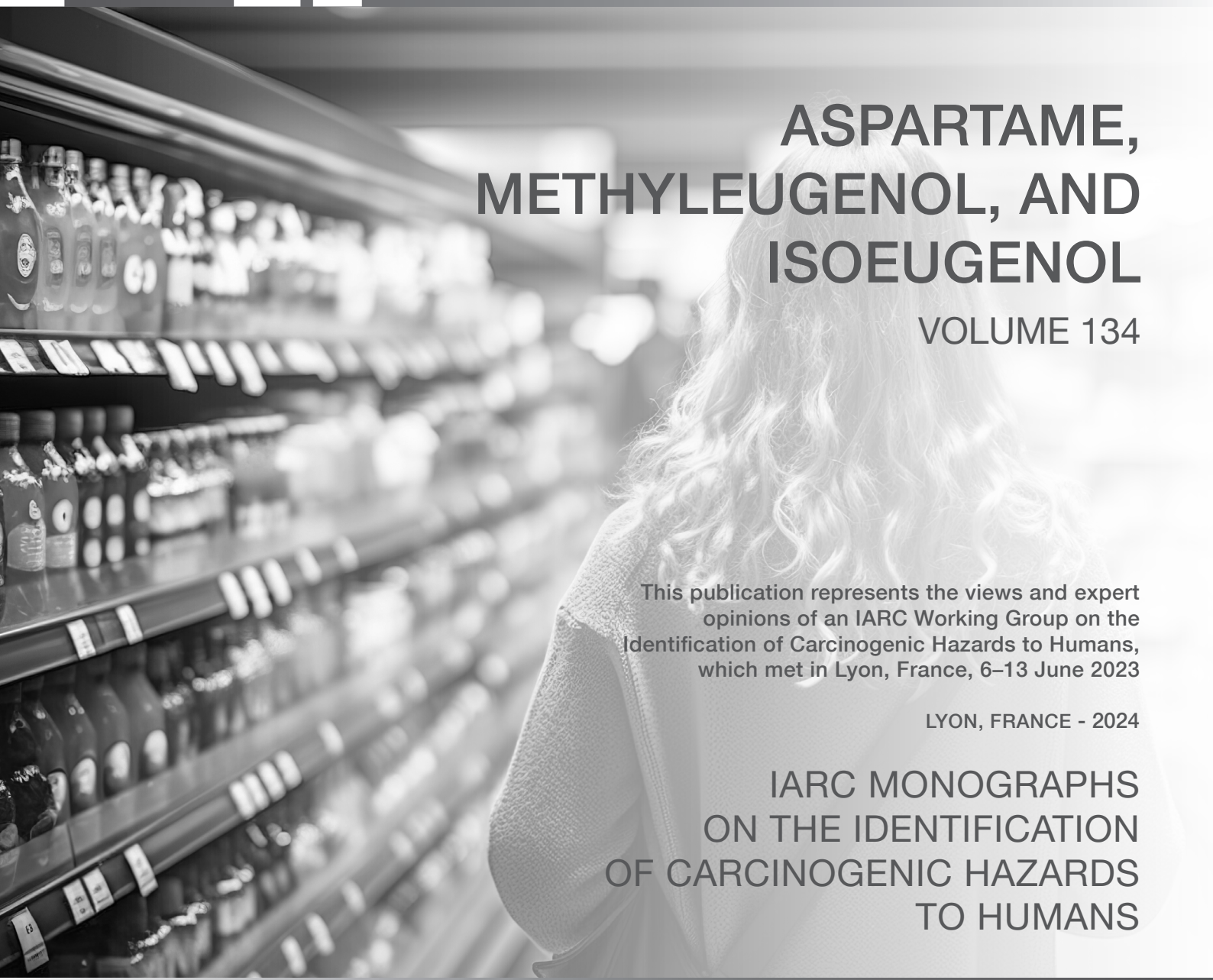
IARC MONOGRAPHS



ASPARTAME, METHYLEUGENOL, AND ISOEUGENOL

VOLUME 134

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IARC MONOGRAPHS ON THE IDENTIFICATION OF CARCINOGENIC HAZARDS TO HUMANS

International Agency for Research on Cancer

World Health Organization

ISOEUGENOL

1. Exposure Characterization

1.1 Identification of the agent

1.1.1 Nomenclature

Chem. Abstr. Serv. Reg. No.: 97-54-1 (*E/Z*); 5912-86-7 (*Z*); 5932-68-3 (*E*) (Chemical Abstracts Service, 2022a)

EC/List No.: 202-590-7 (*E/Z*); 227-633-7 (*Z*); 227-678-2 (*E*) (ECHA, 2023a, b, c)

Chem. Abstr. Serv. name: 2-methoxy-4-(1-propenyl)phenol; *cis*-isoeugenol (*Z*); *trans*-isoeugenol (*E*) (O'Neil, 2006; Chemical Abstracts Service, 2022a)

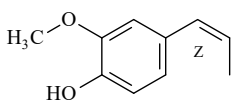
IUPAC systematic name: 2-methoxy-(4-prop-1-enyl)phenol (*E/Z*); 2-methoxy-4-[(*Z*)-prop-1-enyl]phenol (*Z*); 2-methoxy-4-[(*E*)-prop-1-enyl]phenol (*E*) (NCBI, 2022a, b)

Synonyms: 2-methoxy-4-(1-propen-1-yl)phenol (ACI); phenol, 2-methoxy-4-(1-propenyl)-(9CI); phenol, 2-methoxy-4-propenyl- (8CI); 1-(3-methoxy-4-hydroxyphenyl)-1-propene; 2-methoxy-4-(1-propenyl)phenol; 2-methoxy-4-propenylphenol; 3-methoxy-4-hydroxy-1-propenylbenzene; 4-(1-propenyl) guaiacol; 4-hydroxy-3-methoxy-1-propenylbenzene; 4-hydroxy-3-methoxy-β-methylstyrene; 4-propenyl-2-methoxyphenol; 4-propenyl-guaiacol; *iso*-eugenol; isoeugenol; NSC 6769 (Chemical Abstracts Service, 2022c)

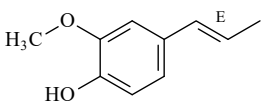
1.1.2 Structural and molecular information

Relative molecular mass: 164.20 (Chemical Abstracts Service, 2022a)

Chemical structure: (Chemical Abstracts Service, 2022a)



Z-(*cis*-) isomer



E-(*trans*-) isomer

Molecular formula: $C_{10}H_{12}O_2$ (Chemical Abstracts Service, 2022a).

1.1.3 Chemical and physical properties

In both nature and commerce, isoeugenol is usually a mixture of *cis*- and *trans*-isomers in the approximate ratio of 1:7 (NTP, 2010). The information in the present monograph pertains to the mixture, unless stated otherwise. [The Working Group noted that there is no evidence to suggest that the *cis*- and *trans*-isomers of isoeugenol have significantly different environmental occurrences.]

Description: The mixture of the isomers is a pale yellow, viscous liquid with a floral odour reminiscent of carnation (Burdock, 2010; NTP, 2010). The *cis*-isomer is a liquid (O'Neil, 2006), but the *trans*-isomer is crystalline, and its odour is more delicate (Fahlbusch et al., 2003).

Odour threshold: 22.54 µg/L in ethanol (46% volume) (Fan and Xu, 2011)

Boiling-point: 266 °C (Chemical Abstracts Service, 2022a)

Melting-point: –10 °C (mixture); 33 °C (*trans*-isomer) (O'Neil, 2006; Chemical Abstracts Service, 2022a)

Density: 1.0869 g/cm^3 at 20 °C (Chemical Abstracts Service, 2022a)

Solubility: slightly soluble in water; soluble in most fixed oils and ether [diethyl ether]; 1:5 in 50% alcohol; insoluble in glycerine (O'Neil, 2006; Burdock, 2010)

Flash-point: > 100 °C (NCBI, 2022a)

Vapour pressure: 0.01–0.02 mm Hg (NCBI, 2022a)

Viscosity: 7.476 cP at 20 °C (NCBI, 2022a)

Octanol/water partition coefficient (P): log K_{ow} = 3.04 (Griffin et al., 1999)

Dissociation constant: pK_a = 9.88 at 25 °C (NCBI, 2022a).

1.1.4 Commercial products and impurities

The commercial product is a mixture of *cis*- and *trans*-isomers (Burdock, 2010), but the pure *trans*-isomer is also available commercially (NTP, 2010). The *trans*-isomer dominates because it is thermodynamically more stable (Panten and Surburg, 2016). Commercial qualities with purities in the range of 90–94%, 95–98%, and ≥ 99% are available (Chemical Abstracts Service, 2022b). [The Working Group noted that there is no publicly available information on impurities. Depending on the specific manufacturing process (see Section 1.2.1), it can be deduced that eugenol or other phenolic compounds such as guaiacol may be present as impurities, especially in lower-purity grades. Possible inorganic impurities include metals used as catalysts during the manufacturing process.]

1.2 Production and use

1.2.1 Production process

Isoeugenol is produced by the alkaline isomerization of eugenol obtained from essential oils rich in eugenol (Burdock, 2010). The conversion involves heating eugenol with potassium hydroxide (Larrañaga et al., 2016) with catalysts such as various metals (Červený et al., 1987; Fahlbusch et al., 2003) or more environmentally friendly hydrotalcite (Kishore and Kannan, 2002). In particular, eugenol – the starting material for the production of isoeugenol – is often obtained from the leaf oil of clove (*Syzygium aromaticum*) (Panten and Surburg, 2015). Another synthetic route starts with the esterification of guaiacol and propionic acid, followed by a Fries rearrangement of the resulting guaiacyl propionate in the presence of aluminium chloride into 4-hydroxy-3-methoxypropiophenone, which is reduced to the corresponding secondary alcohol, and removal of water finally yields isoeugenol (Fahlbusch et al., 2003). [The Working Group was unable to find information about which process is currently preferred to produce isoeugenol. The Working Group noted that, in addition to chemical synthesis, isoeugenol may also be directly extracted from a variety of plant materials by steam distillation or with organic solvents.]

1.2.2 Production volume

In 1983, information from five isoeugenol producers indicated that approximately 21 000 pounds [9 tonnes] were produced (NTP, 2010). [The Working Group noted that NTP (2010)

did not state where the production occurred, presumably in the USA.] In recent years, USA national aggregate production volumes were below 1 000 000 pounds [454 tonnes] for 2016, 2017, 2018, and 2019. One company in the USA reported annual production volumes of 8818 pounds [4.0 tonnes] in 2016, 8818 pounds [4.0 tonnes] in 2017, 6613 pounds [3.0 tonnes] in 2018, and 4409 pounds [2.0 tonnes] in 2019 (US EPA, 2023). In 1990, four isoeugenol importers imported between 12 000 pounds [5.4 tonnes] and 122 000 pounds [55.3 tonnes] (data from US EPA; NTP, 2010). [The Working Group noted that NTP (2010) did not state where the importation occurred, presumably only into the USA.] In 1992, the USA imported approximately 730 000 pounds [330 tonnes] of eugenol or isoeugenol (NTP, 2010).

The total use of isoeugenol in Europe was estimated to be 26 000 kg/year (HERA, 2005).

In 2004, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) assumed an annual production volume of 817 kg for the USA and 327 kg for Europe (data extracted from references for 1970–1999), whereas the annual volume in naturally occurring foods was assumed to be 2162 kg for the USA (no data for Europe were available). The ratio between consumption via natural occurrence in foods and use as a flavouring substance was 7 (WHO, 2004a). [Considering this ratio, the Working Group noted that relying solely on production data may lead to an underestimation of the total amount of isoeugenol use. Therefore, it is important to consider both the production and importation data and the amount of isoeugenol naturally contained in foods to obtain a more accurate estimate of overall exposure.]

1.2.3 Uses

The presence of isoeugenol in products can derive from the addition of the isolated or synthesized agent, but also from its natural occurrence in a wide variety of plants (see Section 1.4). Therefore, in a substantial proportion of the products in which isoeugenol has been detected, the presence of isoeugenol may be unintentional.

Additional fragrance materials can be produced by esterification or etherification of the hydroxy group of isoeugenol (Panten and Surburg, 2016).

Isoeugenol is used as a reagent in the synthesis of compounds such as isoindolo quinolines (Merchán-Arenas et al., 2020). Isoeugenol can be biotechnologically converted to vanillin using several different types of microorganism (Priefert et al., 2001; Ma et al., 2022). For example, a method has been described that gave an 81% yield of vanillin, without overoxidation to vanillic acid or accumulation of undesirable by-products such as acetaldehyde (Yamada et al., 2008). However, although the process of making vanillin by oxidizing isoeugenol was historically significant, this method is no longer commonly used today and has been replaced by more efficient and cost-effective methods (Panten and Surburg, 2016).

The sweet, spicy, floral fragrance of isoeugenol leads to its use as a fragrance or flavouring agent in perfumes, cosmetics, personal hygiene products, household cleaning agents, and foods. Tasting like anise or liquorice, isoeugenol is added to non-alcoholic drinks, baked foods, candies, and chewing gums (NTP, 2010).

Isoeugenol exhibits antibacterial and antifungal properties, inhibiting the growth of a broad range of bacteria and moulds, including *Listeria monocytogenes, Escherichia coli*, *Bacillus licheniformis*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Salmonella* type B, *Staphylococcus aureus, Aspergillus oryzae, Penicillium camemberti*, and *Penicillium roqueforti* (Faith et al., 1992; Wendorff et al., 1993; Mansour et al., 1996; Hyldgaard et al., 2015). [The Working Group noted that it was not clear from the information provided whether the doses of isoeugenol

commonly used in foods were sufficient to exhibit bacteriostatic and fungistatic effects.]

The Consumer Product Information Database (CPID) lists the use of isoeugenol in 86 products, including disinfectant cleaners, laundry detergents, toilet cleaners, air and fabric fresheners, fragrance diffusers, candles, leather lotions, shoe-cleaning creams, moisturizing oils, eau-de-cologne sprays, sunscreen lotions, roll-on deodorants, and various automobile cleaners and fresheners (DeLima Associates, 2022). In Europe, about 60% of the total use of isoeugenol was for household laundry and cleaning products, including laundry detergents, laundry pre-treatment products, fabric softeners, hard-surface cleaners, hand dishwashing products, and toilet cleaners (HERA, 2005).

In medicine, isoeugenol is used as a test reference allergen in epicutaneous patch tests, which are indicated for use in the diagnosis of allergic contact dermatitis (NCBI, 2022a).

Isoeugenol is used as the active ingredient in so-called zero-withdrawal anaesthetics used in the culture and management of finfish and shellfish (NTP, 2010). Isoeugenol-containing anaesthetics prevent struggling and thus maintain muscle quality during the "rested harvesting" of king salmon (NTP, 2010). Isoeugenol can be used as a feed additive for fattening cattle, pigs, or chickens (EFSA, 2012).

[The Working Group noted that the literature is often unclear as to whether isoeugenol itself is used, or whether it has been added indirectly via the use of various plant materials and essential oils.]

1.3 Detection and analysis

Methods for the sampling, identification, and quantification of isoeugenol in air, water, herbs, cosmetics, and food have been developed and used in research and practice. No methods were found for the measurement of isoeugenol in the soil. Some methods are described in a recent review (Dang and Quirino, 2021), and selected publications containing information on sample preparation and method performance data, including the limit of detection (LOD), when reported, are presented in Table 1.1. A European Standard (EN 16274) method for the analysis of isoeugenol in consumer products was approved in 2012 (CEN, 2012). Most studies report the sum of *cis*- and *trans*-isoeugenol, or only the *trans*-isomer. However, there are methods capable of distinguishing between the two isomers (Wisneski et al., 1988; Rodríguez-Bencomo et al., 2008; Martínez-Gil et al., 2018).

1.3.1 Air

Stanfill and Ashley (2000) developed a method combining solid-phase extraction with analysis by selected ion monitoring-gas chromatography-mass spectrometry (SIM-GC-MS) to quantify isoeugenol in mainstream cigarette smoke particulate. Mainstream smoke from a smoking machine was captured into a glass fibre filter and extracted with hexane and 3′,4′-methylenedioxyacetophenone. The LOD was 20.1 ng/cigarette.

Kuo et al. (2009) developed a diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) method to measure isoeugenol in aerosol particles of essential oils in indoor air. The aerosol was collected on aluminium foil for 30 minutes. The DRIFTS results were similar to those of gas chromatography (GC) analysis.

1.3.2 Water

Martínez et al. (2013) developed an analytical method based on headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS) for the simultaneous determination of 76 micropollutants in water samples. The LOD for isoeugenol was 0.100 ng/mL. An immunoassay for the determination of isoeugenol was developed

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

Sample matrix	Sample preparation	Instrument (LOD)	Comments	Reference
Air				
Indoor air	No extraction; collection of aerosol on aluminium foil	DRIFTS (42 ng/mL)	Aerosols of ylang essential oils	Kuo et al. (2009)
Tobacco smoke particulate	Smoking machine; glass fibre (Cambridge) filter; hexane/MDAP extraction, SPE	SIM-GC-MS (20.1 ng/cigarette)		Stanfill and Ashley (2000)
Water				
Surface water, sea water, waste water	Headspace SPME	GC-MS (0.100 ng/mL)		Martínez et al. (2013)
Water	No pre-treatment	EFM-based LFIA (6.02 µg/kg)		Lei et al. (2023)
Food				
Herbs/spices, sauces	Ultrasound extraction of finely chopped samples in methanol, maceration at 50°C for 12 hours	Capillary LC (0.148 mg/L)		Avila et al. (2009)
Peppers	Ultrasound extraction with ethyl acetate	GC-HRMS (10 µg/kg)	*trans*-Isoeugenol validated for black pepper according to SANTE/11813/2017 guidelines	Rivera-Pérez et al. (2020); European Commission (2017b)
Fish fillet	Homogenization, ultrasound extraction in hexane, SPE with ethyl acetate	GC-MS/MS (1.2 µg/kg)		Ke et al. (2016)
Fish fillet	Homogenization in dry ice, four sequential extractions with acetonitrile, SPE	LC (4–14 µg/kg)	LOD range is for 9 freshwater fish species	Meinertz et al. (2008)
Fish and shrimp	Ultrasound extraction in acetonitrile; dispersive SPE with polystyrene-glycidylmethacrylate microspheres, primary secondary amines, and C18; DMSO-assisted concentration	HPLC-UV (13 µg/kg)		Shi et al. (2022)
Smoked sausage, smoked fish	Homogenization in phenol solution, membrane-based microextraction using PVDF-*co*-PTFE membrane impregnated with choline chloride	HPLC-FLD (0.6 µg/kg)		Shishov et al. (2020)
Fish	Homogenization, ultrasound extraction with acetonitrile, air drying in room temperature, dissolved in methanol solution	LFIA (5.9 µg/kg)		Lei et al. (2023)
Medicinal herbs or plants				
Anemopsis californica root	Supercritical fluid extraction (methanol)	GC-MS (NR)		Medina-Holguín et al. (2008)
Myrtus communis L.	Headspace SPME, headspace single-drop microextraction	GC-MS (NR)	*cis*-Isoeugenol	Moradi et al. (2012)
Consumer products				
Creams	Direct contact sorptive tape extraction	GC-MS (190 µg/kg)		Sgorbini et al. (2010)

Table 1.1 (continued)

Sample matrix	Sample preparation	Instrument (LOD)	Comments	Reference
Creams, perfumes, anti-hair loss products, etc.	Headspace-programmable temperature vapourizer	Fast GC-MS (0.014 µg/mL)		del Nogal Sánchez et al. (2010)
Perfumes	Acetonitrile dilution	HPLC (0.13 µg/mL)		Soo Lim et al. (2018)
Insect repellent, massage oil, cream, hair conditioner	Acetonitrile dilution	HPLC (0.10 µg/mL)		Villa et al. (2007)
Perfumes, colognes, toilet waters	NaOH/isooctane extraction	LC-FLD (16 µg/mL for *cis*-isoeugenol; 38 µg/mL for *trans*-isoeugenol)		Wisneski et al. (1988)
Creams and lotions	Dispersive SPE-PLE	GC-MS [0.075 µg/g]		Lamas et al. (2010)

DMSO, dimethyl sulfoxide; DRIFTS, diffuse reflectance infrared Fourier transform spectroscopy; EFM, europium-fluorescent microspheres; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; FLD, fluorescence detection; GC-HRMS, gas chromatography-high resolution mass spectrometry; LC, liquid chromatography; LFIA, lateral-flow immunoassay; LOD, limit of detection; MDAP, 3′,4′-methylenedioxyacetophenone; MeOH, methanol; MS/MS, tandem mass spectrometry; NaOH, sodium hydroxide; NR, not reported; PLE, pressurized liquid extraction; PVDF-co-PTFE, poly(vinylidene fluoride-*co*-tetrafluoroethylene; SIM, selected ion monitoring; SPE, solid-phase extraction; SPME, solid-phase microextraction; UV, ultraviolet.

recently. The LOD was 6.02 µg/kg in water (Lei et al., 2023).

1.3.3 Medicinal herbs or plants

Medina-Holguín et al. (2008) used supercritical fluid extraction and GC-MS to quantify isoeugenol in the roots of the medicinal plant *Anemopsis californica* (yerba mansa or lizard tail). Moradi et al. (2012) measured *cis*-isoeugenol in *Myrtus communis* L. (common myrtle) with GC-MS. In sample preparation, HS-SPME and headspace single-drop microextraction (HS-SDME) were not superior to hydrodistillation.

1.3.4 Consumer products

Sgorbini et al. (2010) determined isoeugenol content in cosmetic creams spread on skin by direct contact sorptive tape extraction and GC-MS. del Nogal Sánchez et al. (2010) determined isoeugenol content in cosmetic products by headspace-programmed temperature vapourization-fast gas chromatography-quadrupole mass spectrometry (HS-PTV-fast GC-MS). The LOD was 0.014 µg/mL.

Villa et al. (2007) and Soo Lim et al. (2018) analysed perfumes and other cosmetics by acetonitrile dilution high-performance liquid chromatography (HPLC) and reported LODs of 0.10 and 0.13 µg/mL, respectively. Lamas et al. (2010) developed a solid-phase dispersion pressurized liquid extraction method followed by GC-MS for the analysis of isoeugenol and other compounds in creams and lotions. For isoeugenol, the LOD was [0.075 µg/g].

1.3.5 Food

Several methods for the quantification of isoeugenol in food have been described. Before extraction, Avila et al. (2009) homogenized herbal samples and macerated them for 12 hours at 50 °C. On-column preconcentration-capillary LC was used to determine isoeugenol in herbs, spices, and sauces. The LOD was 13 ng/mL. With the direct determination method (without preconcentration), the LOD was 0.148 mg/L.

Rivera-Pérez et al. (2020) used gas chromatography coupled to high-resolution mass spectrometry (GC-HRMS-Q-Orbitrap) in combination with a simple ultrasound-assisted extraction method with ethyl acetate to determine simultaneously eight alkenylbenzenes – including *trans*-isoeugenol – in peppers. For *trans*-isoeugenol, the LOD was 10 µg/kg and the limit of quantitation (LOQ) was 200 µg/kg.

For the analysis of isoeugenol in fish, samples were homogenized at room temperature or in dry ice. Hexane (Ke et al., 2016; Shishov et al., 2020) or acetonitrile (Meinertz et al., 2008; Shi et al., 2022) were used as extraction solvents. The use of ultrasound improved extraction yield.

Meinertz et al. (2008) developed an SPE-LC-based method to quantify isoeugenol residue in fillet tissue from 10 species of freshwater fish. The LODs for isoeugenol were between 4 and 14 µg/kg.

Ke et al. (2016) determined isoeugenol content in fish fillets using gas chromatography coupled to tandem mass spectrometry (GC-MS/MS). The LOD was 1.2 µg/kg and the LOQ was 4 µg/kg.

Shishov et al. (2020) used membrane-based microextraction and HPLC-FLD to quantify isoeugenol in smoked sausage and fish. The LOD was 0.6 µg/kg.

Shi et al. (2022) established a dispersive solid-phase extraction method, combined with HPLC-UV, for the simultaneous determination of seven anaesthetics, including isoeugenol, in fish and shrimp. For isoeugenol, the LOD was 13 µg/kg.

An HPLC method with fluorescence detection for the determination of residues of isoeugenol in muscle or skin of Atlantic salmon was developed and validated in a laboratory that was approved for Good Laboratory Practice (GLP). The LOQ for the method was 0.25 mg/kg. The method was sufficiently validated (according to the

requirements of Volume 8 of the Rules governing veterinary medicinal products in the European Union, EU) and verified by the relevant European reference laboratory, which confirmed the suitability of the method (EMA, 2020). Isoeugenol remained quantifiable in salmon plasma (LOD, 25 ng/L) for up to 12 hours after the end of exposure but was no longer detectable in plasma at 24 hours (EMA, 2020).

An immunoassay for the determination of isoeugenol has been developed. The LOD was 5.9 µg/kg in fish (Lei et al., 2023).

1.3.6 Biological specimens

cis-Isoeugenol was measured in human serum by GC-MS (Wang et al., 2010). [The Working Group noted that this study lacked a detailed description of sampling, sample preparation, and detection methods.]

A method based on solid-phase extraction followed by analysis using GC-MS has been reported for measuring isoeugenol in urine (Dills et al., 2001, 2006). The LODs were approximately 0.004 µg/mL.

1.4 Occurrence and exposure

1.4.1 Environmental occurrence

Isoeugenol is a compound that occurs naturally in the essential oils of more than 500 plant species, including cloves (*Syzygium aromaticum*), sweet flag (*Acorus calamus)*, sweet wormwood (*Artemisia annua)*, Ceylon cinnamon (*Cinnamomum verum)*, coffee (*Coffea arabica)*, nutmeg (*Myristica fragrans)*, basil (*Ocimum basilicum)*, perilla (*Perilla frutescens)*, and ylang-ylang (*Cananga odorata*) (USDA, 2021; HMDB, 2022; Wishart et al., 2022). An overview of the occurrence of isoeugenol in plants is given in Table 1.2.

Isoeugenol occurs naturally in foods and beverages and can be added to foods as a flavouring agent. It is present in smoked food products since it is a component of wood smoke. Isoeugenol also occurs in cosmetics and personal care products, and some tobacco and cannabis products. The production and use of isoeugenol can result in its release to the environment through various waste streams. When wood is burned, isoeugenol is released into the air. The direct release of isoeugenol to the environment is anticipated because of its use as a sedative or anaesthetic for fish (NCBI, 2022a).

(a) Air

Isoeugenol is expected to exist almost exclusively as a vapour in the ambient atmosphere (NCBI, 2022a). In the vapour phase, isoeugenol is degraded in the atmosphere by reaction with photochemically generated hydroxyl radicals and with ozone. The atmospheric half-life is estimated to be between 3 and 4 hours (NCBI, 2022a). Occurrence in air is expected in smoke from wood combustion (NCBI, 2022a).

Isoeugenol is primarily produced during high-temperature torrefaction of woody biomass (González Martínez et al., 2018). The emission rate of isoeugenol in wood smoke from the combustion of oak, eucalyptus, and pine wood was reported to be 1.0, 0.5, and 17 mg/kg, respectively (Nolte et al., 2001). The emission rate of isoeugenol in wood smoke from the combustion of pine wood logs, oak wood logs, and synthetic logs was reported to be 8.04 mg/kg, 0.16 mg/kg, and not detected, respectively (Rogge et al., 1998). Isoeugenol, acetoguaiacone, and 4-vinylguaiacol were the dominant phenolic compounds identified in the pyrolysis of *Tectona grandis* (teak) biomass (Balogun et al., 2014). According to a technical specification of the European Committee for Standardization, isoeugenol is one of the most abundant single organic compounds in the product gases of biomass gasification (CEN, 2006).

Table 1.2 Concentrations of isoeugenol in various plant parts

Plant	Part	Concentration (mg/kg)
Acorus calamus	Rhizome	228–12 510
Anethum graveolens	Plant	[a]
Artemisia dracunculus	Plant	[a]
Artemisia annua	Leaf	1–45
Cananga odorata	Flower	[a]
Cinnamomum verum	Stem bark	[a]
Cinnamomum verum	Leaf essential oil	[a]
Cinnamomum verum	Bark	2–8
Cinnamomum aromaticum	Plant	[a]
Coffea arabica	Seed	[a]
Myristica fragrans	Seed essential oil	1000–3800
Myristica fragrans	Seed	40–320
Psiadia argute	Essential oil	565 000
Nicotiana tabacum	Leaf	[a]
Ocimum basilicum	Plant	8–95
Origanum sipyleum	Shoot	3–5
Oryza sativa	Plant	[a]
Perilla frutescens	Leaf essential oil	2500
Pimenta racemosa	Leaf	[a]
Pimenta dioica	Plant	[a]
Pimenta dioica	Leaf essential oil	[a]
Santalum album	Wood	[a]
Satureja parvifolia	Shoot	[a]
Satureja odora	Shoot	525
Scutellaria baicalensis	Root essential oil	[a]
Syzygium aromaticum	Plant	[a]
Thymus vulgaris	Plant	[a]
Vaccinium corymbosum	Fruit	[a]
Vaccinium myrtillus	Fruit juice	[a]
Zingiber officinale	Rhizome	1.48–1.68
Laurus nobilis	Leaves	1000–6000
Strychnos spinosa	Peel of the fruits	4762
Salacca zalacca	Fruit	[a]
Myroxylon pereirae	Resin fraction	8500

[a] No quantitative data reported.
Compiled from Janssens et al. (1990), Duke (2001), Sitrit et al. (2003), Kilic et al. (2004), Wijaya et al. (2005), Schaller and Schieberle (2020), USDA (2021).

(b) Water

Two condensate effluents from a bleached softwood pulp mill in Canada contained isoeugenol at concentrations of 10 µg/L and 121 µg/L (Belknap et al., 2006). Isoeugenol was detected at concentrations ranging from 0 (LOD, not reported) to 28 643 µg/L in six effluents collected from 13 pulp and paper mills in Quebec, Canada (Lavallee et al., 1992).

When released to water, isoeugenol is expected to be adsorbed to suspended solids and sediments. Volatilization from water is expected, with half-lives for a model river and model lake of 13 and 100 days, respectively. Isoeugenol can be degraded in natural waters by reaction with photochemically generated hydroxyl radicals, with an estimated half-life of 21 days. The potential for bioconcentration of isoeugenol in aquatic organisms is moderate (NCBI, 2022a).

(c) Soil

On the basis of its physical properties (see Section 1.1), isoeugenol is expected to have low mobility in soil. Volatilization of isoeugenol from moist soil surfaces is expected to be an important process (NCBI, 2022a). Biodegradation may be an important environmental process in soil, with 79% biodegradation achieved after 28 days (NCBI, 2022a). Isoeugenol is not expected to volatilize from dry soil surfaces because of its vapour pressure (NCBI, 2022a). [The Working Group noted that there are limited data available to support or refute theoretical assumptions about the fate of the compound in soil. Some of the information available appears contradictory (e.g. that isoeugenol volatilizes from moist but not from dry soil) and in need of experimental validation.]

(d) Consumer products

The usual and maximum concentrations of isoeugenol in some cosmetic products were 0.03% and 0.3% in soap, 0.003% and 0.03% in detergents, 0.015% and 0.1% in creams and lotions, and 0.4% and 0.8% in perfumes, respectively (Opdyke, 1975). According to labelling, 27 out of 300 evaluated cosmetics (9%) on the market in the United Kingdom (UK) in 2006 contained isoeugenol (Buckley, 2007). However, a study from Sweden noted during a survey of 45 products that isoeugenol was not detected in 67% of fragrances in which it had been declared by the supplier (Bárány and Lodén, 2000).

Rastogi and colleagues measured the isoeugenol content of different consumer products available on the Danish or European markets (Rastogi et al., 1996, 1998, 1999). Out of 42 cosmetic products based on natural ingredients, isoeugenol was found in 3 products (7%) at 0.0127%, 0.027%, and 0.139% (Rastogi et al., 1996). Out of 22 vapour-spray deodorants with fragrance, 9 (41%) contained isoeugenol (mean, 0.0129 g/100 mL; median, 0.0098 g/100 mL; range, 0.0001–0.0458 g/100 mL); 9 out of 22 aerosol deodorant sprays (41%) contained isoeugenol (mean, 0.0031 g/100 mL; median, 0.0024 g/100 mL; range, 0.0001–0.0104 g/100 mL), and 2 out of 28 roll-on deodorants (8%) contained isoeugenol at 0.0241 and 0.0268 g/100 mL (Rastogi et al., 1998). In an investigation of 25 children's cosmetics, isoeugenol was not detected in shampoos and lotions, but was detected at 0.019% and 0.074% in two out of seven (29%) hydroalcoholic products (Rastogi et al., 1999). Among 25 popular perfume brands, isoeugenol was found in 14 products (56%) (mean, 71 mg/L; median, 80 mg/L; range, 48–193 mg/L) (Rastogi et al., 2007). Among 29 international brands of hydroalcoholic perfumes and aftershaves, 16 products (55%) contained isoeugenol (mean, 71 mg/kg; median, 45 mg/kg; range, 27–203 mg/kg) (Rastogi and Johansen, 2008).

In 59 domestic and professional products for which hand exposure would occur (such as soap, cleaners, laundry agents, hand and dish wash, furniture polish, stain remover, and car shampoo), isoeugenol was detected in three products (5%)

[no quantitative data were provided], and it was concluded that isoeugenol occurred much more frequently in cosmetic products than in household products (Rastogi et al., 2001).

In Taiwan, China, four popular types of traditional worship incense based on agarwood (*Aquilaria agallocha*) or sandalwood (*Santalum album*) contained isoeugenol (mainly in the *trans*-form) at a concentration of between 18 and 29 mg/kg of powder. The emissions also contained the *cis*-isomer produced during the burning process, with a total isoeugenol content between 56 and 80 mg/kg of incense (Kuo et al., 2015).

Isoeugenol has been detected in the essential oil of cannabis (*Cannabis sativa*) Turner et al., 1980).

Isoeugenol is a constituent of smoke condensate from Turkish tobacco (approximately 14.7 mg per 1000 cigarettes) (Rodgman and Cook, 1964). Both *cis*- and *trans*-isomers have been detected in cigarette smoke condensates (Arnarp et al., 1989). In a study of eight commercial cigarette brands in the USA, seven brands were found to contain isoeugenol (mean values, 265–4050 ng/cigarette) in smoke particles from unblocked cigarettes (Stanfill and Ashley, 2000). The effect of blocking the ventilation holes in the cigarette filter was investigated in another brand (containing isoeugenol at 188 ng/cigarette). Isoeugenol was detected in the smoke of the unblocked cigarette and in the smoke when the holes were partially or completely blocked (226, 525, and 1030 ng in the smoke particulate of one cigarette, respectively) (Stanfill and Ashley, 2000).

Some tobacco flavourings contain alkenylbenzenes, including isoeugenol (Stanfill et al., 2003). Isoeugenol was found in only 1 out of 20 brands of bidi cigarettes (small hand-rolled cigarettes produced mainly in India) purchased in the USA; levels ranged from 71 mg/kg to 93 mg/kg. Lower levels of isoeugenol were found in cigarettes produced in the USA, ranging from 0.055 to 0.44 mg/kg (Stanfill et al., 2003). In a study of 68 cigarette brands on the USA market, isoeugenol was found in 4 brands (5.9%) at levels of 0.068–0.38 mg/kg (Stanfill and Ashley, 1999).

(e) Food

Some of the plant species containing isoeugenol are culinary herbs, spices, and edible fruits, such as blueberry, guava, blackberry, tomato, cinnamon, cloves, nutmeg, mace, thyme, tea, coffee, plum, dill, Chinese quince, pimento, bay leaves, ginger, pepper, fennel, and mate (Kilic et al., 2004; Burdock, 2010; Rivera-Pérez et al., 2020; Schaller and Schieberle, 2020; Suleiman, 2020). Processed foods can be flavoured with plant parts, essential oils, or extracts from specific plants that contain isoeugenol (Burdock, 2010). The concentration of isoeugenol in some edible products has been reported to be about 4 mg/kg in beverages, 4–10 mg/kg in foods, and 0.3–1000 mg/kg in gums (Opdyke, 1975). An overview of concentrations of isoeugenol in foods is given in Table 1.3.

Isoeugenol has been reported to be added as a flavouring agent to alcoholic beverages (0.21–0.35 mg/kg), bakery products (10.88–14.38 mg/kg), chewing gum (0.14–0.17 mg/kg), condiments and relishes (up to 0.04 mg/kg), frozen dairy products (4.25–6.63 mg/kg), gelatins and puddings (4.56–6.42 mg/kg), hard candies (3.60 mg/kg), meat products (4.34 mg/kg), nonalcoholic beverages (3.27–5.61 mg/kg), and soft candies (6.22–9.62 mg/kg) (Burdock, 2010).

Isoeugenol was detected in two samples of barley tea at 1 and 22 µg/kg (Tatsu et al., 2020). It was also found in infusions of green, black, and oolong teas (n = 19) at concentrations ranging between 0.5 and 30 µg/kg (semiquantitative data) (Baba and Kumazawa, 2014).

In wines, isoeugenol may occur because of extraction out of oak wood because of either traditional barrel ageing or treatment with oak chips. The final levels of extracted isoeugenol depend on the toast degree of the wood chips and

Table 1.3 Concentrations of isoeugenol in selected food products

Food, drink, or preparation	Isoeugenol concentration
Smoked sausages	6–76 mg/kg
Fillet tissue freshwater fish species exposed to isoeugenol	19–62 mg/kg
Bakery products[a]	11–14 mg/kg
Soft candies[a]	6.2–9.6 mg/kg
Frozen dairy products[a]	4–7 mg/kg
Gelatins and puddings[a]	4.6–6.4 mg/kg
Non-alcoholic beverages[a]	3.3–5.6 mg/kg
Meat products[a]	~4.3 mg/kg
Hard candies[a]	~3.6 mg/kg
Alcoholic beverages[a]	0.2–0.4 mg/kg
Soluble coffee	0.06–0.3 mg/kg
Chewing gum[a]	0.14–0.17 mg/kg
Roasted coffee beans	0.006–0.1 mg/kg
Condiments and relishes[a]	≤ 0.04 mg/kg
Roasted chicory coffee brews	15–45 µg/L
Wine (wood-aged)	4–34 µg/L
Green, black, and oolong teas	0.5–30 µg/kg
Barley tea	1–22 µg/kg
Brewed coffee	~2 µg/L

[a] Flavoured products.
Compiled from Tressl et al. (1978), Meinertz et al. (2008), Viegas et al. (2008), Burdock (2010), Hernández-Orte et al. (2012), Pöhlmann et al. (2012), Hitzel et al. (2013), Baba and Kumazawa (2014), Herrero et al. (2016), Kalschne et al. (2018), Martínez-Gil et al. (2018), Wu and Cadwallader (2019), Pua et al. (2020), Tatsu et al. (2020).

the time of contact with the wine, and levels may be reached of about 12 µg/L for *trans*-isoeugenol and about 1 µg/L for *cis*-isoeugenol (Rodríguez-Bencomo et al., 2008). During the ageing of Chardonnay and Sauvignon Blanc wines in French oak wood barrels, isoeugenol increased from not detectable levels to 4–8 µg/L (Herrero et al., 2016). Wines aged in Colombian oak wood (*Quercus humboldtii*) were found to have higher concentrations of isoeugenol (32–34 µg/L) than did wines aged in European or American oak wood (*Q. alba* and *Q. petrea*) (6–20 µg/L) (Martínez-Gil et al., 2018). In Tempranillo wine, isoeugenol concentrations increased from 4 µg/L to 15 µg/L during malolactic fermentation, but no significant increase was found during malolactic fermentation of Cabernet Sauvignon wines (isoeugenol, 6–8 µg/L) (Hernández-Orte et al., 2012).

In juice, wine, and vinegar produced from hawthorn fruit (*Crataegus tanacetifolia*), isoeugenol was detected at concentrations of 7.23, 166.3, and 199.6 µg/100 mL (Özdemir et al., 2022).

Isoeugenol occurs in smoke flavour preparations as a result of the pyrolysis of lignin. In 15 samples of liquid and solid smoke preparations, isoeugenol was found to be between 0.1% and 1.2% of the total phenolic fraction in 7 samples, between 2.0% and 2.2% of the total phenolic content in smoked ham, and between 2.1% and 2.3% of the phenolic extract in bacon treated with liquid smoke (Tóth and Potthast, 1984). In six commercial liquid smoke preparations, isoeugenol was detected at concentrations of between 1.4 and 15 mg/L (Giri et al., 2017). The isoeugenol content in 19 frankfurter-type sausages and mini salamis experimentally

smoked with different types of wood ranged from 6 to 76 mg/kg [mean, 24 mg/kg; median, 11 mg/kg], which was significantly higher than the content found in 11 non-smoked mini salamis (4 mg/kg) (Hitzel et al., 2013). In another investigation of 24 frankfurter-type sausages experimentally hot-smoked, isoeugenol content ranged from 9 to 29 mg/kg [mean, 19 mg/kg; median, 19 mg/kg] (Pöhlmann et al., 2012).

One sample of roasted *Coffea arabica* coffee contained isoeugenol at 0.1 mg/kg (Tressl et al., 1978). In roasted coffee beans [species not reported] from Columbia, isoeugenol was detected at 0.12 mg/kg (Ho et al., 1993). A roasted *C. canephora* sample contained isoeugenol at 5.7 µg/kg, increasing to up to 7.7 µg/kg after steam treatment (Kalschne et al., 2018). Roasted *C. arabica* beans from Yunnan province, China, contained 0.15% isoeugenol (Zhou et al., 2013a, b) [the percentage probably refers to the percentage in the aroma extract and is not an absolute percentage in the coffee bean]. *C. arabica* coffee beans processed by monsooning (storage in humid air) contained isoeugenol at about 9 µg/kg, irrespective of subsequent irradiation treatment (Variyar et al., 2003). Isoeugenol was also detected in two out of four samples of brewed *C. arabica* coffee from Brazil and Colombia (2.1 µg/L and 1.7 µg/L, respectively) (Pua et al., 2020). It was also detected in a sample of Brazilian soluble coffee at levels between 0.06 and 0.3 mg/kg, depending on the analytical methodology used (Viegas et al., 2008), and in a sample of Brazilian roasted *C. arabica* coffee oil at 0.81 µg/kg (Böger et al., 2021). Isoeugenol was also found in roasted chicory (*Cichorium intybus*) coffee brews (n = 3) at an average concentration of 45 µg/L (*trans*-isoeugenol) and 15 µg/L (*cis*-isoeugenol) (Wu and Cadwallader, 2019).

In fresh king salmon (*Oncorhynchus tshawytscha*), isoeugenol was detected in fish harvested with isoeugenol-containing anaesthetic [no quantitative data provided] (Wierda et al., 2006). In nine freshwater fish species exposed to isoeugenol at 8.5 mg/L for 60 minutes, the fillet tissue contained isoeugenol at between 19 and 62 mg/kg [mean, 42 mg/kg; median, 39 mg/kg] (Meinertz et al., 2008). In a market survey of fish fillets in China, isoeugenol was detected in two samples of grouper fish fillets (86 and 1032 µg/kg) [the total sample number was not reported; the occurrence was probably caused by the use of isoeugenol or clove oil as a veterinary anaesthetic] (Ke et al., 2016).

1.4.2 Occupational exposure

In the USA, the National Institute for Occupational Safety and Health (NIOSH) conducted the National Occupational Exposure Survey (NOES) in 1981–1983. NOES revealed that 35 171 (95% confidence interval, CI, 28 489–41 853) employees (71% females) were potentially exposed to isoeugenol (CDC, 1990). Of the potentially exposed, 29 918 employees worked as hairdressers or cosmetologists (85%), and the other commonly exposed employees included janitors and cleaners, machine operators (1282), and packaging and filling machine operators (875). Firefighters may be exposed to isoeugenol contained in smoke after using extinguishing agents (Dills et al., 2008). Urinary concentrations of isoeugenol were significantly increased after smoke exposure among wildland firefighters (Neitzel et al., 2009).

[The Working Group noted the lack of comprehensive exposure data in an occupational context. The Working Group also noted that, despite lacking exposure data, occupational exposure to isoeugenol by dermal or inhalation routes may additionally occur through the production and use of products containing isoeugenol as a fragrance (e.g. in professional cleaning settings), the production and use of isoeugenol as a flavouring agent in the food industry, and the production and use of isoeugenol-containing veterinary anaesthetics.]

1.4.3 Exposure of the general population

Exposure to isoeugenol is mainly associated with its presence as a fragrance in household products and cosmetics (Buckley et al., 2000; Temesvári et al., 2002; Tanaka et al., 2004; Bruze et al., 2005; White et al., 2007; Ezendam et al., 2011). Avoiding this exposure could be difficult, considering that fragrances are ubiquitous in consumer products (Sánchez-Pujol et al., 2021). Additionally, exposure to isoeugenol is also possible through the consumption of foods that contain plants and plant extracts, such as savoury basil, clove, mace, dill seed, and nutmeg (Smith et al., 2009; Scheman et al., 2014), or foods that contain isoeugenol as a flavouring agent, such as non-alcoholic beverages, candies, and chewing gums (Smith et al., 2009). Isoeugenol has also been demonstrated to be a pyrolysis product of lignin (a structural component of plants) and to be present in the smoke particulate fraction of seven of the eight US commercial cigarette brands analysed (Stanfill and Ashley, 2000).

Smith et al. (2009) estimated the daily per capita intake of isoeugenol as a flavouring agent to be 0.02 µg/kg bw per day in the USA. Burdock (2010) also reported an estimated individual exposure to isoeugenol in flavourings of 0.78 µg/kg bw per day. Exposure of the human population was also estimated as part of a fragrance ingredient safety assessment by the Research Institute for Fragrance Materials. Exposure by inhalation was estimated to account for 0.1 µg/kg bw per day and total systemic exposure (dermal, oral, and inhalation) was 0.4 µg/kg bw per day (Api et al., 2016). JECFA has estimated exposure of the general population; the estimated daily per capita intake was 120 µg in Europe and 40 µg in the USA (WHO, 2004b). The European Food Safety Authority (EFSA) has estimated a daily per capita intake of 0.012 µg/day, based on the maximized survey-derived daily intake (MSDI) approach, not considering possible natural occurrences in food (EFSA, 2011).

No data on biomonitoring levels of isoeugenol in the general population were available to the Working Group. Two biomonitoring studies measured increased levels of isoeugenol in the urine of volunteers after exposure to wood smoke or smoke-flavoured food ingestion in an experimental setting in Washington state, USA (Dills et al., 2001, 2006). Pre-exposure *trans*-isoeugenol urine levels of the participants with no known smoke exposure in the 48 hours before sampling were 0.14 ± 0.20 µg/mL (mean ± standard deviation) in 21 participants (Dills et al., 2001) and 0.014 ± 0.010 µg/mL in 9 participants (Dills et al., 2006). Urinary *cis*-isoeugenol levels were 0.002 ± 0.003 µg/mL in 9 participants (Dills et al., 2006) with no prior smoke exposure. [The Working Group noted that these small studies with volunteers are probably not representative of exposure levels of other population groups. They however give some indication that commonly consumed smoke-flavoured foods and exposure to wood smoke or barbecues contribute to the general population exposure.]

1.5 Regulations and guidelines

In the European Union, isoeugenol is authorized to be used as a flavouring substance in food. This is in accordance with Commission Implementing Regulation (EU) No. 872/2012 of 1 October 2012, adopting the list of flavouring substances provided for by Regulation (EC) No. 2232/96 and introducing isoeugenol in Annex I to Regulation (EC) No. 1334/2008 (European Commission, 2012).

In the USA, isoeugenol was classified as "generally recognized as safe" (GRAS) as a food ingredient by the US Food and Drug Administration (US FDA) under 21 CFR §172.515 (US FDA, 2004) and was also permitted for direct addition in food for human consumption as a synthetic flavouring substance (US FDA, 2010).

Regarding the use of isoeugenol in cosmetics, Annex III of Regulation 1223/2009/EC on Cosmetic Products as amended by Regulation (EU) 2022/1531, established the conditions for the use of isoeugenol (European Commission, 2009, 2022). For use in oral products and cosmetic products other than oral products, the presence of this substance must be indicated in the list of ingredients when its concentration exceeds 0.001% in leave-on products and 0.01% in rinse-off products (European Commission, 2009, 2022). For cosmetic products other than oral products, a maximum threshold of 0.02% was established (European Commission, 2009, 2022). Isoeugenol use was banned from any toys except olfactory board games, cosmetic kits, and gustative games (European Parliament and Council, 2009).

Commission Implementing Regulation (EU) No. 2017/60 authorizes the use of isoeugenol as a feed additive in animal nutrition for pigs, ruminants, and horses, except those producing milk for human consumption, and pets. The substance was specified in the Annex, belonging to the additive category "sensory additives" and to the functional group "flavouring compounds" (European Commission, 2017a).

According to the Annex of Regulation (EU) No. 363/2011, the maximum residue limit of isoeugenol in fin fish species via its use in veterinary medicine is 6000 µg/kg (European Commission, 2011). Similarly, the Republic of Korea has also implemented a revised maximum residue level (MRL) for isoeugenol for fish (including eels) of 0.01 mg/kg, a limit that was effective from 1 July 2018. In Australia and Japan, the MRL for isoeugenol in fish is 100 mg/kg (Australian Government, 2018; Japan Chemical Research Foundation, 2023). In the USA, isoeugenol has not been approved for use in veterinary medicine; consequently, seafood products containing isoeugenol may not be imported into the USA (US FDA, 2023).

Under the CLP regulation (Classification, labelling and packaging of substances and mixtures, Regulation (EC) No. 1272/2008), isoeugenol is classified as a skin sensitizer 1A (ECHA, 2015)

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

1.6 Quality of exposure assessment in key mechanistic studies in humans

See Supplementary Table S1.4 (Annex 1, Supplementary material for Section 1, Exposure Characterization, online only, available from: https://publications.iarc.who.int/627).

One study (Sieben et al., 2001) examined T-cell responses using peripheral blood mononuclear cells and T cells from skin lesions of fragrance-allergic patients after exposure to isoeugenol. The study employed patch testing to confirm fragrance allergy and a second patch test to establish a positive reaction to isoeugenol. The second patch test was performed 3–4 weeks after the first positive skin test. Biopsies were taken from skin lesions 48 hours after topical application of a single fragrance from patients with a second positive patch test reaction. Blood for in vitro investigations was drawn 1–2 weeks after the positive patch test, which is sufficient time to detect immune responses to the exposure. [The certainty of exposure in this study is probably high on the basis of the information provided, since exposure to isoeugenol appears to be controlled with standardized exposure levels.] Final concentrations of isoeugenol ranged from 0.1 to 100 µg/mL, allowing for a range of exposures to be tested, covering both low and high concentrations.

2. Cancer in Humans

No data were available to the Working Group.

3. Cancer in Experimental Animals

See Table 3.1.

3.1 Mouse

In a study of chronic toxicity and carcinogenicity that complied with GLP, groups of 50 male and 50 female B6C3F$_1$ mice (age, 5–7 weeks) were exposed by gavage to isoeugenol (purity, ≥ 99%) in corn oil at doses of 0 (control), 75, 150, or 300 mg/kg body weight (bw) per day, 5 days per week, for 104 (females) or 105 (males) weeks (NTP, 2010). The survival rate of males in the group at the highest dose was significantly decreased (P = 0.019, life-table trend test) compared with the control group. The survival rates of all other exposed groups were similar to those of the vehicle controls. The mean body weights of males and females at the highest dose were less (by 10% and 14%, respectively) than those of the control groups at the end of the study.

In male mice, exposure to isoeugenol significantly increased the incidence of hepatocellular adenoma (multiple) in all treated groups – 10/50 (20%), 26/50 (52%), 28/50 (56%), 20/50 (40%) for the groups at 0 (control), 75, 150, and 300 mg/kg bw per day, respectively ($P \leq 0.01$, poly-3 test, [P = 0.0008, Fisher exact test]; $P \leq 0.01$, poly-3 test, [P = 0.0002, Fisher exact test]; and $P \leq 0.05$, poly-3 test, [P = 0.0243, Fisher exact test], respectively). There was a significant positive trend (P = 0.012, poly-3 trend test) in the incidence of hepatocellular adenoma (includes multiple) – 24/50 (48%), 35/50 (70%), 37/50 (74%), 33/50 (66%) in the groups at 0 (control), 75, 150 and 300 mg/kg bw per day treated groups, respectively – and the incidence was significantly increased in all treated groups (P = 0.015, poly-3 test, [P = 0.0207, Fisher exact test]; P = 0.010, poly-3 test, [P = 0.0067, Fisher exact test]; and P = 0.009, poly-3 test, respectively), exceeding the upper bound of the range observed in historical controls from this laboratory: gavage – 50/100 (50% ± 2.8%); range, 48–52%; all routes: 544/1146 (47.5% ± 14.9%); range, 14–72%.

There was a significant positive trend (P = 0.027, poly-3 trend test; [P = 0.03, Cochran–Armitage trend test]) in the incidence of hepatocellular carcinoma (includes multiple) – 8/50 (16%), 18/50 (36%), 19/50 (38%), 18/50 (36%) for the groups at 0 (control), 75, 150, and 300 mg/kg bw per day, respectively – and the incidence was significantly increased in all treated groups (P = 0.022, poly-3 test, [P = 0.0195, Fisher exact test]; P = 0.017, poly-3 test, [P = 0.0116, Fisher exact test]; and P = 0.012, poly-3 test, [P = 0.0195, Fisher exact test], respectively), exceeding the upper bound of the range observed in historical controls from this laboratory: gavage – 22/100 (22.0% ± 8.5%); range, 16–28%; all routes – 317/1146 (27.7% ± 9.2%); range, 8–48%. There was a significant positive trend ($P < 0.001$, poly-3 trend test, [$P < 0.001$, Cochran–Armitage trend test]) in the incidence of hepatocellular adenoma or carcinoma (combined) – 28/50 (56%), 43/50 (86%), 43/50 (86%), 43/50 (86%) – for the groups at 0 (control), 75, 150, and 300 mg/kg bw per day, respectively – and the incidence was significantly increased in all treated groups ($P \leq 0.003$, poly-3 test, [P = 0.0009, Fisher exact test]), exceeding the upper bound of the range observed in historical controls from this laboratory: gavage – 61/100 (61.0% ± 7.1%); range, 56–66%; all routes – 729/1146 (63.6% ± 15.6%); range, 20–84%.

In female mice, there was a significant positive trend (P = 0.015, poly-3 trend test; [P = 0.008, Cochran–Armitage trend test]) in the incidence of histiocytic sarcoma in multiple tissues, and the incidence – 0/49, 1/50 (2%), 1/50 (2%), 4/50 (8%) – was within the range observed in historical controls from this laboratory: gavage – 0/99; all routes – 31/1249 (2.5% ± 2.5%); range, 0–8%. [The Working Group noted that histiocytic sarcoma is a rare non-Langerhans histiocytic neoplastic disorder with unifocal or multifocal extranodal tumours. It shows highly pleomorphic cells or

Table 3.1 Studies of carcinogenicity in experimental animals exposed to isoeugenol

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (M) 5–7 wk 105 wk NTP (2010)	Gavage Purity, ≥ 99% Corn oil 0, 75, 150, 300 mg/kg bw 5 days/wk for 105 wk 50, 50, 50, 50 39, 38, 36, 27	*Liver* Hepatocellular adenoma (multiple) 10/50 (20%), 26/50** (52%), 28/50** (56%), 20/50* (40%)	**$P \leq 0.01$, poly-3 test; [$P = 0.0008$, Fisher exact test] **$P \leq 0.01$, poly-3 test; [$P = 0.0002$, Fisher exact test] *$P \leq 0.05$, poly-3 test; [$P = 0.0243$, Fisher exact test]	*Principal strengths*: well-conducted GLP study; adequate number of animals; adequate duration; males and females used; multiple doses used. Historical controls: hepatocellular adenoma (includes multiple): gavage – 50/100 (50.0% ± 2.8%), range, 48–52%; all routes – 544/1146 (47.5% ± 14.9%), range, 14–72%; hepatocellular carcinoma (includes multiple): gavage – 22/100 (22.0% ± 8.5%), range, 16–28%; all routes – 317/1146 (27.7% ± 9.2%), range, 8–48%; hepatocellular adenoma or carcinoma (combined): gavage – 61/100 (61.0% ± 7.1%), range, 56–66%; all routes – 729/1146 (63.6% ± 15.6%), range, 20–84%.
		Hepatocellular adenoma (includes multiple) 24/50 (48%), 35/50* (70%), 37/50** (74%), 33/50*** (66%)	$P = 0.012$, poly-3 trend test; *$P = 0.015$, poly-3 test; [$P = 0.0207$, Fisher exact test] **$P = 0.010$, poly-3 test; [$P = 0.0067$, Fisher exact test] ***$P = 0.009$, poly-3 test; [$P = 0.0528$, Fisher exact test]	
		Hepatocellular carcinoma (includes multiple) 8/50 (16%), 18/50* (36%), 19/50** (38%), 18/50*** (36%)	$P = 0.027$, poly-3 trend test; [$P = 0.03$, Cochran–Armitage trend test] *$P = 0.022$, poly-3 test; [$P = 0.0195$, Fisher exact test] **$P = 0.017$, poly-3 test; [$P = 0.0116$, Fisher exact test] ***$P = 0.012$, poly-3 test; [$P = 0.0195$, Fisher exact test]	
		Hepatocellular adenoma or carcinoma (combined) 28/50 (56%), 43/50* (86%), 43/50* (86%), 43/50* (86%)	$P < 0.001$, poly-3 trend test; [$P = 0.001$, Cochran–Armitage trend test] *$P \leq 0.003$, poly-3 test; [$P = 0.0009$, Fisher exact test]	

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Mouse, B6C3F$_1$ (F) 5–7 wk 104 wk NTP (2010)	Gavage Purity, ≥ 99% Corn oil 0, 75, 150, 300 mg/kg bw 5 days/wk for 103 wk 49, 50, 50, 50 34, 39, 38, 33	*Liver* Hepatocellular adenoma (includes multiple) 11/49 (22%), 10/50 (20%), 9/49 (18%), 4/50 (8%) Hepatocellular carcinoma (includes multiple) 3/49 (6%), 8/50 (16%), 9/49 (18%), 6/50 (12%) Hepatocellular adenoma or carcinoma (combined) 13/49 (26%), 16/50 (32%), 15/49 (30%), 9/50 (18%) *Multiple sites* (including liver, ovary, uterus, spleen, lung, lymph nodes, kidney, thymus, and bone marrow) Histiocytic sarcoma 0/49, 1/50 (2%), 1/50 (2%), 4/50 (8%)	NS NS NS P = 0.015, poly-3 trend test; [P = 0.0088, Cochran–Armitage trend test]	*Principal strengths*: well-conducted GLP study; adequate number of animals; adequate duration; males and females used; multiple doses used. Historical controls: hepatocellular adenoma (includes multiple): gavage – 17/99 (17.2% ± 7.4%), range, 12–22%; all routes – 345/1245 (27.8% ± 17.0%), range, 2–62%; hepatocellular carcinoma (includes multiple): gavage – 4/99 (4.1% ± 2.9%), range, 2–6%; all routes – 131/1245 (10.5% ± 7.7%), range, 0–28%; hepatocellular adenoma or carcinoma (combined): gavage – 20/99 (20.3% ± 8.9%), range, 14–27%; all routes – 419/1245 (33.7% ± 19.1%), range, 8–64%; histiocytic sarcoma: gavage – 0/99; all routes – 31/1249 (2.5% ± 2.5%), range, 0–8%.
Full carcinogenicity Rat, F344/N (M) 5–7 wk 105 wk NTP (2010)	Gavage Purity, ≥ 99% Corn oil 0, 75, 150, 300 mg/kg bw 5 days/wk for 105 wk 50, 50, 50, 50 35, 34, 33, 30	*Mammary gland* Carcinoma 0/50, 0/50, 0/50, 2/50 (4%) *Thymus* Benign or malignant thymoma (combined) 0/50, 0/50, 0/50, 2/50 (4%)	P = 0.042, poly-3 trend test; [P = 0.015, Cochran–Armitage trend test] P = 0.047, poly-3 trend test; [P = 0.015, Cochran–Armitage trend test]	*Principal strengths*: well-conducted GLP study; adequate number of animals; adequate duration; males and females used; multiple doses used. Historical controls: mammary gland carcinoma: gavage – 0/100; all routes – 8/1199 (0.7% ± 1.3%), range, 0–4%; thymomas: gavage – 0/94; all routes – 3/1146 (0.3% ± 0.7%), range, 0–2%.

Table 3.1 (continued)

Study design Species, strain (sex) Age at start Duration Reference	Route Purity Vehicle Dose(s) No. of animals at start No. of surviving animals	Tumour incidence	Significance	Comments
Full carcinogenicity Rat, F344/N (F) 5–7 wk 105 wk NTP (2010)	Gavage Purity, ≥ 99% Corn oil 0, 75, 150, 300 mg/kg bw 5 days/wk for 105 wk 50, 50, 50, 50 33, 35, 34, 31	No significant increase in tumour incidence in treated animals		*Principal strengths*: well-conducted GLP study; adequate number of animals; adequate duration; males and females used; multiple doses used. *Other comments*: exposed groups similar to vehicle control groups.

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

spindle cell cytology, mimicking pleomorphic or spindle cell sarcoma.]

Regarding non-neoplastic lesions, in males, the incidence of clear cell foci of the liver was significantly increased in the groups at the lowest and intermediate doses. The incidence of respiratory metaplasia in olfactory epithelium in all exposed groups in males and females and of atrophy and hyaline droplet accumulation in all exposed groups, except in females at the lowest dose, was significantly greater than that observed in controls. The incidence of hyperplasia of the Bowman glands (olfactory glands) was also increased significantly in all exposed groups. The incidence of minimal to mild necrosis of renal papilla and of mild to moderate necrosis of renal tubules was increased significantly in females at the highest dose, but no renal tumours were observed. In male mice, forestomach tumours were not observed, but the incidence of forestomach squamous hyperplasia, inflammation, and ulceration increased with exposure and was significant in the groups at the highest dose. The incidence of glandular stomach ulcers was low but significantly increased in the groups of males and females at the highest dose. [The Working Group noted that this was a well-described study that complied with GLP, used multiple doses, both sexes (with respective control groups), an adequate duration of exposure and observation, and an adequate number of animals per group.]

3.2 Rat

In a study of chronic toxicity and carcinogenicity that complied with GLP, groups of 50 male and 50 female F344/N rats (age, 5–7 weeks) were exposed by gavage to isoeugenol (purity, ≥ 99%) in corn oil at doses of 0, 75, 150, or 300 mg/kg bw per day, 5 days per week, for 105 weeks. There was no difference in survival rates between exposed male and female rats and unexposed control rats. Mean body weights of male rats at the highest dose were 9% greater than those of the controls at the end of the study (NTP, 2010).

In male rats, there was a significant positive trend in the incidence of mammary gland carcinoma – 0/50, 0/50, 0/50, 2/50 (4%) for the groups at 0 (control), 75, 150, and 300 mg/kg bw per day, respectively) (P = 0.042, poly-3 trend test; [P = 0.015; Cochran–Armitage trend test]) – and benign or malignant thymoma (combined) – 0/47, 0/43, 0/49, 2/48 (4%) for the groups at 0 (control), 75, 150, and 300 mg/kg bw per day, respectively (P = 0.047, poly-3 trend test; [P = 0.015; Cochran–Armitage trend test]) – and the incidence of benign and malignant thymoma (combined) exceeded the upper bound of the range observed in historical controls from this laboratory: gavage – 0/94; all routes – 3/1146 (0.3% ± 0.7%), range 0–2%. [Thymomas are mediastinal tumours with a lobulated architecture comprised of cellular lobules intersected by fibrous bands, in which the neoplastic cells are the epithelial cells, and the thymocytes are reactive.]

Regarding non-neoplastic lesions, the rates of minimal atrophy and minimal-to-mild respiratory metaplasia of the olfactory epithelium were increased in males at the intermediate dose and in males and females at the highest dose. The incidence of minimal-to-mild olfactory epithelial degeneration in males at the highest dose was similarly increased. [The Working Group noted that lesions in the olfactory epithelium of the nose are unusual, considering that this was not an inhalation study. The Working Group also noted that this was a well-described and well-conducted study that complied with GLP, used multiple doses, both sexes (with respective control groups), an adequate duration of exposure and observation, and an adequate number of animals per group.]

3.3 Evidence synthesis

The carcinogenicity of isoeugenol has been assessed in one well-conducted GLP study in male and female B6C3F$_1$ mice treated by oral administration (gavage) (NTP, 2010) and in one well-conducted GLP study in male and female F344 rats treated by oral administration (gavage) (NTP, 2010).

In the study that complied with GLP in male and female B6C3F$_1$ mice treated by oral administration (gavage), there was a significant increase in the incidence of hepatocellular adenoma (multiple) in males in all treated groups. Also in males, there was a significant positive trend in the incidence of hepatocellular adenoma (includes multiple) with the incidence being significantly increased in all treated groups. There was a significant positive trend in the incidence of hepatocellular carcinoma (includes multiple), and the incidence was significantly increased in all treated groups. There was a significant positive trend in the incidence of hepatocellular adenoma or carcinoma (combined), and the incidence was significantly increased in all treated groups. In female mice, there was a significant positive trend in the incidences of histiocytic sarcoma in multiple tissues.

In the study that complied with GLP in male and female F344 rats treated by oral administration (gavage), there was a significant positive trend in the incidence of mammary gland carcinoma and benign or malignant thymoma (combined) in males.

4. Mechanistic Evidence

4.1 Absorption, distribution, metabolism, and excretion

This section describes the available evidence on the absorption, distribution, metabolism, and excretion of isoeugenol in humans and experimental animals.

4.1.1 Absorption, distribution, and excretion

(a) Exposed humans

The absorption and distribution of isoeugenol in exposed humans were investigated in a few studies of dermal administration.

As they did for methyleugenol (see the monograph on methyleugenol in the present volume), Sgorbini et al. (2010) quantified the amount of isoeugenol (mixture of *cis*- and *trans*-isomers) penetrating the skin after the application of a skin cream containing isoeugenol at 50 ppm, using a sorptive tape (absorbent polydimethylsiloxane tape) extraction technique in two volunteers. The amount of isoeugenol detected on the stratum corneum decreased by approximately 68.6% 1 hour after the exposure. The estimate was based on the boiling-point (266 °C) and the semi-volatile nature of isoeugenol; thus, a proportion of the loss from the skin surface was probably due to volatilization.

Isoeugenol was also tested for absorption and penetration in excised human epidermis in the presence of several cosmetic and ointment vehicles (Jimbo et al., 1983). Radiolabelled isoeugenol (10 mM) was dissolved in liquid vehicles (i.e. ethanol, ethanol/water, propylene glycol, and liquid paraffin), cosmetic vehicles (lotion, milky lotion, oil/water-type cream, water/oil-type cream, oil/water-type foundation, and oil-type foundation) or ointment vehicles (petrolatum and macrogol). Mixtures of ^{14}C-labelled isoeugenol and various vehicles were applied to portions

of human skin (postmortem excised epidermis from the lower abdominal area) under occlusive conditions for 24 hours. Absorption through the epidermis was measured using a liquid scintillation counter. The highest absorption (penetration) of 10.38 ± 0.63% was observed when isoeugenol was dissolved in a cosmetic vehicle (i.e. milky lotion). Notably, penetration was an order of magnitude lower for ethanol, ethanol/water, or propylene glycol vehicles, which showed penetration percentages of < 1%. [The Working Group noted that the identity of the test material was not provided.]

Liu and Hotchkiss (1997) investigated the percutaneous absorption of ^{14}C-labelled isoeugenol in human and rat (see Section 4.1(b)) skin, using a flow-through diffusion cell in vitro model. Approximately 8.4% and 7.5% of the radioactivity was detected in the human skin and receptor fluid, respectively, 72 hours after application of [^{14}C]isoeugenol at 15.5 mg/cm^2. [The Working Group noted that the study was of low informativeness since it did not report sufficient details, including the identity of the test material.]

Skin sensitization in exposed humans

Madsen et al. (2010) assessed the potency of ethosome formulations of isoeugenol (as a mixture of *cis*- and *trans*-isomers) to enhance skin sensitization in human volunteers. Forty-eight patients with a previous positive patch-test reaction to isoeugenol were selected to be tested for an enhanced reaction when isoeugenol was delivered in an ethosome formulation (a formulation containing phospholipid-based elastic nanovesicles with a high ethanol content) or in an ethanol/water vehicle. The study participants underwent first a patch test (n = 8) with isoeugenol at concentrations of 0, 2.80, or 6.54 mg/mL, followed by a repeated open application test (ROAT) (n = 6) with isoeugenol at 5.66 mg/mL. Both tests were conducted using methyldibromo glutaronitrile as the positive control. Delivery of isoeugenol in an ethosome formulation elicited an enhanced reaction compared with delivery in an ethanol/water vehicle. In contrast, in a follow-up in vitro study, the same group reported that the percutaneous absorption and deposition of isoeugenol in a human skin Franz cell model was decreased when isoeugenol was delivered in the ethosome formulation (Madsen et al.,2011). [The Working Group noted that the number of study participants and groupings were not clearly reported. Furthermore, on the basis of the in vitro results, the increased sensitization potency observed for the ethosome formulation of isoeugenol may not be due to penetration or absorption characteristics.]

(b) Experimental systems

(i) Oral and intravenous routes

Badger et al. (2002) studied the absorption, distribution, metabolism (see also Section 4.1.2(a)(ii)), and excretion of ^{14}C-labelled isoeugenol (as a mixture of *cis*- and *trans*-isomers) in male F344 rats weighing 175–250 g, after exposure by gavage (156 mg/kg bw in corn oil) or intravenous administration (15.6 mg/kg bw in emulphor/ethanol/saline). [The Working Group noted that the number of animals used in this study was not reported.] The disposition of radioactivity in the expired air, blood, urine, faeces, and selected tissues (i.e. heart, kidneys, liver, lungs, muscle, subcutaneous adipose tissue, and testicular adipose tissue) was measured at various intervals up to 72 hours after exposure. Blood and urine samples were analysed by HPLC for parent compound and metabolites.

After gavage exposure, low levels of radioactivity were present in the blood, and no parent ^{14}C-labelled isoeugenol was present at any of the sampling intervals (LOQ, 1.5 ng/mL). However, 40% of the administered radioactivity was detected in the urine within 6 hours and 85% after 24 hours. Low levels of radioactivity were detected in the urine between 24 and 72 hours

after exposure. Approximately 10% of the administered radioactivity was detected in the faeces, < 0.1% was recovered in the expired air, and < 0.2% was observed in tissue samples 72 hours after dosing. Similar patterns were observed after intravenous exposure. For example, within 24 hours, 85% and 10% of the administered radioactivity was observed in the urine and faeces, respectively; and < 0.1% and 0.2% of the administered radioactivity was detected in expired air and tissue samples, respectively. Analysis of the blood samples showed that parent ^{14}C-labelled isoeugenol disappeared rapidly with a half-life of approximately 12.1 minutes and clearance of 1.9 L/min/kg bw after intravenous administration.

The National Toxicology Program (NTP) (NTP, 2010; also summarized in Hong et al., 2013) further explored the dose-, sex-, and species-dependent effects of isoeugenol (as mixture of *cis*- and *trans*-isomers) on various toxicokinetic parameters in male and female rats and mice after gavage and intravenous exposures. Isoeugenol was administered to male and female F344 rats (groups of 21 males and 21 females per dose; age, ~13 weeks) as a single intravenous dose of 17 mg/kg bw or a single gavage dose of 17, 70, or 140 mg/kg bw. In male and female B6C3F$_1$ mice (age, ~13 weeks), isoeugenol was administered as a single intravenous dose of 35 mg/kg bw (41 males and 41 females) or a single gavage dose of 35, 70, or 140 mg/kg bw (groups of 42 males and 42 females per dose). Plasma isoeugenol concentrations were determined at various time intervals in rats and mice up to 6 hours after dosing by intravenous injection and up to 10 hours after dosing by gavage.

After intravenous administration, the following observations were made: (i) female mice showed higher values for the area under the curve $(AUC)_\infty$ and lower values for clearance, compared with male mice; (ii) AUC_∞ and clearance values in male and female rats were not significantly different; and (iii) the apparent volume of distribution at steady state appeared to be higher in mice than in rats.

The apparent volume of distribution at steady state was high in both mice and rats, suggesting distribution to extravascular tissues in both species.

After gavage dosing, isoeugenol was rapidly absorbed in rats and mice; measurable amounts in the plasma were detected within 2 minutes and T_{max} values ranged from 10 to 20 minutes. The reported mean C_{max} values in female rats (i.e. 0.364 ± 0.103, 1.82 ± 0.88, and 5.91 ± 2.28, respectively), were almost twice as high as those reported in male rats (i.e. 0.192 ± 0.022, 1.02 ± 0.41, and 2.06 ± 0.73, respectively) after gavage exposure to isoeugenol as a single dose of 17, 70 or 140 bw. Higher C_{max} values were also reported in female mice (1.94 ± 0.17, 2.54 ± 0.17, and 3.99 ± 2.10, respectively) than in male mice (1.13 ± 0.18, 1.27 ± 0.13, and 1.91 ± 0.14, respectively). However, the absolute bioavailability was greater in mice (~30% for males and females) than in rats (~10% for males; 19% for females). [The Working Group noted that the low absolute bioavailability estimates reported by NTP (2010) and Hong et al. (2013) for mice and rats probably reflected extensive first-pass metabolism.] As the dose increased, clearance decreased in male and female rats, suggesting saturation of isoeugenol metabolism. In contrast, clearance values appeared to increase in male mice as the dose increased. In female mice, clearance appeared to be unaffected by increasing dose. Based on the AUC_T values observed after exposure by gavage, systemic exposure in females appeared to be greater than in males. Notably, secondary peak plasma concentrations of isoeugenol were observed in both species after exposure by gavage. Because of the presence of these "large" secondary peaks, some toxicokinetic parameters were not reported, while others were estimated because they could not be directly calculated. [The Working Group noted that it was speculated that the source of the secondary peaks in the

plasma concentration-versus-time curve, after gavage exposure, was the vehicle used (Hong et al., 2013) or enterohepatic recirculation (NTP, 2010). According to Hong et al. (2013), corn oil may markedly delay, but not diminish, the overall extent of absorption from the gut compared with aqueous vehicles.]

More recently, Zhou et al. (2022) detected isoeugenol in the blood of rats 5 hours after oral exposure to a dried ethanol extract of tsantan sumtang (a traditional Tibetan medicine formula, consisting of *Choerospondias axillaris* (Roxb.) Burtt et Hill, *Santalum album* L., and *Myristica fragrans* Houtt). Sprague-Dawley rats (body weight, 200–250 g) were exposed orally to the tsantan sumtang extract at 2000 mg/kg bw and blood samples were taken 30, 60, 120, 180, 240, and 300 minutes after dosing. The low-molecular-weight compounds of blood serum were analysed using ultra HPLC-MS/MS methods. [The Working Group noted that although the quantification of isoeugenol in the tsantan sumtang preparation and in the blood serum of treated rats was not reported by Zhou et al. (2022), isoeugenol and 10 other tsantan sumtang-related compounds were detected in the serum of exposed rats up to 5 hours after exposure. It was also noted that the isomerism of the test material was not provided in the publication.]

(ii) Dermal route

Liu and Hotchkiss (1998, 1997) described the disposition of isoeugenol in rat skin. In three male F344 rats, Liu and Hotchkiss (1998) applied ^{14}C-labelled isoeugenol at 2.6 mg/cm^2 to the dorsal skin under occluded conditions for 24 hours. Urine samples were collected up until the rats were killed, 24 hours after the dermal exposure. Excised skin and urine were then analysed for the presence of radioactivity. After 24 hours of topical occluded exposure, low levels of radioactivity were detected in the skin (0.8 ± 0.2% of the applied dose); 25.0 ± 1.0% of the applied radioactivity was recovered in the urine as metabolites; and no parent ^{14}C-labelled isoeugenol was recovered in the urine. Liu and Hotchkiss (1997) also described the results of an in vitro study investigating the percutaneous absorption of ^{14}C-labelled isoeugenol in rat skin and in human skin (see Section 4.1(a)). Using a flow-through diffusion cell model, 46.3 ± 4.8% and 15.7 ± 3.5% of the radioactivity was detected in the skin and receptor fluid, respectively, 72 hours after the application of ^{14}C-labelled isoeugenol at 15.5 mg/cm^2 to the skin (Liu and Hotchkiss (1997).] [The Working Group noted that the study was of low informativeness since it did not report sufficient details, including the identity of the test material.]

4.1.2 Metabolism

(a) Humans

(i) Exposed humans

No studies relevant to the metabolism of isoeugenol in exposed humans were available to the Working Group.

(ii) Human cells in vitro

de Sousa et al. (2016) reported that when isoeugenol was administered at concentrations of 2.5, 5.0, and 10.0 μM, > 80% was metabolized by cryopreserved human hepatocyte primary cultures within 60 minutes of incubation. Quantification of "overall metabolism" was determined by quantifying unchanged isoeugenol in the cells using spectroscopy (e.g. absorbance at 270 nm) at various time intervals. [The Working Group noted that the identity of the test material was not provided in the publication.]

(b) Experimental systems

HPLC analysis of pooled urine samples from male rats exposed to ^{14}C-labelled isoeugenol at 156 mg/kg bw showed five discernible peaks (Badger et al., 2002; the study methods are briefly summarized in Section 4.1.1(b)). Each of these peaks was characterized using MS and MS/MS

analysis after treatment with β-glucuronidase and sulfatase. Three of the five peaks were identified as glucuronide (20%) and sulfate (53%) conjugates. The other two peaks were resistant to sulfatase and β-glucuronidase treatment and were not consistent with the peak for parent ^{14}C-labelled isoeugenol. On the basis of the mass spectral data, Badger et al. (2002) proposed a metabolic scheme for orally administered isoeugenol, which is partially accounted for in Fig. 4.1.

The NTP (2010) investigated the effects of repeated oral exposure to isoeugenol (as a mixture of *cis*- and *trans*-isomers) on the activities of 7-ethoxyresorufin-*O*-deethylase (cytochrome P450 family 1 subfamily A member 1, CYP1A1), acetanilide-4-hydroxylase (CYP1A2), and 7-pentoxyresorufin-*O*-deethylase (CYP2B) in rats. Male and female F344 rats (10 of each sex per dose) were exposed to isoeugenol in corn oil at 0, 37.5, 75, 150, 300, or 600 mg/kg bw per day for 31 days (5 days/week). After 31 days of exposure, the rats were killed, and homogenates of the liver were prepared for analysis of enzyme activities. No significant effect on the activity of these enzymes was observed in female rats. However, compared with the controls, treated male rats showed a dose-related and statistically significant decrease in CYP1A1 and CYP2B activities at doses of ≥ 75 mg/kg bw per day. Male rats at 600 mg/kg bw per day also showed a statistically significant decrease in CYP1A2 activity compared with controls. Zhao and O'Brien (1996) also showed that isoeugenol inhibited CYP1A1 activity in 3-methylcholanthrene-induced mouse hepatic microsomes. [The Working Group noted that the magnitude of the CYP inhibition observed was relatively small (e.g. ~70% to 80% of control levels at the highest dose) and may be of limited physiological relevance.]

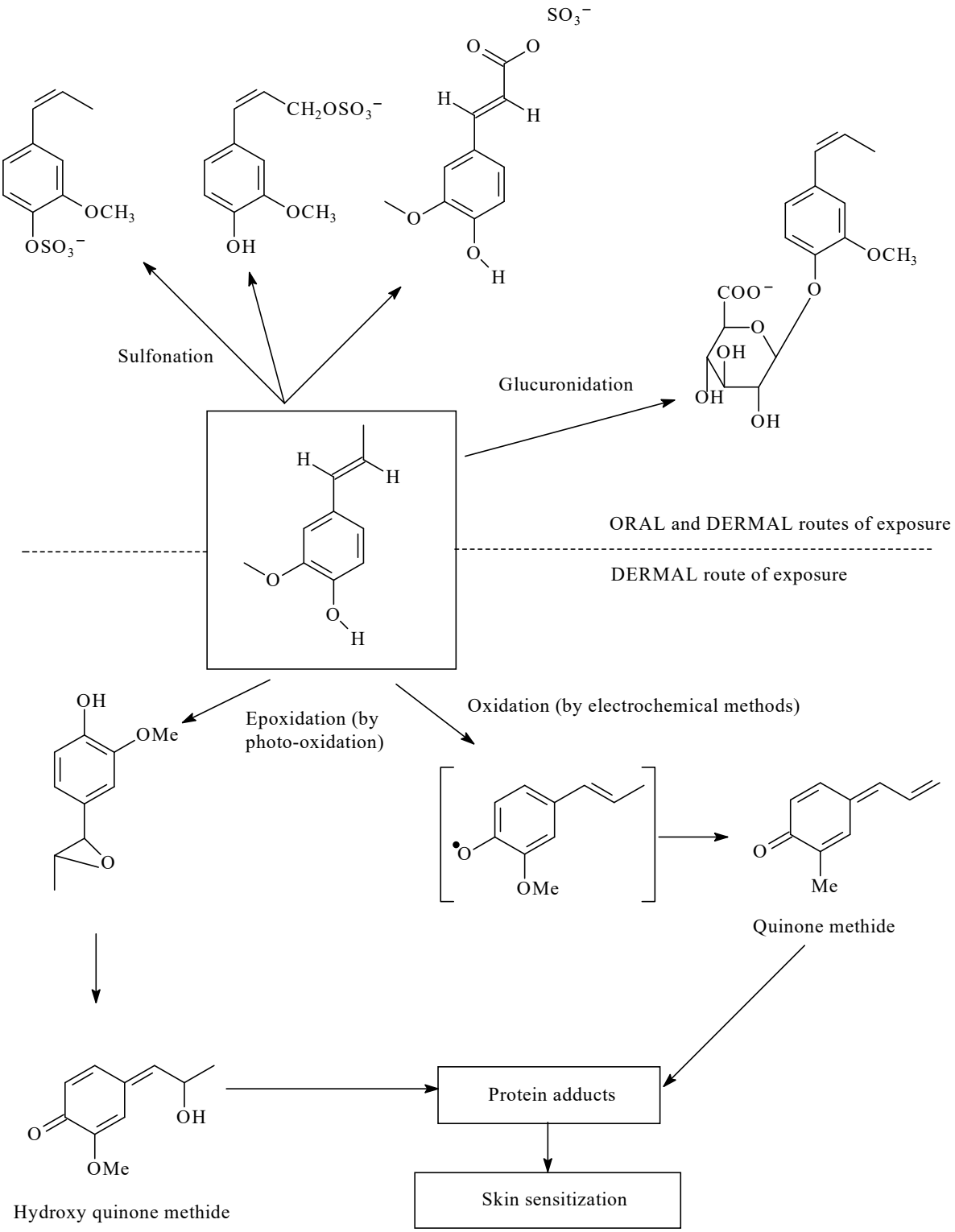
Liu and Hotchkiss (1998; see summary in Section 4.1.1(b) above for study details) used HPLC and GC-MS methods to analyse the urine of male F344 rats after occlusive dermal exposure to ^{14}C-labelled isoeugenol. All urinary metabolites were identified as sulfate or glucuronide conjugates; the sulfate ester of 4-hydroxy-3-methoxy-cinnamic acid (Fig. 4.1) was the predominant urinary metabolite. Of the limited amounts of radioactivity detected in the skin (e.g. 0.8 ± 0.2% of the applied dose), 33.2 ± 4.7% was attributable to the parent ^{14}C-labelled isoeugenol and 58.4 ± 1.9% to metabolites (none specifically identified).

Scholes et al. (1994) examined the effects of CYP modulation on the skin sensitization potency of isoeugenol in a series of studies using the mouse local lymph node assays (LLNA). In the LLNA studies, isoeugenol was co-administered with five CYP1A modulators (i.e. benzo[*a*]pyrene, β-naphthoflavone, and 3-methylcholanthrene were used as inducers, and α-naphthoflavone and clotrimazole were used as inhibitors). However, only the results of co-exposure with clotrimazole could be interpreted, since the other modulators were found to be potent skin sensitizers when administered independently. When co-administered with clotrimazole (an inhibitor of CYP1A activity), the sensitization potency of isoeugenol was increased substantially (~5-fold), suggesting that CYP1A metabolism of isoeugenol decreases its reactivity. [The Working Group noted that the identity of the test material was not provided in the publication.]

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 isoeugenol. Evidence, mostly from studies in experimental systems, was available on whether isoeugenol exhibits the key characteristics "is electrophilic or can be metabolically activated to an electrophile", "is genotoxic", "induces oxidative stress", "induces chronic inflammation", "modulates

Fig. 4.1 Proposed metabolic pathway for isoeugenol after oral and dermal exposure



Adapted from Badger et al. (2002) and Ahn et al. (2023).

receptor-mediated effects", and "alters cell proliferation, cell death, or nutrient supply". The evidence for "alters DNA repair and/or causes genomic instability" was sparse. No data were available for the evaluation of other key characteristics of carcinogens. The exposure assessment for the mechanistic study by Sieben et al. (2001) is reported in Section 1.6 and in Supplementary Table S1.4 (Annex 1, Supplementary material for Section 1, Exposure Characterization, online only, available from: https://publications.iarc.who.int/627).

4.2.1 Is electrophilic or can be metabolically activated to an electrophile

No studies relevant to electrophilicity in humans exposed to isoeugenol were available to the Working Group.

(a) Experimental systems

(i) Formation of DNA adducts

Ishii et al. (2023) investigated the formation of DNA adducts in the liver in male and female B6C3F$_1$ *gpt* delta mice treated with isoeugenol by gavage at doses of 0 (corn oil vehicle only), 150, 300, or 600 mg/kg bw per day for 13 weeks. Another group received estragole as a positive control. Using an adductome approach, the authors analysed DNA by LC-MS (Table 4.1). No specific adducts were detected in isoeugenol-treated mice, and the adductome maps were indistinguishable from those of control mice, whereas the estragole-treated mice showed distinct adducts that were attributable to treatment with this genotoxic carcinogen.

In a turkey egg genotoxicity assay, isoeugenol did not form DNA adducts (as measured by ^{32}P-postlabelling analysis) in the liver of fetuses harvested from White turkey eggs (containing fetuses aged 22–24 days) injected with isoeugenol at doses of 1 or 4 mg/egg per day for 3 days (Kobets et al., 2016) (see also Table 4.2 in Section 4.2.2(b)). [The Working Group noted that, in the same study, methyleugenol gave rise to DNA adducts (see the monograph on methyleugenol in the present volume).]

(ii) Metabolic activation in the skin

Melles et al. (2013) used electrochemical methods, LC, and MS to detect reactive species that may be responsible for the skin sensitization properties (i.e. haptenation) of isoeugenol. The authors used an amperometric thin-layer cell with boron-doped diamond working electrodes and palladium/hydrogen (Pd/H$_2$) as a reference electrode to generate oxidation products from isoeugenol. The oxidation products were then mixed with and without glutathione (GSH) before LC-MS detection. Similarly, to detect protein adducts, β-lactoglobulin A was mixed with the oxidation products of isoeugenol before LC-MS detection. The most abundant oxidation products detected were probably formed via hydroxylation at the aromatic moiety or at the double bond in the side chain. Other products that probably involved demethylation were also detected. After the introduction of GSH, multiple adducts were formed between GSH and isoeugenol oxidation products, including an O-demethylation product. Similarly, three different protein adducts were observed after the introduction of β-lactoglobulin A. [The Working Group noted that these reactive intermediates may also react with other biological matrices, including DNA.] On the basis of the mass spectral data, Melles et al. (2013) proposed an oxidation pathway for dermally administered isoeugenol that included generation of reactive intermediates such as quinone or quinone methide.

Ahn et al. (2020) proposed that isoeugenol is susceptible to abiotic activation (e.g. via photoinduced oxidative conditions) through rapid oxidation to form dimeric 7,4′-oxyneolignan, which is capable of adduct formation with proteins. Subsequently, using kinetic nuclear magnetic resonance (NMR) experiments and in vitro methods (i.e. direct peptide reactivity assay,

Table 4.1 End-points relevant to genotoxicity and related effects in non-human mammals in vivo exposed to isoeugenol

End-point	Species, strain (sex)	Tissue, cell	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Comments	Reference
DNA adducts, LC-MS/MS	Mouse, B6C3F$_1$ *gpt* delta (M, F),	Liver	–	600 mg/kg bw	Gavage for 13 wk: dose range: 150–600 mg/kg bw per day		Ishii et al. (2023)
gpt mutation frequency	Mouse, B6C3F$_1$ *gpt* delta (M, F)	Liver	–	600 mg/kg bw			
Micronucleus formation	Mouse, B6C3F$_1$ (M)	Peripheral blood; normochromatic erythrocytes	–	600 mg/kg bw	Gavage, for 3 mo; dose range, 37.5–600 mg/kg bw per day		NTP (2010)
	Mouse, B6C3F$_1$ (F)	Peripheral blood; normochromatic erythrocytes	+	600 mg/kg bw		Increase of 3.2-fold, with a significant trend, at the highest dose only	

F, female; HID, highest ineffective dose; LED, lowest effective dose; LC-MS/MS, liquid chromatography-tandem mass spectrometry; M, male; mo, month(s); wk, week(s).
[a] +, positive; –, negative.

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

End-point	Species, tissue, cell line	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Unscheduled DNA synthesis	Rat, F344, primary hepatocytes	–	NT	Up to nearly 1 mM	Exact concentrations not stated (displayed graphically with a log scale)	Burkey et al. (2000)
	Mouse, B6C3F$_1$, primary hepatocytes	–	NT			
Chromosomal aberration	Chinese hamster, ovary cells	–	–	200 µg/mL –S9 170 µg/mL +S9		NTP (2010)

HIC, highest ineffective concentration; LEC, lowest effective concentration, NT, not tested; S9, 9000 × *g* supernatant.
[a] –, negative.

KeratinoSens, and a human cell line activation assay), Ahn et al. (2023) further characterized the haptenation pathway of isoeugenol, proposing that a short-lived isoeugenol epoxide might be formed abiotically or enzymatically, followed by isomerization to a hydroxy quinone methide.

(iii) QSAR models

Dimitrov et al. (2005) developed a quantitative structure–activity relationship (QSAR) that incorporates a metabolic simulator designed to mimic enzyme activation (phase I and II reactions) in the skin. On the basis of QSAR predictions, Dimitrov et al. (2005) showed that that isoeugenol undergoes demethylation, followed by oxidation to *o*-quinone, or formation of semiquinone free radicals, or sulfate or glucuronide conjugation. [The Working Group noted that the reactive metabolites of isoeugenol may form quinone methide–protein adducts, which are ultimately responsible for dermal sensitization (Bertrand et al., 1997) and that DNA could similarly be susceptible to electrophilic attack.]

The action of isoeugenol as a skin sensitizer involves covalent modification of skin proteins in the form of isoeugenol–protein adducts, and this reaction in KeratinoSens reporter cells is not dependent on metabolic activation by microsomes from S9 (9000 × *g* supernatant) but is probably the result of spontaneous oxidation (Natsch and Haupt, 2013). Melles et al. (2013) showed that eugenol and isoeugenol form quinones and quinone methides electrochemically and that they are electrophilic and react in an acellular system with thiol groups in proteins. Isoeugenol formed a larger variety of adducts with GSH and proteins than did eugenol. In the acellular study, Ahn et al. (2020) demonstrated that an electrophilic species, a dimeric 7,4′-oxyneolignan, resulted from photo-oxidation of isoeugenol and that it bound to thiol groups. [The Working Group noted that although these studies demonstrated that electrophiles are derived from isoeugenol, these are mainly linked to the skin-sensitizing properties of isoeugenol, resulting in covalent reaction with proteins and subsequent protein modification. Electrophiles were generated either photochemically or electrochemically in acellular systems. In one study in turkey embryos and another in mouse liver, isoeugenol–DNA adducts were not detected.]

4.2.2 Is genotoxic

(a) Humans

(i) Exposed humans

No studies relevant to genotoxicity in humans exposed to isoeugenol were available to the Working Group.

(ii) Human cells in vitro

One study in humans cells in vitro was available to the Working Group. Isoeugenol was identified as a component of the semi-volatile particulate fraction of cigarette smoke and tested for the induction of sister-chromatid exchange (SCE) in human lymphocytes (Jansson et al., 1986). Isoeugenol was found to induce SCE ($P < 0.05$) at concentrations of 0.25 and 0.5 mM. Similar findings were reported in a follow-up report investigating a larger number of cigarette-smoke condensate components (Jansson et al., 1988). [The Working Group noted that the purity of the material tested in these studies ranged between 93.4% and 95%.] [The Working Group also noted that the genotoxicity research community now considers results from the SCE assay to be of less relevance than other currently available genotoxicity tests.]

(b) Experimental systems

See Table 4.1 and Table 4.2.

(i) Non-human mammals in vivo

The frequency of micronucleated erythrocytes did not increase in peripheral blood samples from male B6C3F$_1$ mice after exposure to isoeugenol by gavage at doses ranging from

37.5 to 600 mg/kg bw for 3 months. However, an increase of 3.2-fold in the frequency of micronucleated normochromatic erythrocytes and a significant trend was observed in female mice exposed to isoeugenol at 600 mg/kg bw (NTP, 2010). [The Working Group noted that the highest frequency of micronucleated normochromatic erythrocytes in female mice was similar to that in male mice at the lowest dose.]

Ishii et al. (2023) investigated mutation frequency in the liver of male and female B6C3F$_1$ *gpt* delta mice treated with isoeugenol by gavage at doses of 150, 300, or 600 mg/kg bw per day for 13 weeks. Corn oil was used as the vehicle control. Another group received estragole as a positive control. The *gpt* mutation frequency in isoeugenol-treated mice was not increased above the levels in control mice, whereas there was a significant increase in mutation frequency with the positive control, estragole.

(ii) Non-human mammalian cells in vitro

Isoeugenol at concentrations of up to 200 µg/mL in the cell medium did not induce chromosomal aberrations in cultured Chinese hamster ovary cells, with or without S9 activation (NTP, 2010).]

(iii) Non-mammalian experimental systems

See Table 4.3.

In a turkey egg genotoxicity assay, isoeugenol did not cause DNA strand breaks (measured by comet assay) in the liver of fetuses harvested from White turkey eggs (containing fetuses aged 22–24 days) injected with isoeugenol at doses of 1 or 4 mg/egg per day for 3 days (Kobets et al., 2016) (Kobets et al., 2016). [The Working Group noted that, in the same study, methyleugenol (see the monograph on methyleugenol in the present volume) induced DNA strand breakage.]

Isoeugenol gave negative results in the *Drosophila melanogaster* wing spot somatic mutation and recombination test (SMART) at concentrations of up to 25 mM (Munerato et al., 2005).

In an early study, isoeugenol did not induce an increase in mutation frequency when tested at a dose range of up to 600 µg/plate in *E. coli* WP2 *uvrA* and in *S. typhimurium* strains TA100, TA1535, TA98, TA1537, and TA1538 in the presence or absence of exogenous (S9) metabolic activation (Sekizawa and Shibamoto, 1982).

Similarly, isoeugenol gave negative results for mutagenicity when tested at higher dose range (3–2000 µg/plate) in two independent assays in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 *uvrA* in the presence or absence of exogenous (S9) metabolic activation (NTP, 2010). [The Working Group noted that the highest doses tested were limited by the cytoxicity of isoeugenol.]

When tested in the *Bacillus subtilis* DNA repair test (rec assay) in the absence of S9, isoeugenol gave positive results (concentration, 0.8 mg/disk), with preferential killing of rec$^-$ cells over rec$^+$ cells (Sekizawa and Shibamoto, 1982).

4.2.3 Alters DNA repair or causes genomic instability

(a) Humans

No studies relevant to alteration of DNA repair and/or genomic instability in humans or human cells exposed to isoeugenol were available to the Working Group.

(b) Experimental systems

Isoeugenol did not induce unscheduled DNA synthesis at concentrations of nearly 1 mM in rat or mice hepatocytes (Burkey et al., 2000). [The Working Group noted that the highest concentration was reported inexactly because the graph showing results had a log scale on the *x*-axis.]

Isoeugenol was reported to enhance the cytotoxicity of camptothecin (an inhibitor of topoisomerase 1, TOP1) and etoposide (an inhibitor of TOP2) through a mechanism involving

Table 4.3 End-points relevant to genotoxicity and related effects in non-mammalian experimental systems exposed to isoeugenol

Test system (species, strain)	End-point (assay)	Results[a]		Concentration (LEC or HIC)	Comments	Reference
		Without metabolic activation	With metabolic activation			
Drosophila melanogaster	Somatic mutation and recombination test (SMART)	–	NA	25 mM		Munerato et al. (2005)
Salmonella typhimurium, TA98, TA100, TA1535, TA1537, TA1538	Mutation	–	–	600 µg/plate		Sekizawa and Shibamoto (1982)
Salmonella typhimurium, TA98, TA100, TA1535, TA1537	Mutation	–	–	333 µg/plate: TA1535, TA1537. 1500 µg/plate: TA98, TA100	Isoeugenol was toxic at higher doses	NTP (2010)
Escherichia coli, WP2 *uvrA*	Mutation	–	–	600 µg/plate	Isoeugenol was toxic at higher doses	Sekizawa and Shibamoto (1982)
Escherichia coli, WP2 *uvrA*/pKM101	Mutation	–	–	1000 µg/plate	Isoeugenol was toxic at higher doses	NTP (2010)
Bacillus subtilis, rec H17 and M45 strains	DNA damage, (Rec assay)	+	NR	0.8 mg/disk	Preferential killing of rec^- cells over rec^+	Sekizawa and Shibamoto (1982)
Turkey fetus	DNA adducts (^{32}P-postlabelling)	–	NR	4 mg/egg	Fertilized eggs treated on days 22–24	Kobets et al. (2016)
Turkey fetus	DNA strand breaks (comet assay)	–	NR	4 mg/egg	Fertilized eggs treated on days 22–24	Kobets et al. (2016)

HIC, highest ineffective concentration; LEC, lowest effective concentration, NR, not reported.

[a] +, positive; –, negative.

inhibition of tyrosyl DNA phosphodiesterase 2 (TDP2) catalytic activity. Isoeugenol itself displayed inhibitory activity towards TDP2 but not TDP1 (Elsayed et al., 2016). [The Working Group considered that this study was not particularly informative.]

4.2.4 Induces oxidative stress

See Table 4.4.

(a) Humans

(i) Exposed humans

No studies relevant to oxidative damage in humans exposed to isoeugenol were available to the Working Group.

(ii) Human cells in vitro

In a series of experiments in primary human gingival fibroblasts and in a cell line from a human submandibular adenocarcinoma, an increase in 5-(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDFH-DA) fluorescence, assumed to be caused by reaction with intracellular reactive oxygen species (ROS), was detected with isoeugenol only at concentrations of 500 and 1000 µM. Extensive cytotoxicity (measured as a reduction in metabolic activity) was observed at concentrations greater than 100 µM. No increase in ROS was observed at isoeugenol concentrations of 100 µM or lower. The fluorescence observed with isoeugenol at 1000 µM was comparable to that observed after a parallel exposure to 10 µM H_2O_2. Exposure to isoeugenol at 1000 µM also induced a significant depletion in intracellular GSH (Atsumi et al., 2000, 2005, 2006; Fujisawa et al., 2004). Although no increase in ROS was observed after exposure to isoeugenol at 10 µM, isoeugenol at 5 µM had a synergistic effect on the induction of ROS by visible light and H_2O_2 (Atsumi et al., 2005).

[The Working Group noted that although no cytotoxicity was observed when the cells were treated with visible light, H_2O_2, or 5 µM isoeugenol alone, the cytotoxicity of the combined treatments was not evaluated. Nonetheless, a synergistic ROS induction by isoeugenol and visible light, if properly documented, might be relevant to the skin sensitization findings discussed in Section 4.2.1.]

(b) Experimental systems

Non-human mammals in vivo

See Table 4.5.

Exposure of male Sprague-Dawley rats to isoeugenol at an intraperitoneal dose of 10 mg/kg bw per day for 14 days did not induce consistent membrane lipid peroxidation or cellular responses to oxidative damage as measured by enzyme activities of superoxide dismutase or catalase or glutathione peroxidase in brain, liver, kidney, or heart tissues isolated 5 days after the end of the exposure. Levels of lipid peroxidation and the oxidized form of GSH were decreased in the liver only (Rauscher et al., 2001).

(c) Antioxidant effects

Regarding the role of isoeugenol in oxidative stress, the Working Group identified several studies investigating the potential of isoeugenol to act as an antioxidant.

Rajakumar and Rao reported that the incubation of rat brain homogenates (ex vivo) with isoeugenol reduced the baseline level of membrane lipid peroxidation. The effect was significantly less potent than that reported for equimolar amounts of butylated hydroxytoluene (Rajakumar and Rao, 1993). Isoeugenol solutions were shown to have antioxidant activity comparable to that of well-characterized antioxidants such as α-tocopherol, butylated hydroxytoluene, and butylated hydroxyanisol in the few experiments in which they were used as positive controls (Tominaga et al., 2005; Kadoma et al., 2006; Fındık et al., 2011).

Exposure to lipopolysaccharide (LPS) induced nitric oxide release in the rodent

Table 4.4 End-points relevant to oxidative stress in human cells in vitro exposed to isoeugenol

End-points	Assays	Tissue, cell line	Results[a]	Concentration (LEC or HIC)	Comments	Reference
ROS	CDFH-DA	Human submandibular gland adenocarcinoma cell line (HSG)	No change	1000 µM	Not clear how the authors went from a concentration–response curve (1, 10, 100, 1000 µM) to a single number	Fujisawa et al. (2004)
ROS	ESR	Human submandibular gland adenocarcinoma cell line (HSG)	No change	100 000 µM	Measurement of phenoxyl radicals; dose range, 10 µM to 100 mM	Atsumi et al. (2000)
ROS	CDFH-DA	Human submandibular gland adenocarcinoma cell line (HSG)	↑	500 µM	Substantial cytotoxicity observed with isoeugenol at 100 and 1000 µM	Atsumi et al. (2005)
ROS			↑	5 µM isoeugenol + H_2O_2 (100 µM)/HRP (1 µg/mL)		
ROS			↑	5 µM isoeugenol + visible light		
GSH	Thiols by fluorimetry		↓	1000 µM		
ROS	CDFH-DA	Primary human gingival fibroblasts (HGF) and submandibular gland adenocarcinoma cell line (HSG)	No change	20 µM	Single dose at non-toxic concentration	Atsumi et al. (2006)

CDFH-DA, 5- (and 6-)-carboxy-2′,7′-dichlorofluorescein diacetate; ESR, electron spin resonance spectroscopy; H_2O_2, hydrogen peroxide; HIC, highest ineffective concentration; HRP, horseradish peroxidase; LEC, lowest effective concentration; ROS, reactive oxygen species.

[a] ↓, decrease; ↑, increase.

Table 4.5 End-points relevant to oxidative stress in non-human mammals in vivo exposed to isoeugenol

End-point	Assay	Species, strain (sex) Tissue, cells	Results[a]	Dose (LED or HID)	Route, duration, dosing regimen	Reference
Lipid peroxidation	TBARS assay	Rats, Sprague-Dawley (M) Liver, kidney, brain, and heart	↓ TBARS in heart and liver	10 mg/kg bw per day	Intraperitoneal, for 14 days	Rauscher et al. (2001)
CAT, SOD, GPX or GSR	Enzyme activity		No change in CAT, SOD, GPX and GSR in all tissues			
GSH/GSSG	Protein level		↓ GSSG in liver No change in GSH in all tissues			

bw, body weight; CAT, catalase; GPX, glutathione peroxidase; GSR, glutathione reductase; GSH, glutathione; GSSG, oxidized glutathione; HID, highest ineffective dose; LED, lowest effective dose (units as reported); M, male; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

[a] ↑, increase; ↓, decrease;.

macrophage-like cell line RAW 264.7 through increases in the transcription and expression of nitric oxide synthase (iNOS). In cells co-exposed to LPS and isoeugenol, the transcription and expression of iNOS was significantly reduced, although there were inconsistent results from mechanistic experiments examining the time-course of the responses and the role of transcription factors (e.g. nuclear factor kappa B, NF-κB) known to regulate the response (Choi et al., 2007; Yeh et al., 2011; Murakami et al., 2017) (see Section 4.2.5).

Co-exposure of rat brain tissue (ex vivo) to isoeugenol and acrylamide (Prasad and Muralidhara, 2013), or of rat kidney tissue to isoeugenol and cisplatin (Rao et al., 1999), reduced the lipid peroxidation associated with those agents. In addition, isoeugenol exposure was reported to inhibit lipid peroxidation induced by hydroxy radicals generated by a Fenton reaction system in rat liver microsomes (Taira et al., 1992); however, the mechanism by which isoeugenol interfered with those agents was not established.

Isoeugenol did not protect zebrafish larvae from H_2O_2 toxicity. Isoeugenol protected zebrafish from arsenate toxicity (Endo et al., 2020). [The Working Group noted that it was not clear whether this effect was due to antioxidant activity since arsenate has multiple toxicological modes of action.]

Several groups studied the antioxidant potential of isoeugenol in aqueous and non-aqueous solutions. Although most of the experiments in solution used nonspecific radical sources like 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) or 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (Fındık et al., 2011; Prasad and Muralidhara, 2013; Zuo et al., 2018), others had more specific antioxidant activities, such as trapping superoxide or hydroxyl radical or reduction of ferric compounds (Fındık et al., 2011; Zuo et al., 2018). A series of experiments examined the free radical-scavenging activity of isoeugenol by evaluating the kinetics of radical-initiated polymerization of methylmethacrylate in a non-aqueous system (Fujisawa et al., 2004; Fujisawa and Kadoma, 2006; Kadoma et al., 2006).

[The Working Group considered that the data available for review did not provide evidence that the antioxidant activity of isoeugenol ameliorated cellular or tissue oxidative damage.]

4.2.5 Induces chronic inflammation

(a) Humans

(i) Exposed humans

One study investigated the T-cell response to a mixture of eight fragrances, including isoeugenol, or to isoeugenol only using a skin patch test. Thirty-two fragrance-allergic patients were tested with all eight individual constituents of the mixture, including isoeugenol, using the patch test 3–4 weeks after an initial positive skin test. Positive allergic responses were observed after exposure to isoeugenol in 28.6% of patients (Sieben et al., 2001). After a second skin patch test, the peripheral blood from patients who had a positive allergic response to isoeugenol was taken within 1–2 weeks, and the peripheral blood mononuclear cells were isolated for other tests in vitro. [The Working Group considered this finding to be not highly informative because it examined allergic reactions other than T-cell responses in exposed humans. In addition, the isoeugenol concentration was not reported.]

(ii) Human primary cells

In the same study described above, Sieben et al. investigated the responses of peripheral blood mononuclear cells and T cells from skin lesions of fragrance-allergic patients to isoeugenol exposure. Consistent with findings from the patch test, Sieben et al. (2001) reported a significant increase in lymphocyte transformation, measured as incorporation of [^{3}H]thymidine in the lymphocyte transformation test (LTT), and

the LTT simulation index ranged from 3.2 to 6.4 after exposure to isoeugenol (concentration range, 0.1–100 μg/mL). The authors further investigated the ability of antigen-modified human liver microsomes to stimulate T lymphocytes in the presence of the metabolizing enzyme recombinant CYP1A1 (20 μg/mL) and reported that the T-cell response to isoeugenol was increased in the presence of this enzyme (Sieben et al., 2001) (see Section 4.2.6). [The Working Group considered this finding not very informative to the key characteristic of chronic inflammation since skin sensitization is a local inflammatory process.]

Banerjee et al. measured allergen-induced T-cell proliferation. Concentration-dependent increases in interleukins IL-1α and IL-1β were observed in mononuclear cells derived from human peripheral blood samples exposed to isoeugenol at 0.1–10 ppm (Banerjee et al., 2003). IL-8 release was also stimulated by exposure to isoeugenol (500 μM) in human primary neutrophils, but not in monocytic leukaemia THP-1 cells, in a study that aimed to compare the metabolic activity of the two cell types (Kiorpelidou et al., 2012).

(iii) Human cells in vitro

Levels of IL-8 mRNA levels in the skin were transiently elevated in in vitro models of reconstructed human epidermis exposed to isoeugenol at 3 mg/mL for 4 hours (Frankart et al., 2012) and in human monocyte-derived dendritic cells exposed to isoeugenol at 200 μM for 30 hours (Skazik et al., 2011). In human monocytic leukaemia THP-1 cells, exposure to isoeugenol at 75 μg/mL induced a significant increase in IL-8 mRNA expression (Mitjans et al., 2010); however, the increase in IL-8 secretion observed with isoeugenol at 100 μg/mL was not statistically significant (Mitjans et al., 2010; Galbiati et al., 2012) [The Working Group noted that these models are commonly employed to assess the sensitizing potential of contact allergens.]

Potential proliferative effects and the immune-mediated response were evaluated in normal human keratinocytes (NSK) from neonatal foreskin after treatment with various skin sensitizers and allergens, including isoeugenol. Levels of IL-8 increased after treatment with isoeugenol at concentrations of up to 2 μM (test range, 0.4, 2, 10, or 50 μM) for 24 hours (Bae et al., 2015).

A concentration-dependent increase in IL-18 mRNA was reported in a human keratinocyte cell line (NCTC254) exposed to isoeugenol at 37.5–150 μg/mL for 24 hours (Galbiati et al., 2011).

Furthermore, the p38MAPK pathway, a key regulator of pro-inflammatory cytokine biosynthesis, was shown to be activated in THP-1 cells 15 minutes after isoeugenol treatment at concentrations ranging from 50 to 100 μg/mL (Mitjans et al., 2010). CD1-mediated T-cell activation, indicated by increases in interferon gamma (IFNγ), a pro-inflammatory cytokine, was demonstrated in THP-1 cells treated with isoeugenol at 250 μM (Betts et al., 2017).

Other studies showed an increase in the expression of the cell surface markers cyclins CD86 and CD54 in co-cultures of THP-1 cells and keratinocytes exposed to isoeugenol, as detected by flow cytometry (Hennen et al., 2011; Cao et al., 2012). Consistent upregulation of the expression of cyclins CD86, CD54, and CD40 was also observed in THP-1 cells exposed to isoeugenol in the presence of Aroclor-induced rat liver microsomes (S9) (Chipinda et al., 2011). The expression of CD86 was also confirmed in another study in THP-1 cells, without effects on IL-8 cytokine release (Galbiati et al., 2012). A slight upregulation in the expression of CD83 and elevated expression of CCL27 and TLR2 (toll-like receptor 2) were observed in immature dendritic cells (iDC), and a concentration-dependent upregulation of CD86 was observed in dendritic cell-related cells (DCrc) (Cluzel-Tailhardat et al., 2007; Schreiner et al., 2008).

Some studies reported different or negative results. Lee et al. (2014) did not observe any significant induction of IL-6 or IL-8 release in reconstructed human full-thickness skin with incorporated in vitro-generated immature MUTZ-3-derived Langerhans-like cells (MUTZ-LCs) after exposure to isoeugenol (Lee et al., 2014). No effects were observed in monocyte-derived LC-like cells (Bock et al., 2018).

The reconstructed human full-thickness skin model was employed in another study in which the expression of cell surface markers CD54 and CD86 was evaluated for dendritic cell maturation. Isoeugenol induced a very mild non-significant increase in CD86 and CD54 (Lee et al., 2014).

Guironnet et al. (2000) reported that exposure to isoeugenol did not induce an allergen-specific T-cell response in monocyte-derived dendritic cells (Guironnet et al., 2000).

While assessing the accuracy of a novel in vitro sensitization test, the IL-8 Luc assay, Takahashi et al. (2011) reported inconsistent results when testing isoeugenol, which was ultimately classified as a non-sensitizer.

[The Working Group considered that it was difficult to assess the evidence for chronic inflammation on the basis of results from the above in vitro systems, given the short duration of such assays.]

(b) Experimental systems

(i) Non-human mammals in vivo

Isoeugenol (purity, > 99%) was administered by gavage at doses ranging from 75 to 300 mg/kg bw in a 3-month study and a 2-year study in F344/N rats and B6C3F$_1$ mice exposed under similar conditions (NTP, 2010) (see also Section 3.1). In rats, the incidence of minimal to mild olfactory epithelial degeneration was observed in both sexes at both times of exposure. In mice, a dose-dependent increase in atrophy of the olfactory epithelium was also observed. Additionally, the incidence of forestomach inflammation and ulceration (males only) was significantly increased at the highest dose in the 2-year study. [The Working Group noted that the atrophy observed in the olfactory epithelium could be the result of chronic olfactory inflammation (LaFever et al., 2022).]

In another study, oral exposure of adjuvant-induced arthritic male Wistar rats to isoeugenol at a dose of 10 or 25 mg/kg bw per day for 14 days induced a significant and dose-dependent reduction in the production of prostaglandin E$_2$ (PGE2) and nitric oxide, as well as histological evidence of joint inflammation and leukocyte infiltration, observed 10 days after the end of the exposure (Kaur and Sultana, 2012).

In a series of assays for skin irritation and skin sensitization (the mouse ear swelling test, MEST; in vivo and ex vivo LNNA), repeated dermal exposure to isoeugenol at doses ranging from 0.4% to 50% w/v in acetone:olive oil (4:1) solution applied to the dorsum of the ears of CBA or Balb/c mice induced a consistent significant increase in ear thickness (Garrigue et al., 1994; NTP, 2010, 2020; Bonefeld et al., 2011; Arancioglu et al., 2015).

Topical exposure to isoeugenol (at repeated doses ranging from 1% to 10% w/v) applied to the dorsum of both ears of female CBA/J mice for 3 days in the LLNA induced a proliferative response in the draining lymph node cells (Gerberick et al., 2002) and an increase in the percentage of cells expressing the B-cell marker B220 (Jung et al., 2012). [The Working Group noted that the B220 cell marker is considered to be a secondary end-point for cell proliferation in the LLNA.]

In the same study, Jung et al. (2012) also observed a significant increase in cytokine production (IL-2, IL-6, IFNγ, and TNFα) and an increase, although not significant, in the production of two other proteins involved in the inflammatory response – granulocyte-macrophage colony-stimulating factor (GM-CSF) and monocyte

chemoattractant protein-1 (MCP-1) (Jung et al., 2012). The increase in the production of IFNγ (as previously observed in isolated lymph node cells of mice exposed to various allergens, including isoeugenol at 10%, 25%, and 50% w/v), was considered to be a characteristic profile of the stimulation of a subpopulation of T-helper (Th1) cells (Dearman et al., 1997).

(ii) Non-human mammalian systems in vitro

The effects of exposure to isoeugenol were also investigated in various cell types in vitro. Isoeugenol induced a decrease in iNOS protein expression, as well as significant attenuation of cytokines IL-1b and TNFα in LPS-stimulated murine macrophages (Yeh et al., 2011). Isoeugenol was also shown to suppress, in a concentration-dependent manner, the concanavalin A (ConA)-initiated-lymphoproliferation of $B6C3F_1$ mouse splenocyte cultures and to reduce the production of IL-2 in mice splenocytes and ELA4.IL-2 mouse T cells activated with phorbol 12-myristate 13-acetate (PMA) or ionomycin. The authors also suggested that the effect was mediated by indirect downregulation of the transcription factors nuclear factor of activated T cells (NFAT) and NF-κB (Park et al., 2007). Similar results were obtained in a more recent study in which exposure to isoeugenol inhibited NF-κB-dependent transcriptional activity and DNA-binding activity, as well as signalling upstream of NF-κB activation (e.g. degradation of I-κBα, NF-κB inhibitor alpha) in LPS-stimulated RAW 264.7 cells (Choi et al., 2017). Increases in IL-1α and macrophage inflammatory protein-2 (MIP-2) were observed in murine epidermal keratinocyte cells (HEL-30) exposed to isoeugenol at a concentration range of 1 mL of 0.01, 0.1, 0.5, and 1 × CV_{75} concentration (CV_{75}, concentration that induces 75% of cell viability, which was 318.1 ± 161.2 μg/mL), although only the lowest dose of isoeugenol elicited a significant response in MIP-2 levels relative to the controls (Son et al., 2013).

Murakami et al. (2017) reported that exposure of RAW 264.7 murine macrophages to isoeugenol at concentrations of 0.1 or 1000 μM for 3.5 hours upregulated the expression of nitric oxide synthase 2 (Nos2) and cyclooxygenase 2 (Cox-2) mRNA at the higher concentration, but not the mRNA expression of haem oxygenase 1 (HO-1, encoding an oxidative stress-responsive protein with a key role in tissue resolution). In LPS-stimulated RAW 264.7 cells, isoeugenol at 40 μM (but not at 10 μM) was shown to inhibit Cox-2 mRNA expression (Murakami et al., 2017), although the same authors previously reported that isoeugenol did not inhibit Cox-2 expression or NF-κβ activation in LPS-stimulated macrophages (Murakami et al., 2005). [The Working Group noted that this study was of limited relevance because the measurement of mRNA expression alone is not sufficiently informative to determine whether isoeugenol elicited an inflammatory response in this cell system.]

4.2.6 Modulates receptor-mediated effects

(a) Humans

(i) Exposed humans

No studies relevant to the modulation of receptor-mediated effects in humans exposed to isoeugenol were available to the Working Group.

(ii) Human cells in vitro

Three studies in human cell lines investigated the aryl hydrocarbon receptor (AhR)-mediated effects of isoeugenol exposure and reported positive results (Kalmes et al., 2006; McKim et al., 2010; Kalmes and Blömeke, 2012). Using human immortalized keratinocytes (HaCaT) and a HaCaT-AhR-knock-down cell variant (siAhR HaCaT), Kalmes et al. (2006) demonstrated that isoeugenol (at 60–300 μM) may induce cell cycle arrest (a concentration-dependent increase in arrested cells) by transporting AhR into the nucleus. In a subsequent study, Kalmes and Blömeke (2012) provided corroborative evidence

that isoeugenol exposure (at 300 µM) caused the rapid transport of AhR into the nucleus, induced the expression of AhR-target genes *CYP1A1* and *AHRR* (aryl hydrocarbon receptor repressor) and inhibited the proliferation of HaCaT cells. Isoeugenol also reduced levels of the G1-phase cell cycle-related proteins retinoblastoma (RB, known to interact with AhR) and cyclin-dependent kinase 6 (CDK6) (Kalmes and Blömeke, 2012). In both studies by Kalmes and colleagues, these effects were seen only with isoeugenol at doses up to 300 µM because cytotoxicity (observed as reduced cell viability) was observed at the higher dose (600 µM) tested. Moreover, McKim et al. (2010) reported that isoeugenol (100 and 250 µM) increased CYP1A1 induction and gene expression in HaCaT keratinocytes in a concentration-dependent manner (McKim et al., 2010).

One study employed two regulatory in vitro tests for effects on the androgen receptor (AR): the androgen receptor AR-EcoScreen™ Androgen Receptor TransActivation Assay (AR TA) and the 22Rv1/MMTV_GRKO AR TA assay (described in Organisation for Economic Co-operation and Development, OECD Test Guideline No. 458).

In the AR-EcoScreen™ AR TA assay, the Dual-Glo luciferase assay system was used to detect AR agonist and antagonist activity, including cytoxicity. 5α-Dihydrotestosterone (DHT) at 10 nM and 500 pM was used as the positive standard for the assessment of agonist and antagonist activity, respectively. In the 22Rv1/MMTV_GR-KO AR TA assay, cells were incubated in hormone-deprived conditions, followed by incubation with isoeugenol in the absence (AR agonist screening) or presence (AR antagonist screening) of DHT. The Steady-Glo luciferase assay system was used to measure luciferase activity, using as a positive control 10 nM DHT in the AR agonist assay format, and 800 pM DHT + bicalutamide in the AR antagonist assay format. Isoeugenol exhibited AR antagonism in both assays, although this antagonist activity was decreased significantly in the presence of phase I and II enzymes from pooled rat liver S9 fraction (Park et al., 2021).

(b) Experimental systems

(i) Non-human mammals in vivo

Ishii et al. (2023) recently conducted a study of global gene expression in the liver of male and female B6C3F$_1$ *gpt* delta mice treated with isoeugenol at oral doses of 0, 150, 300, and 600 mg/kg bw per day for 13 weeks. Pathway analyses indicated that the peroxisome proliferator-activated receptor (PPAR) signalling pathway (both PPARα and PPARγ) was activated in the livers of male mice treated with isoeugenol. [The Working Group noted that only global expression data were presented in the paper, and the evidence was less clear in female mice.] This study also used quantitative polymerase chain reaction (qPCR) to confirm mRNA expression of some genes involved in the PPAR pathway, such as *Cd36, Cyp4a10, Cyp4a14*, and *Acadm*, in male mice at 150 mg/kg bw. In female mice, only *Cd36* mRNA expression was significantly increased. *Cth* gene expression significantly increased in male mice treated with isoeugenol at 600 mg/kg bw (Ishii et al., 2023).

(ii) Non-human mammalian cells in vitro

In an AR competitive binding assay performed in vitro with rat AR protein, isoeugenol was found to be a weak agonist on the basis of its reported relative binding affinity (RBA = 0.0015) relative to the median inhibitory concentration (IC_{50}) of the synthetic androgen R1881 (RBA = 1.00) (Fang et al., 2003). [The Working Group noted that the results from the binding affinity assay were unclear.] The capacity of isoeugenol to interact with the mouse olfactory receptor (mOR-EG) in mOR-EG-expressing HEK293 cells was reported in another study that documented different types of interaction according to the chemical state of the molecule (freshly purified versus stored isoeugenol)

(Oka et al., 2004). [The Working Group noted that mOR-EG is not a nuclear receptor, and no evidence was available on its relevance to carcinogenicity.]

4.2.7 Alters cell proliferation, cell death, or nutrient supply

(a) Humans

(i) Exposed humans

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

(ii) Human cells in vitro

Ghosh et al. (2005) assessed the ability of isoeugenol to inhibit the proliferation of melanoma cells. Different cell types, each representing a different stage of disease progression, were tested: Sbcl2 cells, primary melanoma; WM3211 cells, primary radial growth phase; WM98-1, primary vertical growth phase; and WM1205Lu, metastatic melanoma. Cell growth was not inhibited by treatment with isoeugenol at 0.5, 2.5 or 5 μM for 72 hours. [The Working Group noted that no clear information on the origin of the isoeugenol used in the study was provided.]

Similarly, Pisano et al. (2007) evaluated the effects on cell growth in the melanoma cell lines WM266-4, SK-Mel-28, LCP-Mel, LCM-Mel, PNP-Mel, CN-MelA, 13443, and GR-Mel, which were incubated for up to 6 days in the presence of 100 μM isoeugenol. The cells were established as primary short-term cell cultures starting from tumour samples from donor patients diagnosed with melanoma. Treatment with isoeugenol did not inhibit cell growth [%] when compared with untreated cultures, as measured by colorimetric assay.

In a study conducted by Atsumi et al. (2000) in a human submandibular gland tumour cell line and primary human gingival fibroblast cells incubated for 48 hours with serial dilutions of isoeugenol in the medium (10^{-7} to 10^{-3} M), a concentration-dependent decrease in cell viability, significant at 10^{-4} M, was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. IC_{50} values were 26.8 μM and 72.6 μM in the submandibular tumour cell line and fibroblast cells, respectively. In addition, similar concentrations of isoeugenol induced DNA-synthesis inhibition, measured by CyQUANT cell proliferation assay kit, after 24 hours incubation in submandibular gland tumour cells.

Bae et al. (2015) investigated potential proliferative effects and the immune-mediated response in normal human keratinocytes (NSK) from neonatal foreskin after treatment with various skin sensitizers and allergens, including isoeugenol. However, isoeugenol treatment at 0.4, 2, 10, and 50 μM for 24 hours did not induce an increase in cell proliferation but significantly decreased cell viability to 7 ± 6% at 10 μM, as measured by the WST-1 assay. In addition, isoeugenol did not modify the production of vascular endothelium growth factor (VEGF) at any tested concentration, as measured by immunoassay in the supernatants from treated NSK cells compared with those from untreated control cells.

Frankart et al. (2012) investigated the effect of isoeugenol on mRNA expression of IL-8 and its release in a reconstructed human epidermis model. Topical exposure to isoeugenol at 3 mg/mL for 24 hours caused a transient expression of IL-8 mRNA associated with IL-8 release, which also correlated with transient activation of epidermal growth factor receptor (EGFR).

As described in Section 4.2.6(a)(ii), Kalmes et al. (2006) investigated whether AhR mediates the cell cycle arrest induced by isoeugenol in human keratinocytes (HaCat cell line). Cells were incubated with isoeugenol (300 μM) in the presence and absence of an AhR antagonist (3′-methoxy-4′-nitroflavone, MNF). After

exposure to isoeugenol, 32–34% of cells were in G_0/G_1 phase, as assessed by fluorescence-activated cell sorting (FACS) analysis, whereas the induced G_0/G_1 arrest was reduced in the presence of the AhR antagonist MNF. In a follow-up study, the authors confirmed the mediation of AhR in cell cycle regulation. They also observed a reduction in levels of the G_1-phase cell cycle-related proteins retinoblastoma (RB), and cyclin-dependent kinase 6 (CDK6), but not CDK2 and CDK4, and an increase in protein levels of the CDK inhibitor p27Kip1 (CDKN1B) (Kalmes and Blömeke, 2012).

Scozzafava et al. (2015) investigated the capacity of isoeugenol and other phenolic compounds (e.g. vanillin, eugenol, and guaiacol) to inhibit catalytically active human isozymes of the Zn^{2+}-containing carbonic anhydrases. Phenol red was used as an indicator, the hydration of carbon dioxide by carbonic anhydrases was followed for 10–100 seconds, and results were expressed as inhibition constants. Isoeugenol showed inhibition constants of 10.29 µM, 6.73 µM, 9.32 µM, and 9.13 µM with human carbonic anhydrase isozymes hCAI, hCAII, hCAIX, and hCAXII, respectively. [The Working Group noted that this study was not particularly relevant. In this acellular system, isoeugenol showed inhibitory effects on carbonic anhydrases that were similar to those shown by other similar phenolic compounds, including catechol. This metalloenzyme family is involved in numerous pathological and physiological processes in different tissues and organs, including biosynthetic reactions such as gluconeogenesis, lipid and urea synthesis, calcification, lipogenesis, ureagenesis, tumorigenicity, and the growth and virulence of various pathogens.]

(b) Experimental systems

(i) Non-human mammals in vivo

Isoeugenol (purity, > 99%) was administered at doses of 75–300 mg/kg by gavage in a 2-year study in F344/N rats and $B6C3F_1$ mice (NTP, 2010) (see Section 3.1). In male mice, the incidence of hyperplasia of the Bowman glands (olfactory glands) was increased significantly in all exposed groups in the 2-year gavage study. [The Working Group noted that lesions of the olfactory epithelium of the nose are unusual, considering that exposure was not by the inhalation route.] The incidence of forestomach squamous hyperplasia increased in a dose-dependent manner and was significant at the highest dose.

Arancioglu et al. (2015), also described in Section 4.2.5(b)(i), reported a threefold increase in lymphocyte proliferation (measured by LLNA) in the ears of female Balb/c mice after exposure to isoeugenol. Similarly, T-lymphocyte proliferation (measured by LNNA) was reported in the lymph nodes of CBA/Ca mice exposed to isoeugenol (2.5%, 5%, or 10%) dissolved in acetone:olive oil (4:1) daily for 3 days (Kimber et al., 1991). The authors also observed that pre-treatment with a 25% isoeugenol ointment solution applied on the shaved flank for 48 hours, 5 days before the three consecutive days of isoeugenol exposure (5%) on the dorsum of both ears, induced an enhancement in cell proliferation. ter Burg et al. (2014) studied the effects of isoeugenol on cell proliferation in the mandibular lymph nodes by LLNA in male BALB/c mice after daily inhalation exposure for three consecutive days. Isoeugenol was nebulized in acetone to produce an aerosol of liquid droplets at a target concentration of 75 mg/m^3. Isoeugenol increased cell proliferation starting from the first exposure, 45 minutes/day, and induced a threefold increase in cell proliferation at the maximum exposure of 360 minutes/day.

[The Working Group considered that the end-point of cell proliferation from the LLNA was not particularly relevant to the key characteristic

of "alters cell proliferation, cell death, or nutrient supply", because it focused on a localized topical effect.]

(ii) Non-human mammalian cells in vitro

Kim et al. (2016) studied the effects of isoeugenol in cultures of primary myoblasts from mice (C2C12) and rats (L6). Cells were treated with isoeugenol at 30 μM for 3 hours. Glucose uptake was analysed by measuring the uptake of 2-deoxy-D(H^3)-glucose. Intracellular calcium concentration was measured by detecting the fluorescence of cells treated with a calcium-sensitive indicator, fluo-3 AM. The cells were transiently transfected with small interfering RNAs (siRNAs) against genes encoding AMP-activated protein kinases AMPKa2 and AMPKa1 and Akt substrate 160 (AS160). Cell surface expression of Myc-tagged glucose transporter type 4 (Myc-GLUT4) was quantified by an antibody-coupled colorimetric absorbance assay. Isoeugenol stimulated glucose uptake via AMPK phosphorylation in myoblasts of mouse C2C12 cells and, more effectively, in rat L6 cells, with the involvement of both AMPKa2 and AMPKa1. Isoeugenol increased glucose uptake through the calcium-mediated calcium/calmodulin-dependent protein kinase (CaMKK)–AMPK and the protein kinase C-alpha (PKCα) pathways and stimulated GLUT4 translocation through the AMPK–AS160 pathway. [The Working Group noted some limitations of the study because of lack of clarity in the description of the cells' source.]

Liu et al. (2008) reported that the exposure of vascular smooth muscle cells derived from Sprague-Dawley rats to isoeugenol at 50 μM did not inhibit the stimulation of phosphorylation of platelet-derived growth factor receptor beta (PDGFRβ) by PDGF or H_2O_2, thus showing no effect on growth factor signalling that could lead to cell proliferation or migration.

4.2.8 Data relevant to multiple key characteristics

(a) Microarray and omics data

There were 12 papers describing the development of predictive methods for identifying skin sensitizers (Cluzel-Tailhardat et al., 2007; McKim et al., 2010; Vandebriel et al., 2010; Williams et al., 2010; Andreas et al., 2011; Takahashi et al., 2011; Jung et al., 2012; Corsini et al., 2013; Neves et al., 2013; Saito et al., 2013, 2017; Bae et al., 2015). Given that isoeugenol is an OECD reference chemical for skin sensitization, these studies included isoeugenol among the training set of chemicals to develop methods for toxicity prediction. [The Working Group considered that although most of these studies used microarrays to identify predictive gene sets, none provided any mechanistic information.] Isoeugenol was used as a negative training compound to identify gene classifiers for the prediction of hepatocarcinogens in the mouse liver, but no data were provided to show changes in gene expression related to carcinogenesis (Auerbach et al., 2010). [The Working Group noted that this study did not provide any mechanistic insights.]

Ishii et al. (2023) recently examined gene expression changes in the livers of *gpt* delta mice treated with isoeugenol at 150 or 600 mg/kg bw per day for 13 weeks. Genes that were identified as altered in expression included those known to be regulated by PPAR subtypes and involved in fatty acid metabolism. More genes were altered in the livers of male mice than in those of female mice, consistent with males being more susceptible than females to hepatocellular adenoma and carcinoma. [The Working Group noted that since PPARα is the major subtype expressed in the mouse liver, the gene expression changes are likely due to PPARα and not PPARγ.]

(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). Isoeugenol 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, 2024).

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 the supplementary material 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 290 assays in which isoeugenol was tested, it was found to be active and without caution flags in three assays relevant to key characteristics of carcinogens. Isoeugenol was active in one assay mapped to key characteristic 5, "induces oxidative stress". This assay, ATG_NRF2_ARE_CIS_up, is a cell-based, multiplexed-readout assay in HepG2 (a human liver cell line), with measurements taken 24 hours after chemical dosing in a 24-well plate. The assay measures the activation of a reporter gene under the control of the antioxidant responsive element regulated by the oxidant-induced transcription factor NRF2 (NFE2 like bZIP transcription factor 2). The AC_{50} (50% of maximum activity) was 17.11. There are two high-throughput assays that measure activation of Nrf2 (TOX21_ARE_BLA_agonist_ratio, ATG_NRF2_ARE_CIS_up). [The Working Group noted that, given that only one out of the two NRF2 assays gave positive results, the evidence is weak that isoeugenol activates NRF2.]

Isoeugenol gave positive results in two assays mapped to key characteristic 8, "modulates receptor-mediated effects". The two assays are called OT_ER_ERaERb_1440 and ATG_PPARg_TRANS_up. OT_ER_ERaERb_1440 is a cell-based assay that uses HEK293T (a human kidney cell line), with measurements taken 24 hours after chemical dosing in a 384-well plate. The assay measures the interaction of green fluorescent protein (GFP)-tagged estrogen receptor alpha and beta (ERα and ERβ) with nuclear DNA and is one of 17 assays that assess the activity of compounds for the ability to modulate ER activity (Judson et al., 2015). [The Working Group noted that, given that only one out of the 17 ER assays gave positive results, the evidence was very weak that isoeugenol activates ER.]

Isoeugenol gave positive results for the activation of PPARγ. The ATG_TRANS assays are cell-based, multiplexed-readout assays that use HepG2 (a human liver cell line), with measurements taken 24 hours after chemical dosing in a 24-well plate. The assay measures the ability of the compound to activate a reporter gene through the ligand-binding domain of PPARγ. The AC_{50} was 60.36 μM. There are six assays that measure modulation of PPARγ (ATG_PPARg_TRANS_up, ATG_PPRE_CIS_up, OT_PPARg_PPARgSRC1_0480, OT_PPARg_PPARgSRC1_1440, TOX21_PPARg_BLA_Agonist_ratio, and TOX21_PPARg_BLA_antagonist_ratio). [The Working Group noted that, given that only one out of the six PPARγ assays (ATG_PPARg_TRANS_up) gave

positive results, the evidence was very weak that isoeugenol activates PPARγ.]

5. Summary of Data Reported

5.1 Exposure characterization

Isoeugenol is a flavour compound that can be synthesized from eugenol and that also occurs naturally in more than 500 plant species such as cloves, Ceylon cinnamon, sweet flag, nutmeg, basil, perilla, and ylang-ylang. It has been identified as a pyrolysis product of lignin and has been detected in wood smoke, smoked foods, and the smoke particulate fraction of commercial cigarette brands.

In many countries around the world, isoeugenol is approved for use in food, cosmetics, animal feed, and veterinary medicines. It is widely used as a fragrance or flavouring agent in perfumes, cosmetics, personal care products, household cleaners, and various food products. Its sweet and spicy floral aroma adds a distinctive fragrance and flavour to soft drinks, bakery products, confectionery, and chewing gum. Isoeugenol is a skin sensitizer and is commonly used in allergen patch testing. It is also the active ingredient in anaesthetics used in aquaculture.

People in a variety of occupations, including workers involved in the synthesis of isoeugenol, hairdressers, beauticians, janitors, cleaners, and firefighters, may be exposed to isoeugenol by dermal and/or inhalation routes. Exposure of the general population to isoeugenol occurs by several routes, and daily intake through consumption of foods containing isoeugenol is estimated to be in the low-micrograms range. Inhalation and dermal contact contribute to exposure, mainly through the use of personal care products; the presence of isoeugenol as a fragrance in household products and cosmetics is an important source of exposure.

5.2 Cancer in humans

No data were available to the Working Group.

5.3 Cancer in experimental animals

Treatment with isoeugenol caused an increase in the incidence of malignant neoplasms or an appropriate combination of benign and malignant neoplasms in one species (mouse) and a single rare result in three organs, the occurrence of histiocytic sarcoma at multiple sites in female mice and the presence of thymoma and mammary gland carcinoma in male rats.

Isoeugenol was administered by oral administration (gavage) in one study that complied with Good Laboratory Practice (GLP) in male and female B6C3F$_1$ mice. In male mice, isoeugenol caused an increased incidence of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined). In female mice, there was a significant positive trend in the incidence of histiocytic sarcoma (multiple sites).

Isoeugenol was administered by oral administration (gavage) in one study that complied with GLP in male and female F344 rats. In male rats, isoeugenol caused a significant positive trend in the incidence of mammary gland carcinoma and benign or malignant thymoma.

5.4 Mechanistic evidence

Data on the absorption, distribution, metabolism, and excretion of isoeugenol in humans were sparse. Isoeugenol is capable of both permeation and penetration of human skin after dermal exposures. No information on oral and inhalation exposure in humans was available. After oral and dermal exposure in rodents, isoeugenol is rapidly absorbed and excreted, predominantly in the urine as glucuronide or sulfate conjugates, with very little retention in tissues. Lower absorption was observed after dermal exposure

than after oral exposure. Isoeugenol has been shown to inhibit the activity of cytochrome P450 (CYP) enzymes in experimental systems.

Overall, the mechanistic evidence considered for the key characteristics of carcinogens "is electrophilic or can be metabolic activated to an electrophile", "is genotoxic", "alters DNA repair and/or genomic instability", "induces oxidative stress", "induces chronic inflammation", "modulates receptor-mediated effects", and "alters cell proliferation, cell death, or nutrient supply" was sparse or the available results were negative in experimental systems. There was only one study evaluating the allergic reaction to isoeugenol in exposed humans, and this was considered uninformative for the key characteristics of carcinogens.

Regarding the key characteristic "is electrophilic or can be metabolically activated to an electrophile", isoeugenol is a skin sensitizer that can be converted photochemically to electrophiles that form protein adducts. However, in two studies in vivo, one in mouse liver and another in turkey fetuses, isoeugenol–DNA adducts were not detected.

Regarding the key characteristic "is genotoxic", isoeugenol induced sister-chromatid exchange in human lymphocytes and caused an increase in the frequency of micronucleated normochromatic erythrocytes in female mice but not in male mice in one study at the highest dose tested. Isoeugenol gave negative results for *gpt* mutations in the liver of transgenic mice and did not induce unscheduled DNA synthesis in rodent hepatocytes or DNA strand breaks in turkey fetuses. It gave negative results for mutagenicity in two studies in bacteria and in one study in *Drosophila melanogaster*.

Regarding the key characteristic "induces oxidative stress", two in vitro studies, one in human primary cells and one in a human cell line found evidence that isoeugenol exposure increased intracellular ROS and depleted intracellular glutathione, but only at concentrations associated with significant cytotoxicity. There were no studies examining whether ROS associated with isoeugenol exposure caused any type of oxidative stress or damage.

Regarding the key characteristic "induces chronic inflammation", three studies in human primary cells showed that isoeugenol stimulated inflammatory markers, including T-cell responses, releasing interleukins IL-8, IL-1α, and IL-1β. Several studies in human cell lines observed increases in inflammatory cytokines such as IL-8 and interferon gamma (IFNγ). However, a few studies in human cells did not observe any effects on inflammatory cytokines. Additionally, isoeugenol-induced atrophy, attributable to chronic inflammation, was observed in the olfactory epithelium of treated rats and mice of both sexes at two exposure durations (3 months and 2 years). The same study also reported that the incidence of forestomach inflammation and of ulceration (in males only), was significantly increased at the highest dose in the 2-year study.

For the key characteristic "modulates receptor-mediated effects", a few studies in human keratinocytes in vitro indicated that isoeugenol activates the aryl hydrocarbon receptor (AhR), as assessed by increases in CYP1A1 expression. In another study, isoeugenol exhibited androgen receptor (AR) antagonism in in vitro transactivation assays. One microarray study showed that isoeugenol induced the expression of a set of genes known to be under the control of peroxisome proliferator-activated receptor alpha (PPARα) in mouse liver.

Regarding the key characteristic "alters cell proliferation, cell death, or nutrient supply", in human cell lines isoeugenol did not cause cell proliferation. In long-term studies in mice treated by (oral) gavage, hyperplasia was observed in Bowman glands and in the forestomach.

For the other key characteristics, "alters DNA repair or causes genomic instability" and "is immunosuppressive", there was a paucity of data.

Isoeugenol was essentially without effects in the assay battery of the Toxicology in the 21st Century (Tox21) and Toxicity Forecaster (ToxCast) research programmes.

6. Evaluation and Rationale

6.1 Cancer in humans

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

6.2 Cancer in experimental animals

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

6.3 Mechanistic evidence

There is *inadequate* mechanistic evidence.

6.4 Overall evaluation

Isoeugenol is *possibly carcinogenic to humans* (Group 2B).

6.5 Rationale

The Group 2B classification for isoeugenol is based on *sufficient evidence* for cancer in experimental animals. The *sufficient evidence* for cancer in experimental animals is based on an increase in the incidence of malignant neoplasms (liver) and a combination of benign and malignant neoplasms (liver), in one species (mouse) and one sex (males) in one study that complied with Good Laboratory Practice. In addition to the liver malignant neoplasms in male mice, there were significant positive trends (based on the poly-3 trend test) in mammary gland carcinoma and benign or malignant thymoma in male rats and histiocytic sarcomas (multiple sites) in female mice. A minority of the Working Group considered that the evidence for carcinogenicity in experimental animals was *limited*, given that the pairwise comparison with the controls did not reach statistical significance in any of the treated groups, and thus that isoeugenol should be classified as Group 3.

The evidence regarding cancer in humans was *inadequate* because no studies were available. The mechanistic evidence was *inadequate* because the available data were sparse or largely negative.

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