 was informed by data from high-throughput screening assays generated by the Toxicology in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes of the government of the USA (Thomas et al., 2018). Methyleugenol was one of thousands of chemicals tested across the large assay battery of the Tox21 and ToxCast research programmes. Detailed information about the chemicals tested, assays used, and associated procedures for data analysis is publicly available (US EPA, 2023).

The ToxCast/Tox21 high-throughput screening results are presented according to the assays that have been mapped to the key characteristics of carcinogens (Reisfeld et al., 2022). The detailed results are available in supplementary information for this volume (Annex 4, Supplementary material for Section 4, Mechanistic Evidence, online only, available from: https://publications.iarc.who.int/627). Here, for brevity, assays for which there is a positive "hit call" are referred to as "active" assays. A summary of these results is given below as the number of active assays (without any caution flags) out of the total number of key characteristic-related assays for the chemical.

Among the 288 assays in which methyleugenol was tested, it was found to be active and without caution flags in four assays relevant to the key characteristics of carcinogens. Methyleugenol was active in four assays mapped for key characteristic 8, "modulates receptor-mediated effects" (Reisfeld et al., 2022). Three of the assays were cell-based, multiplexed-readout assays in HepG2 (human hepatocellular carcinoma cell line), and measurements were taken 24 hours after chemical dosing in a 24-well plate. The assays that were positive included ATG_RXRb_TRANS_up, ATG_PPARg_TRANS_up, and ATG_PXR_TRANS_up, which measure the ability of the compound to activate a reporter gene through the ligand-binding domain of the nuclear receptors retinoid X receptor beta (RXRβ), peroxisome proliferator-activated receptor gamma (PPARγ), and the pregnane X receptor (PXR), respectively. The AC_{50} (50% of maximal activity) values were 68.11, 10.77, and 35.11 μM, respectively.

The fourth assay, LTEA_HepaRG_CYP2B6_up, is described as a cell-based, multiplexed-readout assay that uses HepaRG (a human liver cell line), and measurements were taken at 48 hours after chemical dosing on a 96-well plate. The assay measures the expression of the *CYP2B6* gene, which is known to be regulated by the constitutive androstane receptor (CAR) and

PXR. In a screen of 1060 compounds, including methyleugenol, *CYP2B6* was found to be the second most frequently altered gene (Franzosa et al., 2021).

The activity of methyleugenol was tested in two (TOX21_RXR_BLA_Agonist_ratio, ATG_RXRb_TRANS_up), six (ATG_PPARg_TRANS_up, ATG_PPRE_CIS_up, OT_PPARg_PPARgSRC1_0480, OT_PPARg_PPARgSRC1_1440, TOX21_PPARg_BLA_Agonist_ratio, TOX21_PPARg_BLA_antagonist_ratio), and three (ATG_PXR_TRANS_up, ATG_PXRE_CIS_up, LTEA_HepaRG_CYP2B6_up) assays for modulation of RXRβ, PPARγ, and PXR, respectively. [The Working Group considered that because methyleugenol was active in only one or two of the total number of assays for each nuclear receptor, the link between methyleugenol and activation of these receptors might be tenuous and should be confirmed with additional appropriate studies in vitro and in vivo.]

5. Summary of Data Reported

5.1 Exposure characterization

Methyleugenol is a liquid with a clove-like odour that can be produced by methylation of eugenol. It occurs naturally in essential oils of various herbs and spices, e.g. basil, lemongrass, and fennel. The compound as such was used as a flavouring agent in various food products and in consumer products such as cosmetics and personal care products, insect attractants, and as an anaesthetic agent in the veterinary context. However, its use for flavouring purposes was prohibited in the European Union (EU) in 2008 and in the USA in 2018. In the EU, methyleugenol must not be added to cosmetics; however, it is still present in various foods and consumer products that contain herbs, spices, or their essential oils. In this case, maximum concentrations of the substance are established in specific legislations.

In occupational settings, workers handling products containing methyleugenol (e.g. aromatherapists and those using essential oils for body massages, workers from food processing industries) are potentially exposed to methyleugenol by dermal and/or inhalation routes. The general population is ubiquitously exposed, mainly through the ingestion of food, or dermally using personal care products containing methyleugenol. The exposure levels of the general population are expected to be less than 1 μg/kg bw per day for each source of exposure.

5.2 Cancer in humans

No data were available to the Working Group.

5.3 Cancer in experimental animals

Treatment with methyleugenol caused an increase in the incidence of malignant neoplasms or an appropriate combination of benign and malignant neoplasms in two species (mouse and rat).

Methyleugenol was administered by oral administration (gavage) in one study in male and female $B6C3F_1$ mice. In both sexes, methyleugenol caused an increase in the incidence of liver tumours (hepatocellular adenoma; hepatocellular carcinoma; hepatocellular adenoma, carcinoma, or hepatoblastoma (combined); combined hepatocellular carcinoma or hepatoblastoma). In females methyleugenol caused an increase in incidence of hepatoblastoma.

Methyleugenol was administered by oral administration (gavage) in one study in male and female F344 rats. In both sexes, methyleugenol caused an increase in the incidence of liver tumours (hepatocellular adenoma; hepatocellular carcinoma; hepatocellular adenoma or carcinoma (combined); hepatocholangioma, and hepatocholangiocarcinoma) and benign and malignant neuroendocrine tumours of the

glandular stomach. In male rats, methyleugenol caused an increase in the incidence of renal tubule adenoma of the kidney, fibroadenoma of the mammary gland, skin fibroma, skin fibroma or fibrosarcoma (combined), and malignant mesothelioma (all organs).

5.4 Mechanistic evidence

There is sparse evidence on the absorption, distribution, metabolism, and excretion of methyleugenol in exposed humans. Methyleugenol is rapidly absorbed after oral exposure and is capable of dermal permeation. There are no data on inhalation exposure. In rodents, methyleugenol is rapidly absorbed, extensively metabolized in the liver, and predominantly excreted in the urine as sulfate or glucuronide conjugates after oral exposure. Methyleugenol undergoes phase 1 oxidation followed quickly by phase 2 conjugation in experimental animals. Human microsomes produce similar metabolites to those found in rodents. Metabolic activation of methyleugenol in rodents involves 1′-hydroxylation followed by sulfonation to form 1′-sulfooxymethyleugenol, which subsequently undergoes sulfate elimination to form a reactive carbonium ion that can react with DNA, RNA, and proteins. Cytochrome P450 (CYP) 1A2, 2E1, and 2C9 are the predominant enzymes for 1′-hydroxylation of methyleugenol in experimental systems. SULT1A isoforms (i.e. SULT1A1 and SULT1A2) are key enzymes involved in the bioactivation of 1′-hydroxymetheugenol to form the reactive carbonium ion.

Data were available for methyleugenol for the following key characteristics of carcinogens: "is electrophilic or can be metabolically activated to an electrophile", "is genotoxic", "alters DNA repair or causes genomic instability", "induces oxidative stress", "induces chronic inflammation", "modulates receptor-mediated effects" and "alters cell proliferation, cell death, or nutrient supply".

There is consistent and coherent evidence that methyleugenol exhibits key characteristics of carcinogens.

Methyleugenol can be metabolically activated to an electrophile. Three studies in humans detected methyleugenol–DNA adducts in the liver and lung. The major adduct is formed at the N^2 position of guanine, with a minor adduct formed at the N^6 position of adenine. These adducts have also been detected in the livers of mice treated with methyleugenol, and the N^6 adenine adduct has been detected in the urine of rats. In genetically modified mice, DNA adduct formation was dependent on the presence of mouse Sult1a1 or human SULT1A1/2 in the liver, caecum, and kidney, but not the stomach. DNA adduct formation in rat liver was also shown to be SULT-dependent. Methyleugenol formed DNA adducts in turkey and chicken embryos. It has also been shown to bind covalently to RNA in the liver of mice and to protein in the liver of rats.

Methyleugenol is genotoxic. No data were available in humans or human primary cells exposed to methyleugenol. There is consistent and coherent evidence for the genotoxicity of methyleugenol in experimental systems. In human cell lines in vitro, methyleugenol and its metabolite 1′-hydroxymethyleugenol caused DNA strand breaks. Methyleugenol induced mutations in rat and mouse liver in vivo. Methyleugenol did not induce micronucleus formation in a human cell line in vitro. Methyleugenol did not increase micronucleated normachromatic erythrocytes in mice. It induced unscheduled DNA synthesis in hepatocytes of male rats and female mice. Methyleugenol and its metabolite 1′-hydroxymethyleugenol caused DNA damage in Chinese hamster lung fibroblasts but did not increase the frequency of micronuclei. In Chinese hamster ovary cells, methyleugenol induced sister-chromatid exchanges in the presence of rat liver S9 (9000 × *g* supernatant) but did not

induce chromosome aberrations. Methyleugenol induced recombination in yeast.

Methyleugenol and 1′-hydroxymethyleugenol were mutagenic in the presence of rat liver S9 in a strain of *Salmonella typhimurium* genetically modified to express human SULT isoforms. Methyleugenol gave positive results in the absence of S9 in a DNA repair test in *Bacillus subtilis*.

Methyleugenol induces cell proliferation, cell death, or alters nutrient supply. No data were available in humans or primary human cells exposed to methyleugenol. There is consistent and coherent evidence for cell proliferation in experimental systems. A series of repeated-dose toxicity studies (3-day, 7-day, 28-day, 90-day, up to 2-year) in mice and rats showed that methyleugenol was able to cause cell proliferation and alter related biomarkers in the liver. In addition, methyleugenol induced hyperplasia in the glandular stomach, nose, and bile duct in mice, and stomach in rats. Gene expression changes in the livers of treated rodents demonstrated modulation of genes involved in cell cycle and cell proliferation.

There is suggestive evidence that methyleugenol induces chronic inflammation. No data were available in humans or human primary cells exposed to methyleugenol. One study showed that methyleugenol reduces matrix metalloproteinase 9 (MMP-9) activity in a human cell line. In 14 weeks of exposure, methyleugenol increased chronic inflammation of the mucosa of the glandular stomach and atrophy in male and female rats. Increased atrophy in rats was also observed at the 6- or 12-month interim kill after a single dose. No evidence of inflammation was observed at the 2-year treatment with methyleugenol in rats; however, a dose-related increase in chronic active inflammation in the glandular stomach was observed in male but not female mice exposed for 2 years.

For the other key characteristics, "alters DNA repair or causes genomic instability", "induces epigenetics alterations", "induces oxidative stress", and "modulates receptor-mediated effects", there is a paucity of data.

Methyleugenol was found to be mostly without effects relevant to the key characteristics of carcinogens in the assay battery of the Toxicology in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes. However, the cells used in the assay battery are not metabolically competent.

6. Evaluation and Rationale

6.1 Cancer in humans

There is *inadequate evidence* in humans regarding the carcinogenicity of methyleugenol.

6.2 Cancer in experimental animals

There is *sufficient evidence* in experimental animals for the carcinogenicity of methyleugenol.

6.3 Mechanistic evidence

There is *strong evidence* that methyleugenol exhibits key characteristics of carcinogens in experimental systems, including humanized mice, and supported by studies in humans.

6.4 Overall evaluation

Methyleugenol is *probably carcinogenic to humans* (Group 2A).

6.5 Rationale

The Group 2A evaluation for methyleugenol is based on *sufficient evidence* for cancer in experimental animals and *strong* mechanistic evidence in experimental systems, including humanized mice and supported by studies in humans. The

sufficient evidence for cancer in experimental animals is based on an increase in the incidence of malignant neoplasms and a combination of benign and malignant neoplasms in two species (mouse and rat) in two studies that complied with GLP. There is *strong evidence* that methyleugenol exhibits multiple key characteristics of carcinogens; methyleugenol is electrophilic; it is genotoxic; and it alters cell proliferation, cell death, or nutrient supply. The supporting data that methyleugenol exhibits these key characteristics come primarily from experimental systems and is supported by evidence of pro-mutagenic methyleugenol-specific DNA adducts in liver and lung of exposed humans and in livers of humanized mice. The evidence regarding cancer in humans was *inadequate* because no studies were available.

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