Australian Government
Department of Health and Aged Care
Australian Industrial Chemicals Introduction Scheme

Benzene, 1-methoxy-4-(2-propenyl)- (estragole)

Evaluation statement

12 October 2022

Draft

Table of contents

Contents

AICIS evaluation statement

Subject of the evaluation

Benzene, 1-methoxy-4-(2-propenyl)- (estragole)

Chemical in this evaluation

Name	CAS registry number
Benzene, 1-methoxy-4-(2-propenyl)-	140-67-0

Reason for the evaluation

Evaluation Selection Analysis indicated a potential human health risk.

Parameters of evaluation

The chemical is listed on the Australian Inventory of Industrial Chemicals (Inventory).

This evaluation is a human health risk assessment for all identified industrial uses of the chemical (except use in e-cigarettes due to absence of relevant hazard data).

Summary of evaluation

Summary of introduction, use and end use

There is no specific information about the introduction, use and end use of the chemical in Australia. However, it is known to be a natural constituent of several aromatic plants and their essential oil fractions, including tarragon, basil, fennel, chervil, and star anise.

Based on international information, the chemical has industrial use as a fragrance ingredient in cosmetic and domestic products, including in perfumes, air fresheners, washing and cleaning products, and polishes and waxes.

An International Fragrance Association (IFRA) Standard applies to the chemical, under which use of the chemical as a fragrance ingredient in finished products is restricted to a concentration limit of up to 1.5%, depending on the product category. IFRA states that the Standards are compulsory for all its members (IFRA 2022).

Human health

Summary of health hazards

The critical health effects for risk characterisation include:

- genotoxicity

- carcinogenicity.

The chemical caused cancer in the liver in multiple studies in rats and mice. The carcinogenic mode of action for the chemical has been well characterised as having a genotoxic mechanism, primarily driven by metabolism of the chemical by the liver to 1'-hydroxyestragole. Sulfoconjucation of the 1'-hydroxyestragole metabolite forms a reactive compound that can bind to DNA, which is considered to contribute to the observed hepatocarcinogenic effects.

This metabolic activation is reported to be primarily facilitated by cytochrome P40 (CYP) enzymes common to both humans and mice, with unscheduled DNA synthesis (UDS) and DNA adduct formation reported in vitro and in vivo studies in animals, and in vitro studies in human cell lines. Based on the weight of evidence, the genotoxic and carcinogenic properties of the chemical are considered relevant to humans. Due to competing detoxification processes, it is possible that a threshold exists for carcinogenic effects. However further data would be required to establish this threshold. It is noted that the genotoxic metabolite, 1-hydroxyestragole, has been identified in the urine of humans and rodents following exposure to low doses.

Based on the available data, the chemical has potential to cause skin sensitisation. Based on positive results from an in chemico (Direct Peptide Reactivity Assay (DPRA: OECD 442C)) and one in vitro (ARE-Nrf2 luciferase test method (KeratinoSens™; OECD TG 442D)) assay that address specific events of the Adverse Outcome Pathway (AOP), the chemical is predicted to be a skin sensitiser, warranting classification. Positive results were reported for in vivo sensitisation studies, although no study details were available. Based on the available in vitro data the chemical is predicted to be irritating to the skin. There is inadequate information available to assess the eye irritation potential of the chemical. While no prediction could be made based on the in vitro data, the need for classification was not ruled out.

The chemical also has moderate acute oral toxicity, with reported oral median lethal dose (LD50) values in rats and mice reported to be between 1008–2000 mg/kg bw. No information is available on acute dermal toxicity or acute inhalation toxicity of the chemical.
While no specific reproductive or developmental toxicity studies are available, treatment related toxicity was observed in the pituitary, testes, and epididymides of male rats (F344/N strain) following repeated oral exposure to the chemical at ≥300 mg/kg bw/day.

Hazard classifications relevant to worker health and safety

The chemical satisfies the criteria for classification according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) (UNECE 2017) for hazard classes relevant for work health and safety as follows. This evaluation does not consider classification of physical hazards and environmental hazards.

Health hazards	Hazard category	Hazard statement
Acute Toxicity	Acute Tox. 4	H302: Harmful if swallowed
Skin Corrosion/Irritation	Skin Irrit. 2	H315: Causes skin irritation
Skin Sensitisation	Skin Sens. 1	H317: May cause an allergic skin reaction
Genotoxicity	Muta. 2	H341: Suspected of causing genetic defects
Carcinogenicity	Carc. 1B	H350: May cause cancer

Summary of health risk

Public

Based on the available use information, the public may be exposed to the chemical at likely concentrations up to 1.5% by:

- direct application of the chemical to the skin
- direct skin contact during use of domestic products
- incidental skin and eye contact with the chemical during use of domestic products
- inhaling aerosols.

Regulatory controls currently apply to preparations of basil oils or fennel oils containing greater than 5% of the chemical. However, no regulatory controls currently apply to the use of the chemical itself or other essential oils that may contain the chemical at high concentrations such as tarragon oil and chervil oil.

Given that the chemical is reported to be a genotoxic carcinogen, the evidence indicates that there is a risk to the public that requires management (see **Proposed means for managing risks** section). The public is already exposed to the chemical through their diets. The increase in carcinogenic risk from use in cosmetics is unknown.

Workers

During product formulation and packaging, dermal, ocular and inhalation exposure might occur, particularly where manual or open processes are used. These could include transfer and blending activities, quality control analysis, and cleaning and maintaining equipment. Worker exposure to the chemical at lower concentrations could also occur while using formulated products containing the chemical. The level and route of exposure will vary depending on the method of application and work practices employed. Good hygiene practices to minimise incidental oral exposure are expected to be in place.

Given the critical systemic long term and systemic acute and local health effects, the chemical could pose a risk to workers. Control measures to minimise dermal and inhalation exposure are needed to manage the risk to workers (see **Proposed means for managing risks** section).

Control measures implemented due to the genotoxicity and carcinogenicity classifications are expected to be sufficient to protect workers from any potential reproductive or developmental toxicity health effects.

Proposed means for managing risk

Public health

Recommendation to Department of Health and Aged Care

It is recommended that the delegate of the Secretary for Poisons Scheduling lists the chemical in the *Poisons Standard — the Standard for the Uniform Scheduling of Medicines and Poisons* (SUSMP).

It is recommended that to manage the potential risks associated with the use of the chemical that the entry:

- prohibits or restricts chemical use to low concentration levels in cosmetic/domestic products.

Consideration should be given to the following:

- the potential use of the chemical in domestic and cosmetic products available in Australia
- the chemical is considered to be a genotoxic carcinogen. Although a threshold may exist for carcinogenic effects, sufficient data are not available to establish this threshold
- the genotoxic metabolite, 1-hydroxyestragole, has been identified in the urine of humans and rodents following exposure to low doses
- current scheduling decisions for preparations of basil oils and fennel oils containing greater than 5% of the chemical were based on concerns including potential carcinogenic effects
- current scheduling of the structurally and toxicologically similar chemical, methyl eugenol, in Schedule 6 at >1% (listed as ‘methyleugenol’)
- the quantity of estragole (as methyl chavicol) in a medicine must be no more than 0.01%
- the IFRA standard restricts the chemical to <0.012% for most leave on skin products and rinse and leave on hair products and 0.05% for household products.

It is recommended that the delegate also consider the need for scheduling of other essential oils that contain high levels of the chemical such as from tarragon, chervil (garden) and sweet chervil (cicely). Consideration should be given to the following:

- the level of the chemical in essential oils from tarragon, chervil and sweet chervil is reported to be 75–85%
- the existing scheduling conditions for preparations of basil oils and fennel oils containing greater than 5% estragole.

In consideration of these recommendations, it is suggested that the delegate determine if a consistent and more appropriate synonym for the chemical be used across all the relevant listings in the *Poisons Standard*; noting that the chemical is commonly referred to by its synonym estragole in the available literature.

Workers

Recommendation to Safe Work Australia

It is recommended that Safe Work Australia (SWA) update the HCIS to include classifications relevant to work health and safety.

Information relating to safe introduction and use

The information in this statement including recommended hazard classifications, should be used by a person conducting a business or undertaking at a workplace (such as an employer) to determine the appropriate controls under the relevant jurisdiction Work Health and Safety laws.

Control measures that could be implemented to manage the risk arising from ocular, dermal or inhalation exposure to the chemical include, but are not limited to:

- using closed systems or isolating operations
- minimising manual processes and work tasks through automating processes
- adopting work procedures that minimise splashes and spills
- cleaning equipment and work areas regularly
- using protective equipment that is designed, constructed, and operated to ensure that the worker does not come into contact with the chemical.

Measures required to eliminate or manage risk arising from storing, handling and using this hazardous chemical depend on the physical form and how this chemical is used.

These control measures may need to be supplemented with health monitoring conducted for any worker who is at significant risk of exposure to the chemical if valid techniques are available to monitor the effect on the worker's health.

Personal protective equipment should not solely be relied upon to control risk and should only be used when all other reasonably practicable control measures do not eliminate or sufficiently minimise risk.

Model codes of practice, available from the Safe Work Australia website, provide information on how to manage the risks of hazardous chemicals in the workplace, prepare an SDS and label containers of hazardous chemicals. Your Work Health and Safety regulator should be contacted for information on Work Health and Safety laws and relevant Codes of Practice in your jurisdiction.

Conclusions

The conclusions of this evaluation are based on the information described in this statement.

Considering the proposed means of managing risks, the Executive Director proposes to be satisfied that the identified human health risks can be managed within existing risk management frameworks. This is provided that all requirements are met under environmental, workplace health and safety and poisons legislation as adopted by the relevant state or territory and the proposed means of managing the risks identified during this evaluation are implemented.

Note: Obligations to report additional information about hazards under *Section 100* of the *Industrial Chemicals Act 2019* apply.

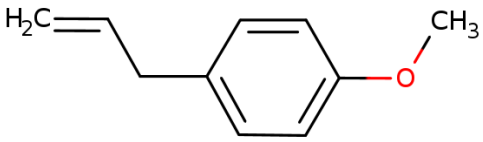
Supporting information

Chemical identity

Estragole is a methoxy-substituted allylbenzene compound, consisting of a benzene ring substituted with one methoxy group and one allyl group.

It is often referred to by its common synonym estragole; however, it is also referred to as methyl chavicol, in some regulations and literature.

Estragole is structurally and functionally related to methyl eugenol (CAS No. 93-15-2), an allylbenzene compound with 2 methoxy substituents. Both estragole and methyl eugenol belong to a group of alkenylbenzenes, including safrole, eugenol and myristicin, which are all naturally occurring substance in essential oils (JECFA 2009; NTP 2011; OEHHA 1999). Methyl eugenol has been assessed by AICIS under the former National Industrial Chemicals Introduction Scheme (NICNAS) program (NICNAS 2015).

Chemical name	Benzene, 1-methoxy-4-(2-propenyl)-
CAS No.	140-67-0
Synonyms	estragole 1-methoxy-4-(2-propen-1-yl)benzene methyl chavicol anisole, p-allyl- 4-allylanisole
Structural formula	
Molecular formula	$C_{10}H_{12}O$
Molecular weight (g/mol)	148.2
SMILES	COC1=CC=C(CC=C)C=C1

Relevant physical and chemical properties

Physical form	Colourless or pale-yellow liquid
Boiling point	216.0°C
Vapour pressure	8.2 kPa (at 20°C) 8.9 kPa (at 25°C) 10.1 kPa (at 37.8°C)
Water solubility	124 mg/L (at 20°C and pH 6.8)

Log Kow	3.4 (at 25°C)

Introduction and use

Australia

No specific Australian information on the introduction, use and end use of the chemical has been identified.

International

Based on international information, the chemical has industrial use as a fragrance ingredient in cosmetics, primarily perfumes. It also reported to be used as a flavouring agent and in air fresheners, washing and cleaning products, and in waxes and polishes (NTP 2011; REACH; SCF 2001). The chemical is listed on the IFRA Transparency list (IFRA). The concentrations specified in the IFRA standard were:

- <0.012% for most leave on skin products and rinse and leave on hair products
- 0.05% for household products
- 0.4% in fine fragrance
- 1.5% in products not intended for direct skin contact.

The chemical is included in the OECD high production volume list of chemicals produced or imported at greater than 1000 tonnes per annum (tpa) (OECD 2007). From 1994–1998, manufacture of the chemical in the USA was reported to be greater than 450 tpa (OEHHA 1999). The chemical is currently reported to be manufactured or imported in the European Union (EU) at volumes between 10–100 tpa (REACH).

Estragole is reported to be detected in cigarettes and tobacco smoke (Stanfill et al. 2003; IARC 2004) and e-cigarettes (Barhdadi et al. 2021; Budzyńska et al. 2020; Peace et al. 2018).

It is a naturally occurring component of several varieties of aromatic plants and their essential oils (EMA 2021; OEHHA 1999; SCF 2001; IFRA 2022); it is reported to be typically present at high concentrations in:

- basil, *Ocimum basilicum* (20-89% of essential oil)
- tarragon, *Artemisia dracunculus* (60-80% of essential oil)
- fennel, *Foeniculum vulgare* (1-20% of essential oil)
- chervil (garden), *Anthriscus cerefolium* (up to 85% of essential oil)
- sweet chervil (cicely), *Myrrhis odorata* (up to 75% of essential oil).

Existing Australian regulatory controls

AICIS

No specific controls are currently available for the chemical.

Public

While no specific controls on the industrial use of the chemical are currently available, preparations of basil oils or fennel oils containing greater than 5% of the chemical (referred to by the synonym, methyl chavicol) are included in Schedule 5 of *the Poisons Standard—the Standard for the Uniform Scheduling of Medicines and Poisons (SUSMP)* as follows (TGA 2022a).

> Schedule 5
> BASIL OIL **except**:
>
> in preparations containing 5 per cent or less of methyl chavicol.
>
> Schedule 5
>
> FENNEL OIL **except:**
>
> c) in preparations containing 5 per cent or less of methyl chavicol.

Schedule 5 chemicals are described as 'Substances with a low potential for causing harm, the extent of which can be reduced through the use of appropriate packaging with simple warnings and safety directions on the label'. Schedule 5 chemicals are labelled with 'Caution'.

Regulatory controls applying to the non-industrial use of the chemical as an excipient in medicines, as listed in the Therapeutic Goods (Permissible Ingredients) Determination No. 4 of 2022 (TGA 2022b), are detailed below.

> Schedule 1—Specified permissible ingredients and requirements applying to these ingredients when contained in a medicine; Volume 4, Item 3303 – METHYL CHAVICOL:
>
> - Permitted for use only in combination with other permitted ingredients as part of a fragrance proprietary excipient formulation
> - The ingredient is not to be included in medicines intended for oral use
> - The quantity of methyl chavicol in a medicine must be no more than 0.01%
> - The total fragrance proprietary excipient formulation in a medicine must be no more than 1%.

Workers

The chemical is not listed on the HCIS and no specific exposure standards are available in Australia (Safe Work Australia).

International regulatory status

European Union

The chemical is prohibited from being added to food in the EU as according to Regulation (EC) No 1334/2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods – Annex III, Part A: Substances which shall not be added as such to food (EU 2008).

Restrictions also apply under this regulation in the EU regarding the detectable amount of the chemical as naturally present in food. A maximum allowable level of the chemical in dairy or fish products, processed fruits, vegetables, nuts and seeds, is 50 mg/kg and for non-alcoholic beverages it is 10 mg/kg.

United States of America

The chemical is currently approved for use as a food additive and as an essential oil by the US Food and Drug Administration (FDA) under Title 21 of the Code of Federal Regulations (CFR) 172.515 and 21 CFR 182.20 (FDA 2021a; FDA 2021b).

Uses of synthetic estragole as a flavouring substance in baked goods, non-alcoholic beverages, condiments, and hard and soft candy are limited to the minimum quantity needed to produce their intended effect, or to concentrations less than or equal to 50 ppm.

Other

The chemical is listed on the International Fragrance Association (IFRA) Standard, with restrictions regarding its use as a fragrance ingredient due to dermal sensitisation and systemic toxicity concerns The acceptable concentrations recommended by IFRA of the chemical in finished products range from 0.0021–1.5% across 12 product categories(IFRA 2022).

Health hazard information

Toxicokinetics

Estragole is reported to be rapidly absorbed, metabolised, and excreted following either oral or intraperitoneal (i.p.) exposure (NTP 2011). Based on the molecular weight and log Kow the chemical is expected to be bioavailable following dermal exposure. Three major metabolic pathways of estragole have been established in rats and mice through both in vitro and in vivo and studies, with no reported difference between species in the metabolites formed.

At low doses, the chemical mainly undergoes O-demethylation, forming 4-allylphenol and ultimately CO2 as the terminal metabolite; this is considered a detoxication pathway. However, as the dose of the chemical increases, O-demethylation is reported to become less prominent and is replaced by 1’-hydroxylation as the main metabolic pathway, forming 1’-hydroxyestragole (EMA 2021; OEHHA 1999).

Subsequent sulfoconjucation of 1'-hydroxyestragole results in the formation of 1’- sulforoxyestragole and a reactive carbocation, reported to be capable of binding to DNA and protein (see **Genotoxicity** section). This metabolism is reported to be primarily facilitated by cytochrome P450 (CYP) enzymes, including CYP1A2, common to both humans and rodents (EMA 2021). It should be noted that a similar activation pathway and formation of reactive metabolites is reported for methyl eugenol (JECFA 2009; NICNAS 2015).

In a study in rats and mice, estragole was administered a single oral dose ranging from 0.5–1000 mg/kg bw/day. 1-Hydroxyestragole was detected in the urine at all dose levels with the amount excreted increased exponentially with increasing dose (OEHHA 1999).

In one available human study, 2 male volunteers ingested a gelatin capsule containing 100 µg of estragole (equivalent to 0.0011 mg/kg bw for each individual) on 2 separate occasions, at least 6 months apart. The administered dose was considered to be roughly equivalent to a normal ingested dietary level of estragole over the same period. The main finding of the study was the formation of 1'-hydroxyestragole, detected in excreted urine at 0.2–0.4% of the administered dose. No excretion of unchanged estragole was detected in any samples collected. The metabolism to 1'-hydroxyestragole was reported to be consistent with results seen in rats and mice, further supporting the conclusion that the metabolic pathway of estragole in mice, rats and humans is similar (EMA 2021; SCF 2001; OEHHA 1999; JECFA 2009).

The 3rd major metabolic pathway is epoxidation of the allyl side chain leading to estragole-2',3'-epoxide. In vitro studies indicate that these metabolites are rapidly metabolised by epoxide hydrolase and glutathione transferase to detoxified metabolites (EMA 2021, NTP 2011; OEHHA 1999).

Acute toxicity

Oral

Based on the available data, the chemical has moderate acute oral toxicity, which warrants hazard classification (see **Hazard classifications relevant to worker health and safety** section).

In an acute oral toxicity study, reported as good laboratory practice (GLP) compliant and conducted in accordance with OECD TG 423, Wistar rats (6 females/dose) were administered a single oral dose of the chemical at either 300 or 2000 mg/kg bw. A 50% mortality rate was observed in the high dose group, while all animals from the low dose group survived. The median lethal dose (LD50) was considered to meet the GHS hazard classification criteria range of 300–2000 mg/kg bw; however, the actual LD50 is likely to be closer to 2000 mg/kg bw. No sublethal signs of toxicity were reported (REACH).

Other reported oral LD50 values (NTP 2011; Opdyke 1976) following acute exposure to the chemical (no further details available) are:

- 1008–1820 mg/kg bw for rats
- 1250 mg/kg bw for mice.

Dermal

No reliable data are available for this chemical. A dermal LD50 >5000 mg/kg bw was reported for rabbits; however, no further details were provided (NTP 2011; Opdyke 1976)

Inhalation

No data are available for this chemical.

Corrosion/Irritation

Skin irritation

Based on the results of the in vitro studies the chemical is considered to be an irritant to skin, warranting classification (see **Hazard classifications relevant to worker health and safety** section).

The undiluted chemical was reported to cause moderate irritation when applied to occluded intact or abraded rabbit skin for an exposure period of 24 hours; no further details were provided. However, in a study in humans, a 3% solution of the chemical in petrolatum did not produce any irritation reactions following occluded exposure for 48 hours; no further details were provided (Opdyke 1976; NTP 2011).

In an in vitro skin corrosion assay, reported as GLP compliant and conducted in accordance with OECD TG 431, the chemical was applied to RhE (skin model EpiDermTM) for up to 60 minutes. The mean tissue viability was reported to be 111.2% after 3 minutes exposure and 21.0% after the 60 minutes exposure period (REACH). Substances that have measured viability ≥50% after 3 minutes and ≥15% after 60 min exposure are considered non-corrosive.

In an in vitro skin irritation study, reported as GLP compliant and conducted in accordance with OECD TG 439 (in vitro reconstructed human epidermis (RhE) test method for skin irritation), the chemical was applied to RhE, for an exposure period of 60 minutes, followed by an observation period of 23 hours. A mean tissue viability value of 16.4% was reported for the chemical in this study, and it was determined to be irritating to the skin due to being below the threshold for cell viability of 50% (REACH). Interpretation of results obtained from OECD TG 439 studies do not allow for distinction between irritation and corrosion.

The mechanistic profiling functionality of the OECD Quantitative Structure-Activity Relationship (QSAR) Application Toolbox did not reveal any skin irritation structural alerts for the chemical (OECD QSAR Toolbox version 4.2).

Eye irritation

There is inadequate information available to assess the eye irritation potential of the chemical. No determination could be made based on the in vitro data, but the need for classification was not ruled out.

In an ex vivo eye corrosivity/irritation study, reported as GLP compliant and conducted according to OECD TG 437, the chemical was applied to bovine corneae (number not specified). The mean in vitro irritancy score (IVIS) was 3.54 indicating ‘no prediction can be made’ for the eye irritation potential of the chemical according to the TG (REACH). The chemical does not meet the TG criteria for either serious eye damage classification (IVIS >55) or chemicals not requiring classification for eye irritation or serious eye damage (IVIS ≤3).

In an in vitro eye corrosion study, reported as GLP compliant and conducted according to OECD TG 492, the chemical was topically applied to reconstructed human cornea-like epithelium (RhCE) (specific test method not indicated) and tissue viability was measured following exposure and a post treatment incubation period. The tissue viability was determined to be 47% (REACH), indicating ‘no prediction can be made’ according to the TG. Chemicals that show tissue viability >60%, require no classification according to the TG.

The mechanistic profiling functionality of the OECD QSAR Application Toolbox did not reveal any eye irritation structural alerts for the chemical (OECD QSAR Toolbox version 4.2).

Sensitisation

Skin sensitisation

Positive results were reported from one in chemico and one in vitro cell based assays that address specific key events of the Adverse Outcome Pathway (AOP) for skin sensitisation. Based on the “2 out of 3” defined approach (OECD Guideline 497) the chemical is predicted to be a skin sensitiser, warranting classification. However, it is not possible to determine potency sub-categorisation. The sensitisation potential is supported by reported positive in vivo animal test results, although, no study details were available.

In chemico/in vitro

The chemical was reported as positive in the first key event assay of the AOP for skin sensitisation, in the in chemico direct peptide reactivity assay (DPRA) conducted in accordance with OECD TG 442C. Mean cysteine and lysine depletion by the chemical was 47.1%; therefore, the assay was considered to indicate peptide binding by the chemical (REACH).

The chemical was reported as positive in the second key event assay of the AOP for skin sensitisation, an in vitro skin keratinocyte activation test (LuSens assay) conducted in accordance with OECD TG 442D at concentrations of 135–1000 µM. Two independent experiments were performed. Test item concentration showed a viability ≥70% at concentrations up to 579 µM in experiment I and 833 µM in experiment II. A substantial and reproducible dose dependent increase in luciferase induction ≥1.5 fold in more than 2 non-cytotoxic test item concentrations was reported for both experiments (REACH).

These tests are part of an Integrated Approach to Testing and Assessment (IATA) which address specific events of the Adverse Outcome Pathway (AOP) leading to development of skin sensitisation (OECD 2017). The tests are thus considered relevant for assessment of the skin sensitisation potential of the chemical. The chemical showed positive responses in 2 of the 3 tests of the AOP for skin sensitisation.

In vivo

The chemical is reported to be positive in a local lymph node assay (LLNA) and Guinea-pig maximisation test (GPMT) (NTP 1999). No study details or results including an EC3 are available.

In silico

The mechanistic profiling functionality of the OECD QSAR Application Toolbox did not reveal any specific skin sensitisation structural alerts for the chemical (OECD QSAR Toolbox version 4.2). The knowledge based expert system, Deductive Estimation of Risk from Existing Knowledge (DEREK) Nexus (version 6.0.1), was utilised to estimate the skin sensitisation potential of the chemical (Lhasa Limited). An alert for skin sensitisation (vinylic or allylic anisole) was reported. The chemical was reported as being in the training set for this QSAR.

Observation in humans

In one study in 25 human volunteers (reported as a maximisation test), a 3% solution of the chemical in petrolatum did not produce any skin sensitisation reactions (Opdyke 1976); no further details were provided.

Repeat dose toxicity

Oral

The chemical is reported to primarily effect the liver in both rats and mice following repeated oral exposure.

In a 3 month repeated oral exposure study, rats (F344/N strain) and mice (B6C3F1 strain) were administered the chemical in corn oil at 37.5, 75, 150, 300, or 600 mg/kg bw/day (10 animals/sex/dose) by oral gavage, for 5 days per week (NTP 2011). The no observed adverse effect level (NOAEL) was reported to be 37.5 mg/kg bw/day in mice. An NOAEL could not be established in rats based on dose-dependent histopathological liver changes seen at all doses.

At 600 mg/kg bw/day, all female mice died during the first week and one male mouse died during week 9 of the study. No mortality was observed in any of the treated rats. Significantly decreased mean body weights were reported in female mice administered ≥75 mg/kg bw/day, and in male and female rats and in male mice administered ≥300 mg/kg bw/day.

In treated rats, changes in blood biochemistry (increased serum levels of alanine transaminase, sorbitol dehydrogenase and bile salt), haematology (anaemia-like) were reported to be generally dose-dependent. Histopathological effects in the liver including hepatocellular hypertrophy, bile duct hyperplasia and chronic periportal inflammation were observed at all doses. In the 600 mg/kg bw/day group, multiple intrahepatic cholangiocarcinomas were identified in 2 male rats, and a hepatocellular adenoma was identified in another male rat (see **Carcinogenicity** section). Significant histopathological changes in the bone marrow, kidney, olfactory epithelium, pituitary and testes were also observed at doses of ≥300 mg/kg bw/day.

Histopathological changes in the liver were observed at ≥75 mg/kg bw/day in female mice and ≥300 mg/kg bw/day in rats. In addition, effects were observed in the stomach (600 mg/kg bw/day) and olfactory epithelium (≥ 300mg/kg bw/day).

A parallel 30 day study was conducted in F344/N strain rats (10 animals/sex/dose) administered the chemical at the same dose levels as described for the 3 month study above.

Gastric gland atrophy was significantly increased in the stomach of rats administered the chemical at ≥300 mg/kg bw/day. Serum gastrin concentration and stomach pH were also significantly increased in rats at the highest dose (600 mg/kg bw/day). Enzyme measurements indicated that CYP-induced hepatic 7-pentoxyresorufin-O-deethylase activity was significantly increased in all treatment groups except for the low dose females (37.5 mg/kg bw/day); this increase was reported to be dose-related (NTP 2011).

Dermal

No data are available for this chemical.

Inhalation

No data are available for this chemical.

Genotoxicity

The available in vitro and in vivo data indicate that the chemical is considered to have genotoxic potential which warrants classification. The chemical caused damage to DNA in somatic cells in vivo and in in vitro assays.

In vitro

The chemical was generally reported to have negative results in bacterial in vitro assays (NTP 2011; OEHHA 1999) as listed below, with any mutagenic effects noted more so in the presence of metabolic activation:

- negative results were reported in a bacterial reverse mutation assay in *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537, with and without metabolic activation
- weak positive results were reported in bacterial reverse mutation assays in *S. typhimurium* strain TA1535, with increased mutagenic activity reported in the presence of metabolic activation enzymes
- negative results were reported in an *Escherichia coli* WP2 uvrA reversion test, with and without metabolic activation
- negative results were reported in a *Bacillus subtilis* repair test (Rec-assay) without metabolic activation.

It is noted that the metabolite, 1'-hydroxyestragole (see **Toxicokinetics** section), returned positive results in a bacterial reverse mutation assay in *S. typhimurium* strain TA100, with and without metabolic activation.

Mostly positive results were reported in the available mammalian cell in vitro assays (Martins et al. 2012; NTP 2011; Müller et al. 1994):

- the chemical and its metabolite, 1'-hydroxyestragole, induced unscheduled DNA synthesis (UDS; indicative of DNA damage) in primary rat hepatocytes at concentrations of 1–1000 µM
- the chemical did not induce chromosomal aberrations in V79 hamster cells at the same range of concentrations in the presence or absence of metabolic activation
- an increase in sister chromatid exchange was reported in V79 hamster cells at concentrations ranging from 250–1000 µM without metabolic activation
- positive results were reported in an alkaline comet assay in V79 hamster cells and in in Chinese hamster ovary (CHO) cell lines (AA8 and EM9) in the absence of metabolic activation, at concentrations ranging from 100–750 µM, indicating DNA strand breakage
- increased formation of DNA adducts were reported in V79 hamster cells following incubation for 2 hours at concentrations ranging from 500–1000 µM in the absence of metabolic activation; the rate of repair at 1000 µM was reported to be inefficient based on persistence of the adducts after a 24 hour recovery period
- accumulation of DNA adducts in human liver HepaRG cells has also been reported following repeated exposure to 50 mM of the chemical (Yang et al, 2021).

It is noted that exposure to the structurally related chemical, methyl eugenol (CAS No. 93-15-2; see **Chemical identity** section) and its 1'-hydroxy metabolite, also induced UDS in rat hepatocytes; the 1'- hydroxy metabolites of both estragole and methyl eugenol were reported to be more potent genotoxins than their parent compounds (NTP 2011).

In vivo

In an in vivo rat study, a dose-related increase in UDS was reported in the liver of male Wistar rats following ingestion of the chemical at 500, 1000 or 2000 mg/kg bw (Müller et al. 1994).

Intraperitoneal (i.p.) injection of the chemical or its metabolite, 1'-hydroxyestragole, was reported to increase DNA adduct formation in the livers of CD-1 female mice, while estragole and methyl eugenol were reported to induce DNA adducts in the liver of newborn male B6C3F1 mice following i.p. injection on days 1, 8, 15 and 22 after birth, at doses ranging from 0.25–3.0 μM (NTP 2011).

Structurally related methyl eugenol (CAS No. 93-15-2) is classified as a hazardous genotoxin (Category 2), with the risk phrase 'Suspected of causing genetic defects' (H341) in HCIS (Safe Work Australia).

Carcinogenicity

Based on the available data, the chemical is considered to be carcinogenic. The weight of evidence from the available repeated dose studies and genotoxicity studies indicate that this is primarily driven by metabolism of the chemical by the liver to 1'-hydroxyestragole. As the metabolic pathways for the chemical are reported to be similar for rats, mice and humans, the carcinogenic potential of the chemical is considered relevant for humans. Available studies (Drinkwater et al. 1976; NTP 2011; Martins et al. 2012) include those summarised below. Hazard classification is warranted.

Male and female pre-weanling CD-1 mice (reported as approximately 50 animals) were administered 370 mg/kg bw/day of the chemical in trioctanoin by oral gavage, twice weekly for 5 weeks. Within 11 to 14 months, a significant increase of hepatocellular carcinomas was observed in male (73%), but not female (9%), mice compared with control group animals (24% and 9%, respectively).

A dose-related significant increase in hepatocellular carcinomas was also reported in 8 week old female CD-1 mice fed diets containing 0.23% or 0.46% of the chemical, or 0.25% of its 1-hydroxyestragole metabolite, for 12 months. At 20 months, 50–70% of the mice developed liver tumours. No tumours were observed in control group animals.

Pre-weanling CD-1 male mice (approximately 50 animals) were administered a total dose of 70 mg/kg bw of the chemical as i.p. injections in the first 3 weeks of life. At 12 months, hepatocellular carcinomas were reported in 65% of treatment group animals compared with 24% in the control animals.

Groups of 50 CD-1 female mice, approximately 8 weeks old, were fed grain diets containing 2300 or 4600 ppm estragole or 2500 ppm 1'-hydroxyestragole for 12 months. This was reported to be equivalent to an average daily intake of 150−300 and 300−600 mg/kg bw/day for estragole, and 180−360 mg/kg bw/day for 1'-hydroxyestragole. Hepatocellular carcinomas were reported in 56% and 71% for mice treated with estragole and 56% for mice treated with 1'-hydroxyestragole. No hepatocellular carcinomas were reported in the control group animals. Angiosarcomas were reported in 4 mice treated with estragole at the highest dose.

Pre-weanling B6C3F1 male mice (groups of about 50 animals) were administered a total dose of 31.8 mg/kg bw of estragole by 4 i.p. injections in the first 3 weeks of life. At 18 months, hepatocellular carcinomas were reported in 83% of treatment group animals compared with 41% in control animals.

In the 3 month repeated oral exposure study in F344/N rats (described in the **Repeated Dose Toxicity** section), animals were administered the chemical in corn oil at 37.5–600 mg/kg bw/day (10 animals/sex/dose) by oral gavage, for 5 days per week (NTP 2011). The chemical was reported to show carcinogenic activity based on the occurrence of two cholangiocarcinomas and one hepatocellular adenoma in the liver of 3 male rats in the high dose group (600 mg/kg bw/day). These findings were considered to be significant evidence for carcinogenicity, with no liver neoplasms of any type in 662 control males and 677 control females in 3 month studies reported to be detected in concurrent NTP subchronic study results. This was further supported by the characterisation of the lesions as differing from spontaneous cholangiomas and cholangiocarcinomas.

Structurally related methyl eugenol (CAS No. 93-15-2) is classified as a hazardous carcinogen (Category 1B), with the risk phrase ‘May cause cancer’ (H350) in HCIS (Safe Work Australia). It should be noted that a similar activation pathway and formation of reactive metabolites is reported for this chemical (NICNAS 2020).

Due to competing detoxification processes, it is possible that a threshold exists for carcinogenic effects. However further data would be required to establish this threshold. It is noted that the genotoxic metabolite, 1-hydroxyestragole, has been identified in the urine of humans and rodents following exposure to low doses (see **Toxicokinetics** section).

Reproductive and development toxicity

While no specific reproductive or developmental toxicity studies are available, treatment related toxicity was observed in the pituitary, testes, and epididymides of male rats (F344/N strain) following repeated oral exposure to the chemical at 300 or 600 mg/kg bw/day (see **Repeated Dose Toxicity** section). Testes weights at ≥300 mg/kg bw/day were reported to be significantly decreased, with marked bilateral degeneration of the germinal epithelium in the testes and bilateral hypospermia in the epididymides reported in all males from these groups (NTP 2011).

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