European Commission

Combined Draft (Renewal) Assessment Report prepared according to Regulation (EC) N° 1107/2009
and
Proposal for Harmonised Classification and Labelling (CLH Report) according to Regulation (EC) N° 1272/2008

EUGENOL
2-methoxy-4-(prop-2-en-1-yl)phenol

Volume 1

Rapporteur Member State: Spain
Co-Rapporteur Member State: Greece

Version History

When	What
July 2022	Initial DRAR-RMS Spain
November 2022	DRAR after CoRMS and Applicant comments
February 2023	RAR updated after EFSA CoCh received on 24/01/2023 and ECHA Accordance check

The RMS is the author of the Assessment Report. The Assessment Report is based on the validation by the RMS, and the verification during the EFSA peer-review process, of the information submitted by the Applicant in the dossier, including the Applicant's assessments provided in the summary dossier. As a consequence, data and information including assessments and conclusions, validated and verified by the RMS experts, may be taken from the applicant's (summary) dossier and included as such or adapted/modified by the RMS in the Assessment Report. For reasons of efficiency, the Assessment Report should include the information validated/verified by the RMS, without detailing which elements have been taken or modified from the Applicant's assessment. As the Applicant's summary dossier is published, the experts, interested parties, and the public may compare both documents for getting details on which elements of the Applicant's dossier have been validated/verified and which ones have been modified by the RMS. Nevertheless, the views and conclusions of the RMS should always be clearly and transparently reported; the conclusions from the applicant should be included as an Applicant's statement for every single study reported at study level; and the RMS should justify the final assessment for each endpoint in all cases, indicating in a clear way the Applicant's assessment and the RMS reasons for supporting or not the view of the Applicant.

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Level 1

EUGENOL

1 STATEMENT OF SUBJECT MATTER AND PURPOSE FOR WHICH THIS REPORT HAS BEEN PREPARED AND BACKGROUND INFORMATION ON THE APPLICATION

1.1 CONTEXT IN WHICH THIS DRAFT ASSESSMENT REPORT WAS PREPARED

1.1.1 Purpose for which the draft assessment report was prepared

The draft assessment report was prepared for the renewal of approval of eugenol in accordance with Regulation (EC) No 1107/2009.

Eugenol was approved as active substance in accordance with Regulation (EC) No 1107/2009 by Commission Implementing Regulation (EU) No 546/2013 of 14 June 2013. The main notifier for the original EU Inclusion was Eden Research plc and the RMS was United Kingdom.

Eugenol is included in AIR 5 program (SANTE-2018- 10048–rev 3, February 2020). Eden Research plc is the main notifier for the renewal of approval of eugenol, the RMS is Spain and the co-RMS is Greece (Commission Implementing Regulation (EU) No 2018/155 of 31 January 2018).

For the implementation of the uniform principles of Annex VI, the conclusions of the review report on eugenol (SANCO/10577/2013 rev 3, 17 May 2013), the Conclusion on the peer review of the pesticide risk assessment of the active substance eugenol (EFSA Journal 2012;10(11):2914) and Outcome of the consultation with Member States, the applicant and EFSA on the pesticide risk assessment for eugenol in light of confirmatory data (EFSA Supporting publication 2017:EN-1165) shall be taken into account.
The content of the dossier is in compliance with the data requirements described in Regulation (EU) No 283/2013 and Regulation (EU) No 284/2013 and according to Commission Regulation (EU) No 844/2012 and prepared according to SANCO/10181/2013 – rev. 5 (12 June 2019, Guidance Document For Applicants On Preparing Dossiers For The Approval Of A Chemical New Active Substance And For The Renewal Of Approval Of A Chemical Active Substance According To Regulation (Eu) No 283/2013 And Regulation (Eu) No 284/2013). Supplementary dossier (SD) including supporting CA and CP dossiers for the active substance and representative formulation Mevalone is provided.

The active substance is for use as a fungicide, for application on grapes and pome fruits. The representative product is Mevalone, a CS formulation containing 33 g/L eugenol, 66 g/L geraniol and 66 g/L thymol. Mevalone is currently authorised in various EU Member States. Mevalone is the common representative product of the three active substances eugenol, geraniol and thymol intended to be renewed at the same time.

1.1.2 Arrangements between rapporteur Member State and co-rapporteur Member State

According to Commission Implementing Regulation (EU) 2018/155 of 31 January 2018 amending Implementing Regulation No 686/2012 allocating to Member States, for the purposes of the renewal procedure, the evaluation of the active substances whose approval expires by 31 December 2024 at the latest, Spain has been designated as the Rapporteur Member State (RMS) and Greece as the Co-rapporteur Member State (Co-RMS).

For the purposes of the renewal procedure, the evaluation of each active substance set out in the first column of the Annex, is allocated to a rapporteur Member State, as set out in the second column of that Annex, and to a co-rapporteur Member State, as set out in the third column of that Annex.

'PART C

ALLOCATION OF THE EVALUATION OF ACTIVE SUBSTANCES WHOSE APPROVAL EXPIRES AFTER 31 DECEMBER 2021 AND NOT LATER THAN 31 DECEMBER 2024

Active substance	Rapporteur Member State	Co-rapporteur Member State
Eugenol	ES	EL

RMS has arranged with the Co-RMS a commenting period before sending the RAR to EFSA

1.1.3 EU Regulatory history for use in Plant Protection Products

Refer to 1.1.1.

1.1.4 Evaluations carried out under other regulatory contexts

Eugenol is registered under the REACH Regulation and is manufactured in and / or imported to the European Economic Area.
This substance is used by consumers, by professional workers (widespread uses), in formulation or re-packing, at industrial sites and in manufacturing.

Consumer Uses
Eugenol is used in the following products: washing & cleaning products, air care products, polishes and waxes, cosmetics and personal care products and perfumes and fragrances. Other release to the environment of this substance is likely to occur from: indoor use as processing aid and outdoor use as processing aid.

Eugenol is registered in US-EPA as pesticide and it is listed as 40 CFR 152.25: Exemptions for pesticides of a character not requiring FIFRA regulation-Active Ingredients, Exempted Minimum Risk Pesticide Products. It is also listed by US-EPA as 2020 CDR TSCA (The Toxic Substances Control Act) Inv Active in the chemicals list which is subject to a data report every 4 years via Chemical Data Reporting (CDR).

References :

EU source: Substance Information - ECHA (europa.eu)
EPA source: System of Registries | US EPA

1.2 APPLICANT INFORMATION

1.2.1 Name and address of applicant(s) for approval of the active substance

Name: Eden Research plc
Address: 67C Innovation Drive
Milton Park
Oxfordshire
OX14 4RQ
UK

Contact: [redacted]
E-Mail:
Phone:

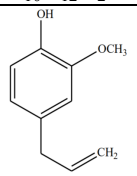
1.2.2 Producer or producers of the active substance

CONFIDENTIAL information – data provided separately-Vol. 4

1.2.3 Information relating to the collective provision of dossiers

Eden Research plc is to the best of their knowledge the only company submitting a dossier for the renewal of eugenol. Therefore, a task force is not required for this submission.
All data is owned by Eden Research plc.

1.3 IDENTITY OF THE ACTIVE SUBSTANCE

1.3.1 Common name proposed or ISO-accepted and synonyms	Eugenol (No ISO common name)
1.3.2 Chemical name (IUPAC and CA nomenclature)	
IUPAC	2-methoxy-4-(prop-2-en-1-yl)phenol
CA	Phenol, 2-methoxy-4-(2-propen-1-yl)-
1.3.3 Producer's development code number	None
1.3.4 CAS, EEC and CIPAC numbers	
CAS	97-53-0
EC	202-589-1
CIPAC	967
1.3.5 Molecular and structural formula, molecular mass	
Molecular formula	$C_{10}H_{12}O_2$
Structural formula	
Molecular mass	164.20 g/mol
1.3.6 Method of manufacture (synthesis pathway) of the active substance	CONFIDENTIAL information - data provided separately (Volume 4)
1.3.7 Specification of purity of the active substance in g/kg	Minimum purity : 990 g/kg
1.3.8 Identity and content of additives (such as stabilisers) and impurities	
Additives	CONFIDENTIAL information - data provided separately (Volume 4)
Significant impurities	CONFIDENTIAL information - data provided separately (Volume 4)
Relevant impurities	Methyl eugenol : max. 1 g/kg
Analytical profile of batches	CONFIDENTIAL information - data provided separately (Volume 4)

1.4 INFORMATION ON THE PLANT PROTECTION PRODUCT

<table>
<tr><td>1.4.1 Applicant</td><td>Eden Research plc</td></tr>
<tr><td>1.4.2 Producer of the plant protection product</td><td>CONFIDENTIAL information – data provided separately-Vol. 4</td></tr>
<tr><td>1.4.3 Trade name or proposed trade name and producer's development code number of the plant protection product</td><td>Trade name: Mevalone
Code number: 3AEY</td></tr>
<tr><td colspan="2">1.4.4 Detailed quantitative and qualitative information on the composition of the plant protection product</td></tr>
<tr><td>Composition of the plant protection product</td><td>
<table>
<tr><th>Chemical name</th><th>g/L</th><th>% w/w</th></tr>
<tr><td>Eugenol (pure, 100%)</td><td>33.0 (29.7 – 36.3)</td><td>3.21 (2.89 – 3.53)</td></tr>
<tr><td>Eugenol technical (99.0%)</td><td>33.3 (30.0 – 36.6)</td><td>3.24 (2.92 – 3.56)</td></tr>
<tr><td>Geraniol (pure, 100%)</td><td>66.0 (59.4 – 72.6)</td><td>6.41 (5.77 – 7.06)</td></tr>
<tr><td>Geraniol technical (98.0%)</td><td>67.3 (60.6 – 74.1)</td><td>6.54 (5.89 – 7.20)</td></tr>
<tr><td>Thymol (pure, 100%)</td><td>66.0 (59.4 – 72.6)</td><td>6.41 (5.77 – 7.06)</td></tr>
<tr><td>Thymol technical (99.0%)</td><td>66.7 (60.0 – 73.3)</td><td>6.48 (5.83 – 7.12)</td></tr>
</table>
FAO tolerance: ± 10%
</td></tr>
<tr><td>Information on the active substances</td><td>
<table>
<tr><th>Type</th><th>Name/Code Number</th></tr>
<tr><td>ISO common name</td><td>No ISO common name for Thymol</td></tr>
<tr><td>CAS No.</td><td>89-83-8</td></tr>
<tr><td>EC No.</td><td>201-944-8</td></tr>
<tr><td>CIPAC No.</td><td>969</td></tr>
<tr><td>Salt, ester anion or cation present</td><td>No</td></tr>
</table>
<table>
<tr><th>Type</th><th>Name/Code Number</th></tr>
<tr><td>ISO common name</td><td>No ISO common name for Eugenol</td></tr>
<tr><td>CAS No.</td><td>97-53-0</td></tr>
<tr><td>EC No.</td><td>202-589-1</td></tr>
<tr><td>CIPAC No.</td><td>967</td></tr>
<tr><td>Salt, ester anion or cation present</td><td>Not applicable</td></tr>
</table>
<table>
<tr><th>Type</th><th>Name/Code Number</th></tr>
<tr><td>ISO common name</td><td>No ISO common name for Geraniol</td></tr>
<tr><td>CAS No.</td><td>106-24-1</td></tr>
<tr><td>EC No.</td><td>203-377-1</td></tr>
<tr><td>CIPAC No.</td><td>968</td></tr>
<tr><td>Salt, ester anion or cation present</td><td>Not applicable</td></tr>
</table>
</td></tr>
<tr><td>Information on the active substances</td><td>CONFIDENTIAL information – data provided separately-Vol. 4</td></tr>
</table>

1.4.5 Type and code of the plant protection product	Capsule Suspension [Code: CS]
1.4.6 Function	Fungicide
1.4.7 Field of use envisaged	Grapes, pome fruits
1.4.8 Effects on harmful organisms	Eugenol has action on contact. Due to the lipophilic nature of the active substance, it disrupts the cell walls, membranes or organelles of micro-organisms.

1.5 DETAILED USES OF THE PLANT PROTECTION PRODUCT

1.5.1 Details of representative uses

Crop and/or situation (a)	**Member State or Country**	**Product name**	**F G or I** (b)	**Pests or Group of pests controlled** (c)	**Preparation**		**Application**				**Application rate per treatment**			**PHI (days)** (m)	**Remarks**
					Type (d-f)	Conc. a.s. (i)	method kind (f-h)	range of growth stages & season (j)	number min-max (k)	Interval between application (min)	g a.s /hL min-max (l)	Water L/ha min-max	g a.s./ha min-max (l)		
Grape vines	*MT, ES, IT, PT*	*3AEY (Mevalone)*	*F*	*Botrytis [BOTRCI]*	*CS*	*eugenol 33 g/L geraniol 66 g/L thymol 66 g/L*	*Tractor-mounted / trailed boom or air blast sprayer. Hand-held knapsack sprayer.*	*BBCH 60-89*	*1-4*	*7*	*Per appl. 13.2 (E) 26.4 (G) 26.4 (T)*	*400-1000*	*1 appl. 52.8 - 132 (E) 105.6 - 264 (G) 105.6 - 264 (T)* *4 appl. 211.2 - 528 (E) 422.4 - 1056 (G) 422.4 - 1056 (T)*	*7*	*The concentration in g a.s./hL is kept constant – the higher application rate is diluted in the higher water volume. Preventative and curative control.*
Grape vines	*ES*	*3AEY (Mevalone)*	*F*	*Powdery mildew [UNCINE]*	*CS*	*eugenol 33 g/L geraniol 66 g/L thymol 66 g/L*	*Tractor-mounted / trailed boom or air blast sprayer. Hand-held knapsack sprayer.*	*BBCH 60-89*	*1-4*	*7*	*Per appl. 13.2 (E) 26.4 (G) 26.4 (T)*	*400-1000*	*1 appl. 52.8 - 132 (E) 105.6 - 264 (G) 105.6 - 264 (T)* *4 appl. 211.2 - 528 (E) 422.4 - 1056 (G) 422.4 - 1056 (T)*	*7*	*The concentration in g a.s./hL is kept constant – the higher application rate is diluted in the higher water volume. Preventative and curative control.*

Crop and/or situation (a)	**Member State or Country**	**Product name**	**F G or I** (b)	**Pests or Group of pests controlled** (c)	**Preparation**		**Application**				**Application rate per treatment**			**PHI (days)** (m)	**Remarks**
					Type (d-f)	Conc. a.s. (i)	method kind (f-h)	range of growth stages & season (j)	number min-max (k)	Interval between application (min)	g a.s /hL min-max (l)	Water L/ha min-max	g a.s./ha min-max (l)		
Grape vines	*EL, BG, CY*	*3AEY (Mevalone)*	*F*	*Grey mould (Botryotinia fuckeliana, Botrytis cinerea) (BOTRCI)*	*CS*	*eugenol 33 g/L geraniol 66 g/L thymol 66 g/L*	*Tractor-mounted / trailed boom or air blast sprayer. Hand-held knapsack sprayer.*	*BBCH 60-89*	*1-4*	*7*	*Per appl. 13.2 (E) 26.4 (G) 26.4 (T)*	*400-1000*	*1 appl. 52.8 -132 (E) 105.6 - 264 (G) 105.6 - 264 (T)* *4 appl. 211.2 - 528 (E) 422.4 - 1056 (G) 422.4 - 1056 (T)*	*7*	*The concentration in kg a.s./hL is kept constant – the higher application rate is diluted in the higher water volume. Preventative and curative control. Table grapes includes use on grapes grown for raisin production.*
*Pome Fruit**	*Central Zone IE, GB, NL, BE, LU, DE, CZ, AT, SI, SK, HU, RO, PL*	*Mevalone*	*F*	*Post-harvest storage diseases* (Phytophthora spp. PHYTSP mainly P. cactorum PHYTCC or P. syringae PHYTSY, ALTESP, Botrytis cinerea BOTRCI)*	*CS*	*eugenol 33 g/L geraniol 66 g/L thymol 66 g/L*	*Tractor-mounted / trailed boom or air blast sprayer. Hand-held knapsack sprayer.*	*BBCH 75-87*	*1-4*	*7*	*Per appl. 13.2 (E) 26.4 (G) 26.4 (T)*	*600-1000*	*1 appl. 79.2 -132 (E) 158 -264 (G) 158 -264 (T)* *4 appl. 317 -528 (E) 634 - 1056 (G) 634 - 1056 (T)*	*1*	*The product is applied so that the concentration in g a.s./hL is kept constant at 13.2 (Eugenol), 26.4 (Geraniol), 26.4 (Thymol) g a.s / hectolitre of spray water volume. Therefore, the higher application rate is diluted in the higher water volume.*

*(apple *Malus domestica* MABSD, pear *Pyrus communis* PYUCO, quince *Cydonia oblonga* CYDOB, crab-apple *Malus sylvestris* MABSY, loquat *Eryobotria japonica* EIOJA, medlar *Mespilus germanica* MSPGE, Nashi pear *Pyrus pyrifolia var. culta* PYUPC, black chokeberry *Aronia melanocarpa* ABOME, mountain ash *Sorbus* sp. SOUSS)

(a) For crops, the EU and Codex classifications (both) should be taken into account; where relevant, the use situation should be described (e.g. fumigation of a structure)
(b) Outdoor or field use (F), greenhouse application (G) or indoor application (I)
(c) *e.g.* biting and sucking insects, soil born insects, foliar fungi, weeds
(d) *e.g.* wettable powder (WP), emulsifiable concentrate (EC), granule (GR), capsule suspension (CS)
(e) CropLife International Technical Monograph no 2, 6th Edition. Revised May 2008. Catalogue of pesticide

(i) g/kg or g/L. Normally the rate should be given for the active substance (according to ISO) and not for the variant in order to compare the rate for same active substances used in different variants (e.g. fluoroxypyr). In certain cases, where only one variant is synthesised, it is more appropriate to give the rate for the variant (e.g. benthiavalicarb-isopropyl).
(j) Growth stage range from first to last treatment (BBCH Monograph, Growth Stages of Plants, 1997, Blackwell, ISBN 3-8263-3152-4), including where relevant, information on season at time of application

(f) All abbreviations used must be explained (g) Method, e.g. high volume spraying, low volume spraying, spreading, dusting, drench (h) Kind, *e.g.* overall, broadcast, aerial spraying, row, individual plant, between the plant- type of equipment used must be indicated	(k) Indicate the minimum and maximum number of applications possible under practical conditions of use (l) The values should be given in g or kg whatever gives the more manageable number (e.g. 200 kg/ha instead of 200 000 g/ha or 12.5 g/ha instead of 0.0125 kg/ha (m) PHI - minimum pre-harvest interval

RMS comment: The uses 1 and 3 are the same, only the MSs are different. They were kept separated for internal strategy from the notifier. It should be emphasized that black chokeberry (*Aronia melanocarpa*) and mountain ash (*Sorbus* sp.) have been applied for the Notifier. However, they are not included in the pome fruits group (see Commission Regulation 2018/62).

1.5.2 Further information on representative uses

Application Rate and Concentration of Active Substance

Grapes	1.6 – 4.0 litres product/hectare 52.8 - 132 g a.s./ha (Eugenol) 105.6 - 264 g a.s./ha (Geraniol) 105.6 - 264 g a.s./ha (Thymol)
Pome fruits	2.4 – 4.0 litres product/hectare 79.2 - 132 g a.s./ha (Eugenol) 158 - 264 g a.s./ha (Geraniol) 158 - 264 g a.s./ha (Thymol)

Dose rate for grapes: Apply 400 mL of Mevalone per 100 L water in an overall volume 400-1000 L water per hectare. DO NOT exceed a volume of 1000 L water per hectare (equivalent to a maximum of 4.0 L of Mevalone per hectare).
Dose rate for pome fruits: Apply 400 mL of Mevalone per 100 L water in an overall volume 600-1000 L water per hectare. DO NOT exceed a volume of 1000 L water per hectare (equivalent to a maximum of 4.0 L of Mevalone per hectare).
Mevalone is a CS formulation containing 3.2 % w/w eugenol, 6.4 % w/w thymol and 6.4 % w/w geraniol. The relative density of the formulation is 1.029 (nominal according to the recipe) at 20 ± 0.5°C. The content of eugenol is therefore, 33 g/L, content in geraniol and thymol is 66 g/L.

Grapes	0.132 g/L (Eugenol) 0.264 g/L (Geraniol) 0.264 g/L (Thymol)
Pome fruits	0.132 g/L (Eugenol) 0.264 g/L (Geraniol) 0.264 g/L (Thymol)

Method of Application

Method of application:	High volume broadcast spray
Type of equipment used:	Tractor-mounted/trailed boom or air blast sprayer with hydraulic nozzles or using hand held equipment (hydraulic or air blast knapsack sprayer)
Volume of diluent (water) per unit of area or volume	Grapes: 1.6 – 4.0 L product/ha in 400 – 1000 litres water. Pome fruits: 2.4 – 4.0 L product/ha in 600 – 1000 litres water.

For preventative control, a maximum of 4 applications can be made between growth stages BBCH 60-89 on grapes and BBCH 75-87 on pome fruits. Applications must be made at least 7 days apart and at the last application should not be made later than 7 days before harvest of grapes, and 1 days before harvest of pome fruits.
For curative control a maximum of 4 applications can be made between growth stages BBCH 60-89 on grapes and BBCH 75-87 on pome fruits. Applications must be made at least 7 days apart and at the last application should not be made later than 7 days before harvest of grapes, there is no pre-harvest interval on pome fruits. Apply weekly when mycelial growth and active sporulation is observed on post veraison fruit.
For preventative control application can be made before signs of infestation.
For curative control apply weekly when mycelial growth and active sporulation is observed on post veraison fruit.
Each application will provide protection for up to 14 days. Mevalone is best applied with multiple applications, as described above. The duration of control will then be up to harvest.

Number and Timings of Applications and Duration of Protection

Not applicable, grape vines and pome fruits are perennial crops not grown in rotation.

Proposed Instructions for Use

The representative formulation is already registered in the EU (please refer to Doc D2). Example of labels for some Member States are available in Doc C.

1.5.3 Details of other uses applied for to support the setting of MRLs for uses beyond the representative uses

Summary of additional intended uses , that in addition to the uses above, have also been considered to support the MRL application that accompanies this submission. Regulation (EC) N° 1107/2009 Article 8.1(g))

Important note: efficacy, environmental risk and risk to humans by exposure other than via their diet have not been assessed for these uses

Crop and/or situation (a)	Member State or Country	Product name	F G or I (b)	Pests or Group of pests controlled (c)	Preparation		Application				Application rate per treatment			PHI (days) (m)	Remarks
					Type (d-f)	Conc. a.s. (i)	method kind (f-h)	range of growth stages & season (j)	number min-max (k)	Interval between application (min)	g a.s /hL min-max (l)	Water L/ha min-max	g a.s./ha min-max (l)		
Grape vines	Northern residue zone	3AEY (Mevalone)	F	Botrytis [BOTRCI] Powdery mildew [UNCINE] Grey mould (*Botryotinia fuckeliana, Botrytis cinerea*) [BOTRCI]	CS	eugenol 33 g/L geraniol 66 g/L thymol 66 g/L	Tractor-mounted/ trailed boom or air blast sprayer. Hand-held knapsack sprayer.	BBCH 60-89	1-4	7	Per appl.: 13.2 (E) 26.4 (G) 26.4 (T)	400-1000	1 appl.: 52.8 - 132 (E) 105.6 - 264 (G) 105.6 - 264 (T) 4 appl.: 211.2 - 528 (E) 422.4 - 1056 (G) 422.4 - 1056 (T)	7	The concentration in g a.s./hL is kept constant – the higher application rate is diluted in the higher water volume. Preventative and curative control.

Crop and/or situation (a)	Member State or Country	Product name	F G or I (b)	Pests or Group of pests controlled (c)	Preparation		Application				Application rate per treatment			PHI (days) (m)	Remarks
					Type (d-f)	Conc. a.s. (i)	method kind (f-h)	range of growth stages & season (j)	number min-max (k)	Interval between application (min)	g a.s /hL min-max (l)	Water L/ha min-max	g a.s./ha min-max (l)		
Pome Fruit*	Southern residue zone	Mevalone	F	Post-harvest storage diseases* (*Phytophthora* spp. PHYTSP mainly *P. cactorum* PHYTCC or *P. syringae* PHYTSY, ALTESP, *Botrytis cinerea* BOTRCI)	CS	eugenol 33 g/L geraniol 66 g/L thymol 66 g/L	Tractor-mounted/ trailed boom or air blast sprayer. Hand-held knapsack sprayer.	BBCH 75-87	1-4	7	Per appl.: 13.2 (E) 26.4 (G) 26.4 (T)	600-1000	1 appl.: 79.2 - 132 (E) 158 -264 (G) 158 -264 (T) 4 appl.: 317 -528 (E) 634 - 1056 (G) 634 - 1056 (T)	3	The product is applied so that the concentration in g a.s./hL is kept constant at 13.2 (Eugenol), 26.4 (Geraniol), 26.4 (Thymol) g a.s / hectolitre of spray water volume. Therefore, the higher application rate is diluted in the higher water volume.

* Apple *Malus domestica* MABSD, pear *Pyrus communis* PYUCO, quince *Cydonia oblonga* CYDOB, crab-apple *Malus sylvestris* MABSY, loquat *Eryobotria japonica* EIOJA, medlar *Mespilus germanica* MSPGE, Nashi pear *Pyrus pyrifolia var. culta* PYUPC. **It should be emphasized that black chokeberry (*Aronia melanocarpa*) and mountain ash (*Sorbus* sp.) have been applied for the Notifier. However, they are not included in the pome fruits group (see Commission Regulation 2018/62).**

(a) For crops, the EU and Codex classifications (both) should be taken into account; where relevant, the use situation should be described (e.g. fumigation of a structure)
(b) Outdoor or field use (F), greenhouse application (G) or indoor application (I)
(c) *e.g.* biting and sucking insects, soil born insects, foliar fungi, weeds
(d) *e.g.* wettable powder (WP), emulsifiable concentrate (EC), granule (GR), capsule suspension (CS)
(e) CropLife International Technical Monograph no 2, 6th Edition. Revised May 2008. Catalogue of pesticide
(f) All abbreviations used must be explained
(g) Method, e.g. high volume spraying, low volume spraying, spreading, dusting, drench
(h) Kind, *e.g.* overall, broadcast, aerial spraying, row, individual plant, between the plant- type of equipment used must be indicated
(i) g/kg or g/L. Normally the rate should be given for the active substance (according to ISO) and not for the variant in order to compare the rate for same active substances used in different variants (e.g. fluoroxypyr). **In certain cases, where only one variant is synthesised, it is more appropriate to give the rate for the variant (e.g. benthiavalicarb-isopropyl).**
(j) Growth stage range from first to last treatment (BBCH Monograph, Growth Stages of Plants, 1997, Blackwell, ISBN 3-8263-3152-4), including where relevant, information on season at time of application
(k) Indicate the minimum and maximum number of applications possible under practical conditions of use
(l) The values should be given in g or kg whatever gives the more manageable number (e.g. 200 kg/ha instead of 200 000 g/ha or 12.5 g/ha instead of 0.0125 kg/ha
(m) PHI - minimum pre-harvest interval

1.5.4 Overview on authorisations in EU Member States

List of currently authorized uses and extent of use

Country	Trade name	A.S Content	Reg. Number	Registered uses
Albania	Mevalone	66 g/L	650	Grape (wine)
Albania	Mevalone	66 g/L	650	Grape (table)
Albania	Mevalone	66 g/L	650	Aubergine
Albania	Mevalone	66 g/L	650	Pomegranate
Albania	Mevalone	66 g/L	650	Spring onion
Albania	Mevalone	66 g/L	650	Kiwi
Bulgaria	Mevalone	66 g/L	01354 - PPP-1 / 15.02.2016	Grape (wine)
Bulgaria	Mevalone	66 g/L	01354 - PPP-1 / 15.02.2016	Grape (table)
Cyprus	Mevalone	66 g/L	3333	Grape (wine)
Cyprus	Mevalone	66 g/L	3333	Grape (table)
Cyprus	Mevalone	66 g/L	3333	Aubergine
Cyprus	Mevalone	66 g/L	3333	Pomegranate
Cyprus	Mevalone	66 g/L	3333	Spring onion
Cyprus	Mevalone	66 g/L	3333	Kiwi
Cyprus	Mevalone	66 g/L	3333	Tomato
Cyprus	Mevalone	66 g/L	3333	Olives
France	Mevalone	66 g/L	2161080	Grape (wine)
France	Mevalone	66 g/L	2161080	Grape (table)
France	Mevalone	66 g/L	2161080 (conclusions provided, waiting for final decision)	Pome Fruit
Greece	Mevalone	66 g/L	60467	Grape (wine)
Greece	Mevalone	66 g/L	60467	Grape (table)
Greece	Mevalone	66 g/L	60467	Aubergine
Greece	Mevalone	66 g/L	60467	Pomegranate
Greece	Mevalone	66 g/L	60467	Spring onion
Greece	Mevalone	66 g/L	60467	Kiwi
Greece	Mevalone	66 g/L	60467	Tomato
Greece	Mevalone	66 g/L	60467	Olives
Italy	3logy	66 g/L	16480	Grape (wine)
Italy	3logy	66 g/L	16480	Grape (table)
Italy	3logy	66 g/L	16480	Kiwi
Italy	3logy	66 g/L	16480	Strawberry and small fruits
Italy	3logy	66 g/L	16480	Pomegranate
Malta	Mevalone	66 g/L	2015-05-18 P02 (SZ)	Grape (wine)
Malta	Mevalone	66 g/L	2015-05-18 P02 (SZ)	Grape (table)
Portugal	Araw	66 g/L	1012	Grape (wine)
Portugal	Araw	66 g/L	1012	Grape (table)
Romania	Mevalone	66 g/L	684C	Grape (wine)
Spain	Araw	66 g/L	ES-00108	Grape (wine)
Spain	Araw	66 g/L	ES-00108	Grape (table)
Spain	Araw	66 g/L	ES-00108	Veg - numerous
Non EU Countries				
Australia	Novellus	66 g/L	87197/117745	Grape (wine)
Australia	Novellus	66 g/L	87197/117745	Grape (table)
FYROM	Mevalone	66 g/L	25-972/16	Grape (wine)

Country	Trade name	A.S Content	Reg. Number	Registered uses
FYROM	Mevalone	66 g/L	25-972/16	Grape (table)
FYROM	Mevalone	66 g/L	25-972/16	Aubergine
FYROM	Mevalone	66 g/L	25-972/16	Pomegranate
FYROM	Mevalone	66 g/L	25-972/16	Spring onion
FYROM	Mevalone	66 g/L	25-972/16	Kiwi
Kenya	Hawk	66 g/L	PCPB(CR) 1412	Snow Peas
Kenya	Hawk	66 g/L	PCPB(CR) 1412	Squash
Kenya	Hawk	66 g/L	PCPB(CR) 1412	French beans
Kenya	Hawk	66 g/L	PCPB(CR) 1412	Roses
Serbia	Mevalone	66 g/L	321-01-2092 / 2019-11	Grape (wine)
Serbia	Mevalone	66 g/L	321-01-2092 / 2019-11	Grape (table)

Level 2

EUGENOL

2 SUMMARY OF ACTIVE SUBSTANCE HAZARD AND OF PRODUCT RISK ASSESSMENT

Summary of methodology proposed by the applicant for literature review and for all sections

2.1 IDENTITY

2.1.1 Summary or identity

Eugenol technical source submitted in the renewal dossier is the same than the source included in DAR 2013.No new sources of TGAI are presented in this RAR. Details of the assessment of the identity is include in the Volume IV of this RAR. Method of manufacturer was described, **however notifier is called to detail the QC critical points for the manufacture of eugenol TGAI**, such as temperature, pressure and pH.

Minimum purity has been declared as 990 g/kg, purity of eugenol as manufactured is not relevant to a pilot plant.

The certified limits for the active substance are based on the value derived from the mean of the five-batch analysis study (EPP00510 McPherson, 2021) minus three standard deviations and rounded to 99%.

Eugenol technical grade active substance does not contain any additives.

Impurities present in eugenol at levels above 1 g/kg are included in the proposed specification for the active substance and are presented in Volume IV. Methyleugenol is a relevant impurity in eugenol. It is present at a maximum limit of 0.025 g/kg. However, the specification for methyleugenol is proposed at 0.1% w/w, in line with that agreed in the previous EU Review. At this level there will be no adverse effect on the toxicological profile of eugenol. Furfural and dichloromethane are theoretical relevant impurities. However, they were not found in any of the eugenol batches screened

2.2 PHYSICAL AND CHEMICAL PROPERTIES [EQUIVALENT TO SECTION 7 OF THE CLH REPORT TEMPLATE]

2.2.1 Summary of physical and chemical properties of the active substance

Table 1: Summary of physicochemical properties of the active substance

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20°C and 101,3 kPa	Pale straw coloured, oily liquid. Strong clove oil odour	White G.A., 2007 Report J16548 *B.2.3/01*	Visual examination Smelling
Melting/freezing point	Melting point: < - 40°C	White G.A., 2007 Report J16548 *B.2.1/01*	Measured
Boiling point	Boiling point: 254.70 °C	White G.A., 2007 Report J16548 *B.2.1/02*	Measured
Relative density	D^{20}_4 = 1.069 at 20 ± 0.5°C	White G.A., 2007 Report J16548 *B.2.14/01*	Measured
Vapour pressure	Vapour pressure: 2.7 Pa at 20°C	White G.A., 2007 Report J16548 *B.2.2/01*	Measured
Surface tension	As a pure product:	White G.A., 2007	Measured

<table>
<tr><th>Property</th><th>Value</th><th>Reference</th><th>Comment (e.g. measured or estimated)</th></tr>
<tr><td></td><td>36.6 mN/m at 20°C
35.2 mN/m at 40°C

<u>As a 90% saturated solution in water</u>:
41.2 mN/m at 20°C
41.5 mN/m at 40°C

Eugenol is surface active.</td><td>Report J16548
B.2.12/01</td><td></td></tr>
<tr><td>Water solubility</td><td>pH 4: 1.85 g/L
pH 7: 1.78 g/L
pH 9: 2.10 g/L
All measured at 20 ± 1°C</td><td>White G.A., 2007
Report J16548
B.2.5/01</td><td>Measured</td></tr>
<tr><td>Partition coefficient n-octanol/water</td><td><table><tr><td>pH</td><td>4</td><td>7</td><td>9</td></tr><tr><td>Log_{10} P_{ow}</td><td>2.47</td><td>2.49</td><td>2.44</td></tr></table>All measured at 21.5 ± 0.5°C.</td><td>White G.A., 2007
Report J16548
B.2.7/02</td><td>Measured</td></tr>
<tr><td>Henry’s law constant</td><td>0.24 $Pa.m^3$ mol^{-1} at 20°C</td><td>White G.A., 2007
Report J16548
B.2.2/02</td><td>Measured</td></tr>
<tr><td>Flash point</td><td>Flash point = 127°C</td><td>White G.A., 2007
Report J16548
B.2.10/01</td><td>Measured</td></tr>
<tr><td>Flammability</td><td>Not flammable. (FP>60°C)</td><td>White G.A., 2007
Report J16548
B.2.9/01</td><td>Measured</td></tr>
<tr><td>Explosive properties</td><td>Not explosive</td><td>White G.A., 2007
Report J16548
B.2.11/01</td><td>Measured</td></tr>
<tr><td>Self-ignition temperature</td><td>Autoflammability: 376°C</td><td>White G.A., 2007
Report J16548
B.2.9/02</td><td>Measured</td></tr>
<tr><td>Oxidising properties</td><td>Not oxidizing.</td><td>White G.A., 2007
Report J16548
B.2.13/01</td><td>Expert assessment</td></tr>
<tr><td>Granulometry</td><td>Not Applicable</td><td>NA</td><td>NA</td></tr>
<tr><td>Solubility in organic solvents and identity of relevant degradation products</td><td>n-Heptane: >250 g/L
p-Xylene: >250 g/L
1,2-Dichloroethane: >250 g/L
Methanol: >250 g/L
Acetone: >250 g/L
Ethyl acetate: >250 g/L

All measured at 20 ± 1°C</td><td>White G.A., 2007
Report J16548
B.2.6/01</td><td>Measured</td></tr>
<tr><td>Dissociation constant</td><td>pKa = 10.27</td><td>White G.A., 2007
Report J16548</td><td>Measured</td></tr>
</table>

Property	Value	Reference	Comment (e.g. measured or estimated)
		B.2.8/01	
Viscosity	No data available		
Spectra (UV/VIS, IR, NMR, MS), molar extinction at relevant wavelengths, optical purity	UV/Vis spectrum is typical of Eugenol. UV/Vis: no absorption > 290 nm in acidic and neutral conditions. Minor absorption at 297 nm under alkaline conditions (ε= 3780; maximum absorbance = 0.118).	White G.A., 2007 Report J16548 *B.2.4/01*	Measured
	IR spectrum is typical of Eugenol.	White G.A., 2007 Report J16548 *B.2.4/02*	Measured
	NMR spectrum is typical of Eugenol.	White G.A., 2007 Report J16548 *B.2.4/03*	Measured
	MS spectrum is typical of Eugenol.	White G.A., 2007 Report J16548 *B.2.4/04*	Measured

2.2.1.1 Evaluation of physical hazards [equivalent to section 8 of the CLH report template]

2.2.1.1.1 Explosives *[equivalent to section 8.1 of the CLH report template]*

Table 2: Summary table of studies on explosive properties

Method	Results	Remarks	Reference
ASTM ES37-02 (DSC)	According to Differential scanning Calorimetry (DSC) graphs, no exothermic reaction was observed in the temperature range from 30ºC to 400ºC	. DSC is not a standard technique for assessing explosive properties according to CLP criteria.	White G.A., 2007 Report number J16548

2.2.1.1.1.1 Short summary and overall relevance of the provided information on explosive properties

According to Differential Scanning Calorimetry (DSC) graphs, no exothermic reaction was observed in the temperature range from 30°C to 400°C with eugenol. However, DSC is not a standar technique for assessing explosive properties according to CLP Regulation.

2.2.1.1.1.2 Comparison with the CLP criteria

According to Differential Scanning Calorimetry (DSC) graphs, no exothermic reaction was observed in the temperature range from 30°C to 400°C for eugenol. However, this test method is not comparable to the test procedures for the classification of explosives described in Part I of the UN RTDG, Manual of Tests and Criteria.
According to point 2.1.4.3 of Annex I of CLP Regulation, eugenol contains an olephinic group (C-C unsaturation) which is a chemical group associated with explosive properties as specified in Table A6.1 in Appendix 6 of the UN Recommendations on Transport of Dangerous Goods (RTDG), Manual of Tests and Criteria. However,the calculated Oxygen Balance of eugenol was

determined as being - 233.6 which is less than the threshold limit of - 200. Therefore, eugenol does not meet the criteria for classification as an explosive substance.

2.2.1.1.1.3 Conclusion on classification and labelling for explosive properties
No classification is required for this hazard class according to CLP criteria.

2.2.1.1.2 Flammable gases (including chemically unstable gases) *[equivalent to section 8.2 of the CLH report template]*

Table 3: Summary table of studies on flammable gases (including chemically unstable gases)

Method	Results	Remarks	Reference
Not applicable			

2.2.1.1.2.1 Short summary and overall relevance of the provided information on flammable gases (including chemically unstable gases)
Not applicable. The active substance is not a gas.

2.2.1.1.2.2 Comparison with the CLP criteria
Not applicable.

2.2.1.1.2.3 Conclusion on classification and labelling for flammable gases
Not applicable
.

2.2.1.1.3 Oxidising gases *[equivalent to section 8.3 of the CLH report template]*

Table 4: Summary table of studies on oxidising gases

Method	Results	Remarks	Reference
Not applicable			

2.2.1.1.3.1 Short summary and overall relevance of the provided information on oxidising gases
Not applicable. The active substance is not a gas.

2.2.1.1.3.2 Comparison with the CLP criteria
Not applicable

2.2.1.1.3.3 Conclusion on classification and labelling for oxidising gases
Not applicable

2.2.1.1.4 Gases under pressure *[equivalent to section 8.4 of the CLH report template]*

Table 5: Summary table of studies on gases under pressure

Method	Results	Remarks	Reference
Not applicable			

2.2.1.1.4.1 Short summary and overall relevance of the provided information on gases under pressure
Not applicable. The active substance is not a gas.

2.2.1.1.4.2 Comparison with the CLP criteria
Not applicable.

2.2.1.1.4.3 Conclusion on classification and labelling for gases under pressure
Not applicable.

2.2.1.1.5 Flammable liquids *[equivalent to section 8.5 of the CLH report template]*

Table 6: Summary table of studies on flammable liquids

Method	Results	Remarks	Reference
ASTM E537 (DSC method)	Boiling point: 254.70°C	> 35°C	White 2007, Report number J16548
EEC A9 (closed cup)	Not flammable. Flash point: 127°C	FP>60°C	White G.A., 2007 Report number J16548

2.2.1.1.5.1 Short summary and overall relevance of the provided information on flammable liquids
The boiling point and flash point were determined. Eugenol is unlikely to be flammable.

2.2.1.1.5.2 Comparison with the CLP criteria
No flammability classification required according to CLP criteria. The flash point of eugenol was determined as being 127°C which is more than the threshold limit of 60°C. Moreover, the substance is not a halogenated substance. Therefore eugenol should not be classified as flammable liquid.

2.2.1.1.5.3 Conclusion on classification and labelling for flammable liquids
No flammability classification required according to CLP criteria.

2.2.1.1.6 Flammable solids *[equivalent to section 8.6 of the CLH report template]*

Table 7: Summary table of studies on flammable solids

Method	Results	Remarks	Reference
Not applicable.			

2.2.1.1.6.1 Short summary and overall relevance of the provided information on flammable solids
Not applicable. The active substance is not a solid.

2.2.1.1.6.2 Comparison with the CLP criteria
Not applicable.

2.2.1.1.6.3 Conclusion on classification and labelling for flammable solids
Not applicable.

2.2.1.1.7 Self-reactive substances *[equivalent to section 8.7 of the CLH report template]*

Table 8: Summary table of studies on self-reactivity

Method	Results	Remarks	Reference
DSC, ASTM E537-02	According to Differential Scanning Calorimetry (DSC) graphs, no exothermic reaction was observed in the temperature range from 30°C to 400°C. Oxygen Balance of eugenol was determined as being - 233.6.		White G.A., 2007 Report number J16548
EEC A15	Autoflammability: 376°C	Unlikely to self-ignite under normal storage conditions.	White G.A., 2007 Report number J16548

2.2.1.1.7.1 Short summary and overall relevance of the provided information on self-reactive substances
A test according to UN test series as noted in CLP criteria is not available. The followng information relevant for this hazard class is provided:
According to Differential Scanning Calorimetry (DSC) graphs, no exothermic reaction was observed in the temperature range from 30°C to 400°C.
According to the EEC A15 eugenol is not autoinflammable.

2.2.1.1.7.2 Comparison with the CLP criteria

According to CLP point 2.8.4.2 of Annex I of CLP Criteria, the hazard class does not apply if there are no chemical groups in the molecule associated with explosive or self reactive properties. Howevere eugenol contains an olephinic group (unsaturation) which is a chemical group that indicates self-reactive properties as specified in Table A6.3 in Appendix 6 of the UN Recommendations on Transport of Dangerous Goods (RTDG), Manual of Tests and Criteria. Consequently self-reactivity of eugenol cannot be discarded considering chemical groups in the substance.
Additionally, for a single organic substance such as eugenol, if the the estimated SADT for a 50 kg package is greater than 75 ºC or the exothermic decomposition energy is less than 300 J/g the substance does not need to be classify. The decomposition energy for eugenol has been calculated using DSC, a suitable technique according to CLP criteria as established in point 2.8.4.2.b) of Annex I of CLP criteria, with no exothermic reaction oberverd in the range of temperatre from 30 ºC to 400 ºC. Consequenlty, classification as self-reactive substance is not required for eugenol.

2.2.1.1.7.3 Conclusion on classification and labelling for self-reactive substances
Conclusive but not sufficient for classification.

2.2.1.1.8 Pyrophoric liquids *[equivalent to section 8.8 of the CLH report template]*

Table 9: Summary table of studies on pyrophoric liquids

Method	Results	Remarks	Reference
No data available	--	--	--

2.2.1.1.8.1 Short summary and overall relevance of the provided information on pyrophoric liquids
No suitable test data are available for eugenol to evaluate this hazard class. Experience in manufacture and handling shows that the substance eugenol does not ignite spontaneously on coming into contact with air at normal temperature.

2.2.1.1.8.2 Comparison with the CLP criteria
Experience in manufacture and handling shows that the substance eugenol does not ignite spontaneously on coming into contact with air at normal temperature.

2.2.1.1.8.3 Conclusion on classification and labelling for pyrophoric liquids
Eugenol is not a pyrophoric liquid. No classification for this hazard class is proposed.

2.2.1.1.9 Pyrophoric solids *[equivalent to section 8.9 of the CLH report template]*

Table 10: Summary table of studies on pyrophoric solids

Method	Results	Remarks	Reference
Not applicable.			

2.2.1.1.9.1 Short summary and overall relevance of the provided information on pyrophoric solids
Not applicable. The active substance is not a solid.

2.2.1.1.9.2 Comparison with the CLP criteria
Not applicable.

2.2.1.1.9.3 Conclusion on classification and labelling for pyrophoric solids
Not applicable.

2.2.1.1.10 Self-heating substances *[equivalent to section 8.10 of the CLH report template]*

Table 11: Summary table of studies on self-heating substances

Method	Results	Remarks	Reference
DSC, ASTM E537-02	According to Differential Scanning Calorimetry (DSC) graphs, no exothermic reaction was observed in the temperature range from 30°C to 400°C. Oxygen Balance of eugenol was determined as being - 233.6.		White G.A., 2007 Report number J16548
DSC, ASTM E537-02	Melting point:< - 40°C		White G.A., 2007 Report number J16548
EEC A15	Autoflammability: 376°C	Unlikely to self-ignite under normal storage conditions.	White G.A., 2007 Report number J16548

2.2.1.1.10.1 Short summary and overall relevance of the provided information on self-heating substances

Eugenol is a liquid and unlikely to be self-heating and the test for pyrophoricity according to UN Test N.4 described in Part III, Section 33 of the UN Recommendations on the Transport of Dangerous Goods, Manual of Tests and Criteria should not be performed.

2.2.1.1.10.2 Comparison with the CLP criteria

Eugenol is a liquid, has an autoflammability temperature of 376°C and according to Differential Scanning Calorimetry (DSC) graphs, no exothermic reaction was observed in the temperature range from 30°C to 400°C. The surface of liquids is not large enough for reaction with air and the test method is not applicable to liquids. Therefore liquids as eugenol are not classified as self-heating.

2.2.1.1.10.3 Conclusion on classification and labelling for self-heating substances

Eugenol is not a self-heating substance.No auto-flammability classification required according to CLP criteria.

2.2.1.1.11 Substances which in contact with water emit flammable gases *[equivalent to section 8.11 of the CLH report template]*

Table 12: Summary table of studies on substances which in contact with water emit flammable gases

Method	Results	Remarks	Reference
No data			

2.2.1.1.11.1 Short summary and overall relevance of the provided information on substances which in contact with water emit flammable gases

No suitable data to evaluate this hazard class are available (UN test series N.5). Eugenol is a natural substance that is naturally synthesised in plants including high-water content fruits such as grapes. Eugenol does not emit flammable gases when in contact with water.

Moreover water solubility determination, experience in manufacture and handling shows that the substance eugenol does not emit flammable gases on coming into contact with water at normal temperature.

2.2.1.1.11.2 Comparison with the CLP criteria

According to CLP point 2.12.4.1 of Annex I of CLP Criteria, the hazard class does not apply as the chemical structure of eugenol does not contain metal or metalloids, experience in production or handling shows that the subtance does not react with water and eugenol is known to be soluble in water (water solubility value of 1.78 g/L at pH 7). Therefore, eugenol does not meet the criteria for classification for this hazard class.

2.2.1.1.11.3 Conclusion on classification and labelling for substances which in contact with water emit flammable gases

No classification is proposed for this hazard class.

2.2.1.1.12 Oxidising liquids *[equivalent to section 8.12 of the CLH report template]*

Table 13: Summary table of studies on oxidising liquids

Method	Results	Remarks	Reference
EEC A17 and scientific reasoned case	Not oxidizing. The molecular structure of eugenol does not contain any oxidising groups.	The molecule does not contain fluorine or chlorine, and only contains oxygen atoms bonded to carbon. Also the structure is not a peroxide, therefore subsequent classification does not apply (column 2 of Annex VII to REACH).	White G.A., 2007 Report number J16548

2.2.1.1.12.1 Short summary and overall relevance of the provided information on oxidising liquids

Eugenol is unlikely to be oxidising according to the method EEC A17.

The required test according to CLP Regulation is not available: UN Test O.2 as described in Part III, Section 34 of the UN Recommendations on the Transport of Dangerous Goods, Manual of Tests.

2.2.1.1.12.2 Comparison with the CLP criteria

Eugenol is unlikely to be oxidising according to the method EEC A17. According to Section 2.13.4.1 of Annex I of CLP Regulation, eugenol is not an oxidising liquid as it does not contain chlorine or fluorine and it contains oxygen atoms bonded only to carbon and hydrogen. Therefore, eugenol does not meet the criteria for classification for this hazard class.

2.2.1.1.12.3 Conclusion on classification and labelling for oxidising liquids

Eugenol is not an oxidising liquid. No classification is required for this hazard class according to CLP criteria.

2.2.1.1.13 Oxidising solids *[equivalent to section 8.13 of the CLH report template]*

Table 14: Summary table of studies on oxidising solids

Method	Results	Remarks	Reference
Not applicable			

2.2.1.1.13.1 Short summary and overall relevance of the provided information on oxidising solids

Not applicable. The active substance is not a solid.

2.2.1.1.13.2 Comparison with the CLP criteria

Not applicable.

2.2.1.1.13.3 Conclusion on classification and labelling for oxidising solids

Not applicable.

2.2.1.1.14 Organic peroxides *[equivalent to section 8.14 of the CLH report template]*

Table 15: Summary table of studies on organic peroxides

Method	Results	Remarks	Reference
Not applicable			

2.2.1.1.14.1 Short summary and overall relevance of the provided information on organic peroxides

Not applicable. Eugenol structure is not of a peroxide.

2.2.1.1.14.2 Comparison with the CLP criteria

Not applicable.

2.2.1.1.14.3 Conclusion on classification and labelling for organic peroxides
Not applicable.

2.2.1.1.15 Corrosive to metals *[equivalent to section 8.15 of the CLH report template]*

Table 16: Summary table of studies on the hazard class corrosive to metals

Method	Results	Remarks	Reference
No data			

2.2.1.1.15.1 Short summary and overall relevance of the provided information on the hazard class corrosive to metals
No data derived in accordance with the recommended test method in CLP (test in Part III, sub-section 37.4 of the UNRTDG Manual of Tests and Criteria) are available. Eugenol contains no functional groups that are either acidic or alkaline. Eugenol does not dissociate at environmental pHs and eugenol has very limited water solubility (< 2.4 g/L at pH 4, 7 and 9).
Eugenol is unlikely to be corrosive to metal and the test for metal corrosive properties according to UN Test C.1 of the UN Recommendations on the Transport of Dangerous Goods, Manual of Tests and Criteria should not be performed.

2.2.1.1.15.2 Comparison with the CLP criteria
No data derived following the UN test C.1 are available for eugenol. According to the ECHA Guidance on the Application of the CLP Criteria (version 5.0 July 2017), properties such as chemical nature of the substances (e.g. strong acids) and pH values may be helpful to evaluate corrosive properties. Eugenol contains no functional groups that are either acidic or alkaline. Eugenol does not dissociate at environmental pHs and eugenol has very limited water solubility (< 2.4 g/L at pH 4, 7 and 9). Therefore, eugenol is not expected to be corrosive to metals.

2.2.1.1.15.3 Conclusion on classification and labelling for corrosive to metals
No classification is proposed for this hazard class.

2.2.2 Summary of physical and chemical properties of the plant protection product

Mevalone (3AEY) is a capsule suspension containing geraniol (66 g/L), thymol (66 g/L) and eugenol (33 g/L). Physical characteristics of Mevalone correspond to that of a capsule suspension. Mevalone is a dark cream/beige viscous liquid which is not explosive, not oxidising and non-flammable up to temperatures of 400°C. It has a pH of around 5.8 when in a 1% suspension. Stability data indicate a shelf-life of at least 2 years at ambient temperature.

The intended concentration of use is 0.4 L Mevalone per 100 L of spray, equivalent to 0.4% v/v.

2.3 DATA ON APPLICATION AND EFFICACY

2.3.1 Summary of effectiveness

Terpene compounds such as eugenol, geraniol and thymol generally possess antifungal activity, having effects on spore germination, hyphal penetration, mycelial growth and hyphal growth.

All terpene compounds are reported to have direct effects on cell walls, membranes, which is associated with the capability of the compounds to dissolve lipids and results in leakage of cellular substances leading to cell death. Studies have confirmed that cyclic terpene hydrocarbons accumulate in the cell membrane causing a loss of membrane integrity, with associated changes in composition of fatty acids and phospholipids. This is thought to occur as a result of lesion formation in the cytoplasmic membrane with reductions in ergosterol content due to the disruption of biosynthesis.

Due to these effects on membranes, there is also thought to be an impact on processes involving ATP and active transport of molecules across membranes, leading to depletion of the ATP pool and leakage of cellular substances, with impairment of energy metabolism. Mitochondrial structure disorganization may occur and the effects on membranes have been shown to cause partial dissipation of the pH gradient and electrical potential.

Terpenes have also been observed to cause changes in the hyphal wall. Some effects on enzyme activity have also been reported, including interference with respiratory enzymes and enzymes responsible for cell wall synthesis. There is also evidence to suggest that the synthesis of genetic material is affected.

For the uses grapes/BOTRCI and UNCINE, the representative formulation, MEVALONE, is currently commercially available and supported by efficacy data evaluated under Uniform Principles for national registrations.

Regarding pome fruits/postharvest storage diseases (PHYTSP, ALTESP and BOTRCI), currently, this use is not registered, however, it is considered that the GAP is realistic from an efficacy point of view considering the studies provided by the applicant (studies submitted for new registration in Central zone in July 2021, for further details, see MCP6 doc). The conclusions on the demonstration of the effectiveness are left to subsequent product dossiers assessed under Uniform Principles, nevertheless, in a first approach, "*in nearly all trials, the efficacy level of all treatment was low to moderate. This can be explain by the fact that no post harvest treatment was performed. However, in most trials, some fungicide programs including MEVALONE performed better than the reference. MEVALONE applied alone performed quite poorly and it is therefore important to include it in a fungicide program (from orchard to post harvest treatments)*."

2.3.2 Summary of information on the development of resistance

Eugenol is a contact action fungicide. It prevents the development of fungal mycelium from spores or destroys existing mycelium by a direct action on the cell membranes. Due to the mode of action, no problems with resistance or cross-resistance are expected. Eugenol, is a plant extract included in the terpene alcohols chemical group, classified by FRAC into plant oils, FRAC codes F7: cell membrane disruption /46, with resistance not known.

2.3.3 Summary of adverse effects on treated crops

Due to eugenol's mode of action, no adverse effects on field crops are expected. Phytotoxic symptoms were regularly checked in all trials provided on grapes and on apples, and MEVALONE demonstrated a high crop safety.
MEVALONE formulation has been applied in various EU member states for many years without reports of adverse effects on treated crops. Available efficacy used to obtain registration of the representative formulation in various countries shows the absence of phytotoxicity when the product is used according to the GAP. Consequently, no negative impact is expected on treated crops when used according to recommendations.

2.3.4 Summary of observations on other undesirable or unintended side-effects

There is no evidence of any undesirable or unintended side-effects.

2.4 FURTHER INFORMATION

2.4.1 Summary of methods and precautions concerning handling, storage, transport or fire

The applicant has proposed:

a) Handling:
Avoid contact with skin and eyes. Avoid inhalation of vapour or mist. Normal measures for preventive fire protection.

b) Storage:
Keep container tightly closed in a dry and well-ventilated place Containers which are opened must be carefully resealed and kept upright to prevent leakage.

c) Transportation:
Not classified as hazardous for transport.

d) Fire:
Not flammable or combustible.
Extinguishing media: Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.
Fire fighting: Wear self-contained breathing apparatus for firefighting if necessary.
Fire and explosion hazards: Hazardous decomposition products formed under fire conditions. - Carbon oxides.

2.4.2 Summary of procedures for destruction or decontamination

Product: Offer surplus and non-recyclable solutions to a licensed disposal company. Contact a licensed professional waste disposal service to dispose of this material. Dissolve or mix the material with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber.
Contaminated packaging: Dispose of as unused product.

2.4.3 Summary of emergency measures in case of an accident

a) Personal Precautions:
Use personal protective equipment. Avoid breathing vapors, mist or gas. Ensure adequate ventilation. Evacuate personnel to safe areas.
b) Environmental Precautions:
Prevent further leakage or spillage if safe to do so. Do not let product enter drains. Discharge into the environment must be avoided.
c) Methods of Cleaning Up:
Soak up with inert absorbent material and dispose of as hazardous waste. Keep in suitable, closed containers for disposal.

2.5 METHODS OF ANALYSIS

2.5.1 Methods used for the generation of pre-authorisation data

2.5.1.1. Analysis of the active substance as manufactured

Eugenol content is determined in the Eugenol technical material after dilution in acetone using GC-FID, employing acetanilide as an internal standard.

2.5.1.2. Formulation analysis

- **Eugenol**

Total eugenol is determined and quantified in 3AEY after dilution in methanol using gas chromatography with a flame-ionisation detector and external standards.
Free eugenol is determined and quantified in 3AEY after extraction in hexane using gas chromatography with a flame-ionisation detector and 1-nonanol as internal standard.

- **Methyleugenol**

Methyleugenol is determined and quantified in 3AEY after dilution in methanol using gas chromatography with a MS detector and external standards, with a Limit of Quantification of 16.8 mg/kg. 1-nonanol can be used as internal standard with a Limit of Quantification of 1 mg/kg.

2.5.1.3. Methods for risk assessment

2.5.1.3.1. Plants and plant products

Grapes:

The Limit of Quantification was set at 0.01 mg/kg.

- **Eugenol**

For total eugenol, samples are homogenised and mixed with acetone. Homogenised samples are dried over anhydrous sodium sulphate and centrifuged, then filtrated prior to quantification by GC-MS (monitored ions: 164 m/z, 149 m/z and 131 m/z). Ethyl acetate is also a suitable extraction solvent. In the most recent studies, extraction is performed using acetonitrile and solid-phase extraction salts for clean-up followed by centrifugation, evaporation under nitrogen and reconstitution of the extract in acetonitrile prior quantification by GC-MS (monitored ions: 164 m/z, 149 m/z and 133 m/z).
For free eugenol, samples are de-stalked but not homogenised prior to extraction using acetone followed by filtration and quantification by GC-MS (monitored ions: 164 m/z, 149 m/z and 131 m/z).

- **Methyleugenol**

Methyleugenol is extracted from homogenised samples using acetonitrile and solid-phase extraction salts for clean-up followed by centrifugation, evaporation under nitrogen and reconstitution of the extract in acetonitrile prior quantification by GC-MS (monitored ions: 178 m/z, 163 m/z and 147 m/z)

Apples:

The Limit of Quantification was set at 0.01 mg/kg.

- **Eugenol**

Homogenized apple samples are extracted in acetonitrile, cleaned up using solid phase extraction and concentrated under nitrogen prior to quantification by GC-MS (monitored ions: 164 m/z, 149 m/z and 133 m/z).

- **Methyleugenol**

Homogenized apple samples are extracted in acetonitrile, cleaned up using solid phase extraction and concentrated under nitrogen prior to quantification by GC-MS (monitored ions: 178 m/z, 163 m/z and 147 m/z).

2.5.1.3.2. Food of animal origin

There are no methods available for the quantification of eugenol in food of animal origin.

2.5.1.3.3. Soil

- **Eugenol**

The DT_{90} of eugenol in soil has been found to be < 3 days therefore, a method for the quantification of residues in soil is not required for eugenol.

- **Methyleugenol**

The DT_{90} of eugenol in soil has been found to be < 3 days therefore, a method for the quantification of residues in soil is not required for methyleugenol.

2.5.1.3.4. Water

Medium used in fish toxicity studies:
Samples of fish medium are diluted in acetonitrile and analysed directly by GC-MS. The lowest Limit of Quantification validated was 0.163 mg/L for eugenol in fish test medium.
Medium used in daphnia toxicity studies:
Samples of daphnia medium are diluted in acetonitrile and analysed directly by GC-MS. The lowest Limit of Quantification validated was 0.163 mg/L for eugenol in daphnia test medium.
Eugenol is extracted with toluene and final determination was performed by GC-MS, monitoring three ions of m/z >100. The Limit of Quantification with this new method was 3.5 µg/L for eugenol in aqueous test medium.
Medium used in algal toxicity studies:
Samples of fish medium are diluted in acetonitrile and analysed directly by GC-MS. The Limit of Quantification was 0.323 mg/L for eugenol in algae test medium.
Water stock solution in ecotoxicological studies:
Eugenol is extracted from test medium (sugar feeding solution and water stock solution) with acetonitrile prior to analysis by HPLC-DAD. The Limit of Quantification was 100.6 mg/L in water stock solution.
Water in physicochemical studies:
The contents of eugenol in aqueous solutions were determined by HPLC with UV detection after dilution in acetonitrile.

2.5.1.3.5. Air

- **Eugenol**

Eugenol is quantified in air sampling cartridges (pre-packed XAD-2 cartridges) by sonication with ethyl acetate and final determination was performed by GC-MS (three ions monitored). The Limit of Quantification was 1.2 $\mu g/m^3$ for eugenol content in air.

- **Methyleugenol**

Methyleugenol is quantified in air sampling cartridges (pre-packed XAD-2 cartridges) by sonication with ethyl acetate and final determination was performed by GC-MS (three ions monitored). The Limit of Quantification was 1.2 $\mu g/m^3$ for methyleugenol content in air.

2.5.2 Methods for post control and monitoring purposes

2.5.2.1 Plants and plant products

Grapes:
The Limit of Quantification was set at 0.01 mg/kg.

- **Eugenol**

For total eugenol, samples are homogenised and mixed with acetone. Homogenised samples are dried over anhydrous sodium sulphate and centrifuged, then filtrated prior to quantification by GC-MS (monitored ions: 164 m/z, 149 m/z and 131 m/z). Ethyl acetate is also a suitable extraction solvent. In the most recent studies, extraction is performed using acetonitrile and solid-phase extraction salts for clean-up followed by centrifugation, evaporation under nitrogen and reconstitution of the extract in acetonitrile prior quantification by GC-MS (monitored ions: 164 m/z, 149 m/z and 133 m/z).
For free eugenol, samples are de-stalked but not homogenised prior to extraction using acetone followed by filtration and quantification by GC-MS (monitored ions as above).

- **Methyleugenol**

Methyleugenol is extracted from homogenised samples using acetonitrile and solid-phase extraction salts for clean-up followed by centrifugation, evaporation under nitrogen and reconstitution of the extract in acetonitrile prior quantification by GC-MS (monitored ions: 178 m/z, 163 m/z and 147 m/z).

Apples:
The Limit of Quantification was set at 0.01 mg/kg.

- **Eugenol**

Homogenized apple samples are extracted in acetonitrile, cleaned up using solid phase extraction and concentrated under nitrogen prior to quantification by GC-MS (monitored ions: 164 m/z, 149 m/z and 133 m/z).

- **Methyleugenol**

Homogenized apple samples are extracted in acetonitrile, cleaned up using solid phase extraction and concentrated under nitrogen prior to quantification by GC-MS (monitored ions: 178 m/z, 163 m/z and 147 m/z).

2.5.2.2 Food of animal origin

There are no methods available for the quantification of eugenol in food of animal origin.

2.5.2.3 Soil

- **Eugenol**

The DT_{90} of eugenol in soil has been found to be < 3 days therefore, a method for the quantification of residues in soil is not required for eugenol.

- **Methyleugenol**

The DT_{90} of methyleugenol in soil has been found to be < 3 days therefore, a method for the quantification of residues in soil is not required for methyleugenol.

2.5.2.4 Water

- **Eugenol**

Eugenol is quantified by direct injection of water samples by LC-MS/MS (two chromatographic methods used). The Limit of Quantification was 0.1 µg/L for eugenol in surface water.

- **Methyleugenol**

The methyleugenol content is extracted in surface water via steam distillation and quantified by GC-MS (three ions monitored). The Limit of Quantification was 0.1 µg/L for methyleugenol in surface water.

2.5.2.5 Air

- **Eugenol**

Eugenol is quantified in air sampling cartridges (pre-packed XAD-2 cartridges) by sonication with ethyl acetate and final determination was performed by GC-MS (three ions monitored). The Limit of Quantification was 1.2 µg/m^3 for eugenol content in air.

- **Methyleugenol**

Methyleugenol is quantified in air sampling cartridges (pre-packed XAD-2 cartridges) by sonication with ethyl acetate and final determination was performed by GC-MS (three ions monitored). The Limit of Quantification was 1.2 µg/m^3 for methyleugenol content in air.

2.5.2.6 Body fluids and tissues

- **Eugenol**

Samples of plasma and urine are extracted in acetonitrile, cleaned up using solid phase extraction followed by primary secondary amine prior to quantification by GC-MS (monitored ions: 164 m/z, 149 m/z and 133 m/z). The Limit of Quantification was set at 0.01 mg/L for both plasma and urine.
Homogenized samples of meat and liver are extracted in acetonitrile, cleaned up using solid phase extraction followed by primary secondary amine prior concentration and quantification by GC-MS (monitored ions: 164 m/z, 149 m/z and 133 m/z). The Limit of Quantification was set at 0.01 mg/kg for both meat and liver.

- **Methyleugenol**

Samples of plasma and urine are extracted in acetonitrile, cleaned up using solid phase extraction followed by primary secondary amine prior to quantification by GC-MS (monitored ions: 178 m/z, 163 m/z and 147 m/z). The Limit of Quantification was set at 0.01 mg/L for both plasma and urine.
Homogenized samples of meat and liver are extracted in acetonitrile, cleaned up using solid phase extraction followed by primary secondary amine (for liver) prior concentration and quantification by GC-MS (monitored ions: 178 m/z, 163 m/z and 147 m/z). The Limit of Quantification was set at 0.01 mg/kg for both meat and liver.

2.6 EFFECTS ON HUMAN AND ANIMAL HEALTH

2.6.1 Summary of absorption, distribution, metabolism and excretion in mammals *[equivalent to section 9 of the CLH report template]*

Table 17: Summary table of toxicokinetic studies

<table>
<tr><th>Method</th><th>Results / Remarks</th><th>Reference</th></tr>
<tr><td>Absorption, excretion and metabolism in vivo (single dose)

No guidance

Human volunteers (4♂,4♀)

Single oral dose 150 mg eugenol (purity not stated)

Urine collected (over 24h period)

GLP: No

Supporting information</td><td><u>Absorption & Excretion</u>:
Relatively rapid and almost complete based on urinary excretion
An average 94.8 % eliminated in urine after 24 h post-dosing.

<u>Metabolic profile</u>:
> 55% applied dose was excreted as eugenol conjugates (glucuronide/sulphate)
7% applied dose was excreted as conjugated cis/trans isoeugenol (metabolites II and III)
11% applied dose was excreted as a thiolphenol derivative (metabolite X): mercapto-4-hydroxy-3-methoxyphenyl-propane
13% applied dose was excreted as epoxide-diol pathway derivatives (metabolites XII and XI)
5% applied dose was excreted as a propionic acid derivative (metabolite IX) via the allylic oxidation pathway</td><td>Fischer, I.U. et al.
(1990)
(AS)
B.6.1.1.1</td></tr>
<tr><td>Metabolism and excretion in vivo (single dose)

Pre-guidance

Wistar albino rats (♀)

Single oral doses (stomach tube) of 0.5, 5, 50 and 1000 mg/kg bw

Eugenol, purity/batch no not stated

Urine and faeces collected for 3 days

Urinary metabolites analysed

GLP: No

Supporting information</td><td><u>Excretion</u>:
Urinary excretion ranged between 75-80 % total administered dose
10 % excreted in faeces

<u>Metabolic profile</u>:
Eugenol conjugates (glucuronide/sulphate) corresponded to ~ 50 % applied dose
Major metabolite 3,4-dihydroxypropyl benzene excreted as conjugate (glucuronide/sulphate)
Minor 4-Hydroxy-3-methoxypropyl benzene
10% applied dose unidentified

Sulphate conjugation predominant at lower doses vs glucuronidation at high doses.

<table><tr><th colspan="4">% Radioactivity in 0-24h urine</th></tr><tr><th>Dose (mg/kg)</th><th>Eugenol conjugates</th><th>3,4-dihydroxypropyl benzene</th><th>4-Hydroxy-3-methoxypropyl benzene</th></tr><tr><td>0.5</td><td>50</td><td>15</td><td>3</td></tr><tr><td>5</td><td>60</td><td>5</td><td>1</td></tr><tr><td>50</td><td>55</td><td>15</td><td>1</td></tr><tr><td>1000</td><td>60</td><td>Not determined</td><td>Not determined</td></tr></table></td><td>Sutton, J.D. et al.
(1985)
(AS)
B.6.1.1.2</td></tr>
<tr><td>Metabolism and excretion in vivo (single dose) and in vitro

Pre-guidance

Wistar rats (♂)

Single ip dose of 200 mg/kg bw eugenol

Urine collected every 2h for 24h.

Microsomes prepared from rats pre-treated i.p. with</td><td><u>In vitro study</u>:
Incubation of rat liver cells with eugenol resulted in the formation of the allyl epoxide.

Incubation of eugenol with hepatic microsomes resulted in the formation of the dihydrodiol derivative, which may be an indication of the epoxide-diol pathway.

<u>In vivo study</u>:
Detection of both epoxides (allylcatechol and eugenol epoxide) and their dihydrodihydroxy derivatives were observed in urine but not liver homogenates of rats pre-treated with eugenol.</td><td>Delaforge M. et al.
(1980)
(AS)
B.6.1.1.3</td></tr>
</table>

Method	Results / Remarks	Reference
phenobarbital for 3 days at a dose of 80 mg/kg bw/day. Incubation of rat adult liver cell culture with 1 mg eugenol in 5mL HAM F10 medium GLP: No **Supporting information**		
Metabolism *in vitro* in rat and mouse liver microsomes No specific testing regulation/guidelines Rat (Fischer) and mouse (CD-1) liver microsomes Eugenol: Purity not stated Incubation of eugenol (2mM in 0.1 mL ethanol) with liver microsomes (rat/mouse) GLP: No **Study acceptable**	<u>Formation of 2',3'-epoxy eugenol in mice :</u> Male : 0.7 ± 0.3 (7) nmol per mg protein per hour Female : ≤ 1.3 (4) <u>Formation of 2',3'-epoxy eugenol in rats :</u> Male : ≤ 1.9 (4) Female : 0.4 ± 0.1 (4) Eugenol is a poor substrate for epoxidation under the conditions of the study. The epoxide formed was rapidly hydrolysed by microsomal epoxide hydrolase.	Swanson, A.B. *et al.* (1981) (AS) B.6.1.1.4
Metabolism *in vitro* in rat No specific testing regulation/guidelines Rat (Sprague-Dawley) liver and lung microsomes Eugenol: Purity not stated [^{3}H]Eugenol: Purity not stated Incubation of 1mg/mL microsomal protein with 1mM eugenol or 0.5 µCi [^{3}H]eugenol (1mM) and 5mM glutathione and NADPH-regenerating system GLP: No **Study acceptable**	<u>Metabolism</u> Lung and liver microsomes catalyse the formation of three glutathione conjugates, two tentatively identified as nucleophilic substitution at the benzylic and allylic positions. Reaction was dependent on NADPH and oxygen. The reaction was inhibited by cytochrome P450 inhibitors. 3-Methylcholanthrene but not phenobarbital induces the enzyme responsible for eugenol oxidation. The glutathione conjugation involves the formation of a highly reactive quinone methide intermediate that is susceptible to react with sulphidryl groups of proteins or glutathione.	Thompson, D. *et al.* (1990) (AS) B.6.1.1.5
Metabolism *in vitro* in rat No specific testing regulations/guidelines Male Sprague-Dawley rat hepatocytes Eugenol: Purity not stated [^{3}H]Eugenol: Radiopurity: 3.4 mCi/nmol	<u>*In vitro* metabolism</u>: Incubations with 1mM eugenol generated three major metabolites: eugenol-glucuronide (major, 200 nmol formed), eugenol-sulphate (minor, 25nmol formed) and eugenol-glutathione conjugate (minor, 25nmol formed). <u>Cytotoxicity:</u> Concentration of 1mM caused 85% cell death over 5h and depletion of 90% glutathione. Addition of N-acetylcysteine prevented the cell loss. Pre-treatment of cells with diethylmaleate increased the cytotoxic effects of eugenol.	Thomson, D.C. *et al.* (1991) (AS) B.6.1.1.6

<table>
<tr><th>Method</th><th>Results / Remarks</th><th>Reference</th></tr>
<tr><td>Incubations with eugenol at concentrations 0.5, 1, 1.5 mM, dissolved in DMSO (<1% v/v).
Incubation period: 5h
GSH conjugation and covalent binding was assessed.

Study acceptable</td><td><u>Covalent binding</u>:
Dose-dependent binding to cellular protein up to 3h. Loss of glutathione and cytotoxicity observed.</td><td></td></tr>
<tr><td>Comparative in vitro metabolism

No specific testing regulations/guidelines

Human (mixed gender), mouse (male) and rat (male) microsomes and S9 fractions

Dosage: 20 µM (1.8 µCi) [^{14}C]-Eugenol, purity not stated
Eugenol, purity not stated

Incubation time: 180 min
NADPH generating system or UDPGA
Protein binding: GSH

GLP: No

Study acceptable</td><td><u>Metabolism in vitro</u>:
Two metabolites identified in all three species: 1'-hydroxy glucuronide and phenoxy-glucuronide.

<u>Phase I metabolite – 1'hydroxylation (microsomes + NADPH)</u>:
- Human: 16.4 % ± 0.4
- Mouse: 22.0 % ± 0.8
- Rat: 7.1 % ± 0.5
<u>Phase I covalent binding (microsomes + NADPH):</u>
- Human: 13.8 % ± 4.5
- Mouse: 25.9 % ± 1.0
- Rat: 20.0 % ± 3.3
The addition of GSH to microsomes markedly decreased the covalent binding.
<u>Phase II glucuronidation (S9+UDPGA):</u>
- Human: 84.3 % ± 1.3
- Mouse: 93.7 % ± 2.6
- Rat: 55.5 % ± 4.5
<u>Phase II glucuronidation (S9+NADPH+ UDPGA):</u>
- Human: 78.1 % ± 1.1
- Mouse: 91.9 % ± 0.4
- Rat: 47.2 % ± 1.9

Limited metabolism in lung compared to liver. Significant glucuronidation observed in rat lung S9 compared to human lung S9.</td><td>Minet, E.F. et al. (2012)
(AS)
B.6.1.1.7</td></tr>
<tr><td>Pharmacokinetic study in rats (single dose)

Comparable to OECD TG 417 (2010)

Male Sprague-Dawley rat, 12 animals in total

Single oral dose (gavage) of 40 mg/kg bw

Blood and plasma samples were collected from the jugular vein and plasma centrifugation, respectively

GLP: No

Supporting information</td><td>The mean plasma concentration profile of eugenol displayed double peaks at 0.25 and 4 hours after dosing and then declined very slowly over the 24 h kinetic study. Eugenol profile in blood included an initial rapid decline followed by a steady decrease afterwards.

<table>
<tr><th>PK parameters</th><th>Plasma</th><th>Blood</th></tr>
<tr><td>$AUC_{0\text{-}t}$ (µg h/mL)</td><td>0.384 (42.4 %)</td><td>0.342 (31.0 %)</td></tr>
<tr><td>$AUC_{0\text{-}inf}$ (µg h/mL)</td><td>0.577 (51.0 %)</td><td>0.518 (38.8 %)</td></tr>
<tr><td>C_{max} (µg/mL)</td><td>0.123 (25.7 %)</td><td>0.270 (80.5 %)</td></tr>
<tr><td>T_{max} (h)*</td><td>2.13 (0.25-4.00)</td><td>0.25 (0.25-0.50)</td></tr>
<tr><td>$T_{1/2}$ (h)</td><td>14.0 (58.3 %)</td><td>18.3 (37.3 %)</td></tr>
<tr><td>CL/F (L/h/kg)</td><td>86.7 (50.0 %)</td><td>86.8 (34.9 %)</td></tr>
<tr><td colspan="3">Mean (%CV) values, n = 6 blood and n = 6 plasma
*Median (Minimum-Maximum)</td></tr>
</table></td><td>Guenette, S.A. et al. (2007)
(AS)
B.6.1.1.8</td></tr>
<tr><td><u>REACH DATA</u>
Basic toxicokinetics – absorption by inhalation
Reliability 2

Eugenol

Mouse, Swiss (♀)</td><td>Trace amounts of eugenol (<0.1 ng/mL of serum) were detected in the blood samples obtained following inhalation exposure to mice (time points at which trace levels were seen was not reported).

Based on trace amounts of eugenol detected in blood samples at up to 90 minutes post-dosing, absorption following inhalation exposure is considered to be low.</td><td>Buchbauer, G. et al. (1993)
"Fragrance compounds and essential oils with sedative effects upon inhalation"
(REACH Registration Dossier data not provided</td></tr>
</table>

Method	Results / Remarks	Reference
4 mice/group exposed via inhalation to eugenol for 1h (dose not reported). Blood samples collected at 0, 30, 60 and 90 mins following inhalation		by applicant and not assessed by the RMS)
REACH DATA Basic toxicokinetics – distribution and excretion Reliability 2 ^{14}C-Eugenol Rats, Wistar (♂) Single i.p. injection of 0.45 g/kg bw	Distribution ^{14}C-eugenol (10 to 20 ng per milligram tissue) was observed in the omentum (however, the authors reported that this may be an artifact due to the intraperitoneal method of administration of the ^{14}C-eugenol). ^{14}C-eugenol also was detected in the small and large intestines, kidney, liver, adrenal gland, stomach, testes, lung, skin, pancreas, heart, spinal cord, and brain (1 ng/mg). This pattern was observed among all tested rats and this pattern was maintained for several hours after the injection. The activity in various organs then rapidly declined, except for a small region in the vicinity of the injection site. Trace activity was detected in all tissues, even after 100 hours. The level of radioactivity detected in the sera of the rats after injection was low and was reduced considerably after 4 hours. The circulating blood cells showed low activity but this was significantly higher than that found in the serum. A major portion of the ^{14}C-recovered material from all tissues corresponded to unaltered ^{14}C-eugenol. Both ether-soluble and aqueous-soluble ^{14}C materials were recovered from most tissues. Excretion Both ether-soluble and aqueous-soluble ^{14}C materials were recovered in the excreta. The urine was radioactive for the next 100 hours. Of the total ^{14}C injected in 24 hours, 0.2 to 1% was detected in the carbon dioxide in the breath of the rats.	Weinberg, J.E. *et al.* (1972) "14C-Eugenol: I. Synthesis, Polymerization and Use" (REACH Registration Dossier data not provided by applicant and not assessed by the RMS)
REACH DATA Basic toxicokinetics – Reliability 2 Eugenol Rats, Sprague-Dawley (♂) Single i.v. dose (6 animals/group dose) Overall study: 0 (vehicle), 5, 10, 20, 40 and 60 mg/kg bw Plasma exposure: 20 mg/kg bw Vehicle: 0.5 mL cremophor, 1 mL ethanol (99%) and 8 mL saline (9%)	The mean systemic clearance for eugenol in plasma and blood were reported to be 157 and 204 mL/min/kg, respectively. AUC: 116 µg min/mL (blood) from time 0 to the last measurable concentration value Half-life 1st: 7.05 min (plasma) The presence of conjugated metabolites (i.e., sulfate and glucuronide) were detected in the urine.	Guenette, S.A. *et al.* (2006) "Pharmacokinetics and anesthetic activity of eugenol in male Sprague-Dawley rats" (REACH Registration Dossier data not provided by applicant and not assessed by the RMS)

2.6.1.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification(s)

A total of eight studies have been submitted for the renewal of approval of the active substance eugenol. Most of these studies were previously evaluated in the original DAR (2011) except for an *in vitro* comparative metabolism study and a pharmacokinetic study, which have been submitted for the renewal process. None of the studies are GLP compliant.

The absorption and excretion of eugenol in human is relatively rapid and almost complete based on urinary excretion (Fischer, I.U. *et al.*, 1990; B.6.1.1.1). An average 94.8 % was eliminated in urine at 24 h post-dosing, of which a mean 53 % was excreted as eugenol conjugates (glucuronide/sulphate). The metabolic profile of eugenol in humans

include the isomerisation (7% *cis/trans* isoeugenol), epoxide-diol pathway (13% applied dose), allylic oxidation (5% applied dose) and reaction with thiol components (11 % applied dose) (Figure 2.6.1.1).

In the rat, urinary and faeces excretion following oral administration of eugenol ranged between 75-80 % and 10 % of administered dose, respectively (Sutton, J.D. *et al.*, 1985; B.6.1.1.2). The metabolic profile of eugenol in the rat following oral doses of 0.5, 5, 50 and 1000 mg/kg bw radiolabelled eugenol includes O-demethylation and allyl reduction reactions, with their corresponding conjugates. Furthermore, sulphation is the predominant conjugation at low doses whereas glucuronidation is predominant at high doses. The metabolites identified included eugenol conjugates (sulphate and glucuronide) corresponding to 50 % applied dose, 3,4-dihydroxypropyl benzene (excreted as glucuronide and sulphate conjugates) and the minor metabolite 4-hydroxy-3-methoxypropyl benzene (Sutton, J.D. *et al.*, 1985; B.6.1.1.2).

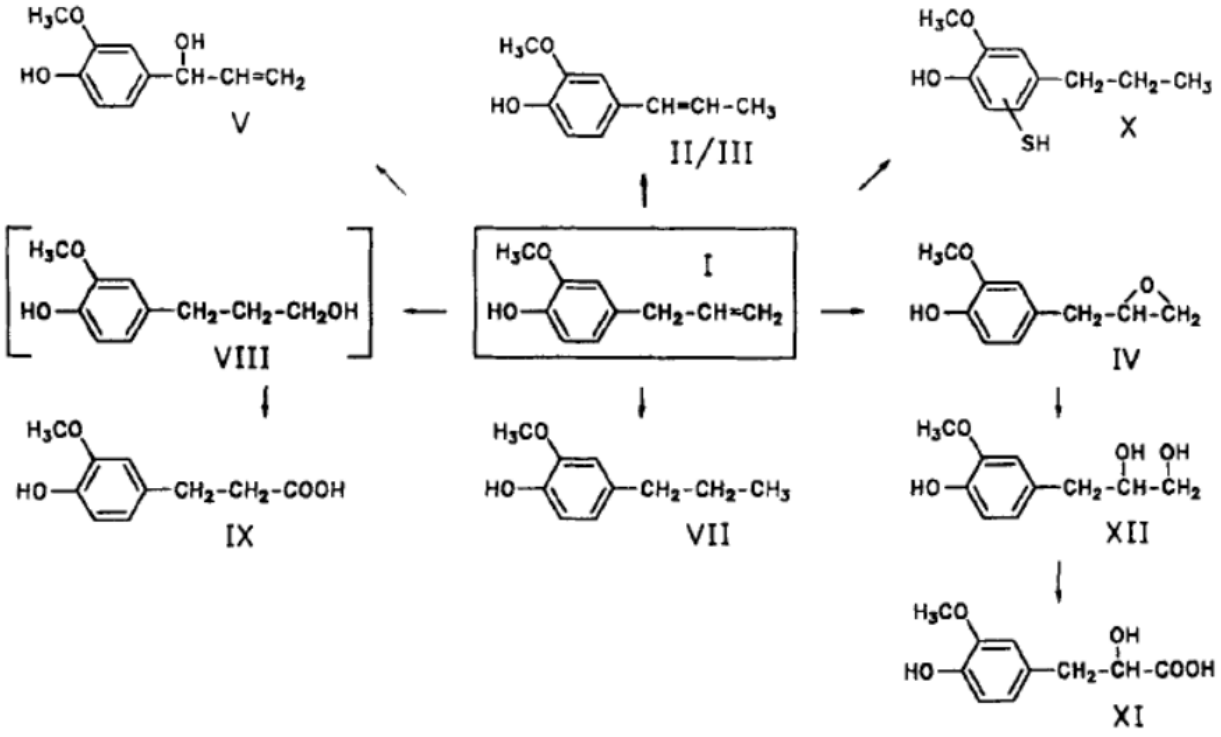
The epoxide-diol pathway was investigated in rats following a single intraperitoneal injection of 200 mg/kg bw eugenol and *in vitro* using liver microsomes (Delaforge, M. *et al.*, 1980; B.6.1.1.3). Eugenol epoxide and its dihydroxy-derivative were detected both *in vitro* and *in vivo* (urine). The O-demethylated allylcatechol epoxide and its dihydroxy-derivative were identified in urine and *in vitro* following incubation of liver microsomes with eugenol.

The formation of eugenol-2',3'-epoxide was investigated *in vitro* using rat and mouse liver microsomes (Swanson, A.B. *et al.*, 1981; B.6.1.1.4). Under the conditions of the study, eugenol was a poor substrate for epoxidation *in vitro* both in mouse and rat liver microsomes.

The metabolism of eugenol *in vitro* was further investigated using rat lung and liver microsomes (Thompson, D. *et al.*, 1990; B.6.1.1.5). Lung and liver microsomes catalyse the formation of three glutathione conjugates, two tentatively identified as nucleophilic substitution at the benzylic and allylic positions. This reaction was NADPH- and oxygen-dependent, and the reaction mechanism involves the formation of a highly reactive quinone methide intermediate that is susceptible to react with sulphidryl groups of proteins or glutathione.

The metabolism of eugenol *in vitro* was investigated using rat hepatocytes (Thompson, D.C. *et al.*, 1991; B.6.1.1.6). Incubations with 1 mM eugenol generated three major metabolites: eugenol-glucuronide (major, 200 nmol formed), eugenol-sulphate (minor, 25 nmol formed) and eugenol-glutathione conjugate (minor, 25 nmol formed). A dose-dependent covalent binding to cellular proteins and depletion of glutathione up to 90 % was observed. The addition of N-acetylcysteine seemed to have a cytoprotective effect.

Figure 2.6.1.1: Proposed metabolic pathway of eugenol based on urinary metabolites at 24h found in human following oral exposure to eugenol (data from study B.6.1.1.1).



A comparative *in vitro* metabolism study was carried out with eugenol in rats, mice and human microsomes (Minet, E.F. *et al.*, 2012; B.6.1.1.7). Two metabolites were identified in all three species after incubation of microsomes and eugenol: 1'-hydroxy glucuronide and phenoxy-glucuronide. These metabolites were further quantified:

Phase I metabolite – 1'hydroxylation (microsomes + NADPH):
- Human: 16.4 % ± 0.4
- Mouse: 22.0 % ± 0.8
- Rat: 7.1 % ± 0.5

Phase I covalent binding (microsomes + NADPH):
- Human: 13.8 % ± 4.5
- Mouse: 25.9 % ± 1.0
- Rat: 20.0 % ± 3.3

The addition of GSH to microsomes markedly decreased the covalent binding.

Phase II glucuronidation (S9+UDPGA):
- Human: 84.3 % ± 1.3
- Mouse: 93.7 % ± 2.6
- Rat: 55.5 % ± 4.5

Phase II glucuronidation (S9+NADPH+ UDPGA):
- Human: 78.1 % ± 1.1
- Mouse: 91.9 % ± 0.4
- Rat: 47.2 % ± 1.9

Based on the results of the study, eugenol is comparably metabolized in mouse and human whereas slight differences were observed in rats such as the extent of glucuronidation (47-55 % in rats against 78-84 % in humans) and formation of 1'-hydroxy-eugenol (7.1 % in rats against 16.4 % in humans).

The pharmacokinetic parameters in the rat were investigated following a single oral administration of eugenol (Guenette, S.A. *et al.*, 2007; B.6.1.1.8). The mean plasma concentration profile of eugenol displayed double peaks at 0.25 and 4 hours after dosing and then declined very slowly over the 24 h kinetic study. Eugenol profile in blood included an initial rapid decline followed by a steady decrease afterwards.

Data from REACH registration dossier were included in the summary table that are relevant for ADME properties, since it is an ECHA requirement for the proposal of harmonised classification (CLH) according to Regulation (EC) no. 1272/2008 (CLP). However, it has to be underlined that these data were not available in the applicant submission dossier for the renewal and consequently they have not been evaluated by the RMS and not included in Volume 3 of this RAR.

Residue definition for body fluids and tissues:

Considering the available information, residues in body fluids and tissues could be defined as the active substance and its sulphate and glucuronide conjugates, measured in urine samples.

2.6.2 Summary of acute toxicity

2.6.2.1 Acute toxicity - oral route [equivalent to section 10.1 of the CLH report template]

Table 18: Summary table of animal studies on acute oral toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance, dose levels, duration of exposure	Value LD_{50}	Reference
Acute oral toxicity study in rats, mice, guinea pigs Prior to OECD TG 401 (1987) Deviations from OECD TG 420 (2001): Test substance not characterised.	Species: Osborne-Mendel rats 10 rats evenly divided by sex Guinea pig (male, female) (number of animals not specified) Mice (strain and	Eugenol (undiluted) Purity: Not indicated Oral by intubation Single dose Up to 14-day observation period	Rats: **Eugenol LD_{50}: 2680 mg/kg** Coma soon after treatment. Time of death approx. 1h Mice: **Eugenol LD_{50}: 3000 mg/kg** Severe depression immediately after treatment. Time of death: few min-2 days	Jenner, P.M. *et al.* (1964) (AS) B.6.2.1.1

<table>
<tr><th>Method, guideline, deviations if any</th><th>Species, strain, sex, no/group</th><th>Test substance, dose levels, duration of exposure</th><th>Value
LD_{50}</th><th>Reference</th></tr>
<tr><td>Poorly described method and results, no necropsy.

GLP: No (prior to GLP enforcement)

Supporting information</td><td>number of animals not indicated)</td><td></td><td>Guinea pigs:
Eugenol LD_{50}: 2130 mg/kg
Depression. Time of death: 4h-2 days</td><td></td></tr>
<tr><td>Acute oral toxicity study in rats

Prior to OECD TG 401 (1987)
Deviations from OECD TG 420 (2001): Test substance not characterised

GLP: No (prior to GLP enforcement)

Study acceptable</td><td>Species: Rats
Strain: Albino
12 rats/group (male and female)</td><td>Eugenol (undiluted)
Purity: Not stated
Oral gavage
Preliminary dose-range experiment with 2 rats/group dose.

Single doses (mL/kg): 1.5, 1.6, 1.75, 1.9, 2.0, 2.1 and 2.2, equivalent to 1597.5, 1704, 1863.75, 2023.5, 2130, 2236.5 and 2343 mg/kg bw
10-day observation period</td><td>Mortality:<table><tr><th>Dose ml/kg bw</th><th>Mortality (dead/total)</th></tr><tr><td>1.5</td><td>4/12</td></tr><tr><td>1.6</td><td>7/12</td></tr><tr><td>1.75</td><td>3/11</td></tr><tr><td>1.75</td><td>3/11</td></tr><tr><td>1.9</td><td>4/12</td></tr><tr><td>2.0</td><td>8/11</td></tr><tr><td>2.1</td><td>9/11</td></tr><tr><td>2.2</td><td>11/11</td></tr></table>Clinical signs: Weakness of hind legs, paralysis of lower extremities, lethargy, coma.

Necropsy: Congestion of liver and kidneys. Glandular stomach contained mucus, clotted blood and areas of mucosal erosion. Diffuse inflammation with congestion in the viscera.
An LD_{50} value was derived statistically and it was found to be 1.8 mL/kg, equivalent to 1930 mg/kg bw (relative density of 1.065 g/mL)
Eugenol LD_{50}: 1930 mg/kg (male and female rats)</td><td>Sober, H.A. et al. (1950)
(AS)
B.6.2.1.2</td></tr>
<tr><td>Acute oral toxicity study in rats

No guideline stated

Deviations from current OECD TG 420 (2001):
-No necropsy
-Limited level of reporting

GLP: No (prior to GLP enforcement)

Supporting information</td><td>Species: Rats
Strain: F344/N

5 rats/group (male and female)</td><td>Eugenol
Purity: > 99%
Batch No.: 36483
Oral gavage
Vehicle: 1% carboxymethylcellulose in saline

Single doses: 150, 250, 500, 1000 and 2000 mg/kg bw
16-day observation period</td><td>Body weight changes were unaffected

Mortality:
1/5 female in 2000 mg/kg bw dose

LD_{50} > 2000 mg/kg bw</td><td>NTP (1983)
(AS)
B.6.2.1.3</td></tr>
<tr><td>Acute oral toxicity</td><td>Species: Mice</td><td>Eugenol</td><td>Body weight changes were</td><td>NTP (1983)</td></tr>
</table>

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance, dose levels, duration of exposure	Value LD_{50}	Reference
study in mice No guideline stated Deviations from current OECD TG 420 (2001): -No necropsy -Limited level of reporting GLP: No (prior to GLP enforcement) **Supporting information**	Strain: B6C3F1 5 animals/group (male and female) No control group tested	Purity: > 99% Batch No.: 36483 Oral gavage Vehicle: 1% carboxymethylcellulose in saline Single doses (mL/kg): 180, 375, 750, 1500 and 3000 mg/kg bw 16-day observation period	unaffected Mortality: 1/5 male in 750 mg/kg bw dose 2/5 males and 5/5 females in 3000 mg/kg bw dose **LD_{50} between 1500 and 3000 mg/kg bw**	(AS) B.6.2.1.4
Acute oral toxicity study in dogs Prior to OECD TG 401 (1987) Deviations from current OECD TG 420/423 (2001): Test substance not characterised, poorly described method and results, 5 dogs used in 20 experiments. GLP: No (prior to GLP enforcement) **Supporting only**	Species: Dogs Strain: Mongrel 5 Female dogs for 20 experiments Single doses: 2% or 5% eugenol in 50mL or 100 mL, equivalent to 100, 200, 250 and 500 mg/kg bw.	Eugenol (aqueous emulsion stabilised with 5% gum acacia) Purity: Not stated Oral (instillation into stomach via Levin tube)	Mortality: Dose (mg/kg bw) / Mortality 500 / 2 250 / 0 200 / 0 100 / 0 Necropsy: Congestion and hyperaemia of both liver and kidneys. Clinical signs: Marked motor dysfunction (ataxia) primarily of the hind limbs, vomiting, slight decrease in body temperature and an increase in pulse rate. **No LD_{50} value can be established** (female dogs)	Lauber, F.U. and Hollander, F. (1950) (AS) B.6.2.1.5

Table 19: Summary table of human data on acute oral toxicity

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
Clinical case report 2-year-old male	**Clove oil**	-Accidental ingestion of 5-10 ml of clove oil. **Clinical effects**: Coma, liver toxicity, disseminated intravascular coagulation. *Biochemistry* ▪ (↑) alanine aminotransferase (ALT). ▪ (↑) fibrin degradation products. **Treatment**: Patient was treated with fresh frozen plasma, heparin, antithrombin III, protein C and factor VII.	Full recovery was observed on day 6. This clinical case predates subsequent studies performed by Thompson *et al.* (Thompson, DC., *et al.*, 1998, B.6.8.1.2.1; and 1991, B.6.1.1.6) that revealed that N-acetyl-cysteine (NAC) administration prevents eugenol hepatotoxicity. Nowadays, NAC therapy is used as curative primary treatment for clove oil or eugenol containing products ingestion.	Hartnoll, G., *et al.* (1993) (AS) B.6.9.3.1

Table 20: Summary table of other studies relevant for acute oral toxicity

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
Micronucleus test in mice Deviations from the current OECD TG 474 (2016): - No characterisation of the test substance - No historical control data reported -No data about animal housing -Oral dose exceeds the 2000 mg/kg bw recommended by guidance -3 different concentrations should be tested -Only 1000 PCE were scored per. GLP: No **Supporting information**	Eugenol Purity: not reported Batch no. not reported	Test system: adult ♂ Swiss-Webster mice, 12 animals/group Dose selection: Preliminary determination of LD_{50} LD_{50}: 1109.6 mg/kg (ip). Oral LD_{50} > 14794.4 mg/kg (no mortality in the study) Route: Intraperitoneal or oral Dosage: 739.7 mg/kg bw (80 % LD_{50}) or 147.9 mg/kg bw (25% LD_{50}) (ip injection) 14794.4 mg/kg bw (oral) Vehicle: saline Sampling Second dose at 24 hours 1000 PCEs scored Negative control: saline Positive control: Quinacrine dihydrochloride	**Positive (ip)** **Positive (oral)** Cytotoxicity: PCE/ total erythrocytes is bigger than control	Woolverton C. *et al.* (1986) (AS) B.6.4.2.1.2

2.6.2.1.1 Short summary and overall relevance of the provided information on acute oral toxicity

All available acute oral toxicity studies were evaluated in the previous DAR (2011).

All studies are pre-guideline and prior to GLP enforcement. Method deficiencies include no characterisation of the test substance, poorly described method, no necropsy performed or the use of five animals in 20 experiments.

Only one study (Sober, H.A. *et al.,* 1950; B.6.2.1.2) is considered acceptable. In this study, the calculated LD_{50} is 1930 mg/kg bw for male and female rats. The result of this study is the one considered for the assessment of the acute oral toxicity of eugenol.

In mice, a reported LD_{50} value between 1500 and 3000 mg/kg bw has been reported (NTP, 1983; B.6.2.1.4). A remarkable difference was obtained in a separate study (Woolverton, C. et al, 1986; B.6.4.2.1.2) in which the reported oral LD_{50} value was greater than 14794.4 mg/kg bw. However, the latter study follows a test method dated from 1938 and the methodology may be question as to the reliability of the results.

Regarding oral toxicity, several accidental oral ingestion cases of clove oil (>80% eugenol) had been described in the literature. Clinical effects usually course with coagulation and hepatic impairment (Hartnoll *et al.*, B.6.9.3.1). The currently routine clinical practice uses N-acetyl-cysteine (NAC) as primary curative therapy based on Thompson *et al.* studies (1998, B.6.8.1.2.1; and 1991, B.6.1.1.6), that revealed that NAC administration prevents eugenol hepatotoxicity.

2.6.2.1.2 Comparison with the CLP criteria regarding acute oral toxicity

The reported Acute Toxicity Estimate (ATE) value of eugenol is 1930 mg/kg bw (according to the study B.6.2.1.2) for male and female rats. This LD_{50} value falls between the established threshold values of 300 and 2000 mg/kg bw/day for classification as acute (oral) Toxicity 4 (Acute Tox.4; H302).

2.6.2.1.3 Conclusion on classification and labelling for acute oral toxicity

Based on the ATE of 1930 mg/kg bw in male and female rats for eugenol, and according to the criteria under Regulation (EC) No. 1272/2008, eugenol is classified as **acute (oral) toxicity, category 4, Acute Tox. 4 (H302)**.

2.6.2.2 Acute toxicity - dermal route [equivalent to section 10.2 of the CLH report template]

Table 21: Summary table of animal studies on acute dermal toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels, duration of exposure	Value LD_{50}	Reference
No data available					

Table 22: Summary table of human data on acute dermal toxicity

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No data available				

Table 23: Summary table of other studies relevant for acute dermal toxicity

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No data available				

2.6.2.2.1 Short summary and overall relevance of the provided information on acute dermal toxicity

No acute dermal toxicity studies have been submitted for eugenol.

According to Regulation (EC) 283/2013, an acute dermal toxicity study is not deemed necessary when the acute oral toxicity is over 2000 mg/kg bw. Eugenol has an oral LD_{50} of 1930 mg/kg bw and is severely irritating to rabbit skin (B.6.2.4.1). The reported oral LD_{50} value is close enough to the cut off value of 2000 mg/kg bw and subsequently, a low acute dermal toxicity could be expected. For this reason, an acute dermal toxicity study is deemed not necessary.

2.6.2.2.2 Comparison with the CLP criteria regarding acute dermal toxicity

No acute dermal toxicity study is available for eugenol. The acute oral toxicity of eugenol is 1930 mg/kg bw, which is close enough to the cut.off value of 2000 mg/kg bw to expect a low acute dermal toxicity. For this reason, it is expected that the acute dermal toxicity of eugenol may exceed the limit for classification for this hazard class.

2.6.2.2.3 Conclusion on classification and labelling for acute dermal toxicity

No classification for acute dermal toxicity is proposed for eugenol (data lacking).

2.6.2.3 Acute toxicity - inhalation route [equivalent to section 10.3 of the CLH report template]

Table 24: Summary table of animal studies on acute inhalation toxicity

<table>
<tr><th>Method, guideline, deviations if any</th><th>Species, strain, sex, no/group</th><th>Test substance, form and particle size (MMAD), dose levels, duration of exposure</th><th>Value LC_{50}</th><th>Reference</th></tr>
<tr>
<td>Acute inhalation toxicity study in rats

Comparable to OECD TG 403 (1981)

Deviations from OECD TG 403 (2009): Test material not characterised

GLP: No

Study acceptable</td>
<td>Species: Rat

Strain: Sprague-Dawley

5 rats/sex/group

3 Treatment groups</td>
<td>Eugenol; Purity: 99%

Test atmosphere:
<table>
<tr><th>Parameters</th><th>Group 1</th><th>Group 2</th><th>Group 3</th></tr>
<tr><td>Dose</td><td>2.58 mg/L</td><td>1.37 mg/L</td><td>0.77 mg/L</td></tr>
<tr><td>Nominal concentration</td><td>4.48 mg/L</td><td>2.39 mg/L</td><td>1.53 mg/L</td></tr>
<tr><td>MMAD</td><td>0.82 µm</td><td>0.88 µm</td><td>0.9 µm</td></tr>
<tr><td>GSD</td><td>2.26</td><td>2.05</td><td>1.87</td></tr>
</table>
MMAD and GSD: 0.82 µm (σg 2.26) (Group 1), 0.88 µm (σg 2.05) (group 2) and 0.9 µm (σg 1.87) (group 3)
Exposure: 4-h (whole body)

Clinical signs: during exposure, increase salivation and restlessness observed in the high dose group. Some gasping was noted which disappeared within 2 h, as did all clinical signs except restlessness at the lower eugenol levels. Immediately following exposure, all high dose rats were lethargic and many high and intermediate dose rats had wet snouts and red-brown staining of their fur. Reduced food and water intake and weight loss was also noted. During the 1st day of the 14-day observation period, all clinical signs disappeared in all groups and all rats appeared normal.
Histological examination of the lungs (after 14-day observation period) was normal.</td>
<td>No mortality

LC_{50} > 2.58 mg/L</td>
<td>Clark, G.C (1988) (AS) B.6.2.3</td>
</tr>
</table>

Table 25: Summary table of human data on acute inhalation toxicity

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No data available				

Table 26: Summary table of other studies relevant for acute inhalation toxicity

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No data available				

2.6.2.3.1 Short summary and overall relevance of the provided information on acute inhalation toxicity

One acute inhalation toxicity study (Clark, G.C., 1988; B.6.2.3) has been carried out with **eugenol**, which has been evaluated and accepted in the previous DAR (2011).

In an acute toxicity study by whole body 4-hour inhalation exposure (Clark, G.C., 1988; B.6.2.3), eugenol was delivered to rats using a submicron aerosol generation system capable of delivering up to 4.48 mg/L (nominal). The measured concentrations achieved in the exposure chambers were 0.77, 1.37 and 2.58 mg/L with a mass median aerodynamic diameter of less than 1 micron. Temporary, readily reversible signs of toxicity were noted, including signs of local irritation in the lung such as moderately increased salivation, restlessness and irregular breathing, weight loss, and reduced food and water intake. There were no deaths and no evidence of blood in the respiratory tract (haemoptysis). The rats were normal in all respects within 48 h. Histological examination of the lungs after a 14-day observation period was normal.

The study is considered relevant to evaluate the acute inhalation toxicity of eugenol in rats.

2.6.2.3.2 Comparison with the CLP criteria regarding acute inhalation toxicity

Eugenol: the 4-hour inhalation study in rats reported an LC_{50} > 2.58 mg/L (maximum attainable concentration).

According to classification criteria under Regulation (EC) No. 1272/2008, the threshold for no classification for acute inhalation toxicity is an LC_{50} > 5.0 mg/L for dusts or mists. However, considering that the maximum attainable concentration did not produce any mortality, no classification for acute inhalation toxicity is therefore proposed.

2.6.2.3.3 Conclusion on classification and labelling for acute inhalation toxicity

Acute inhalation toxicity data available indicate that **eugenol** does not require classification for acute inhalation toxicity (data conclusive but not sufficient for classification).

2.6.2.4 Skin corrosion/irritation [equivalent to section 10.4 of the CLH report template]

Table 27: Summary table of animal studies on skin corrosion/irritation

Method, guideline, deviations if any	**Species, strain, sex, no/group**	**Test substance, dose levels, duration of exposure**	**Results** **- Observations and time point of onset[2]** **- Mean scores/animal** **- Reversibility**	**Reference**
Skin irritation/corrosion in rabbits No guideline stated Deviations from current OECD TG 404 (2015) : -Test item not fully characterised -Dose level was 0.1 g of test item (instead of 0.5 mL/0.5 g required by guidance) -24h exposure to the chemical instead of 4h -Exposure to test item was not done in a patch under occlusion -Multiple application of the test item onto the skin -No observation at 30 min was performed. -Reversibility of effects was not assessed (no observation period of 14 days) -Non-standard scoring method -No individual results provided, only the summary of the overall result GLP: No (prior to GLP Requirement) **Supporting information**	Species: rabbit Strain: Albino 6 rabbits	Eugenol Purity: > 95% Batch No: Not stated Two applications of 0.1 g of the test item undiluted Exposure: 24 h and 30 min (second application) Positive control: hexadecane	Irritancy score **3** Severely irritating to the rabbit skin Positive control reported to have an irritancy score of 3.	Motoyoshi *et al.* (1979) (AS) B.6.2.4.1
Skin irritation/corrosion in guinea pigs No guideline stated Deviations from current OECD TG 404 (2015) : -Test item not fully characterised -Dose level was 0.1 g of test item (instead of 0.5 mL/0.5 g required by guidance) -24h exposure to the chemical instead of 4h	Species: Guinea pig Strain: Hartle 6 males	Eugenol Purity: > 95% Batch No: Not stated Two applications of 0.1 g of the test item undiluted	Irritancy score **2** Moderately irritating to the guinea pig skin Positive control reported to have an irritancy score of 3.	Motoyoshi *et al.* (1979) (AS) B.6.2.4.2

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance, dose levels, duration of exposure	Results - Observations and time point of onset[2] - Mean scores/animal - Reversibility	Reference
-Exposure to test item was not done in a patch under occlusion -Multiple application of the test item onto the skin -No observation at 30 min was performed. -Reversibility of effects was not assessed (no observation period of 14 days) -Non-standard scoring method -No individual results provided, only the summary of the overall result GLP: No (prior to GLP Requirement) **Supporting information**		Exposure: 24 h and 30 min (second application) Positive control: hexadecane		
Skin irritation/corrosion in miniature swine No guideline stated Deviations from current OECD TG 404 (2015) : -Test item not fully characterised -Dose level was 0.05 g of test item (instead of 0.5 mL/0.5 g required by guidance) -48h exposure to the chemical instead of 4h -No observation at 30 min was performed. -Reversibility of effects was not assessed (no observation period of 14 days) -Non-standard scoring method and non-standard species -No individual results provided, only summary of overall result GLP: No (prior to GLP Requirement) **Supporting information**	Species: Miniature swines Strain: Pitman-Moore 6 animals	Eugenol Purity: > 95% Batch No: Not stated One application of 0.05 g of the test item under a 15 mm diameter patch Exposure: 48 h Positive control: hexadecane	Irritancy score **1** Mildly irritating to the miniature swine skin Positive control reported to have an irritancy score of 3.	Motoyoshi *et al.* (1979) (AS) B.6.2.4.3

Table 28: Summary table of human data on skin corrosion/irritation

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
Human patch test No guideline GLP: No (prior to GLP Requirement) **Supporting information**	Eugenol Purity: > 95% Batch No: Not stated	One application of 32% eugenol in acetone Eugenol technical was applied to a panel of 50 volunteers and individuals with known allergic reactions were excluded Exposure: 48 h Positive control: hexadecane	Irritancy score **2** Moderately irritating Positive control reported to have an irritancy score of 3.	Motoyoshi *et al.* (1979) (AS) B.6.2.4.3

Table 29: Summary table of other studies relevant for skin corrosion/irritation

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
<u>REACH DATA</u> Skin irritation study Reliability 2	Eugenol	New Zealand White rabbits (4♀) Volume: 0.5 mL Exposure: 4h Semi-occlusive	Average erythema scores from 24, 48 and 72 h time point scorings: 2, 1.7, 2, 2 Average oedema scores from 24, 48 and 72 h time point scorings: 1.3, 1, 0.67, 1 In 1 out of 4 animals signs of continued irritation and slight scaling continued after 7 days. Conclusion: non-irritant	Unnamed (1988) (REACH registration dossier data not provided by applicant and not assessed by the RMS)

2.6.2.4.1 Short summary and overall relevance of the provided information on skin corrosion/irritation

All the available skin corrosion/irritation data for eugenol were included and assessed in the previous DAR (2011). Eugenol has a measured pKa of 10.27.

Eugenol has been tested in a non-standard assay in the skin of rabbits, guinea pigs, miniature pigs and humans (Motoyoshi *et al.*, 1979; B.6.2.4.1-B.6.2.4.4). The summary of the findings is displayed in the following table:

Relative irritancy of 100% and 32% concentration of eugenol in the skin of:			
Rabbit	**Guinea pig**	**Miniature swine**	**Human**
3	2	1	2
Scoring: 0 = negative, 1 = mildly irritating, 2 = moderately irritating, 3 = severely irritating			

The study is a peer reviewed publication and deviations from the current OECD TG 404 (2015) include:
-Test item not fully characterised
-Dose level was 0.1 g of test item (instead of 0.5 mL/0.5 g required by guidance) (rabbit and guinea pig study)
-24h exposure to the chemical instead of 4h (rabbit and guinea pig study)
-Exposure to test item was not done in a patch under occlusion
-Multiple application of the test item onto the skin (rabbit and guinea pig study)
-No observation at 30 min was performed.
-Reversibility of effects was not assessed (no observation period of 14 days)
-Non-standard scoring method
-No individual results provided, only the summary of the overall result

Based on the method deficiencies, these data are deemed as supporting information but not acceptable for classification purposes.

Data from REACH registration dossier were included in the summary table of other studies relevant for skin corrosion/irritation, since it is an ECHA requirement for the proposal of harmonised classification (CLH) according to Regulation (EC) no. 1272/2008 (CLP). However, it has to be underlined that these data were not available in the applicant submission dossier for the renewal and consequently they have not been evaluated by the RMS and not included in Volume 3 of this RAR.

2.6.2.4.2 Comparison with the CLP criteria regarding skin corrosion/irritation

The skin irritation study (Motoyoshi *et al.*, 1979; B.6.2.4.1-B.6.2.4.4) has a few method deficiencies and for instance, both the scoring and the lack of assessment of the reversibility of effects are not compliant with the current guideline. Furthermore, exposure to the test item was longer than 4h. The overall call is ‘severely irritant ‘to rabbit skin and moderately irritant to human skin (only 32 % eugenol applied).

The outcome of a skin irritation study available from the Eugenol REACH Registration Dossier indicates that eugenol is not-irritant to the rabbit skin. However, the reversibility of effects at 7 days is questionable and the quality of these data has not been adequately assessed.

The criteria for classification as Category 1 include destruction of skin tissue, visible necrosis through the epidermis and into the dermis, in at least one tested animal after exposure ≤ 4h. This type of data has not been reported in any of the studies considered to address this point. Likewise, all available data from animal studies do not contain information to apply the CLP criteria for classification as Category 2. However, based on the precautionary principle and a weight of evidence approach, the skin irritation data available for eugenol is indicative of skin irritation category 2.

2.6.2.4.3 Conclusion on classification and labelling for skin corrosion/irritation

Based on the data available for eugenol, there is evidence that it causes skin irritation but the extent of the lesions are yet to be evaluated. According to the criteria under Regulation (EC) No. 1272/2008, the RMS proposes the classification of this active substance as **Skin Irritant, Category 2, Skin Irrit. 2 (H315).**

2.6.2.5 Serious eye damage/eye irritation [equivalent to section 10.5 of the CLH report template]

Table 30: Summary table of animal studies on serious eye damage/eye irritation

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance	Dose levels duration of exposure	Results - Observations and time point of onset[2] - Mean scores/animal - Reversibility	Reference
No data available					

Table 31: Summary table of human data on serious eye damage/eye irritation

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No data available				

Table 32: Summary table of other studies relevant for serious eye damage/eye irritation

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
REACH DATA Eye irritation study Reliability 2	Eugenol	New Zealand White rabbits (6♀) Volume: 0.1mL	Test article appeared to affect the rabbit eyes 1 day following application; however, the effects were reversible as the eye irritation grades decreased over the 7-day observation period. Eugenol appears to be severely irritating in the first 24h post-exposure, moderately to mildly irritating after 24 and 72h post-exposure, and practically non-irritating after 7 days post-exposure.	Unnamed (1977) (REACH registration dossier data not provided by applicant and not assessed by the RMS)

2.6.2.5.1 Short summary and overall relevance of the provided information on serious eye damage/eye irritation

No eye irritation/corrosion data have been submitted for eugenol. Data are available for the skin irritation/corrosion properties of eugenol, which is reported to be severely irritant to the rabbit skin (Motoyoshi *et al.*, 1979; B.6.2.4.1).

Data from REACH registration dossier were included in the summary table of other studies relevant for eye damage/irritation, since it is an ECHA requirement for the proposal of harmonised classification (CLH) according to Regulation (EC) no. 1272/2008 (CLP). However, it has to be underlined that these data were not available in the applicant submission dossier for the renewal and consequently they have not been evaluated by the RMS and not included in Volume 3 of this RAR.

The RMS is of the opinion that based on skin irritation properties of eugenol, it is expected that at minimum, eugenol may cause irritation to the eye. However, eugenol is a severe irritant to the rabbit skin (reported in the rabbit skin irritation study, B.6.2.4.1) and therefore, the potential to cause severe eye damage cannot be disregarded. For this reason, in the absence of suitable data to determine the extent of the damage to the eye, a data requirement is established for this hazard class. The RMS recommends that for a more certain assessment, a tiered testing strategy as specified in Section 5.2.5 of Regulation (EU) No. 283/2013 should be followed.

2.6.2.5.2 Comparison with the CLP criteria regarding serious eye damage/eye irritation

No eye damage/eye irritation data are available for eugenol. Data available for the skin irritation/corrosion properties indicate that eugenol is a skin irritant and therefore, it may be expected that it may be an eye irritant, according to section 3.3.2.2.2 of Regulation (EC) 1272/2008. Furthermore, data from the Eugenol REACH Registration Dossier indicate that it causes severe eye irritation although reversibility and the extent of the lesions have not been verified by the RMS. A data requirement is therefore established to evaluate the extent of the lesions and reversibility of effects.

The criteria for classification as Category 1 include in at least one animal effects on the cornea, iris or conjunctiva that are not expected to reverse or have not fully reversed within an observation period of normally 21 days. This type of data has not been reported in the study available to address this point. Likewise, all available data from animal studies do not contain information to apply the CLP criteria for classification as Category 2. However, based on the precautionary principle and a weight of evidence approach, the eye irritation data available for eugenol is indicative of eye irritation category 2.

2.6.2.5.3 Conclusion on classification and labelling for serious eye damage/eye irritation

Based on a weight of evidence and according to the criteria under Regulation (EC) No. 1272/2008, the RMS proposes the classification of eugenol as **Eye Irritant Category 2, Eye Irrit. 2 (H319).**

2.6.2.6 Respiratory sensitisation [equivalent to section 10.6 of the CLH report template]

Table 33: Summary table of animal studies on respiratory sensitisation

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance, Dose levels, duration of exposure	Results	Reference
No data available				

Table 34: Summary table of human data on respiratory sensitisation

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No data available				

Table 35: Summary table of other studies relevant for respiratory sensitisation

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No data available				

2.6.2.6.1 Short summary and overall relevance of the provided information on respiratory sensitisation

There are no data available.

2.6.2.6.2 Comparison with the CLP criteria regarding respiratory sensitisation

There are no data available.

2.6.2.6.3 Conclusion on classification and labelling for respiratory sensitisation

Respiratory sensitisation is not assessed for harmonization of classification and labelling due to data lacking.

2.6.2.7 Skin sensitisation [equivalent to section 10.7 of the CLH report template]

Table 36: Summary table of animal studies on skin sensitisation

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance, Dose levels duration of exposure	Results	Reference
Local Lymph Node Assay: BrdU-ELISA (LLNA) Comparable to OECD TG 442B (2010)	Female CBA/JN mice 4 animals/group	Eugenol Purity: > 95 % Batch No: EG0704	Dose-dependent induction of lymph node cell proliferative activity **EC_3 = 25.1 (Sensitiser)**	Takeyoshi *et al.* (2004) (AS) B.6.2.6.1.1

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance, Dose levels duration of exposure	Results	Reference
Deviations from OECD TG 442B (2017): -No positive control -Injection of BrdU was done on day 4 instead of day 5 as required per guidance -Limited level of reporting GLP: No **Supporting information**		Doses: 0, 1%, 6%, 15% and 30% (3 applications) Vehicle: 4:1 acetone:olive oil Volume: 25 µL Exposure: 3 days		
Local Lymph Node Assay (LLNA) Comparable to OECD TG 429 (2002) Deviations from OECD TG 429 (2010): -Male instead of female mice used -No positive control -Limited level of reporting (clinical signs, body weights) GLP: No **Study acceptable**	Male CBA/Ca mice 4 animals/group	Eugenol Purity: 99.9 % Batch No: not stated Doses: 0, 1%, 3%, 10%, 30% and 50% (3 applications) Vehicles: DEP, 1:3 EtOH:DEP, 3:1 EtOH:DEP and EtOH Volume: 25 µL Exposure: 3 days	Dose-dependent induction of lymph node cell proliferative activity **DEP - EC_3 = 15.1** **1:3 EtOH:DEP - EC_3 = 10.5** **3:1 EtOH:DEP - EC_3 = 5.3** **EtOH - EC_3 = 10.7** **Sensitiser in all vehicles**	Lalko *et al.* (2004) (AS) B.6.2.6.1.2
Local Lymph Node Assay (LLNA) Comparable to OECD TG 429 (2002) Deviations from OECD TG 429 (2010): No characterisation of the test item, CBA/Ca strain, no positive controls, and no calculation of the SI or EC_3 values provided. GLP: No **Supporting information**	Female BALB/c mice 4 animals/group	Eugenol Purity not stated Doses: 0, 5%, 10% and 25% (3 applications) Vehicle: 4:1 acetone:olive oil	Dose-dependent induction of lymph node cell proliferative activity **Sensitiser**	Hilton *et al.* (1996) (AS) B.6.2.6.1.3
Maximisation test in guinea pig (Magnusson & Kligman) Method: Similar to OECD 406 (GPMT) Deviations from OECD 406 (2021): -No detailed information of the preliminary dose-range finding test -Adjuvant not stated	Female Hartley guinea pigs 10 animals/group	Eugenol Purity: > 95 % Batch No: EG0704 Preliminary dose-range finding (no information given) Intradermal induction (5%, maximum non-	**Sensitisation rate: 20 % (mild sensitiser)**	Takeyoshi *et al.* (2004) (AS) B.6.2.6.2.1

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance, Dose levels duration of exposure	Results	Reference
-No control group tested - Individual results not reported GLP: No. **Supporting information**		irritant concentration) Vehicle: olive oil		
Maximisation test in guinea pig Method: Similar to OECD 406 (GPMT) Deviations from current OECD TG 406 (2021): -Test substance not characterised -Adjuvant not stated -No individual results reported -No positive control used in the assay GLP: No. **Study acceptable**	Female Albino Dunkin-Hartley guinea pigs 10 animals/test group 5 animals/control group	Eugenol Purity not stated Batch no.: not stated Intradermal induction: 0.1% eugenol in dobs/saline (6 injections) Topical induction: 100% eugenol Challenge: 25% eugenol in acetone/PEG	**30 %** positive response animals Mean erythema score from positive animals: 0.8 **Sensitiser**	Hilton *et al.* (1996) (AS) B.6.2.6.2.2
In vitro skin sensitisation Method: comparable to OECD 442D (2018) Deviations from OECD 442D (2018): -Non-standard assay (new methodology proposed), *i.e.* not currently approved within the scope of the guidance. -Limited level of reporting. GLP: No. **Supporting information**	PHK 16-0b cell line	Eugenol Purity not stated Batch no.: not stated In vitro eugenol dosage level: 0-2000 µM	α-Sens value for eugenol: 2.7±0.52 (*Optimal cutoff value to distinguish sensitisers from non-sensitisers=1.6)* The accuracy, sensitivity and specificity of α-Sens against LLNA were 96.4 %, 95.0 %, and 100.0 %, respectively, and those against human data were all 100%. **Sensitiser**	Maeda *et al.* (2020) (AS) B.6.2.6.4
IgE test No guideline **Supporting information**	Female BALB/c strain mice 6 mice/dose	Eugenol in 4:1 acetone: olive oil (AOO) Volume: 50 µL Concentration: 2.5%, 5% and 10 % Positive control: TMA	IgE values (µg mL^{-1}) – Eugenol Values | 2.5% | 5% | 10% Median | 0.856* | 0.295 | 0.492 *Statistically different from control **Non sensitiser**	Hilton, J. *et al.* (1996) (AS) B.6.2.6.3

Table 37: Summary table of human data on skin sensitisation

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
Maximization test in 25 humans volunteers	**Eugenol** Purity: 8%	Eugenol extra (undefined) was tested in 25 human volunteers using a maximisation test protocol. A patch test was performed using 8% eugenol extra for 48 hours using an occlusive patch. The tested concentration was an arbitrary decision to use twice the concentration of materials than is conventionally found in a product applied to the skin.	Eugenol displayed a total score of 0/25; there were no positive reactions in any of the volunteers. Therefore, eugenol was classified as non-sensitizer.	Greif, N. *et al.* (1967) (AS) B.6.9.2.1
Human skin sensitization data from patch test	**Eugenol** Purity: 100% **Clove leaf:** Purity: 84-88% **Clove stern:** Purity: 82-95% **Clove bud**: Purity: 70-84% **Cinnamon leaf Ceylon**: Purity: 80-88% **Bay leaf:**, Purity: 50-65% **Pimenta berry (allspice):** Purity: 65-90%	Results from a total of 11632 patch tests on eugenol itself, on various consumer products containing eugenol and/or clove leaf oil, and on fragrance blends containing eugenol and/or clove leaf oil were collected from fragrance and formulation companies.	-One instance of induced hypersensitivity at patch-test concentrations of 0.05%. -One instance of pre-existing sensitisation at eugenol patch-test concentrations of 0.09%. The survey indicates that, at the concentration present in consumer products eugenol alone or as part of clove leaf oil has a very low potential either to elicit pre-existing sensitisation or to induce hypersensitivity.	Rothenstein, A.S. *et al.* (1987) (AS) B.6.9.2.2
Clinical case I 46-year-old woman	Liquid used in endodontics (containing **eugenol** at unknown concentration). -Fragrance mix and colophony.	Skin tests were carried out applying compounds in the upper back area for 48 h following the GEIDC (Spanish Contact Dermatitis Research Group) standard battery, the Chemotechnique dental screening series and her own products. Observation and positivity was checked at 72 and 168 hours.	Positivity was observed in the compound test with eugenol 2%, endodontics liquid, and fragrance mix and colophony. The eugenol concentration and source of the endodontics liquid, fragrance mix and colophony was unknown.	De Frutos, F.J.O. *et al.* (2004) (AS) B.6.9.2.3
Clinical case II 32-year-old man	Fragrance essential oils.	Patch testing with the GEIDC (Spanish Contact Dermatitis Research Group) standard battery, fragrance essential oils series.	Positivity was observed in the compound test with eugenol and clove oil 1%. However, the eugenol and clove oil concentration in massage products such as balsam and creams was unknown.	Sánchez-Pérez, J. *et al.* (1999) (AS) B.6.9.2.4

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
		Reactions were measured at 2 and 4 days.		

Table 38: Summary table of other studies relevant for skin sensitisation

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
REACH DATA Skin sensitisation study (LLNA) Reliability 2	Eugenol	Mice, CBA/Ca (4♀/dose) Dose: 0, 2.5, 5, 10, 25 and 50% Vehicle: 1:3 ethanol:diethyl phthalate	EC3 = 5.4% Dose-dependent induction of lymph node cell proliferative activity Sensitiser	Lalko, J. and Api A.M. (2006) "Investigation of the dermal sensitization potential of various essential oils in the local lymph node assay". (REACH registration dossier data not provided by applicant and not assessed by the RMS)

2.6.2.7.1 Short summary and overall relevance of the provided information on skin sensitisation

Data from three LLNA studies, two GPMT and one *in vitro* assay have been submitted. Additionally, a mouse IgE measurement study has been evaluated (B.6.2.6.3).

Eugenol gave a positive response in the Local Lymph Node Assay. In a non-radioactive LLNA study, a dose-dependent induction was achieved and the corresponding EC_3 of 25.1 % was calculated (Takeyoshi *et al.*, 2004; B.6.2.6.1.1). Positive responses were obtained in male CBA/Ca mice with eugenol using various vehicles (DEP and ethanol) (Lalko *et al.*, 2004; B.6.2.6.1.2) for which EC_3 values ranging from 5.3 (3:1 EtOH:DEP) to a higher 15.2 (DEP) were derived. In another Local Lymph Node Assay (Hilton, J. *et al.*, 1996; B.6.2.6.3) eugenol tested up to 25 % showed a dose-response induction of lymph node cell proliferative activity, hence, sensitising activity.

Despite some deviations from each respective guidelines (e.g. test substance not characterised, no positive controls run in any of the assays, no individual data provided or the use of a different mice strain in the LLNA), the studies are considered valid to evaluate the skin sensitisation potential of eugenol.

Eugenol showed positive responses in the Guinea Pig Maximisation Test. Eugenol elicited a sensitisation response rate of 20 % in the Magnusson & Kligman maximisation test (Takeyoshi *et al.*, 2004; B.6.2.6.2.1). In the guinea pig maximisation study by Hilton *et al.* (1996; B.6.2.6.2.2) the concentration of the intradermal injection was 0.1% eugenol in 0.01% dodecyloxybenzene sulphate in 0.9% sodium chloride. Topical induction was done 6-8 days later with 100% eugenol during 48 h. Two weeks later, a 25% concentration of eugenol in acetone/PEG was tested. A 30% positive response was observed with a mean erythema score from positive animals of 0.8. Based on the outcome of the study, eugenol is a mild to moderate sensitiser. Deviations from the current OECD TG 406 (1992) include poorly described method and results, although these studies are deemed valid to evaluate the sensitisation of eugenol.

Eugenol was also positive in the α-Sens *in vitro* assay (Maeda *et al.*, 2020; B.6.2.6.4). The study of Maeda *et al.*, described a new method based on a dual luciferase system that measures simultaneously antioxidant response and

cytotoxicity. The α-Sen value for eugenol was 2.7±0.52 with a cell viability of 63% (*Optimal cutoff value to distinguish sensitisers from non-sensitisers was estab1ished in 1.6)*. The accuracy, sensitivity and specificity of α-Sens against LLNA were 96.4 %, 95.0 %, and 100.0 %, respectively, and those against human data were all 100%. These values were better than KeratinoSens and LuSens to LLNA or human data.

Eugenol at concentrations 2.5%, 5% and 10% in 4:1 acetone: olive oil was applied on both flanks of BALB/c mice (Hilton, J. *et al.*, 1996; B.6.2.6.3). A statistically-significant increase of IgE was detected in the 2.5% treatment group whereas at the two high dose groups, eugenol failed to influence significantly serum concentrations of IgE following dermal exposure.

Regarding data collected on humans, one maximization test using eugenol 8% displayed negative results in 25 human volunteers. On the other hand, maximization test carried out in guinea pigs (B.6.2.6.2.1) that used >95% eugenol showed eugenol as skin sensitizer. In addition, data collected from 11632 patch tests on eugenol itself, or various consumer products containing eugenol, displayed one instance of induced hypersensitivity and one instance of pre-existing sensitisation at eugenol patch-test concentrations of 0.05 and 0.09% in consumer products and fragrance blends, respectively. Moreover, two clinical cases that reported skin sensitization courses in individuals who usually used eugenol-containing products such as endodontic liquid and fragrance essential oils, revealed positive results in patch test for eugenol screened products following the GEIDC (Spanish Contact Dermatitis Research Group) protocol. However, the eugenol concentration in endodontic liquid and fragrance essential mixtures was not specified.

Data from REACH registration dossier were included in the summary table of other studies relevant for skin sentisation, since it is an ECHA requirement for the proposal of harmonised classification (CLH) according to Regulation (EC) no. 1272/2008 (CLP). However, it has to be underlined that these data were not available in the applicant submission dossier for the renewal and consequently they have not been evaluated by the RMS and not included in Volume 3 of this RAR. An ongoing CLH proposal is led by Denmark, which contains more data/references on skin sensitisation than in this assessement.

2.6.2.7.2 Comparison with the CLP criteria regarding skin sensitisation

Under the assessment of Spain this hazard class in not opened for classification and labelling as Denmark has submitted a separate CLH proposal on skin sensitisation of eugenol. Please refer to the CLH dossier by Denmark where Skin Sens. 1A (H317) is proposed.

2.6.2.7.3 Conclusion on classification and labelling for skin sensitisation

It has to be noted that currently there is a CLH Report submitted by Denmark to the ECHA focused only in skin sensitisation with a proposal of classification as Skin Sens. 1A. Consequently, **this hazard class is not assessed for harmonization of the classification and labelling in this dossier. Please refer to the CLH dossier by Denmark where Skin Sens. 1A (H317) is proposed.**

2.6.2.8 Phototoxicity

Table 39: Summary table of studies on phototoxicity

Method, guideline, deviations[1] if any	Test substance	Dose levels duration of exposure	Results	Reference
No data available				

Table 40: Summary table of human data on phototoxicity

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No data available				

Table 41: Summary table of other studies relevant for phototoxicity

Type of study/data	Test substance	Observations	Reference
UV/Visible spectroscopy	Eugenol (98.8 % pure)	**No absorption > 290 nm in acidic or neutral conditions** Alkaline: Extinction Coef. = 3780 at λ = 297 nm	White, G.A., (2007) (AS) (B.2.4)

According to Regulation (EU) No. 283/2013, no phototoxicity testing is required based on the results of the study. No absorption > 290 nm in acidic and neutral conditions (White G.A., 2007). Minor absorption at 297 nm under alkaline conditions.

2.6.2.9 Aspiration hazard [equivalent to section 10.13 of the CLH report template]

Table 42: Summary table of evidence for aspiration hazard

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No data available				

2.6.2.9.1 Short summary and overall relevance of the provided information on aspiration hazard

No data are available to evaluate this endpoint.

2.6.2.9.2 Comparison with the CLP criteria regarding aspiration hazard

No data are available to evaluate this hazard. A substance is classified in Category 1: (a) based on reliable and good quality human evidence or (b) if it is a hydrocarbon and has a kinematic viscosity of 20,5 mm 2 /s or less, measured at 40ºC.
Eugenol does not belong to the hydrocarbon chemical class although it is described as an oily liquid. No kinetic viscosity data are available and therefore, no conclusion can be drawn for this hazard class.

2.6.2.9.3 Conclusion on classification and labelling for aspiration hazard

Aspiration hazard is not assessed for harmonization of the classification and labelling due to data lacking.

2.6.2.10 Specific target organ toxicity-single exposure (STOT SE) [equivalent to section 10.11 of the CLH report template]

Table 43: Summary table of animal studies on STOT SE (specific target organ toxicity-single exposure)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
Acute oral toxicity study in rats Prior to OECD TG 401 GLP : No (prior to GLP enforcement) Deviations : Test substance not characterised Rat, Albino 12 rats/group (male and female) **Study acceptable** *Guideline value for classification:* *STOT SE 1≤ 300 mg/kg bw/day* *STOT SE 2≤ 2000 mg/kg bw/day*	Eugenol (undiluted) Purity: Not stated Oral gavage Preliminary dose-range experiment with 2 rats/group dose. Single doses (mL/kg): 1.5, 1.6, 1.75, 1.9, 2.0, 2.1 and 2.2, equivalent to 1597.5, 1704, 1863.75, 2023.5, 2130, 2236.5 and 2343 mg/kg bw 10-day observation period	**Clinical signs** All animals experienced weakness of the hind legs after 5min post-dosing and by 15 min there was complete paralysis of the lower extremities. 86% of the animals (70 animals) experienced lower jaw relaxed that remained open for a period of time. A total of 70% (64 animals) finally became prostrate unable to move. A total of 54% animals went into a coma. The narcotic effects were observed in surviving animals for as long as 3 days. The authors report all surviving animals recovered by the 5th day. **Necropsy**: Congestion of liver and kidneys. Glandular stomach contained mucus, clotted blood and areas of mucosal erosion. Diffuse inflammation with congestion in the viscera. **LD_{50}: 1930 mg/kg bw (1.8 mL/kg)**	Sober, H.A. *et al.* (1950) (AS) B.6.2.1.2

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
Acute oral toxicity study in dogs Prior to OECD TG 401 GLP : No (prior to GLP enforcement) Deviations: Test substance not characterised, poorly described method and results, 5 dogs used in 20 experiments. **Supporting only** *Guideline value for classification:* *STOT SE 1≤ 300 mg/kg bw/day* *STOT SE 2≤ 2000 mg/kg bw/day*	Eugenol (aqueous emulsion stabilised with 5% gum acacia) Purity: Not stated Oral (instillation into stomach via Levin tube) Species: Dogs Strain: Mongrel 5 Female dogs for 20 experiments Single doses: 2% or 5% eugenol in 50mL or 100 mL, equivalent to 100, 200, 250 and 500 mg/kg bw	**Clinical signs** Marked motor dysfunction (ataxia) primarily of the hind limbs observed in 3 dogs in the high dose group (500 mg/kg bw). This effect was observed at 2-3h post dose and only one surviving dog showed total recovery at 3.5-4h after dose. Retching and vomiting (observed at doses 250 and 500 mg/kg bw), slight decrease in body temperature and an increase in pulse rate. **Necropsy** Congestion and hyperaemia of both liver and kidneys. **LD_{50} < 2000 mg/kg bw**	Lauber, F.U. and Hollander, F. (1950) (AS) B.6.2.1.5
Acute inhalation toxicity study in rats Similar to OECD TG 403 (1981) GLP: No **Study acceptable**	Eugenol;Purity: 99% Species: Rat Strain: Sprague-Dawley 5 rats/sex/group 3 Treatment groups Exposure: 4-h (whole body) Nominal concentration: (group 1), 2.39 mg/L (group 2) and 1.53 mg/L (group 3) MMAD and GSD: 0.82 µm (σg 2.26) (Group 1), 0.88 µm (σg 2.05) (group 2) and 0.9 µm (σg 1.87) (group 3)	No mortality **Clinical signs** During exposure, increase salivation and restlessness observed in the high dose group. Some gasping was noted which disappeared within 2 h, as did all clinical signs except restlessness at the lower eugenol levels. Immediately following exposure, all high dose rats were lethargic and many high and intermediate dose rats had wet snouts and red-brown staining of their fur. During the 1st day of the 14-day observation period, all clinical signs disappeared in all groups and all rats appeared normal. **LD_{50} > 2.58 mg/L**	Clark, G.C (1988) (AS) B.6.2.3

Table 44: Summary table of human data on STOT SE (specific target organ toxicity-single exposure)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
No evidence of effects in humans relevant for STOT SE (specific target organ toxicity-single exposure) in the available data				

Table 45: Summary table of other studies relevant for STOT SE (specific target organ toxicity-single exposure)

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
There are no other available relevant studies for STOT SE (specific target organ toxicity-single exposure)				

2.6.2.10.1 Short summary and overall relevance of the provided information on specific target organ

toxicity – single exposure (STOT SE)

Neither the occupational medical surveillance data nor the available acute inhalation study in rats (Clark, G.C, 1988; B.6.2.3) refers to effects that would lead to the classification of these substances as irritant to the respiratory tract.

In an acute oral toxicity study in rats (Sober, H.A. *et al.*, 1950; B.6.2.1.2), groups of 12 rats/sex were dosed with eugenol at concentrations 1.5, 1.6, 1.75, 1.9, 2.0, 2.1 and 2.2 mL/kg, equivalent to 1597.5, 1704, 1863.75, 2023.5, 2130, 2236.5 and 2343 mg/kg bw. At all doses, clinical effects were reported as weakness of the hind legs 5 min after dosing and by 15min there was complete paralysis of the lower extremities. Further clinical signs as lower jaw relaxation and prostration in 64 animals were reported.

In an acute oral toxicity study in dogs (Lauber, F.U. and Hollander, F., 1950; B.6.2.1.5), marked motor dysfunction (ataxia) was observed in dogs in the top dose group (500 mg/kg bw).

In an acute inhalation toxicity study in rats (Clarck, G.C, 1988; B.6.2.3), clinical effects during exposure included gasping, increased salivation and restlessness in the high dose group. Lethargy was observed in the high dose group following exposure and many high and intermediate dose group rats had wet snouts and red-brown staining of their fur. All clinical signs disappeared in all groups and all rats appeared normal. No mortality was observed in the study.

2.6.2.10.2 Comparison with the CLP criteria regarding STOT SE (specific target organ toxicity-single exposure)

STOT SE 3

STOT SE3 includes narcotic effects and respiratory tract irritation. These are target organ effects for which a substance does not meet the criteria to be classified in Categories 1 or 2.

According to the results of the acute inhalation study (Clark, G.C, 1988; B.6.2.3), respiratory tract irritation was not observed upon administration of eugenol.

Effects observed in acute oral toxicity studies with eugenol, such as motor activity dysfunction and ataxia in rats (Sober, H.A. *et al.*, 1950; B.6.2.1.2) and dogs (Lauber, F.U. and Hollander, F., 1950; B.6.2.1.5), respectively, and also the lethargy observed in the acute inhalation study may be indicative of narcotic effects during acute exposure to eugenol. In rats, the effects were observed in surviving animals up to the 5th day after dose and lasted a maximum of three days. In dogs, these effects were observed at 2-3h post dose and in one suriving dog they ceased after 3.5-4h post dose. Therefore, based on the data available and according to criteria of Regulation (EC) 1272/2008, **eugenol** is classified as **Specific Target Organ Toxicity – single exposure Category 3 (STOT SE 3; H336)**.

STOT SE 1 and 2

STOT-SE Category 1 and 2 is assigned on the basis of findings of 'significant' or 'severe' toxicity. In this context, 'significant' means changes which clearly indicate functional disturbance or morphological changes which are toxicologically relevant. 'Severe' effects are generally more profound or serious than 'significant' effects and are of considerably adverse nature with significant impact on health. Both factors have to be evaluated by weight of evidence and expert judgement.

Based on available single exposure data, no classification is proposed for the category STOT SE 1 and 2 for eugenol.

2.6.2.10.3 Conclusion on classification and labelling for STOT SE (specific target organ toxicity-single exposure)

Based on available acute toxicity data of **eugenol**, and according to the criteria under Regulation (EC) No. 1272/2008, **eugenol** is classified as **Specific Target Organ Toxicity – single exposure Category 3 (STOT SE 3; H336).**

2.6.3 Summary of repeated dose toxicity (short-term and long-term toxicity) [section 10.12 of the CLH report]

2.6.3.1 Specific target organ toxicity-repeated exposure (STOT RE) [equivalent to section 10.12 of the CLH report template]

Table 46: Summary table of animal studies on repeated dose toxicity (short-term and long-term toxicity) STOT RE (specific target organ toxicity - repeated exposure)

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results
- NOAEL/LOAEL
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr><td colspan="4">Short-term toxicity</td></tr>
<tr><td>14-day oral study in rats
<u>GLP</u>: No
<u>Method</u>: Non-stated.
<u>Rat strain:</u>
F344/N rats: ♂ and ♀
<u>No. animals</u>:
5 rats/dose

<u>Deviations from current test guideline (OECD TG 407, 2008):</u>
-No control group was used.
-14 days treatment was assayed instead of 28 days.
- Clinical signs, food consumption, haematology, clinical chemistry, necropsy, organ weights and histopathology not performed.
- Statistical analysis was not performed in bodyweight.

Study acceptable as supportive information.

Guideline value for classification extrapolated to 4-week study:
STOT RE 1 ≤ 64.2 mg/kg bw/day
STOT RE 2 ≤ 642.8 mg/kg bw/day</td><td><u>Test substance:</u>
Eugenol: Batch No.: 36483, Purity: >99%

Eugenol :Oral (diet)

Doses:
<u>Males/Females:</u> 0, 6000, 12500, 25000, 50000, 100000 ppm (equivalent to 0, , 720, , 1500. 3000, 6000 and 12000 mg/kg bw/day).

14-day feed exposure.</td><td>Survival: All female rats that received the high dose died, whereas only one male died at high dose group.

<table><tr><th></th><th colspan="2">% Survival</th></tr><tr><th>Dose (ppm)</th><th>Male</th><th>Females</th></tr><tr><td>6000</td><td>5/5 (100%)</td><td>5/5 (100%)</td></tr><tr><td>12500</td><td>5/5 (100%)</td><td>5/5 (100%)</td></tr><tr><td>25000</td><td>5/5 (100%)</td><td>5/5 (100%)</td></tr><tr><td>50000</td><td>5/5 (100%)</td><td>5/5 (100%)</td></tr><tr><td>100000</td><td>4/5 (80%)</td><td>0/5 (0%)</td></tr></table>
100000 ppm (equivalent to 12000 mg/kg bw/day for ♂/♀)

<u>Bodyweight</u>
▪ (↓) bw in ♂ at termination (27%, compared with initial bw).
<u>Bodyweight gain</u>
▪ (↓) bwg in ♂ (157%, compared with initial bw).
▪
50000 ppm (equivalent to 6000 mg/kg bw/day for ♂/♀)

<u>Bodyweight gain</u>
▪ (↓) bwg in ♂/♀ (72/75%, compared with initial bw).

25000 ppm (equivalent to 3000 mg/kg bw/day for ♂/♀)

<u>Bodyweight gain</u>
▪ (↓) bwg in ♂/♀ (22/31%, compared with initial bw).</td><td>NTP (1983)
(AS)
B.6.3.1.1</td></tr>
<tr><td>14-day oral study in mice</td><td><u>Test substance:</u>
Eugenol</td><td>Survival: All male and female mice that received the high dose died. At 50000 ppm 3/5 males died,</td><td>NTP (1983)
(AS)
B.6.3.1.2</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, route of exposure, dose levels, duration of exposure</th><th>Results
- NOAEL/LOAEL
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr>
<td>GLP: No
Method: Non-stated.
Mice strain:
B6C3F1: ♂ and ♀
No. animals:
5 mice/dose

Deviations from current test guideline (OECD TG 407, 2008):
-No control group was used.
-14 days treatment was assayed instead of 28 days.
- Clinical signs, food consumption, haematology, clinical chemistry, necropsy, organ weights and histopathology not performed.
- Statistical analysis was not performed in bodyweight.

Study acceptable as supportive information.

Guideline value for classification extrapolated to 4-week study:
STOT RE 1 ≤ 64.2 mg/kg bw/day
STOT RE 2 ≤ 642.8 mg/kg bw/day</td>
<td>Eugenol :Oral (diet)

Doses:
Males/Females: 0, 6000, 12500, 25000, 50000, 100000 ppm (equivalent to 0, 1200, 2500, 5000, 10000 and 20000 mg/kg bw/day).

14-day feed exposure.</td>
<td>however, no deaths occurred at 50000 ppm female group.

<table>
<tr><th></th><th colspan="2">% Survival</th></tr>
<tr><th>Dose (ppm)</th><th>Male</th><th>Females</th></tr>
<tr><td>6000</td><td>5/5 (100%)</td><td>5/5 (100%)</td></tr>
<tr><td>12500</td><td>5/5 (100%)</td><td>5/5 (100%)</td></tr>
<tr><td>25000</td><td>5/5 (100%)</td><td>5/5 (100%)</td></tr>
<tr><td>50000</td><td>2/5 (40%)</td><td>5/5 (100%)</td></tr>
<tr><td>100000</td><td>0/5 (0%)</td><td>0/5 (0%)</td></tr>
</table>
50000 ppm (equivalent to 10000 mg/kg bw/day for ♂/♀)

Bodyweight
▪ (↓) bw in ♂ at termination (35%, compared with initial bw).
▪ (↓) bw in ♀ at termination (30%, compared with initial bw).

Bodyweight gain
▪ (↓) bwg in ♂/♀ (333/600%, compared with initial bw).

25000 ppm (equivalent to 5000 mg/kg bw/day for ♂/♀)

Bodyweight
▪ (↓) bw in ♂ at termination (15%, compared with initial bw).
▪ (↓) bw in ♀ at termination (12%, compared with initial bw).
Bodyweight gain
▪ (↓) bwg in ♂/♀ (200/300%, compared with initial bw).

12500 ppm (equivalent to 1875 mg/kg bw/day for ♂/♀)

Bodyweight
▪ (↓) bw in ♂ at termination (5%, compared with initial bw).
Bodyweight gain
▪ (↓) bwg in ♂ (133%, compared with initial bw).</td>
<td></td>
</tr>
<tr>
<td>Oral 21-day toxicity study in dogs
GLP: No
Method: Non-stated.
Dog breed:
Mongrel
No. animals:
1-3

Deviations from</td>
<td>Test substance:
Eugenol

Eugenol :Oral (gavage)

Doses:
Females: 2g eugenol/dog at intervals of 48-72 hours for 10 times (equivalent to 100 mg/kg bw/day).</td>
<td>Mortality: No mortality or morbidity signs were observed during the study.

Clinical signs: No clinical signs were observed during the study.

Necropsy (data not shown in the study): No relevant findings were reported.

Histopathology (data not shown in the study): No relevant findings were reported.</td>
<td>Lauber, F.U. and Hollander, F. (1950) (AS)
B.6.3.1.3</td>
</tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
current test guideline (OECD TG 407, 2008): -Test material not characterised (batch and purity not reported). -Dogs used instead of rodents. -No control groups were used. -Only one dose level was tested. -Test substance was administered in dose-intervals of 48-72 h. -No bodyweight, food consumption, clinical chemistry and haematology were performed. -Data of necropsy, organ weights and histopathology were not presented in the study. **Study acceptable as supportive information.** Guideline value for classification extrapolated to 4-week study: STOT RE 1 ≤ 42.8 mg/kg bw/day STOT RE 2 ≤ 428.5 mg/kg bw/day	21-day exposure.	**-LOAEL= -** **-$NOAEL_{toxicity}$=** 100 mg/kg bw/day -Critical effects at the LOAEL: - Target tissue/organ: None	
Oral 28-day study in rats GLP: No Method: Non-stated. Rat strain: F344/N rats: ♂ No. animals: 5 males/dose Deviations from current test guideline (OECD TG 407, 2008): -Test substance not characterized (purity, batch	Test substance: Eugenol Eugenol :Oral (diet) **Doses:** Males: 2% (equivalent to 2000 mg/kg bw/day). 28-day exposure.	**Survival:** No mortality or morbidity signs were observed during the study. **Clinical signs**: No clinical signs were observed during the study. **Bodyweight *(data not shown in the study)*:** A reduction of 10-15 % was observed in rats treated with 2% eugenol. **Liver weight (data not shown in the study):** No differences were described between treated group and controls. **Histopathology *(data not shown in the study)*:** No gross or histological changes were found in the forestomach. **-LOAEL=** 2000 mg/kg bw/day	WHO/FAO Joint Expert Committee on Food Additives (JECFA). WHO Food additive series No. 56, 155-200. (2006) (review from Hirose *et al.*, 1987) (AS) B.6.3.2.1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
number and certificate of chemical analysis are not reported). - Only 5 male rats were used. - Only one dose level was assayed. - Food consumption, haematology and clinical chemistry were not performed. -Only livers and stomachs were subjected to gross examinations and histopathological analysis. -Bodyweight and histopathology data not presented. **Study acceptable as supportive information.** Guideline value for classification extrapolated to 4-week study: STOT RE 1 ≤ 32.1 mg/kg bw/day STOT RE 2 ≤ 321.4 mg/kg bw/day		**-NOAEL= -** -Critical effects at the LOAEL: ↓ bodyweight. Target tissue/organ: None	
Oral 34-day study in rats GLP: No Method: Non-stated. Rat strain: Osborne-Mendel rats: ♂ No. animals: 20 males/dose Deviations from current test guideline (OECD TG 407, 2008): -Test substance not characterised (purity, batch number and certificate of chemical analysis are not reported). - Only male rats	Test substance: Eugenol Eugenol :Oral (gavage) **Doses:** Males: 1400-4000 mg/kg bw/day. 34-day exposure	**Survival**: A few deaths were reported at 2000 mg/kg bw per day, and the number increased with increasing doses. The survival rate was 40% (8/20 animals) at termination study. **Clinical signs**: No clinical signs were observed during the study. **Bodyweight *(data not shown in the study)*:** No differences were observed between treated groups and controls. **Food consumption *(data not shown in the study)*:** No differences were observed between treated groups and controls. **Haematology *(data not shown in the study)*:** No differences were observed between treated groups and controls. **Necropsy *(data and dose-effects not shown in the study)***: ▪ (↑) adrenal enlargement, with marked yellow discolouration. ▪ (↑) slight enlargement of the liver. ▪ (↑) coalescent areas covered with a thick,	WHO/FAO Joint Expert Committee on Food Additives (JECFA). WHO Food additive series No. 56, 155-200. (2006) (review from Hagan *et al.*, 1965) (AS) B.6.3.3.1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
were assayed. -The dose pattern used between the dose-range applied is not specified. -The dosage levels in which the adverse effects were described are not stated. - No clinical biochemistry analysis was performed. -No data of bodyweights, food consumption, haematology, necropsy, organ weights and histopathological analysis were presented. **Study acceptable as supportive information.** Guideline value for classification extrapolated to 4-week study: STOT RE 1 ≤ 26.4 mg/kg bw/day STOT RE 2 ≤ 264.7 mg/kg bw/day		flaky, white material punctuated with minute ulcers in the forestomach. **Histopathology *(data and dose-effects not shown in the study)*:** *Forestomach:* ▪ (↑) Moderately-severe hyperplasia and hyperkeratosis of the stratified squamous epithelium associated with focal ulceration. *Liver:* ▪ (↑) slight liver-cell enlargement. *Bones:* ▪ (↑) mild osteoporosis. **-LOAEL= -** **-NOAEL= -.** Target tissue/organ: Forestomach and liver	
13-week oral study in rats GLP: No Method: Non-stated. Rat strain: F344/N rats: ♂ and ♀ No. animals: 10 rats/dose Deviations from current test guideline (OECD TG 408, 2018): -Food consumption, haematology, clinical chemistry and ophtalmological examination were not performed. - Statistical analysis	Test substance: Eugenol Batch No.: 36483, Purity: >99% Eugenol :Oral (diet) **Doses:** Males/Females: 0, 800, 1500, 3000, 6000, 12500 ppm (equivalent to 0, 72, 135, 270, 540 and 1125 mg/kg bw/day). 91-day feed exposure.	**Survival:** No differences in survival were noted between treated groups and controls. **12500 ppm (equivalent to 1125 mg/kg bw/day for ♂/♀)** Bodyweight and bodyweight gain ▪ (↓) bw in ♂ at termination (10%). ▪ (↓) bwg in ♂ at termination (12%). **-LOAEL**= 12500 ppm (~1125 mg/kg bw/day). **-NOAEL$_{toxicity}$**= 6000 ppm (~540 mg/kg bw/day) -Critical effects at the LOAEL: ↓ bodyweight and bodyweight gain. Target tissue/organ: None	NTP (1983) (AS) B.6.3.4.1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
was not performed. -Organ weights were not recorded. - Data of necropsies and histopathology were not presented. - Circulating thyroid hormones (T4, T3, TSH) levels were not measured. **Study acceptable as supportive information.**			
13-week oral study in mice GLP: No Method: Non-stated. Mice strain: $B6C3F_1$: ♂ and ♀ No. animals: 10 mice/dose Deviations from current test guideline (OECD TG 408, 2018): -Food consumption, haematology, clinical chemistry and ophtalmological examination were not performed. - Statistical analysis was not performed. -Histopathology examinations were only performed in the control and high dose groups. -Organ weights were not recorded. - Data of necropsies and histopathology were not presented. - Circulating thyroid hormones (T4, T3, TSH) levels were not measured. **Study acceptable as supportive information.**	Test substance: Eugenol. Batch No.: 36483, Purity: >99% Eugenol :Oral (diet) **Doses:** Males/Females: 0, 400, 800, 1500, 3000, 6000 ppm (equivalent to 0, 80, 160, 300, 600 and 1200 mg/kg bw/day). 91-day feed exposure.	**Survival:** No differences in survival were noted between treated groups and controls. **6000 ppm (equivalent to 1200 mg/kg bw/day for ♂/♀)** Bodyweight and bodyweight gain ▪ (↓) bwg in ♂ at termination (10%). **-LOAEL**= 6000 ppm (~1200 mg/kg bw/day). **-$NOAEL_{toxicity}$**= 3000 ppm (~600 mg/kg bw/day) -Critical effects at the LOAEL: ↓ bodyweight gain. Target tissue/organ: None	NTP (1983) (AS) B.6.3.4.2
Oral 19-week study in rats GLP: No	Test substance: Eugenol Commercially available eugenol	**Survival:** No mortality or morbidity signs were observed during the study.	Hagan *et al.* (1967) (AS) B.6.3.5.1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
Method: Non-stated. Rat strain: Osborne-Mendel rats: ♂ and ♀ No. animals: 10 rats/dose Deviations from current test guideline (OECD TG 408, 2018): -Test substance not characterised (purity, batch number and certificate of chemical analysis are not reported). - Only two dose levels were assayed. -The doses used between the dose-range applied are not specified. - No clinical biochemistry analysis was performed. -No data of bodyweights, food consumption, haematology, necropsy, organ weights and haematological analysis were presented. **Study acceptable as supportive information.** Guideline value for classification extrapolated to 4-week study: STOT RE 1 ≤ 6.75 mg/kg bw/day STOT RE 2 ≤ 67.5 mg/kg bw/day	used as food additive (purity: unknown) Eugenol :Oral (diet) **Doses:** Males/Females: 0, 1000 and 10000 ppm (equivalent to 0, 90 and 900 mg/kg bw/day. 133-day exposure.	**Clinical signs:** No clinical signs were observed during the study. **Bodyweight (*data not shown in the study*):** No differences were observed between treated groups and controls. **Food consumption (*data not shown in the study*):** No differences were observed between treated groups and controls. **Haematology (*data not shown in the study*):** No differences were observed between treated groups and controls. **Necropsy (*data not shown in the study*):** No differences were observed between treated groups and controls. **Histopathology (*data not shown in the study*):** No differences were observed between treated groups and controls. **-LOAEL**= - **-NOAEL$_{toxicity}$**= 10000 ppm (~900 mg/kg bw/day). -Critical effects at the LOAEL: - Target tissue/organ: None	
Long-term toxicity and carcinogenicity			
2-year carcinogenicity study in rat Rat strain: F344/N rats: ♂ and ♀ No. animals:	Test substance: Eugenol Eugenol :Oral (diet) **Doses:**	*Only effects relevant for STOT RE* **6000/12500 ppm** (equivalent to 260 and 648 mg/kg bw/day for ♂ and ♀) Neoplastic changes: ▪ (↑) incidence of uterine endometrial stromal	NTP (1983) (AS) B.6.5.1

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
50 mice/dose **Study acceptable as supportive information.** *See table 53 for more information.* Guideline value for classification extrapolated to 4-week study: STOT RE 1 ≤ 1.2 mg/kg bw/day STOT RE 2 ≤ 12.2 mg/kg bw/day	Males: 0, 3000 and 6000 ppm (equivalent to adjusted value by RMS of 0, 128 and 260 mg/kg bw/day, or default value of 0, 150 and 300 mg/kg bw/day). Females: 0, 6000 and 12500 ppm (equivalent to adjusted value by RMS of 0, 302 and 648 mg/kg bw/day, or default value of 0, 300 and 625 mg/kg bw/day) 105-week feed exposure.	polyp/sarcoma in ♀ (34% vs 15% in controls; ns). ▪ (↑) incidence of alveolar/bronchiolar adenoma / carcinoma in ♂ (4% vs 0% in controls; ns; ndr). ▪ (↓) incidence of thyroid C-cell adenoma in ♀ (4% vs 8% in controls; ns; ndr). ▪ (↓) incidence of thyroid C-cell adenoma in ♂ (0% vs 10% in controls). Non-neoplastic changes (*statistical analysis not performed*): ▪ (↑) cystic hyperplasia in the uterus (22% vs 3% in controls). ▪ (↑) spleen haemosiderosis in ♀ (16% vs 2% in controls). ▪ (↑) kidney chronic inflammation in ♂ (86% vs 73% in controls; ncdr). ▪ (↓) kidney chronic inflammation in ♀ (4% vs 10% in controls). ▪ (↑) Thyroid C-cell hyperplasia in ♂ (8% vs 3% in controls). ▪ (↓) Thyroid C-cell hyperplasia in ♀ (10% vs 15% in controls). ▪ (↑) Prostate inflammation suppurative (11% vs 8% in controls; ndr). ▪ (↑) Prostate inflammation chronic suppurative (4% vs 0% in controls; ndr). **3000/6000 ppm** (equivalent to 128 and 302 mg/kg bw/day for ♂ and ♀) Neoplastic changes: ▪ (↑) incidence of alveolar/bronchiolar adenoma / carcinoma in ♂ (10% vs 0% in controls; ndr). ▪ (↑) incidence of thyroid C-cell adenoma in ♀ (22% vs 8% in controls; ndr). Non-neoplastic changes (*statistical analysis not performed*): ▪ (↑) cystic hyperplasia in the uterus in ♀ (4% vs 3% in controls). ▪ (↑) kidney chronic inflammation in ♂ (92% vs 73% in controls; ncdr). ▪ (↓) kidney chronic inflammation in ♀ (6% vs 10% in controls). ▪ (↓) Thyroid C-cell hyperplasia in ♂ (2% vs 3% in controls). ▪ (↓) Thyroid C-cell hyperplasia in ♀ (14% vs 15% in controls). ▪ (↑) Prostate inflammation suppurative in ♂ (18% vs 8% in controls; ndr). ▪ (↑) Prostate inflammation chronic suppurative in ♂ (10% vs 0% in controls; ndr). Target tissue/organ: Uterus and spleen.	
2-year carcinogenicity study in mice Mice strain: B6C3F1: ♂ and ♀ No. animals: 50 mice/dose	Test substance: Eugenol Eugenol :Oral (diet) **Doses:** Males/Females: 0, 3000 and 6000 ppm	*Only effects relevant for STOT RE* **6000 ppm** (equivalent to adjusted value by RMS of 1250/1546 for ♂/♀, or default value of 900 mg/kg bw/day for ♂/♀) Neoplastic changes: ▪ (↑) increase incidences of hepatocellular	NTP (1983) (AS) B.6.5.2

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
Study acceptable as supportive information. *See table 53 for more information.* Guideline value for classification extrapolated to 4-week study: STOT RE 1 ≤ 1.2 mg/kg bw/day STOT RE 2 ≤ 12.2 mg/kg bw/day	(equivalent to adjusted value by RMS of 0, 632/784 and 1250/1546 for ♂/♀, or default values of 0, 450 and 900 mg/kg bw/day). 106-week feed exposure.	adenoma or carcinoma in ♀ (18% vs 4% in controls). ▪ (↑) increase incidences of hepatocellular adenoma or carcinoma in ♂ (37% vs 28% in controls; ns; ndr.). ▪ (↑) increase incidences of hepatocellular adenoma in ♂ (20% vs 8% in controls; ns ndr.). ▪ (↓) increase incidences of hepatocellular carcinoma in ♂ (18% vs 20% in controls; ns; ndr). Non neoplastic changes (*statistical analysis not performed*): ▪ (↑) increase incidences of focal inflammation of the kidney in ♂ (16% vs 0% in controls). ▪ (↓) decrease incidences of kidney nephrosis in ♂ (8% vs 61% in controls), and ♀ (27% vs 36% in controls). ▪ (↑) focal granulomatous inflammation of lung in ♀ (51% vs 36% in controls). ▪ (↑) adenomatous hyperplasia of lung in ♀ (54% vs 44% in controls). ▪ (↓) thyroid cystic degeneration in ♂ (0% vs 23% in controls). ▪ (↓) Skin chronic inflammation in ♂ (0% vs 14% in controls; ndr); and ♀ (0% vs 14% in controls). ▪ (↑) ovary follicular cyst (33% vs 22% in controls). **3000 ppm** (equivalent to adjusted value by RMS of 632/784 for ♂/♀, or default value of 450 mg/kg bw/day for ♂/♀) Neoplastic changes: ▪ (↑) increase incidences of hepatocellular adenoma or carcinoma in ♀ (14% vs 4% in controls; ns). ▪ (↑) increase incidences of hepatocellular adenoma or carcinoma in ♂ (56% vs 28% in controls; ndr.). ▪ (↑) increase incidences of hepatocellular adenoma in ♂ (26% vs 8% in controls; ndr.). ▪ (↑) increase incidences of hepatocellular carcinoma in ♂ (40% vs 20% in controls; ndr). Non neoplastic changes (*statistical analysis not performed*): ▪ (↑) focal granulomatous inflammation of lung in ♀ (39% vs 36% in controls). ▪ (↑) adenomatous hyperplasia of lung in ♀ (45% vs 44% in controls). ▪ (↓) decrease incidences of kidney nephrosis in ♂ (52% vs 61% in controls), and ♀ (33% vs 36% in controls). ▪ (↓) thyroid cystic degeneration in ♂ (6% vs 23% in controls). ▪ (↑) Skin chronic inflammation in ♂ (22% vs 14% in controls; ndr), and (↓) in ♀ (0% vs 14% in controls). Target tissue/organ: Kidney, lung and ovary.	
Carcinogenicity	Test substance:	*Only effects relevant for STOT RE*	Miller *et al.* (1983)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
study in mice (18 months) Mice strain: CD-1 ♀ No. animals: 30 females/dose **Study acceptable as supportive information.** *See table 53 for more information.* Guideline value for classification extrapolated to 4-week study: STOT RE 1 ≤ 1.8 mg/kg bw/day STOT RE 2 ≤ 17.8 mg/kg bw/day	Eugenol Eugenol :Oral (diet) Phenobarbital: Oral (drinking water) **Doses:** Females: 0 (negative control), 0.5% eugenol, 0.5% eugenol + 0.05% phenobarbital and 0.05% phenobarbital (positive control) 72-week feed exposure.	Neoplastic changes ▪ (↑) Thymic lymphoma (7%) vs 0% in controls. ▪ (↑) Mammary adenocanthoma (3%) vs 0% in controls. Target tissue/organ: Thymus and mammary gland.	(AS) B.6.5.3
Reproductive toxicity			
Developmental toxicity study in rat. Rat strain: Sprague-Dawley (CD® –Crl : (SD) BR) Sex: 25 mated females/group. **Study acceptable** *See table 60 for more information.* Guideline value for classification extrapolated to 4-week study: STOT RE 1 ≤ 64.2 mg/kg bw/day STOT RE 2 ≤ 642.8 mg/kg bw/day	Test substance: Eugenol Eugenol :Oral (gavage) Dose levels: ♂/♀: 0, 100, 250 and 600 mg/kg bw/day throughout day 5-19 of gestation.	*Only effects relevant for STOT RE* No findings were observed in the necropsies of the dams and in consequence no effects for STOT RE were noted in this study.	[redacted] (2004) (AS) B.6.6.2.5
Developmental toxicity study in rabbit. Rabbit strain: New Zealand White Sex: 24 mated females/group. **Study acceptable as supportive information.** *See table 60 for more information.*	Test substance: Eugenol Eugenol :Oral (gavage) Dose levels: ♂/♀: 0, 100, 250 and 500/350 mg/kg bw/day throughout day 5-28 of gestation.	*Only effects relevant for STOT RE* No findings were observed in the necropsies of the dams and in consequence no effects for STOT RE were noted in this study.	[redacted] (2004) (AS) B.6.6.2.6

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
Guideline value for classification extrapolated to 4-week study: STOT RE 1 ≤ 39.1 mg/kg bw/day STOT RE 2 ≤ 391.3 mg/kg bw/day			

ns: no significative; ndr: no dose-response; ncdr: no clearly dose-response

Table 47: Summary table of human data on repeated dose toxicity STOT RE (specific target organ toxicity-repeated exposure)

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
No human data relevant on repeated dose toxicity STOT RE				

Table 48: Summary table of other studies relevant for repeated dose toxicity STOT RE (specific target organ toxicity-repeated exposure)

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
Reproductive toxicity				
Teratology study in rats with clove oil. Rat strain: Wistar-rats Sex: 23-25 mated females/group. **Study acceptable as supportive information** *See table 60 for more information.*	Test substance: **Clove oil**	**Clove oil** :Oral (gavage) Dose levels: ♀: 0, 2.8, 13, 60 and 280 mg/kg bw/day throughout day 6-15 of gestation.	*Only effects relevant for STOT RE* No findings were observed in the necropsies of the dams and in consequence no effects for STOT RE were noted in this study.	Morgareidge K. *et al.* (1973a) (AS) B.6.6.2.1
Teratology study in rabbits with clove oil. Rabbit strain: Dutch-belted rabbits Sex: 11-15 mated females/group. **Study acceptable as supportive information** *See table 60*	Test substance: **Clove oil**	**Clove oil** :Oral (gavage) Dose levels: ♀: 0, 1.72, 7.99. 37.1 And 172 mg/kg bw/day throughout day 6-18 of gestation.	*Only effects relevant for STOT RE* No findings were observed in the necropsies of the dams and in consequence no effects for STOT RE were noted in this study.	Morgareidge K. *et al.* (1973b) (AS) B.6.6.2.2

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
for more information.				
Teratology study in mice with clove oil. Mice strain: Albino CD-1 Sex: 23-25 mated females/group. **Study acceptable as supportive information** *See table 60 for more information.*	Test substance: **Clove oil**	**Clove oil** :Oral (gavage) Dose levels: ♀: 0, 2.2, 10, 46 and 215 mg/kg bw/day throughout day 6-15 of gestation.	*Only effects relevant for STOT RE* No findings were observed in the necropsies of the dams and in consequence no effects for STOT RE were noted in this study.	Morgareidge K. *et al.* (1973c) (AS) B.6.6.2.3
Teratology study in hamsters with clove oil. Hamster strain: Golden hamsters Sex: 25-28 mated females/group. **Study acceptable as supportive information** *See table 60 for more information.*	Test substance: **Clove oil**	**Clove oil** :Oral (gavage) Dose levels: ♀: 0, 1.8, 8.2, 38.2 and 177 mg/kg bw/day throughout day 6-15 of gestation.	*Only effects relevant for STOT RE* No findings were observed in the necropsies of the dams and in consequence no effects for STOT RE were noted in this study.	Morgareidge K. *et al.* (1973d) (AS) B.6.6.2.4

2.6.3.1.1 Short summary and overall relevance of the provided information on specific target organ toxicity – repeated exposure (short-term and long-term toxicity)

Short-term toxicity studies, together with long-term and reproductive toxicity studies (further evaluated in *sections 2.6.5 and 2.6.6,* respectively) have been included in the table according to the template and consequently evaluated in this summary for STOT RE.

Six short-term studies, that were previously considered in the previous DAR 2011 (no classification for STOT RE of eugenol), have been presented and reassessed to support the renewal of the active substance eugenol. Accordingly, all the short-term toxicity studies reviewed were non-GLP compliant and presented important methodological deviations. Therefore, they were considered acceptable as supportive.

Six short-term toxicity studies together with three long-term toxicity studies and two developmental toxicity studies are accordingly summarized for STOT RE as follows:

Studies in rats:

-In the 14-day oral study in rats (B.6.3.1.1) eugenol was tested at 0, 6000, 12500, 25000, 50000, and 100000 ppm dose levels (equivalent to 0, 720, 1500, 3000, 6000 and 12000 mg/kg bw/day), in which 5 animals of each sex were subjected to 14-day feed exposure. This study is a range finding study to select appropriate doses for chronic toxicity studies. It presents important deviations, such as no control group was included, and clinical signs, food

consumption, haematology, clinical chemistry, necropsy, organ weights and histopathology were not evaluated. A reduction in survival was observed in the top dose groups, in which 4/5 (80%) males survived at termination, whereas none female survived at the end of the study. On the other hand, a reduction in bodyweight was observed only in the top dose male group. Besides, bodyweight gain decreases were noted from 12500 ppm male group and from 25000 ppm female group, compared with low dose group.

-In the 28-day oral study (B.6.3.2.1), eugenol was tested at single dose of 2 % (equivalent to 2000 mg/kg bw/day) in 5 male Fisher F344/N rats via feed diet. Neither mortality or morbidity symptoms, nor clinical signs were detected in any animal during test substance administration. Bodyweights were decreased (10-15%) in the treated group. Only livers and forestomach were subjected to histopathological examinations, and no alterations in both organs were detected compared with controls. A LOAEL = 2000 mg/kg bw/day could be established based on bodyweight reduction, so a NOAEL cannot be derived.

-In the 34-day oral study (B.6.3.3.1), twenty male weanling Osborne-Mendel rats received 1400 mg/kg bw/day of eugenol by stomach tube, and the dose was gradually increased to 4000 mg/kg bw/day during the 34-day study. A few deaths were reported at 2000 mg/kg bw/day, and the number increased with increasing doses. Only eight animals survived at termination study (40%). However no clinical signs treatment-related were recorded. Regarding bodyweights, no differences were noted in the treated groups compared with controls. Eugenol treatment caused slight adrenal enlargement, with marked yellow discolouration, and slight enlargement of the liver, which was found microscopically to be due to slight liver-cell enlargement. Macroscopic examination of the forestomach showed that the mucosa contained coalescent areas covered with a thick, flaky, white material punctuated with minute ulcers. Furthermore, histopathological forestomach examinations revealed moderately-severe hyperplasia and hyperkeratosis of the stratified squamous epithelium associated with focal ulceration. The doses in which effects were observed had not been specified in the study. RMS deems that the MTD is probably exceed in this study, so reliability of findings should be taken cautiously.

The extrapolated cut-off value for STOT RE 2 classification for a 4-week dose-repeated study is 264.7 mg/kg bw/day. Consequently the mentioned effects in forestomach from the dose of 1400 mg/kg bw/day are not regarded for STOT RE classification.

-In the 13-week oral study in rats (B.6.3.4.1), eugenol was tested at 0, 800, 1500, 3000, 6000 and 12500 ppm dose levels (equivalent to 0, 72, 135, 270, 540 and 1125 mg/kg bw/day), in which 10 animals of each sex were subjected to 91-day feed exposure. This study was conducted to evaluate the cumulative toxicity of the test material, to identify organs affected, and to determine the most appropriate doses for the two-year studies. Some endpoints such as food consumption, haematology, clinical chemistry and organ weights were not measured. Neither gross mortality or morbidity signs, nor treatment related clinical signs were observed in any dose group. A decreased in bodyweight and bodyweight gain (10% and 12%, respectively) were recorded in the top dose male group compared with controls, whereas no differences were noted in the female dose groups. On the other hand, necropsy and histopathology examinations did not reveal differences between treated groups and controls. A **NOAEL** could be established at **6000 ppm (540 mg/kg bw/day**) based on the decreased bodyweight and bodyweight gain in males at study termination.

-In the 19-week oral study (B.6.3.5.1), eugenol was tested at 0, 1000 and 10000 (equivalent to 0, 90 and 900 mg/kg bw/day) via feed diet for 19-week (133 days), in which 10 male and 10 female Osborne-Mendel rats were assigned to each dose group. The daily observations and the explorations post-mortem did not revealed any adverse effect related to test substance administration. A **NOAEL of 10000 ppm** (equivalent to **900 mg/kg bw/day**) could be established due to no adverse effects were noted at high dose tested.

-In the 2-year carcinogenicity study (B.6.5.1) conducted in F344/N rats, statistically significant increase trend in the incidence of uterine endometrial stromal polyp or sarcoma was observed in the high female dose group (15%, 12% and 34% for controls, low and high dose groups, respectively). In the 12500 ppm dose female group, the incidence was non-statistically significant compared to controls (p=0.051), but as noted, the p value was just in the border of the statistical significance. On the other hand non-neoplastic increase incidences of cystic hyperplasia in uterus and spleen haemosiderosis were observed in high dose female group. A **NOAEL for toxicity** was considered to be **6000 ppm,** equivalent to 260/302 mg/kg bw/day for males/females (adjusted value by RMS) or 300 mg/kg bw/day (default value).

The extrapolated cut-off value for STOT RE 2 classification for a 4-week dose-repeated study is 12.2 mg/kg bw/day. Consequently the mentioned effects in uterus and spleen of females at dose of 12500 ppm (648 mg/kg bw/day) are not regarded for STOT RE classification.

-In the developmental toxicity study (B.6.6.2.5) in rats, no remarkable findings were observed in the necropsies of the dams and in consequence no effects for STOT RE were noted in this study.

Studies in mice:

-In the 14-day oral study in mice (B.6.3.1.2), eugenol was tested at 0, 6000, 12500, 25000, 50000, and 100000 ppm dose levels (equivalent to 0, 1200, 2500, 5000, 10000 and 20000 mg/kg bw/day), in which 5 animals of each sex were subjected to 14-day feed exposure. This study is a range-finding study to select appropriate doses for chronic toxicity studies. It presents important deviations, such as no control group was included, and clinical signs, food consumption, haematology, clinical chemistry, necropsy, organ weights and histopathology were not evaluated. A reduction in survival was observed in the 50000 ppm males, in which only 2/5 (40%) animals survived at termination. In the high dose groups (100000 ppm), no animals of both sexes survived at the end of the study. On the other hand, a lost in bodyweight >10% compared with initial bodyweight was observed from 25000 ppm dose groups, and bodyweight gain decreases were noted from 12500 ppm male group and from 25000 ppm female group, compared with low dose group.

-In the 13-week oral study in mice (B.6.3.4.2), eugenol was tested at 0, 400, 800, 1500, 3000, and 6000 ppm dose levels (equivalent to 0, 80, 160, 300, 600 and 1200 mg/kg bw/day), in which 10 animals of each sex were subjected to 91-day feed exposure. This study was conducted to evaluate the cumulative toxicity of the test material, to identify organs affected, and to determine the most appropriate doses for the two-year studies. Some endpoints such as food consumption, haematology, clinical chemistry and organ weights were not evaluated. Neither gross mortality or morbidity signs, nor treatment related clinical signs were observed in any dose group. A decrease in bodyweight gain was detected in the top male dose group (10%), compared with controls. On the other hand, necropsy and histopathology examinations did not reveal differences between treated groups and controls. A **NOAEL** could be established at **3000 ppm (600 mg/kg bw/day)** based on the decreased bodyweight gain (10%) in males at study termination.

-In the 2-year carcinogenicity study (B.6.5.2) conducted in $B6C3F_1$ mice, equivocal signs of carcinogenicity were found in both sexes. Firstly, increase incidences of hepatocellular adenomas and carcinomas (combined) were reported in the high dose female group, and they were considered an equivocal evidence of carcinogenicity due to both incidences did not display statistical significant results when were analysed individually, and they were within the overall historical range of the two largest and most complete reported datasets from NTP Carcinogenesis Program (see *section 2.6.5* for more details). Moreover, increased incidences of hepatocellular adenomas and carcinomas were described in the low dose male group, however, the occurrences did not show a dose response pattern, and were within the range of the historical control datasets. Regarding non-neoplastic effects, increase incidences of focal inflammation of the kidney, focal granulomatous inflammation and adenomatous hyperplasia of lung, and ovary follicular cyst were recorded in high dose male and female groups. Therefore, a **NOAEL for toxicity** was considered to be **3000 ppm,** equivalent to 632 mg/kg bw/day (adjusted value by RMS) or 450 mg/kg bw/day (default value).
The extrapolated cut-off value for STOT RE 2 classification for a 4-week dose-repeated study is 12.2 mg/kg bw/day. Consequently the mentioned effects in liver at 3000 ppm (632/784 mg/ kg bw/day for males/females) and in the kidney, lung and ovary at 6000 ppm (1250/1546 mg/ kg bw/day for males/females) are not regarded for STOT RE classification.

-In the 18-months carcinogenicity study (B.6.5.3) conducted in CD-1 female mice, a single dose of eugenol was tested (2%, equivalent to 750 mg/kg bw/day). At this dose, a slight increase incidence of thymic lymphomas and mammary gland adenocanthomas were described compared with controls. However, these incidences were <10% and did not display statistical significance compared with controls. These findings reported in the eugenol treated group were not reproduced in the further two-year study in mice, and therefore, they were considered not relevant for overall long-term toxicity assessment. A NOAEL cannot be established based on the increased incidences of thymic lymphomas and mammary gland adenocanthomas at single dose tested (750 mg/kg bw/day).

The extrapolated cut-off value for STOT RE 2 classification for a 4-week dose-repeated study is 17.8 mg/kg bw/day. Consequently the mentioned effects in thymus and mammary gland at 750 mg/kg bw/day are not regarded for STOT RE classification.

Studies in rabbits:

-In the developmental toxicity study (B.6.6.2.6) in rabbits, no remarkable findings were observed in the necropsies of the dams and in consequence no effects for STOT RE were noted in this study.

Studies in dogs:

-In the oral short-term toxicity study in dogs (B.6.3.1.3), 2g of eugenol/dog were administered at intervals of 48-72 hours for 10 times to 1-3 healthy Mongrel female dogs via Levin tube (equivalent to 100 mg/kg bw/day). Forty-eight hours after the last administration, at day 23, the dogs were sacrificed and subjected to autopsy. Histopathology analysis was also performed. Neither gross mortality or morbidity signs, nor treatment related clinical signs were observed in the dose group. Moreover, no relevant findings were reported after necropsy and histopathological examinations. **A NOAEL** of **100 mg/kg bw/da**y could be established based on no adverse effects detected.

Therefore, based on the available data, the **overall oral short-term NOAEL** was **540 mg/kg bw/day** (13-week dietary study in rats).

Table 49: Extrapolation of equivalent effective dose for toxicity studies of greater or lesser duration than 90 days [if adequate, otherwise please delete]

Study reference	Effective dose (mg/kg/day)	Length of exposure	Extrapolated effective dose when extrapolated to 90-day exposure	Classification supported by the study
NTP (1983) Study in rats (AS) B.6.3.1.1	No effect	14 days	-	No classification
NTP (1983) Study in mice (AS) B.6.3.1.2	No effect	14 days	-	No classification
Lauber, F.U. *et al.* (1950) Study in dogs (AS) B.6.3.1.3	No effect	21 day	-	No classification
WHO/FAO Joint Expert Committee on Food Additives (JECFA). WHO Food additive series No. 56, 155-200. (2006) (review from Hirose *et al.*, 1987) (AS) B.6.3.2.1	No effect	28 days	-	No classification
Hagan *et al.* Study in rats (AS) B.6.3.5.1	No effect	133 days	-	No classification
NTP (1983) Study in rats (AS) B.6.5.1	648/625 adjusted/default (uterus and spleen)	105 weeks	5290/5100 adjusted/default	No classification
NTP (1983) Study in mice (AS) B.6.5.2	1250/900 adjusted/default (kidney, lung and ovary)	106 weeks	10250/7380 adjusted/default	No classification

Study reference	Effective dose (mg/kg/day)	Length of exposure	Extrapolated effective dose when extrapolated to 90-day exposure	Classification supported by the study
Miller *et al.* (1983) Study in mice (AS) B.6.5.3	750 (thymus and mammary gland)	18 months (72 weeks feed exposure)	4200	No classification
[redacted] (2004) Study in rats (AS) B.6.6.2.5	No effect	14 days	-	No classification
[redacted] (2004) Study in rabbits (AS) B.6.6.2.6	No effect	23 days	-	No classification

2.6.3.1.2 Comparison with the CLP criteria regarding STOT RE (specific target organ toxicity-repeated exposure)

-In the 34-oral study in rats, slight cell morphology alterations were observed in rat livers. However, no specific details on it are provided and no organ weight or clinical biochemistry alterations were reported. Besides, this effect was not recorded in other repeated dose-studies and was considered of low toxicological relevance.

On the other hand, in this study hyperkeratosis and hyperplasia were observed in rat forestomach. The forestomach is the non-glandular part or the stomach. In the rat, mouse and hamster the forestomach occupies about two-thirds of the proximal area of the stomach and is lined by cornified, stratified squamous epithelium, and acts as a storage organ releasing relatively undigested food into glandular stomach in response to energy demand. This organ is also present in ruminants and camelids as dilation and modification of the esophagus. Thus, humans lack forestomach and consequently, the observed changes in rats do not present toxicological relevance for humans. In all likelihood, the effects observed in rats were triggered by eugenol gavage administration. Eugenol is classified as skin sensitizer (H317), and causes severe/moderate/mild skin effects in human, rabbit and pig, and likely the direct eugenol application on forestomach mucosa causes epithelium damage.

-In the 18-months toxicity studies in mice, low incidence of thymic lymphomas and mammary adenocanthomas were detected. These neoplastic incidences were not reproduced in the other 2-year long-term toxicity study in mice, and were not supplemented by clinical biochemistry data or organ macroscopic observations.

-In the 2-year toxicity study in rats, the most relevant findings were located in uterus (increased incidences of polyps/sarcoma and cystic hyperplasia). However, no uterus alterations were found in other species tested in which repeated toxicity studies were conducted. Besides, reproductive performance was not affected in any of evaluated studies. The relevance of this finding is further discussed in *long-term toxicity and endocrine toxicity sections 2.6.5 and 2.6.6*, in which a potential endocrine disruption property of eugenol cannot be ruled out, and further data is needed to clarify this subject.

Regarding spleen haemosiderosis, neither clinical biochemistry nor haematology were evaluated in this study to support this observation. Besides, organ macroscopic evaluation did not show additional spleen changes, and hence, this effect was not taken into account.

-In the 2-year toxicity study in mice, increased inflammation incidences in kidney (focal), together with granulomatous inflammation and adenomatous hyperplasia in lung were detected. These findings were discarded due to they do not constitute severe organ toxicity and were not recorded in other repeated-dose studies. On the other hand, increase incidences of ovary follicular cysts were noted in female top dose group. This finding was not support by statistical analysis and historical control data was not provided, however, due to alterations were also noted in the uterus of rats, RMS deems that further data are needed to evaluate the potential endocrine disruption property of eugenol.

No signs of specific target organ toxicity were noted in the short-term or reproductive toxicity studies with eugenol.

Therefore, according to CLP criteria Regulation 1272/2008 and after evaluation of repeated toxicity studies provided with eugenol, no evidences of specific target organ toxicity (repeated exposure) were found.

2.6.3.1.3 Conclusion on classification and labelling for STOT RE (specific target organ toxicity-repeated exposure)

Based on the data available for eugenol, STOT RE classification is not proposed.

2.6.4 Summary of genotoxicity / germ cell mutagenicity [equivalent to section 10.8 of the CLH report template]

Table 50: Summary table of genotoxicity/germ cell mutagenicity tests *in vitro*

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations /Results	Reference
Bacterial gene mutation (Ames test, pre-incubation) Comparable to OECD TG 471 (1983) Deviations from the current OECD TG 471 (2020): Characterisation of the test substance, TA102 or *E.Coli* WP2 uvrA not tested, no individual data reported (summary only), no positive control reported and historical control data. GLP: No **Supporting information**	Eugenol Purity 100% Batch No. not specified Solvent : DMSO *S. typhimurium* TA92, TA1535, TA1537, TA94, TA98 and TA100 Rat S9	Six concentrations tested (not detailed in the study) Maximum dose 2000 µg/plate Dose selection: The maximum dose represents the highest non-cytotoxic dose used.	**Negative ± S9**	Ishidate M. *et al.* (1984) (AS) B.6.4.1.1.1
Bacterial gene mutation (Ames test, pre-incubation) Pre-guideline Comparable to OECD TG 471 (1983) Deviations from the current OECD TG 471 (2020): only 4 strains used. Statistical analysis not reported, no historical control data. An additional indicator of S9-mix efficacy should be used in combination with 2-aminoanthracene GLP: No **Supporting information**	Eugenol Purity: not reported Batch No. C8A Solvent : DMSO *S. typhimurium* TA1535, TA1537, TA98 and TA100 Rat and Hamster S9	Preliminary dose-range study with TA100 ± S9 up to 10000 µg/plate 3.3-333.3 µg/plate Positive controls (-S9): 4-Nitro-o-phenylenediamine (NOPD) for TA98 2 Sodium azide for TA100 and TA1535 9-Aminoacridine (9AA) for TA1537. Positive controls (+S9): 2-Aminoanthracene (2AA) for S. typhimurium: TA98, TA100, TA1535 and TA1537	**Negative ± S9** Cytotoxicity at 333.3 µg/plate (-S9) in strains TA100 and TA1537	Haworth, S. *et al.* (1983) (AS) B.6.4.1.1.2

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations /Results	Reference
Bacterial gene mutation (Ames test) Prior to OECD TG 471 (1983) Deviations from the current OECD TG 471 (2020): - No characterisation and stability determination of the test item - Only four (not recommended) S. typhimurium tested strains (instead of five strains). These strains are not the recommended by the guide. -Individual data not provided. Very limited information on the study results. - The enzyme-inducing agent for S9 is not reported - No historical control data GLP: No **Not acceptable**	Eugenol Purity : not reported Batch No. : not reported Solvent : not reported *S. typhimurium* TA1530, TA1531, TA1532 and TA1964 (direct bacterial assay and microsomal assay) *S. typhimurium* TA1534, TA1950, TA1951 and TA1952 (host-mediated assay)	<u>Direct bacterial assay</u> Concentration: 1000-5000 µg/plate <u>Microsomal mutagenesis assay</u> S9 from ♂ mice (C3H/HeJ). Concentration : 0.02 and 0.2 M <u>Host-mediated Assay</u> ♂ mice (C3H/HeJ) Dose: 200 mg/kg Volume : 0.1 mL <u>Positive controls :</u> Direct Bacterial Assay: N-methyl-N-nitrosoguanidine Microsomal Assay and Host-mediated Assay: Dimethylnitrosamine	<u>Direct bacterial assay</u> **Negative** <u>Microsomal mutagenesis assay</u> **Negative** <u>Host-mediated Assay</u> **Negative**	Green, N. and Savage, J.R., (1978) (AS) B.6.4.1.1.3
Bacterial gene mutation (Ames test) Prior to OECD TG 471 (1983) Deviations from the current OECD TG 471 (2020): - Batch of test chemical not reported - Only four *Salmonella ty.* strains tested (instead of five strains). - No justification provided for the use of ethanol as solvent -Individual data not provided. Very limited information on the study results. - Only one concentration tested - No historical control data	Eugenol Purity ≥ 97% Batch No.: not reported Solvent: Ethanol *S. typhimurium* TA98, TA100, TA1535 and TA1537 Rat liver S9 derived from Aroclor 1254 or 3-methylcholanthrene Male Sprague-Dawley rats	Concentration : 3µmol/plate, equivalent to 450µg/plate (Single dose) Positive control (-S9): N-methyl-N-nitrosoguanidine Positive control (+S9): 2-aminoanthracene	**Negative ± S9** <u>Cytotoxicity</u>: Not observed	Florin, I. *et al.* (1980) (AS) B.6.4.1.1.4

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations /Results	Reference
GLP: No **Not acceptable**				
Bacterial gene mutation (Ames test) Prior to OECD TG 471 (1983) Deviations from the current OECD TG 471 (2020): - No characterisation and stability determination of the test item - Only three strains tested instead of five - No information on concentrations tested -Individual data not provided. Very limited information on the study results. - No further information on S9 -No positive control and historical control data provided GLP: No **Not acceptable**	Eugenol Purity: not reported Batch No.: not reported Solvent: DMSO *S. typhimurium* TA97, TA98 and TA100 S9 (no further information)	Concentration : not stated but indicated to be non-toxic. Incubation time: 120 min Positive control: not stated Negative control : DMSO	**Negative ± S9**	Azizan, A. and Blevins, R.D. (1995) (AS) B.6.4.1.1.5
TK+/- mutation assay in L5178Y cells (mouse lymphoma assay) Prior to OECD TG 476 (1984) Deviations from the current OECD TG 490 (2016): Characterisation of test item not determined (purity), test carried out without metabolic activation only, historical control data not provided GLP : No **Supporting information**	Eugenol Purity not stated Batch No.: not stated L5178Y $TK^{+/-}$ Solvent : Ethanol Positive control: Methyl methanesulphonate (MMS) Negative control: ethanol	First trial (-S9) : 0, 20, 30, 40, 60, 80, 120 nL/mL Second trial (-S9) : 0, 30, 40, 60 nL/mL Preliminary cytotoxicity test indicated that doses above 120 nL/mL were highly cytotoxic	**Negative - S9** Dose-dependent increase in MF at doses although the mutant frequency did not exceed the global evaluation factor (GEF) at doses with less than 10 % total growth (high cytotoxicity). Also, acceptable criteria not met (MF of untreated controls does not fall within range of guideline).	Myhr, B.C. and Caspary, W.J. (1991) (AS) B.6.4.1.2

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations /Results	Reference
Mammalian cell chromosome aberration test Deviations from current OECD TG 473 (2016): No detailed experimental results data reported, only 100 metaphases scored, short-term treatments (±S9) were not performed, gaps not evaluated, no historical control data available GLP: No **Supporting information**	Eugenol Purity : 100 % Batch No. : not reported Chinese hamster lung fibroblasts (CHL) Solvent: DMSO No metabolic activation used	Three doses up to 125 µg/mL based on preliminary cytotoxicity assay 48 h expression time	**Positive -S9**	Ishidate M. *et al.* (1984) (AS) B.6.4.1.3.1
Mammalian cell chromosome aberration test Deviations from current OECD TG 473 (2016): No detailed experimental results data and criteria for reported, only 200 metaphases scored, only two concentrations tested, no historical control or positive control data available GLP: No **Supporting information**	Eugenol Purity : not stated Batch No. : not reported Chinese hamster ovary cells (CHO) Solvent: MEM Rat liver S9	0.05 and 0.4 mg/mL Experiments ± S9 Effects of transition metals : Cupric sulphate solution Manganese Chloride. Eugenol concentration : 0.2 mg/mL 3h exposure period	**Positive ± S9** <u>Cytotoxicity</u>: Reported mitotic inhibition at 0.4 mg/mL (+S9)	Stich H.F. *et al.* (1981) (AS) B.6.4.1.3.2
Mammalian chromosome aberrations test Comparable to OECD TG 473 (1983) Deviations from the current OECD TG 473 (2016) -No characterisation of the test item --Solvent not stated --Exposure period of 2h instead of the minimum 3h (+S9 experiment)	Eugenol Purity : not stated Batch No. : not reported Clone Chinese hamster ovary cells (CHO-W-B1) Solvent: not stated Rat liver S9	(198-300 µg/mL -S9) (201-324 µg/mL +S9) <u>Dose selection</u>: Doses were chosen based on a preliminary test of cell survival 24 h after treatment. The doses were spaced more closely than half-log series and extended into the toxic range 2h exposure period +S9	**Negative –S9** **Positive + S9** Cytotoxicity observed at the highest dose (198-300 µg/mL -S9)	Galloway S.M. *et al.* (1987) (AS) B.6.4.1.3.3

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations /Results	Reference
-Only 100 metaphases were scored. No historical data and positive control data reported GLP: No **Supporting information**				
Mammalian chromosome aberrations test Comparable to OECD TG 473 (1997) Deviations from the current OECD TG 473 (2016) include no characterisation of the test item, 100 metaphases scored (instead of 300), test results reported as average of two experiments and no historical control data provided. GLP: No **Supporting information**	Eugenol Purity: 99 % Batch No.: not stated V79 Chinese Hamster cells Solvent: DMSO	**Chromosome aberration test** 0, 100, 1000, 2500 and 3000 µM Positive control : MMC and CP 3h exposure period **Induction of Endoreplication test** 0, 500, 1000, 1500, 2000 and 2500 µM	**Chrom. Abs : Positive ± S9** **Endoreplication : Positive ± S9**	Maralhas A. *et al.* (2006) (AS) B.6.4.1.3.4
Mammalian chromosome aberrations test Comparable to OECD TG 473 (1997) Deviations from the current OECD TG 473 (2016) include no characterisation of the test substance, only 100 metaphases scored, 24h exposure instead of 3-6 h in the non-activation assay, no indication of how the rat post-mitochondria supernatant was derived and no positive control data/historical control data provided GLP: No	Eugenol Purity: >95 % Batch No.: not stated Syrian Hamster Embryo cells (SHE) Solvent : DMSO	**Non-activation assay :** 0, 65, 195 and 650 µM 24 h exposure period **Activation assay :** 0, 6.5, 20 and 65 µM 3h exposure period	**Positive ± S9** Cytotoxicity: Relative colony forming efficiency 58 % at 650 µM (-S9). Not tested +S9.	Hikiba H. *et al.* (2005) (AS) B.6.4.1.3.5

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations /Results	Reference
Supporting information				
Mammalian chromosome aberrations test Comparable to OECD TG 473 (1997) Deviations from the current OECD TG 473 (2016) GLP: No **Supporting information**	Eugenol Purity: >95 % Batch No.: not stated Human dental pulp cells (D824) Solvent : DMSO No metabolic activation (-S9)	0, 100, 300, 1000 µM Two experiments : 3h and 30h exposure periods Positive control : Cyclophosphamide (tested with and without 5% rat post-mitochondrial supernatant) Dose selection: Cytotoxicity pre-test.	3h exposure : **Negative –S9** 30h exposure : **Positive –S9** Cytotoxicity: A concentration-dependent growth inhibition was observed.	Someya H. *et al.* (2008) (AS) B.6.4.1.3.6
Mammalian DNA damage – Sister chromatid exchange No guideline Deviations from OECD TG 479 (deleted 2014) : no characterisation of the test chemical, very poor level of reporting, non-activation assay only, GLP : No **Not acceptable**	Eugenol Purity : 99.6 % Batch No. : not stated Human lymphocyte cells Solvent : DMSO No metabolic activation (S9)	Concentrations : 0-0.5 mM Positive control : Styrene-7,8-oxide	**Negative –S9**	Jansson T. *et al.* (1986) (AS) B.6.4.1.4.1
Mammalian DNA damage **Sister Chromatid Exchange** Similar to OECD TG 479 – deleted in 2014 - No characterisation of the test substance - Solvent not stated specifically - Positive control not stated specifically - Method and results poorly described - No historical data were reported GLP: No **Supporting information**	Eugenol Purity : not stated Batch No. : not reported Clone Chinese hamster ovary cells (CHO-W-B1) Solvent: not stated Rat liver S9	11-123 µg/mL -S9 273-326 µg/mL +S9 Dose selection: Doses were chosen based on a preliminary test of cell survival 24 h after treatment. The doses were spaced more closely than half-log series and extended into the toxic range 2h exposure period +S9 5-Bromodeoxyuridine (BrdUrd)	**Positive ± S9** This increase in sister chromatid exchange occurred at doses that caused severe cell cycle delay.	Galloway S.M. *et al.* (1987) (AS) B.6.4.1.4.2
DNA Adducts and 8-OH-dG formation	Eugenol	DNA Adduct formation :	**DNA adduct formation**	Bodell W.J. et al. (1998)

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations /Results	Reference
No guideline GLP : No **Supporting information**	Purity : not stated Batch No. : not reported HL-60 Cells Calf thymus DNA Peroxidase enzymes : HRP or MPO	Incubations of HL-60 with eugenol in DMSO at concentrations 100, 250, 500 and 1000 µM for 24h. Also incubations of eugenol (100 µM) with H_2O_2 (100 µM) Formation of 8-OH-dG Incubations of 100 µM eugenol with 500 µg calf thymus DNA under various activation systems: Horseradish peroxidase (HRP), myeloperoxidase (MPO), H_2O_2 and $CuSO_4$	**correlated with treatment concentration** **Formation of 8-OH-dG increased with activation systems**	(AS) B.6.4.1.4.3
Direct genotoxicity in repair proficient and repair deficient cells : Comet assay and DNA strand breaks No guidance GLP : No **Supporting information**	Eugenol Purity : not stated Batch No. : not reported AA8 CHO cells EM9 CHO cells Solvent : DMSO	MTT cell viability assay 3 and 24 h exposure 50-2000 µM *In vitro* alkaline comet assay 50-500 µM 50 cells/slide γ-H2AX assay 250-750 µM 1h exposure Detection by immunofluorescence	MTT cell viability assay 3h – no significant effects 24h – 50% reduction from doses > 500 µM Comet assay : DNA damage induced in AA8 cells (linear trend with no statistical significance) No DNA damage caused in EM9 cells γ-H2AX assay Significant response of DNA strand breaks in AA8 cells	Martins, C. *et al.* (2011) (AS) B.6.4.1.4.4
REACH DATA Bacterial gene mutation assay Reliability 1 -S9: plate incorporation +S9: preincubation	Eugenol Purity 98.9%	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 and *E.coli* WP2 Rat S9 (Sprague-Dawley) Concentration: 0, 60, 120, 300 and 600 µg/plate Solvent: DMSO	Negative ± S9	Sekizawa J. and Shibamoto, T. (1982) "Genotoxicity of safrole-related chemicals in microbial test systems". (REACH registration dossier data not provided by applicant and not assessed by the RMS)
REACH DATA DNA damage and repair assay, unscheduled DNA synthesis in mammalian cells Reliability 2	Eugenol Purity not reported	Rat hepatocytes, Fischer 344 (♂) Solvent: DMSO Concentration: 0, 1 to 1000 µM	Negative	Howes A.J. et al. (1990) "Structure-specificity of the genotoxicity of some naturally occurring alkenylbenzenes determined by the unscheduled DNA

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study including rationale for dose selection (as applicable)	Observations /Results	Reference
				synthesis assay in rat hepatocytes" (REACH registration dossier data not provided by applicant and not assessed by the RMS)
REACH DATA Bacterial gene mutation assay Reliability 2	Eugenol Purity not reported	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538 Rat S9 Concentration: up to 2 mg/plate Solvent: DMSO	Negative ± S9	Nestmann E.R. *et al.* (1980) "Mutagenicity of constituents identified in pulp and paper mill effluents using the Salmonella/mammalian microsome assay". (REACH registration dossier data not provided by applicant and not assessed by the RMS)

Table 51: Summary table of genotoxicity/mutagenicity tests in mammalian somatic or germ cells *in vivo*

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study (as applicable)	Observations/Results	Reference
Micronucleus test Deviations from the current OECD TG 474 (2016) -Batch number is not reported -A single sex is used -Intraperitoneal injection is not a recommended route of administration -Only 1000 PCE were scored per animal (4000 are recommended in the current guideline GLP: No **Supporting information**	Eugenol Purity: >98% Batch No.: not reported	Test system: ddY mice (♂), 6 animals/group dose. Femoral marrow cells Dose selection: preliminary test (no information given in the study) Route: intraperitoneal injection Dosage: Single administration 100, 200, 400, 800 mg/kg bw Multiple administration 400 mg/kg bw Vehicle: olive oil Sampling: 1000 PCEs scored 24 h post-dosing Negative control: Vehicle Positive control: Mitomycin C	**Negative** Mortality (4/6) observed at 800 mg/kg (i.p) Cytotoxicity: PCE/ total erythrocytes is lower than controls	Hayashi M. *et al.* (1988) (AS) B.6.4.2.1.1
Micronucleus test Deviations from the current OECD TG 474 (2016): - No characterisation of the test substance - No historical control data reported -No data about animal housing	Eugenol Purity: not reported Batch no. not reported	Test system: adult ♂ Swiss-Webster mice, 12 animals/group Dose selection: Preliminary determination of LD_{50} LD_{50}: 1109.6 mg/kg (ip). Oral LD_{50} > 14794.4 mg/kg (no mortality in the study)	**Positive (ip)** **Positive (oral)** Cytotoxicity: PCE/ total erythrocytes is bigger than control	Woolverton C. *et al.* (1986) (AS) B.6.4.2.1.2

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study (as applicable)	Observations/Results	Reference
-Oral dose exceeds the 2000 mg/kg bw recommended by guidance -3 different concentrations should be tested -Only 1000 PCE were scored per. GLP: No **Supporting information**		Route: Intraperitoneal or oral Dosage: 739.7 mg/kg bw (80 % LD_{50}) or 147.9 mg/kg bw (25% LD_{50}) (ip injection) 14794.4 mg/kg bw (oral) Vehicle: saline Sampling Second dose at 24 hours 1000 PCEs scored Negative control: saline Positive control: Quinacrine dihydrochloride		
Anti-mutagenic activity assessed in micronucleus test Deviations from the current guideline OECD TG 474 (2016) -Batch is not reported -No historical control data reported -3 different concentrations should be tested -Only 1000 PE were scored per animal GLP: No **Supportive information**	Eugenol Purity: > 99% Batch No. not reported Mutagens tested: MMC CP EMS B[a]P	Test system: adult Swiss mice (♂), 30 animals/group (feeding study) and 5 animals/group for the treatment with mutagens Femoral marrow cells Route: Oral Dosage: Eugenol fed in diet *ad libitum* for 14 days (approx.. 680 mg/kg bw) CP: 25 mg/kg bw (i.p) MMC: 1.5 mg/kg bw (i.p) EMS: 300 mg/kg bw (i.p) B[a]P: 250 mg/kg bw (i.p) Vehicle: saline or olive oil Sampling 24h after mutagen dose. 1000 PCE's scored Negative control: diet	Reduction of micronuclei induction of polychromatic erythrocytes by cyclophosphamide in animals fed with eugenol for 14 days: Control: **28.4 ± 8.7** Eugenol-fed diet: **15.6 ± 3.7**	Rompelberg C.J.M. *et al.* (1995) (AS) B.6.4.2.1.3
Micronucleus test Deviations from the current OECD TG 474 (2016) -No characterisation of the test substance -Only one sex is tested -Limited detail of reporting -Only 1000 PCE were scored per animal -No historical control data reported GLP: No **Supporting information**	Eugenol Purity: not stated Batch no: not stated	Test system: B6C3F1 mice (♂), 5 or 7 animals/group Bone marrow Route: Intraperitoneal Dose selection: preliminary toxicity study Dosage: 3 daily doses of 0, 150, 300 or 600 mg/kg Vehicle: corn oil Sampling: 24h after the last dose 2000 PCEs scored Positive control: mitomycin C and dimethylbenzanthracene	**Negative** Toxicity: No mortality. Cytotoxicity: Not observed.	Shelby M.D. *et al.* (1993) (AS) B.6.4.2.1.4

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study (as applicable)	Observations/Results	Reference
Micronucleus test Deviations from the current OECD TG 474 (2016) -No characterisation of the test substance -Single sex only -Only 1000 PE scored per animal -No historical data GLP: No **Supporting information**	Eugenol Purity: not stated Batch no: not stated	Test system: CF1 mice (♂), 8 animals/group Bone marrow Route: Intraperitoneal Dose selection: preliminary study of 10 mice/groups at dose levels of 440, 500, 600, 700, 800, 900, 1000 or 1500 mg/kg bw. Dosage: single ip doses of 100, 400 or 600 mg/kg bw (25 mL/kg bw) Three groups were tested with an ip dose of 400 mg/kg bw (different sampling times). Vehicle: corn oil Sampling: Single dose: 30 h after the last dose Second experiment – different sampling times: 24h, 30h and 48h 1000 PCEs scored Positive control: MMS Negative control: vehicle	**Positive (main study)** **Positive at 24h, 30h or 48h sampling times at doses over 400 mg/kg bw**	Ellahueñe M.F. *et al.* (1994) (AS) B.6.4.2.1.5
Micronucleus test Deviations from the current OECD TG 474 (2016) -No characterisation of the test substance -Single sex only -Only 1000 PE scored per animal -Limited level of reporting -No historical data GLP: No **Supporting information**	Eugenol Purity: 99% Batch No. not stated	Test system: Wistar rats ♂, 8 rats/group dose Femoral bone marrow Route: oral gavage Dosage: 500, 1000 mg/kg bw Vehicle: corn oil Dose selection: Described in Rompelberg *et al.* 1993 Sampling: 24 h after dosing (1000 PCE scored) Control: Mitomycin C (i.p)	**Negative** Mortality: 1 rat at 1000 mg/kg bw dose group died	Rompelberg C.J.M. *et al.* (1996a) (AS) B.6.4.2.1.6
Micronucleus test Deviations from the current OECD TG 474 (2016) -No characterisation of the test substance -Only 4 animals per group (single sex) -1000 PCE scored	Eugenol Purity: not stated Batch no: not stated	Test system: Sprague-Dawley rats (♀) Bone marrow Route: oral Dosage: 0, 335, 670 and 1340 mg/kg bw. Half the dose was given 30h and the other half 6h prior to sacrifice. Vehicle: physiological saline	**Negative**	Maura A.C. *et al.* (1989) (AS) B.6.4.2.1.7

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study (as applicable)	Observations/Results	Reference
-Sampling at 6h after last dose GLP: No **Supporting information**		Dose selection: acute oral study described by Jenner *et al.* (1964) Sampling: 6h after dose (1000 PCE scored) Negative control: vehicle (control) Positive control: triethylenemelamine		
Micronucleus test OECD TG 474 (2016) Deviations from the guideline: -No characterisation of the test substance -Limited level of reporting of results, clinical signs GLP: No **Study acceptable**	Eugenol Purity: not stated Batch No.: not reported	Test system: Sprague-Dawley rats (♂) Peripheral blood Route: oral (gavage) Dosage: 0, 400, 800 and 1200 mg/kg bw. Volume: 5 mL/kg bw Vehicle: corn oil Dose selection: not reported Sampling: 0h and 48h fter dose (4000 PCE scored) Negative control: sodium saccharin in 1% sodium carboxymethyl cellulose Positive control: EMS, CP, COL and ENU	**Negative**	Chen Y. *et al.* (2021) (AS) B.6.4.1.8
Transgenic rodent somatic cell gene mutation assay Deviations from current OECT TG 488 (2020): --Non-standard assay -Dose selection not indicated -Very poor level of reporting - No estimation or evidence of liver exposure to eugenol GLP: No **Supporting information**	Eugenol Purity: 99 % Batch No. not reported B[*a*]P (purity and batch not stated)	Test system: λ-lacZ-transgenic mice strain 40.6 (♂) 8 mice per treatment group (diet) On day 10, 4 mice/group were treated with single injection of B[*a*]P Dosage and duration: Daily in take of 673.60 mg/kg/bw/day of eugenol in feed (0.4 % eugenol in feed) during 58 days Vehicle: olive oil (ip) Negative control: diet (no eugenol) and olive oil	**Eugenol fed in mice did not affect the mutant frequency in mice treated with a single ip dose of B[*a*]P** **Oral doses of eugenol did not result in an increase of mutant frequency compared to control group** **DNA adduct formation showed larger amounts in mice treated with B[*a*]P**	Rompelberg, C.J.M. *et al.* (1996b) (AS) B.6.4.2.2
Mammalian alkaline elution assay (Comet) Deviations from current OECT TG 489 (2016):	Eugenol Purity: not stated Batch no: not stated	Test system: Sprague Dawley rats (♀) Dosage: 1340 mg/kg bw Dose selection ½ LD_{50} (Jenner *et al.*, 1964)	**No induction of DNA fragments**	Maura, A.C. *et al.* (1989) (AS) B.6.4.2.3

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study (as applicable)	Observations/Results	Reference
-No characterisation of the test item -Poor description of the method -Very limited level of reporting --No evidence of exposure to liver and kidney provided GLP: No **Supporting information**		Route: oral Vehicle: Physiological saline Interval analysis: 2, 24 and 48h Tissues: liver and kidney		
DNA adducts No test guidance available GLP: No **Supporting information**	Eugenol Purity: not stated Batch no: not stated Safrole (purity not stated, batch no. not stated)	Test system: B6C3F1 mice (♂) Dosage: Repeat dose experiment: Total of 4.25 µmoles over 22 days: 0.25 µmoles (day 1), 0.5 µmoles (day 8), 1.0 µmoles (day 15) and 3.0 µmoles (day 22). Single dose experiment: 2 or 10 mg in 0.1 mL vehicle Route: intraperitoneal injection Vehicle (second experiment): trioctanoin Positive control: Safrole Sampling: DNA from liver	**Eugenol did not produce detectable DNA adducts under the conditions of this study.** **Safrole was used as a positive control**	Phillips, D.H. (1990) (AS) B.6.4.2.4
Human study (various parameters) No guidance, non-standard assay GLP: No **Supporting information**	Eugenol Purity: food grade Batch no.: not stated	Test system: human volunteers (♂) (n = 10) Dosage: 50 mg eugenol 3 times per day for 7 consecutive days Vehicle: Dried starch Route: oral (capsules) Duration of the study: 22 days On days 8 and 22, after fasting overnight, volunteers ingested 500 mg paracetamol. Blood samples at 5h post dose were taken and plasma was measured to calculate clearance of paracetamol Blood samples taken on days 8 and 22 to analyse WBD, PLT, HBG, HCT, ASAT, ALAT, γ-GT, LDH, AP AND GST. Cytogenetic analysis of human erythrocytes extracted from volunteers treated in vitro with	**Eugenol did not have an effect in haematology or clinical biochemistry parameters or on the clearance of paracetamol.** **Eugenol did not cause an increase in the background level of micronuclei or chromosomal aberrations** **Eugenol was detected in urine by NMR spectroscopy**	Rompelberg *et al.* (1996c) (AS) B.6.4.2.5.1

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study (as applicable)	Observations/Results	Reference
		known mutagens (vinblastine and mitomycin C) Urine was collected on days 7 and 21 and submitted for analysis.		
Host-mediated mutagenicity assay No guidance available GLP: No **Supporting information**	Eugenol Purity: Not stated Batch No.: Not stated Eugenol metabolite II [3-piperidyl-1-(3'-methoxy-4'-hydroxyphenyl)-1-propanone] Eugenol metabolite III [3-pyrrolidinyl-1-(3'-methoxy-4'-hydroxyphenyl)1-propanone]	Test system: C3H/HeJ mice (♂) 3 mice/treatment *S.Typhimurium* TA1530, TA1531, TA1532 and TA1964 strains Dosage: 2mL solution of *S.Typhimurium* strains and 0.1 mL of eugenol or metabolites at a dose of 200 mg/kg bw. Route: Intraperitoneal injection Incubation period: 2 days	**Under the conditions of the study, eugenol or its metabolites did not induce an increase in mutant frequency of any of the strains tested**	Green, N. and Savage, J.R., (1978) (AS) B.6.4.2.5.2
REACH DATA Transgenic rodent somatic and germ cell gene mutation Reliability 2	Eugenol Purity: 99%	Mouse (6♂) λ-lacZ-transgenic mouse strain 40.6 Dose: 0.4% diet (1000 mg/kg bw/day nominal, equivalent to actual 580 mg/kg bw/day) Exposure: 58 days	Negative Clinical signs: growth retardation, reduction in body weight gain.	Unnamed (1996) (REACH registration dossier data not provided by applicant and not assessed by the RMS)
REACH DATA Mammalian erythrocyte micronucleus test Reliability 2	Eugenol Purity not reported	Test system: Sprague-Dawley rats (5♂/dose) Polychromatic erythrocytes from bone marrow cells Route: oral (gavage) Dosage: 0, 1340 mg/kg bw (single administration or once/day for 2 days) Volume: 5 mL/kg bw Vehicle: distilled water with 1% carboxymethylcellulose 1000 PCE scored	Negative	Allavena A. *et al.* (1992) "Evaluation in a battery of in vivo assays of four in vitro genotoxins proved to be non-carcinogens in rodents (REACH registration dossier data not provided by applicant and not assessed by the RMS)
REACH DATA DNA damage and/or repair (unscheduled DNA synthesis test) Reliability 2	Eugenol Purity not reported	Rat, hepatocytes, Sprague-Dawley (5♂) Route: oral (gavage) Dosage: 0, 1340 mg/kg bw (single administration or once/day for 2 days) Volume: 5 mL/kg bw	Negative	Allavena A. *et al.* (1992) "Evaluation in a battery of in vivo assays of four in vitro genotoxins proved to be non-carcinogens in rodents

Method, guideline, deviations[1] if any	Test substance	Relevant information about the study (as applicable)	Observations/Results	Reference
		Vehicle: distilled water with 1% carboxymethylcellulose 50 cells/slide		(REACH registration dossier data not provided by applicant and not assessed by the RMS)
REACH DATA Gene mutation in non-somatic cell – COMET (turkey egg genotoxicity assay) Reliability 2	Eugenol Purity not reported	White turkey (Meleagris gallopavo) eggs (22-24 days old) Concentration: 1, 2.5 and 5mg/egg) 3 daily injections on days 22 to 24 Vehicle: 20% aqueous solution of Kolliphor HS15 Liver cells	Negative	Kobets T. *et al.* (2016) "Structure-activity relationships for DNA damage by alkenylbenzenes in turkey egg fetal liver". (REACH registration dossier data not provided by applicant and not assessed by the RMS)

Table 52: Summary table of human data relevant for genotoxicity / germ cell mutagenicity

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No data available				

2.6.4.1 Short summary and overall relevance of the provided information on genotoxicity / germ cell mutagenicity

Most data to address this point were presented in the original DAR (2011) in support of the inclusion of eugenol in Annex I of Directive 91/414/EEC and were deemed acceptable following evaluation and peer review at EU level.

A total of 29 studies have been submitted for the renewal process to evaluate the genotoxicity of eugenol, of which 16 correspond to studies *in vitro* and 13 correspond to studies *in vivo*. Only three new genotoxicity studies (*in vitro* chromosome aberration, B.6.4.1.3.6, *in vitro* comet, B.6.4.1.4.4, and *in vivo* micronucleus assay, B.6.4.2.1.8) have been submitted for the renewal process and the remaining studies were presented and evaluated as part of the original DAR (2011).

In Commission Regulation (EU) No. 283/2013 in vitro photomutagenicity studies may be indicated by the structure of a molecule. If the ultraviolet/visible (UV/VIS) molar extinction/absorption coefficient of the active substance and its major metabolites is less than 1000 L x mol^{-1} x cm^{-1} photomutagenicity testing is not required. In the case of eugenol (alkaline), the molar extinction/absorption coefficient is 3780 L x mol^{-1} x cm^{-1} at 297 nm, although maximum absorption occurs at 200 nm (39846 3780 L x mol^{-1} x cm^{-1}) (White, G.A., 2007). The neutral or acid forms of eugenol do not absorb at UV/vis spectrum. Due to the minimal absorption of the alkaline form in the UV/vis spectrum no photomutagenicity testing is considered to be necessary.

***In vitro studies*:**
Eugenol gave negative results *S.Typhimurium* strains TA92, TA94, TA98, TA100, TA1535 and TA1537 in the presence and absence of metabolic activation (Ishidate *et al.*, 1984, B.6.4.1.1.1; Haworth *et al.*, 1983; B.6.4.1.1.2; Florin I. *et al.*, 1980; B.6.4.1.1.4). These studies did not include a strain to test for cross-linking mutagens. Additional studies have been submitted in which eugenol is reported negative in *S.Typhimurium* strains TA97, TA98 and TA100, (Azizan and Blevins, 1995; B.6.4.1.1.5) and the non-standard strains TA1530, TA1531, TA1532 and TA1964 (Green *et al.*, 1980; B.6.4.1.1.3). However, based on method deficiencies and deviations from current OECD TG 471 guideline, these studies are deemed as not acceptable to assess the bacterial gene mutation potential of eugenol.

Based on the available information, it can be concluded that eugenol is not mutagenic in bacteria gene mutation assays when tested in strains TA98, TA100, TA1535 and TA1537. However, the current guideline requires a fifth strain to check for cross-linking mutagens. For this reason, the assessment of bacterial gene mutation remains incomplete.

Eugenol induced an increase in mutation frequency in the mouse lymphoma assay in the absence of metabolic activation (Myhr and Caspari, 1991, B.6.4.1.2). The dose-dependent increase in mutant frequency was observed from 20 nL/mL up to 120 nL/mL although high cytotoxicity was observed at the highest dose (RTG < 10 %). According to the criteria from current OECD TG 490 (2016), positive results obtained with less than 10 % total growth would not be considered positive. Also, the global evaluation factor (GEF) calculated for this study is 117 and 116, for the first and second experiment, respectively. The mutation frequency does not exceed this value and therefore, the positive result is not considered biologically relevant. The experiment did not account for metabolic activation and therefore, the mammalian gene mutation potential of eugenol remains incomplete.

A number of *in vitro* mammalian chromosome aberration tests have been submitted to evaluate the clastogenicity potential of eugenol although all are deemed as supporting information based on deviations from the guideline. Positive results in the absence of metabolic activation were reported in CHL cells (Ishidate *et al.*, 1984, B.6.4.1.3.1). Eugenol was also reported positive in the presence and absence of metabolic activation in an *in vitro* chromosome aberration test in CHO cells (Stich *et al.*, 1981; B.6.4.1.3.2). Positive results in the chromosome aberration test in the presence (but not absence) of metabolic activation in cloned CHO cells were reported (Galloway *et al.*, 1987; B.6.4.1.3.3). Eugenol also induced chromosomal aberrations in V79 cells and SHE cells both in the presence and absence of metabolic activation (Maralhas *et al.*, 2006; B.6.4.1.3.4; Hikiba *et al.*, 2005; B.6.4.1.3.5). Eugenol did not induce chromosome aberrations in human D284 cells following a 3-hour exposure without metabolic activation, whereas it was positive at 30-hour exposure without metabolic activation (Someya *et al.*, 2008; B.6.4.1.3.6). In summary, based on available evidence, eugenol gives a positive response with and without metabolic activation in the chromosomal aberration assay *in vitro*. Although the studies are all deemed as supporting information, the overall assessment is that eugenol is positive in the *in vitro* chromosome aberration assay.

Eugenol also produced sister chromatid exchanges in the presence of metabolic activation (Jansson *et al.*, 1986; B.6.4.1.4.1; Galloway *et al.*, 1987; B.6.4.1.4.2) although based on deviations from guideline and poor level or reporting, the first study is not acceptable and the second is deemed as supporting information only. Based on the available data, evidence suggests that eugenol induces chromosomal aberration and SCE's *in vitro*.

Eugenol formed DNA adducts and formed 8-OH-dG *in vitro* in the presence of an activation system in HL-60 cells (Bodell *et al.*, 1998; B.6.4.1.4.3). DNA reactivity was assessed in the alkaline comet assay and eugenol showed a positive trend in Chinese Ovary AA8 cells (Martins *et al.*, 2011; B.6.4.1.4.4). In this study, eugenol showed the formation of DNA strand breaks too.

Data from REACH registration dossier were included in the summary table of other studies relevant for genotoxciity *in vitro*, since it is an ECHA requirement for the proposal of harmonised classification (CLH) according to Regulation (EC) no. 1272/2008 (CLP). However, it has to be underlined that these data were not available in the applicant submission dossier for the renewal and consequently they have not been evaluated by the RMS and not included in Volume 3 of this RAR.

***In vivo*:**

In vivo MN

A series of *in vivo* micronucleus assays have been provided in various species and *via* various routes of administration. Most of the results following i.p. administration are positive whereas oral administration of eugenol has given conflicting results in this assay for which negative results are reported in rats and positive in mice.

Eugenol was reported negative in the mouse (dYY mice) micronucleus assay following single i.p doses of eugenol at 100, 200, 400 and 800 mg/kg bw or multiple doses of 400 mg/kg bw (Hayashi *et al.*, 1988; B.6.4.2.1.1). Positive results were reported both following i.p. (147.9 or 739.7 mg/kg bw) or oral (14794.4 mg/kg bw) administration of eugenol in Swiss-Webster mice (Woolverton *et al.*, 1986; B.6.4.2.1.2). The oral dose in this study exceeds the 2000 mg/kg bw dose recommended by guidance and therefore, this result may only be considered as supporting information only. Eugenol was negative in an *in vivo* MN study in which 3 consecutive doses up to 600 mg/kg bw were administered intraperitoneally to B6C3F1 mice (Shelby *et al.*, 1993; B.6.4.2.1.4). Eugenol gave positive results in an *in vivo* MN assay in mice following ip administration at doses of 400 or 600 mg/kg bw (Ellahueña *et al.*, 1994; B.6.4.2.1.5). Eugenol was negative in an *in vivo* MN study in rats dosed orally at 500 or 1000 mg/kg bw [Rompelberg *et al.*, 1996a; B.6.4.2.1.6]. Eugenol did not induce MPE in rats treated orally with doses up to 1340 mg/kg bw [Maura *et al.*, 1989; B.6.4.2.1.7] or up to 1200 mg/kg bw [Chen *et al.*, 2021; B.6.4.1.8]. Most of these studies have

methodological deficiencies and deviations from the current OECD TG 474 (2016) and therefore, they have been deemed as supporting information only, with the exception of Chen *et al*. (2021; B.6.4.1.8)..

The anti-mutagenic activity of eugenol has been assessed. Mice treated with eugenol in the feeding diet for 14 days reduced the cytogenicity in bone marrow cells observed by the decrease in MPE following a single intraperitoneal injection of cyclophosphamide (Rompelberg *et al.*, 1995; B.6.4.2.2.3). Eugenol did not have an effect with other mutagens (MMC, EMS or B[*a*]P. In a Transgenic gene mutation assay, oral doses of eugenol (0.4 % w/w equivalent to 674 mg/kg bw) did not have an effect in mutant frequency following i.p. dose of B[*a*]P in Muta Mouse (Rompelberg *et al.*, 1996b; B.6.4.2.2).

Eugenol did not induce DNA damage in rat hepatocytes and kidney cells when dosed orally at 1340 mg/kg bw doses in a DNA alkaline elution assay *in vivo* (Maura *et al.*, 1989, B.6.4.2.3).

No DNA binding was identified in eugenol-dosed B6C3F1 mice (Phillips D.H., 1990; B.6.4.2.4). In humans, a non-standard cytogenetic assay was performed whereby oral doses of eugenol did not increase binucleated and chromosomal aberrations of erythrocytes in the treatment group (Rompelberg *et al.*, 1996c; B.6.4.2.5.1). In this study, biochemical parameters were also investigated and eugenol did not show any significant differences against the control group. In a host-mediated assay, eugenol or two metabolites did not increase the mutant frequency in *Salmonella typhimurium* strains TA1950, TA1951, TA1952 and TA1964 (Green and Savage, 1978; B.6.4.2.5.2).

Based on all the available genotoxicity data, negative results in bacterial gene mutation are reported although eugenol has not been tested in either *E.coli* or TA102 as per guidance requirement. Mammalian gene mutation *in vitro* has not been fully investigated and the study is incomplete (no metabolic activation experiment was carried out). Eugenol gives positive results in the *in vitro* chromosomal aberration test and produces DNA adducts *in vitro*. *In vivo* cytogenetic data indicates that eugenol gives positive results in mice both via ip or oral administration and has been reported negative in rats after oral administration. However, these studies are only considered supporting information based on method deficiencies and deviations from the current guidance. Mammalian gene mutations were investigated in the *in vivo* transgenic assay in Muta mouse and evidence suggests eugenol is negative in this assay. Similarly, eugenol is negative in the alkaline elution assay *in vivo*.

In conclusion, eugenol was negative *in vitro* for gene mutation (Ames test and MLA). Positive results were obtained *in vitro* for structural chromosome aberration, sister chromatid exchange and DNA adducts formation. On the other hand, results from *in vivo* assays were mostly negative (micronucleus, chromosomal aberration, gene mutation and DNA damage tests). The only positive results were obtained in two micronucleus tests performed by intraperitoneal administration or after oral eugenol administration at a very high dose (14794.4 mg/kg orally).

Data from REACH registration dossier were included in the summary table of other studies relevant for genotoxciity *in vivo*, since it is an ECHA requirement for the proposal of harmonised classification (CLH) according to Regulation (EC) no. 1272/2008 (CLP). However, it has to be underlined that these data were not available in the applicant submission dossier for the renewal and consequently they have not been evaluated by the RMS and not included in Volume 3 of this RAR.

Overall, based on all the available information and using a weight of evidence approach, it may be concluded that eugenol is considered to be genotoxic *in vitro* but not *in vivo.*

2.6.4.2 Comparison with the CLP criteria regarding genotoxicity / germ cell mutagenicity

No human data are available for eugenol, hence a classification as Category 1 is not possible. Also, there are no *in vivo* germ cell tests in mammals that could justify Cat 1B.

The classification in Category 2 is based on:
- Positive evidence obtained from experiments in mammals and/or in some cases from *in vitro* experiments, obtained from:
- Somatic cell mutagenicity tests *in vivo*, in mammals; or
- Other *in vivo* somatic cell genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assays.

Most of the available *in vivo* somatic cells mutagenicity assay data do not meet the criteria for classification. The overall body of toxicological data from a number of *in vitro* and *in vivo* assays indicates that eugenol is of no genotoxic concern, hence no classification and labelling for mutagenicity under CLP Regulation is required.

2.6.4.3 Conclusion on classification and labelling for genotoxicity / germ cell mutagenicity

Based on the data available for **eugenol** and according to the criteria under Regulation (EC) No 1272/2008, **no classification of genotoxicity / germ cell mutagenicity can be drawn (data conclusive but not sufficient for classification).**

2.6.5 Summary of long-term toxicity and carcinogenicity [equivalent to section 10.9 of the CLH report template]

Table 53: Summary table of animal studies on long-term toxicity and carcinogenicity

<table>
<tr><th>Method, guideline, deviations[1] if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
- NOAEL/LOAEL
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr>
<td>2-year carcinogenicity study in rats

GLP: No

Method: None stated.

Rat strain: F344/N rats: ♂ and ♀

No. animals:
50 rats/dose
40 rats in control group

Deviations from current test guideline (OECD TG 453, 2018):

-No satellite groups to monitor the reversibility of toxicological changes were incorporated.

- Only two dose levels were assayed, when at least three dose levels should be used.

-Only 40 animals per sex were used in control group (OECD TG 453 requires at least 50 animals/sex).

- No haematology, urinalysis and clinical chemistry were performed.

-Organ weight data was not recorded.

-Individual bodyweight data were not presented.

-Statistical analysis was not</td>
<td>Test substance: Eugenol. Batch No.: 36483 and 26068, Purity: >99%

Eugenol :Oral (diet)

Doses:
Males: 0, 3000 and 6000 ppm (equivalent to adjusted value by RMS of 0, 128 and 260 mg/kg bw/day, or default value of 0, 150 and 300 mg/kg bw/day).
Females: 0, 6000 and 12500 ppm (equivalent to adjusted value by RMS of 0, 302 and 648 mg/kg bw/day, or default value of 0, 300 and 625 mg/kg bw/day)

105-week feed exposure.</td>
<td>Survival: No significant differences were detected between treated groups and controls.

Survival at termination of study (105 weeks)

<table>
<tr><th></th><th colspan="2">survival</th></tr>
<tr><th></th><th>♂</th><th>♀</th></tr>
<tr><td>Control</td><td>58% (23/40)</td><td>75% (30/40)</td></tr>
<tr><td>Low dose (3000 ppm ♂/ 6000 ppm ♀)</td><td>52% (26/50)</td><td>72% (36/50)</td></tr>
<tr><td>High dose (6000 ppm ♂/ 12500 ppm ♀)</td><td>74% (37/50)</td><td>88% (44/50)</td></tr>
</table>

Clinical signs: No compound-related clinical signs were observed.

6000/12500 ppm (equivalent to 260/648 mg/kg bw/day for ♂/♀)
Bodyweight(statistical analysis not performed)
▪ (↓) bw in ♀ throughout week 51-102 (10-15%).

Food consumption (statistical analysis not performed): No differences in food consumption were observed in treated groups for both sexes.

Histopathology
Neoplastic changes
Uterus
▪ (↑) Uterine endometrial stromal polyp or sarcoma (34% vs 15% in controls; ns).
▪ (↑) Uterine endometrial stromal polyp ♀ (32% vs 15% in controls; ns).
▪ (↑) Uterine endometrial stromal sarcoma ♀ (2% vs 0% in controls; ns).
Lung
▪ (↑) alveolar/bronchiolar adenoma or carcinoma (combined) in ♂ (4% vs 0% in controls; ns; ndr).
Thyroid
▪ (↓) Thyroid C-cell adenoma in ♀ (4% vs 8% in controls; ns; ndr).
▪ (↓) Thyroid C-cell adenoma in ♂ (0% vs 10% in controls).
▪ (↓) Thyroid C-cell adenoma/carcinomas in ♂ (4% vs 18% in controls).
Mammary gland
▪ (↓) Mammary gland fibroadenoma in ♀ (12% vs 35% in controls).

Summary of neoplastic incidences in the rats 2-year feed study with eugenol

<table>
<tr><th colspan="3">Neoplastic incidences</th></tr>
<tr><th>Control (%)</th><th>low dose (%)(3000 ppm ♂/ 6000 ppm ♀)</th><th>high dose (%)(6000 ppm ♂/ 12500 ppm ♀)</th></tr>
</table>
</td>
<td>NTP (1983) (AS)
B.6.5.1</td>
</tr>
</table>

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference

performed in bodyweight, food consumption and non-neoplastic lesions.

Study acceptable as supportive information.

	males	females	males	females	males	females
Alveolar/ bronchiolar adenoma / carcinoma	0/40 (0%)	1/39 (3%)	**5/49 (10%)* (↑10%)**	1/50 (2%)	2/50 (4%)	0/50 (0%)
Alveolar/ bronchiolar adenoma	0/40 (0%)	0/39 (0%)	2/49 (4%)	1/50 (2%)	2/50 (4%)	0/50 (0%)
Alveolar/ bronchiolar carcinoma	0/40 (0%)	1/39 (3%)	3/49 (6%)	0/50 (0%)	0/50 (0%)	0/50 (0%)
Thyroid C-cell adenoma/ carcinoma	**7/40 (18%)#**	7/40 (18%)	8/50 (16%)	12/49 (24%)	**2/50 (4%)* (↓14%)**	6/50 (12%)
Thyroid C-cell adenoma	**4/40 (10%)#**	3/40 (8%)	5/50 (10%)	**11/49 (22%)* (↑14%)**	**0/50 (0%)* (↓10%)**	2/50 (4%)
Thyroid C-cell carcinoma	3/40 (8%)	4/40 (10%)	3/50 (6%)	1/49 (2%)	2/50 (4%)	4/50 (8%)
Uterine endometrial stromal polyp /sarcoma		**6/40 (15%)#**		6/50 (12%)		17/50 (34%)
Uterine endometrial stromal polyp	–	6/40 (15%)	–	6/50 (12%)	–	16/50 (32%)
Uterine endometrial stromal sarcoma	–	0/40 (0%)	–	0/40 (0%)	–	1/50 (2%)
Mammary gland fibroadenoma	0/40 (0%)	14/40 (35%)	2/50 (4%)	**7/50 (14%)* (↓21%)**	2/50 (4%)	**6/50 (12%)* (↓23%)**

**p<0.05*
#p<0.05 (Cochran-armitage trend test)

Non-neoplastic changes (*statistical analysis not performed)*

- (↑) cystic hyperplasia in the uterus in ♀ (22% vs 3% in controls).
- (↑) spleen haemosiderosis in ♀ (16% vs 2% in controls).
- (↑) kidney chronic inflammation in ♂ (86% vs 73% in controls; ncdr).
- (↓) kidney chronic inflammation in ♀ (4% vs 10% in controls).
- (↑) Thyroid C-cell hyperplasia in ♂ (8% vs 3% in controls; ncdr).
- (↓) Thyroid C-cell hyperplasia in ♀ (10% vs 15% in controls).
- (↑) Prostate inflammation suppurative (11% vs 8% in controls; ndr).
- (↑) Prostate inflammation chronic suppurative (4% vs 0% in controls; ndr).

Summary of non-neoplastic incidences in the rats 2-year feed

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference

study with eugenol

	non-neoplastic incidences					
	Control (%)		low dose (%)		high dose (%)	
	males	females	males	females	males	females
Uterus cystic hyperplasia	–	1/40 (3%)	–	2/50 (4%)	–	11/50 (22%)
Spleen haemosiderosis	0/40 (0%)	1/40 (2%)	0/50 (0%)	1/50 (2%)	0/50 (0%)	8/50 (16%)

Historical control data from NTP Carcinogenesis Program and Southern Research Institute

	HCD from NTP feeding carcinogenicity studies (1990-1997)					
	Male			Female		
	nº animals /total	Rate (%)#	Range (%)	nº animals /total	Rate (%)#	Range (%)
Alveolar/bronchiolar adenoma / carcinoma	45/1352	3,3	0-14	26/1351	1,9	0-10
Alveolar/bronchiolar adenoma	35/1352	2,6	0-14	22/1351	1,6	0-10
Alveolar/bronchiolar carcinoma	10/1352	0,7	0-4	4/1351	0,3	0-2
Thyroid C-cell adenoma/carcinoma	199/1347	14,8	0-35	184/1347	13,6	0-22
Thyroid C-cell adenoma	175/1347	13	2-35	158/1347	11,7	4-22
Thyroid C-cell carcinoma	24/1347	1,8	0-6	26/1347	1,9	0-4
Uterine endometrial stromal polyp / sarcoma	–	–	–	199/1351	14,7	0-30
Uterine endometrial stromal polyp	–	–	–	192/1351	14,2	2-30
Uterine endometrial stromal sarcoma	–	–	–	7/1351	0,5	0-4
Mammary gland fibroadenoma	58/1354	4.3	0-12	556/1351	41,2	8-60
	HCD from NTP feeding carcinogenicity studies (1983)					
Alveolar/bronchiolar adenoma / carcinoma	55/2305	2,4	n.a.	27/2354	1,2	n.a.
Alveolar/bronchiolar adenoma	35/2305	1,5	n.a.	18/2354	0,8	n.a.
Alveolar/bronchiolar carcinoma	20/2305	0,9	n.a.	9/2354	0,4	n.a.
Thyroid C-cell adenoma/carcinoma	198/2230	8,9	n.a.	192/2265	8,5	n.a.
Thyroid C-cell adenoma	114/2230	5,1	n.a.	111/2265	4,9	n.a.
Thyroid C-cell carcinoma	84/2230	3,8	n.a.	81/2265	3,6	n.a.
Uterine endometrial stromal polyp / sarcoma	–	–	–	449/2318	19,4	n.a.
Uterine endometrial stromal polyp	–	–	–	424/2318	18,3	n.a.
Uterine endometrial stromal sarcoma	–	–	–	25/2318	1,1	n.a.
Mammary gland fibroadenoma	51/2320	2.2	n.a.	572/2370	24,1	n.a.
	HCD from Southern Research Institute (1983)					
	Male			Female		
	nº animals /total	Rate (%)#	Range (%)	nº animals /total	Rate (%)#	Range (%)
Alveolar/bronchiolar adenoma / carcinoma	15/438	3	n.a.	n.a.	n.a.	n.a.
Uterine endometrial stromal polyp / sarcoma	–	–	–	66/438	15	n.a.
Mammary gland fibroadenoma	n.a.	n.a.	n.a.	120/439	27	n.a.

 # percentage of animals with tumors
NOTE In order to completeness, all the available information is presented, however, HCD from Southern Research Institute was the only accepted by RMS, due to it was the laboratory in which the study was conducted, and the time frame is within the 5-recommended years.

<table>
<tr><th>Method, guideline, deviations[1] if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
- NOAEL/LOAEL
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr><td></td><td></td><td>3000/6000 ppm (equivalent to 128/302 mg/kg bw/day for ♂/♀)

<u>Histopathology</u>
Neoplastic changes
Lung
▪ (↑) alveolar/bronchiolar adenoma or carcinoma (combined) in ♂ (10% vs 0% in controls; ndr).
Thyroid
▪ (↑) Thyroid C-cell adenoma in ♀ (22% vs 8% in controls; ndr).
▪ (↓) Thyroid C-cell adenoma/carcinomas in ♂ (16% vs 18% in controls; ns).
Mammary gland
▪ (↓) Mammary gland fibroadenoma in ♀ (14% vs 35% in controls).

Non-neoplastic changes (statistical analysis not performed)
Uterus
▪ (↑) cystic hyperplasia in the uterus in ♀ (4% vs 3% in controls).
▪ (↑) kidney chronic inflammation in ♂ (92% vs 73% in controls; ncdr).
▪ (↓) kidney chronic inflammation in ♀ (6% vs 10% in controls).
▪ (↓) Thyroid C-cell hyperplasia in ♂ (2% vs 3% in controls).
▪ (↓) Thyroid C-cell hyperplasia in ♀ (14% vs 15% in controls).
▪ (↑) Prostate inflammation suppurative in ♂ (18% vs 8% in controls; ndr).
▪ (↑) Prostate inflammation chronic suppurative in ♂ (10% vs 0% in controls; ndr).

-LOAEL_{toxicity}= 12500 ppm (~648 mg/kg bw/day)
-LOAEL_{carcinogenicity}= -
-NOAEL_{toxicity}= 6000 ppm (~260/302 mg/kg bw/day for ♂/♀)
NOAEL_{carcinogenicity} = 6000 ppm (~260/302 mg/kg bw/day for ♂/♀)

-Critical effects at the LOAEL: ↑ incidence of uterine endometrial stromal polyp/sarcoma and cystic hyperplasia in the uterus, ↑ spleen haemosiderosis, ↓ bodyweight in ♀.

<u>Target tissue/organ</u>: Uterus and spleen.</td><td></td></tr>
<tr><td>2-year carcinogenicity study in mice

<u>GLP</u>: No

<u>Method</u>: None stated.

<u>Mice strain:</u>
B6C3F₁: ♂ and ♀

<u>No. animals</u>:
50 mice/dose

<u>Deviations from current test guideline (OECD TG 453, 2018):</u>

-No satellite groups to monitor the reversibility of toxicological changes were</td><td><u>Test substance:</u>
Eugenol Batch No.: 36483 and 26068, Purity: >99%

Eugenol :Oral (diet)

Doses:
<u>Males/Females:</u>
0, 3000 and 6000 ppm (equivalent to adjusted value by RMS of 0, 632/784 and 1250/1546 for ♂/♀, or default values of 0, 450 and 900 mg/kg bw/day).</td><td><u>Survival</u>: No significant differences were detected between treated groups and controls. A reduction in survival was detected in low and high dose male groups (12%), and in low dose female group (6%), compared with controls at termination of the study.

Survival at termination of study (106 weeks)
<table><tr><th></th><th colspan="2">Survival (%)</th></tr><tr><th></th><th>♂</th><th>♀</th></tr><tr><td>Control</td><td>82% (41/50)</td><td>86% (43/50)</td></tr><tr><td>3000 ppm</td><td>70% (35/50)</td><td>80% (40/50)</td></tr><tr><td>6000 ppm</td><td>70% (35/50)</td><td>90% (45/50)</td></tr></table>
<u>Clinical signs</u>: No compound-related clinical signs were observed.

6000 ppm (equivalent to 1250/1546 for ♂/♀).
<u>Bodyweight</u> (statistical analysis not performed)
▪ (↓) bw in ♀ at week 101 (14%; ns) and 104 (11%; ns).

<u>Food consumption</u> (statistical analysis not performed)<u>:</u> No differences in food consumption were observed in treated groups</td><td>NTP (1983) (AS) B.6.5.2</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations[1] if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
- NOAEL/LOAEL
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr><td>incorporated.
- Only two dose levels were assayed, when at least three dose levels should be used.
- No haematology, urinalysis and clinical chemistry were performed.
-Organ weight data was not recorded.
-Statistical analysis was not performed in bodyweight, food consumption and non-neoplastic lesions.
-Individual bodyweight data were not presented.
-Animal cages were not arranged in such a way that possible effects due to cage placement were minimized.
Study acceptable as supportive</td><td>106-week feed exposure.</td><td>for both sexes.
<u>Histopathology</u>
Neoplastic changes
Liver:
▪ (↑) Hepatocellular adenoma or carcinoma in ♀ (18% vs 4% in controls).
▪ (↑) Hepatocellular adenoma or carcinoma in ♂ (37% vs 28% in controls; ns; ndr.).
▪ (↑) Hepatocellular adenoma ♂ (20% vs 8% in controls; ns; ndr).
▪ (↓) Hepatocellular carcinoma ♂ (18% vs 20% in controls; ns; ndr).
Thyroid
▪ (↑) Follicular cell adenoma in ♂ (6% vs 0% in controls; ns).
Summary of neoplastic incidences in the mice 2-year feed study with eugenol
<table>
<tr><th></th><th colspan="6">neoplastic incidences</th></tr>
<tr><th></th><th colspan="2">Control (%)</th><th colspan="2">3000 ppm (%)</th><th colspan="2">6000 ppm (%)</th></tr>
<tr><th></th><th>males</th><th>females</th><th>males</th><th>females</th><th>males</th><th>females</th></tr>
<tr><td>Hepatocellular adenoma</td><td>4/50 (8%)</td><td>0/50 (0%)</td><td>13/50 (26%)* (↑18%)</td><td>4/49 (8%)</td><td>10/49 (20%)</td><td>3/49 (6%)</td></tr>
<tr><td>Hepatocellular carcinoma</td><td>10/50 (20%)</td><td>2/50 (4%)</td><td>20/50 (40%)* (↑20%)</td><td>3/49 (6%)</td><td>9/49 (18%)</td><td>6/49 (12%)</td></tr>
<tr><td>Hepatocellular adenoma/ carcinoma</td><td>14/50 (28%)</td><td>2/50 (4%)#</td><td>28/50 (56%)* (↑28%)</td><td>7/49 (14%)</td><td>18/49 (37%)</td><td>9/49 (18%)* (↑14%)</td></tr>
<tr><td>Thyroid follicular cell adenoma</td><td>0/48 (0%)#</td><td>2/48 (4%)</td><td>0/49 (0%)</td><td>0/47 (0%)</td><td>3/49 (6%)</td><td>1/49 (2%)</td></tr>
</table>
* p<0.05
# p<0.05 (Cochran-Armitage trend test)
Non-neoplastic changes (no statistical analysis performed)
Kidney
▪ (↑) Focal inflammation in ♂ (16% vs 0% in controls).
▪ (↓) Nephrosis in ♂ (8% vs 61% in controls), and ♀ (27% vs 36% in controls).
Lung
▪ (↑) focal granulomatous inflammation of lung in ♀ (51% vs 36% in controls).
▪ (↑) adenomatous hyperplasia of lung in ♀ (54% vs 44% in controls).
Thyroid
▪ (↓) thyroid cystic degeneration in ♂ (0% vs 23% in controls).
Skin
▪ (↓) Skin chronic inflammation in ♂ (0% vs 14% in controls; ndr); and ♀ (0% vs 14% in controls).
Ovary
▪ (↑) ovary follicular cyst (33% vs 22% in controls).
Summary of non-neoplastic incidences in the mice 2-year feed study with eugenol
<table>
<tr><th></th><th colspan="6">non-neoplastic incidences</th></tr>
<tr><th></th><th colspan="2">Control (%)</th><th colspan="2">3000 ppm (%)</th><th colspan="2">6000 ppm (%)</th></tr>
<tr><th></th><th>males</th><th>females</th><th>males</th><th>females</th><th>males</th><th>females</th></tr>
<tr><td>Kidney, focal inflammation</td><td>0/49 (0%)</td><td>0/50 (0%)</td><td>0/50 (0%)</td><td>0/49 (0%)</td><td>8/49 (16%)</td><td>0/49 (0%)</td></tr>
<tr><td>lung focal granulomatous inflammation</td><td>12/49 (24%)</td><td>18/50 (36%)</td><td>14/49 (29%)</td><td>19/49 (39%)</td><td>0/50 (0%)</td><td>25/49 (51%)</td></tr>
</table>
</td><td></td></tr>
</table>

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference

Historical control data of liver and thyroid gland tumors from NTP Carcinogenesis Program and Southern Research Institute

		HCD from NTP feeding carcinogenicity studies (1990-1997)					
		Male			Female		
		nº animals /total	Rate (%)#	Range (%)	nº animals /total	Rate (%)#	Range (%)
Liver	Adenoma/ carcinoma	638/1350	47,3	4-60	347/1350	25,7	0-50
	Adenoma	397/1350	29,4	4-60	234/1350	17,3	2-50
	Carcinoma	241/1350	17,9	6-29	113/1350	8,4	0-20
Thyroid gland	Follicular cell adenoma/ carcinoma	26/1343	1,9	0-4	26/1340	1,9	0-8
	Follicular cell adenoma	20/1343	1,5	0-4	24/1340	1,8	0-8
	Follicular cell carcinoma	6/1343	0,4	0-2	2/1340	0,1	0-2

		HCD from NTP feeding carcinogenicity studies (1983)					
		Male			Female		
		nº animals /total	Rate (%)#	Range (%)	nº animals /total	Rate (%)#	Range (%)
Liver	Adenoma/ carcinoma	743/2386	31	0-58	208/2519	8	0-18
	Adenoma	242/2386	10	0-22	102/2519	4	0-18
	Carcinoma	501/2386	21	6-36	106/2519	4	0-15
Thyroid gland	Follicular cell adenoma/ carcinoma	27/2178	1,2	n.a.	46/2203	2,1	n.a.
	Follicular cell adenoma	22/2178	1	n.a.	40/2203	1,8	n.a.
	Follicular cell carcinoma	5/2178	0,2	n.a.	6/2203	0,3	n.a.

		HCD from Southern Research Institute (1983)					
		Male			Female		
		nº animals /total	Rate (%)#	Range (%)	nº animals /total	Rate (%)#	Range (%)
Liver	Adenoma/ carcinoma	145/446	32	n.a.	30/445	7	n.a.
	Adenoma	42/446	9	n.a.	14/445	3	n.a.
	Carcinoma	103/446	23	n.a.	16/445	4	n.a.

 # percentage of animals with tumors
NOTE In order to completeness, all the available information is presented, however, HCD from Southern Research Institute was the only accepted by RMS, due to it was the laboratory in which the study was conducted, and the time frame is within the 5-recommended years.

3000 ppm (equivalent to 632/784 for ♂/♀).

Histopathology
Neoplastic changes
Liver
- (↑) Hepatocellular adenoma/carcinoma in ♂ (56% vs 28% in controls; ndr).
- (↑) Hepatocellular adenoma/carcinoma in ♀ (14% vs 4% in controls; ns).

<table>
<tr><th>Method, guideline, deviations[1] if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
- NOAEL/LOAEL
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr><td></td><td></td><td>▪ (↑) Hepatocellular adenoma in ♂ (26% vs 8% in controls; ndr).
▪ (↑) Hepatocellular carcinoma in ♂ (40% vs 20% in controls; ndr).

Non-neoplastic changes (no statistical analysis performed)
Lung
▪ (↑) focal granulomatous inflammation of lung in ♀ (39% vs 36% in controls).
▪ (↑) adenomatous hyperplasia of lung in ♀ (45% vs 44% in controls).
Kidney
▪ (↓) incidences of kidney nephrosis in ♂ (52% vs 61% in controls), and ♀ (33% vs 36% in controls).
Thyroid
▪ (↓) thyroid cystic degeneration in ♂ (6% vs 23% in controls).
Skin
▪ (↑) Skin chronic inflammation in ♂ (22% vs 14% in controls; ndr), and (↓) in ♀ (0% vs 14% in controls).

-LOAEL_{toxicity}=6000 ppm (~ 1250 mg/kg bw/day)
-LOAEL_{carcinogenicity}= -
-NOAEL_{toxicity}= 3000 ppm (~632 mg/kg bw/day)
-NOAEL_{carcinogenicity}= 6000 ppm (~1250 mg/kg bw/day).

-Critical effects at the LOAEL: ↑ increase incidences of focal inflammation of the kidney in males, and focal granulomatous inflammation and adenomatous hyperplasia of lung and ovary follicular cyst in females.

Target tissue/organ: Kidney, lung and ovary.</td><td></td></tr>
<tr><td>Carcinogenicity study in mice (18 month)
GLP: No
Method: None stated.
Mice strain: CD-1 ♀
No. animals: 30 females/dose
Deviations from current test guideline (OECD TG 453, 2018):
-Test substance not characterised (batch number and certificate of chemical analysis are not reported).
-The duration of the experiment was not 24 month for carcinogenicity assessment.
-No satellite</td><td>Test substance: Eugenol

Eugenol :Oral (diet)
Phenobarbital: Oral (phenobarbital was administered in drinking-water for 18 months)

Doses:
Females: 0 (negative control), 0.5% eugenol, 0.5% eugenol + 0.05% phenobarbital and 0.05% phenobarbital (positive control)

-12 month treatment + 6 month of the recovery period with grain diet</td><td>Survival: No differences were detected between eugenol-fed animals without phenobarbital treatment and control animals. A reduction in survival (20%) at 18 months was detected between eugenol-fed animals with phenobarbital and control animals.
Survival at study time-points
<table><tr><th></th><th colspan="3">Survival (no mice alive)</th></tr><tr><th></th><th>start</th><th>10 months (%)</th><th>18 months (%)</th></tr><tr><td>control</td><td>30</td><td>30 (100%)</td><td>30 (100%)</td></tr><tr><td>Phenobarbital (PB)</td><td>30</td><td>30 (100%)</td><td>29 (97%)</td></tr><tr><td>Eugenol</td><td>30</td><td>29 (97%)</td><td>29 (97%)</td></tr><tr><td>Eugenol + PB</td><td>30</td><td>25 (83%)</td><td>24 (80%)</td></tr></table>Statistical analysis not performed
Clinical signs: No clinical signs were reported in this study.
0.5% eugenol (750 mg/kg bw/day for ♀)
Bodyweight gain
▪ (↓) bwg at 4 (13%) and 8 (15%) months compared with controls.
<table><tr><th></th><th colspan="3">Bodyweight gain (g/mouse)</th></tr><tr><th></th><th>1 month (% vs control)</th><th>4 months (% vs control)</th><th>8 months (% vs control)</th></tr><tr><td>control</td><td>4.9</td><td>9.9</td><td>13</td></tr></table></td><td>Miller et al., (1983) (AS) B.6.5.3</td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations[1] if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
- NOAEL/LOAEL
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr>
<td>groups to monitor the reversibility of toxicological changes were incorporated.
-Only 30 female mice/dose were used (OECD TG 453 requires at least 50 animals/sex/dose).
- Only one dose level was assayed, when at least three dose levels should be used.
- Bodyweight and food consumption were not measured. Bodyweight gain was only measured at 1, 4 and 8 months.
- No haematology, urinalysis, clinical chemistry or organ weights were performed.
- Only liver, lung, skin, pleural and peritoneal cavity were subjected to necropsy and histopathological examinations.
-Statistical analysis not performed in survival and bodyweight gain measurements.
Study acceptable as supportive information.</td>
<td>without any supplement</td>
<td>
<table>
<tr><td>Phenobarbital (PB)</td><td>5.5 (112%)</td><td>8.5 (85%)</td><td>11 (85%)</td></tr>
<tr><td>Eugenol</td><td>5.1 (104%)</td><td>8.6 (87%)</td><td>11 (85%)</td></tr>
<tr><td>Eugenol + PB</td><td>6 (122%)</td><td>7.9 (80%)</td><td>10 (77%)</td></tr>
</table>
Statistical analysis not performed

Histopathology
Neoplastic changes
▪ (↑) Thymic lymphoma (7%; ns) vs 0% in controls.
▪ (↑) Mammary gland adenocanthoma (3%; ns) vs 0% in controls.

<table>
<tr><th></th><th colspan="4">Neoplastic incidences</th></tr>
<tr><th></th><th>control</th><th>PB</th><th>Eugenol</th><th>Eugenol + PB</th></tr>
<tr><td>Pulmonary adenoma</td><td>1/30 (3%)</td><td>1/30 (3%)</td><td>1/30 (3%)</td><td>0</td></tr>
<tr><td>Haemangioendothe liosarcoma</td><td>0</td><td>1/30 (3%)</td><td>0</td><td>0</td></tr>
<tr><td>Thymic lymphoma</td><td>0</td><td>0</td><td>2/30 (7%)</td><td>0</td></tr>
<tr><td>Mammary adenoacanthoma</td><td>0</td><td>0</td><td>1/30 (3%)</td><td>0</td></tr>
</table>
PB Phenobarbital
Statistically significant differences were not found

0.5% eugenol (750 mg/kg bw/day for ♀) + 0.05% phenobarbital

Bodyweight gain
▪ (↓) bwg at 4 (20%) and 8 (23%) months compared with controls.

-LOAEL= 750 mg/kg bw/day
-NOAEL_{toxicity}= -
-NOAEL_{carcinogenicity}= -

-Critical effects at the LOAEL: ↑ incidences of thymic lymphoma and mammary gland adenocanthoma.

Target tissue/organ: Thymus and mammary gland.
</td>
<td></td>
</tr>
</table>

Table 54: Summary table of human data on long-term toxicity and carcinogenicity

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No data				

Table 55: Summary table of other studies relevant for long-term toxicity and carcinogenicity

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No data				

2.6.5.1 Short summary and overall relevance of the provided information on long-term toxicity and carcinogenicity

Three long-term/carcinogenicity toxicity studies, all previously included in the original DAR (2011), have been submitted and reassessed to support the renewal of the active substance eugenol (one in rats, B.6.5.1, and two in mice, B.6.5.2 and B.6.5.3). These three studies present important deviations, as using only one or two dose levels, the lack of important measurements (haematology, clinical chemistry, urinalysis and organ weights), or that statistical analysis were not extended to all the endpoints.

In the 2-year oncogenicity study in rats (B.6.5.1), eugenol was tested at dose levels of 0, 3000/6000 ppm for males (equivalent to 128/260 mg/kg bw/day) , and 6000/12500 ppm for females (equivalent to 302/648 mg/kg bw/day).

Neither mortality nor clinical signs were observed in any dose group.

No differences in mean bodyweights were detected in male treated groups. In the female high dose group a reduction >10% (10-15%) was observed in the mean bodyweight from week 51 to almost the end of the study (week 102).

No differences in food consumption were observed in treated groups for both sexes compared with controls. The average daily feed consumption was 99% and 96% for low and high dose male groups, and 98% and 95% for low and high dose female groups, respectively.

Histopathological evaluation showed statistically significant increased incidence of alveolar/bronchiolar adenomas and carcinomas (combined) in the 3000 ppm dose male group compared with controls (10% vs 0% in controls), but no in the 6000 ppm dose male group (4%), so a dose-related trend was not observed. The incidence of alveolar/bronchiolar adenomas and carcinomas (combined) noted in the low dose male group was higher than mean incidence of HCD reported by Southern Research Institute (3%), and NTP Carcinogenesis Program (2.4% for 1983 period and 3.3% for 1990-1997 period), but within the range of the NTP Carcinogenesis Program 1990-1997 (0-14%) [Haseman *et al.*, *Toxicol Pathol.*, 1984: 12(2): 126-35; and 1998, 26(3):428-41].

On the other hand, statistically significant increased incidence of thyroid C-Cell adenoma was described in the 6000 ppm female dose group compared with controls (22% vs 8% in controls), however, the incidence in the 12500 ppm female dose group was even lower (4%) than described in the low and control groups, so a dose-related trend was not observed. Accordingly, the incidence of thyroid C-Cell adenomas noted in the low dose female group (22%) was higher than mean incidence and in the range limit of the HCD reported by NTP Carcinogenesis Program (mean incidence= 4.9%; range: n.a. for 1983 period; and 11.7%; range= 4-22% for 1990-1997 period). In contrast, a statistically significant decrease in the incidence of thyroid C-Cell adenoma (10%, 10% and 0% for controls, low and high dose groups) and adenoma/carcinoma combined (18%, 16% and 4% for controls, low and high dose groups) was detected in the thyroids of high dose male group compared with controls.

The most relevant finding was recorded in the uterus of female rats. In this organ, a statistically significant increase trend in the incidence of uterine endometrial stromal polyp or sarcoma was observed in the high dose female group (15%, 12% and 34% for controls, low and high dose groups). In the high dose group, the p value was in the border of statistical significance (p=0.051). This occurrence was higher than mean incidence of HCD reported by Southern Research Institute (15%) and NTP Carcinogenesis Program (19.4% for 1983 period and 14.7% for 1990-1997 period), and out of the range recorded by NTP Carcinogenesis Program 1990-1997 (0-30%). If we analyse the incidences individually, the incidence of uterine endometrial stromal polyps was 15, 12 and 32% for control, low and high dose female groups, respectively. The occurrence in the top dose female group was also higher than mean and out of the range of HCD reported by NTP Carcinogenesis Program (mean incidence= 18.3%; range: n.a. for 1983 period; and mean incidence=14.2%; range 2-30% for 1990-1997 period). On the other hand, the incidence of uterine endometrial stromal sarcoma was 0, 0 and 2% for control, low and high dose female groups, respectively. The result observed in the high dose female group was higher than mean, but within the HCD reported by NTP Carcinogenesis Program (mean= 1.1%; range = n.a. for 1983 period; and mean=0.5%; range 0-4% for 1990-1997 period).

In addition, and also in the reproductive system, a statistically significant decrease in the incidence of mammary gland fibroadenomas was detected in the low and high dose females group compared with controls (35%, 14% and 12% for control, low and high dose group, respectively). The possible protective role of eugenol that could exert in this type of neoplasm is unknown.

No further differences were recorded in other neoplastic alterations between treated groups and controls.

Regarding non-neoplastic changes, increase incidences of cystic hyperplasia in the uterus (22% vs 3%) and spleen haemosiderosis (16% vs 2%) were observed in the high dose female groups compared with controls. Moreover, male dose groups showed a non-clear dose-related increase of kidney chronic inflammation (73%, 92% and 86% for control, low and high dose groups, respectively). No other relevant non-neoplastic lesions were detected in other organs or tissues in the low or high dose groups of both sexes. It is likely that presence of cystic hyperplasia in the uterus was related with the endometrial stromal polyp or sarcoma previously described.

NOAEL for carcinogenicity was considered to be **6000 ppm,** equivalent to 260/302 mg/kg bw/day for males/females, based on no evidence of carcinogenicity. **NOAEL for toxicity** was considered to be **6000 ppm,** equivalent to 260/302 mg/kg bw/day for males/females, based on increase incidences of endometrial stromal polyp/sarcoma and cystic hyperplasia in uterus, and spleen haemosiderosis in females.

In the 2-year oncogenicity study in mice (B.6.5.2), eugenol was tested at dose levels of 0, 3000 and 6000 ppm, equivalent to 632/784 and 1250/1546 mg/kg bw/day for males/females.

Survival in the high dose male group was slightly lower than low dose and control group after week 38, whereas the survival in the low dose female group was lower than high dose and control group after week 80. A reduction in survival was detected in low and high dose male groups (12%), and in low dose female group (6%), compared with controls at termination of the study.

No differences in bodyweights were detected in male treated groups. In the female high dose group, a reduction >10% was observed in the mean bodyweight at weeks 101 (14%) and 104 (11%), respectively.

No differences in food consumption were observed in treated groups for both sexes compared with controls. The average daily feed consumption was 97% and 93% for low and high dose male groups, and 106% and 90% for low and high dose female groups, respectively, compared with controls.

Statistically significant ($p<0.05$) increased incidence of hepatocellular adenomas and carcinomas were recorded in the 3000 ppm male dose group (26% and 40% for adenomas and carcinomas, respectively) compared with controls (8% and 20% for adenomas and carcinomas, respectively). In the high dose male group, the incidence was lower than observed in the low dose group (20% and 18% for adenomas and carcinomas, respectively) and did not display statistical significance, so a dose-related trend was not observed.

The incidence of hepatocellular adenomas noted in the low dose male group (26%) was higher than mean, and slightly outside the range of the historical control datasets documented until 1983 by the NTP Carcinogenesis Program (mean=10%; range=0-22%) and the Southern Research Institute (mean= 9%; range= n.a.). Conversely, and taken into account a further HCD published by the NTP Carcinogenesis Program encompassing period 1990-1997 (Haseman et al., *Toxicol Pathol.*, 1998, 26(3):428-41), the occurrence observed in the 3000 ppm male group was lower than mean incidence and within the range of this HCD (mean= 29.4%; range= 4-60%).

On the other hand, the hepatocellular carcinoma incidence observed in the low dose male (40%) group was higher than mean incidences and out of the range of the three reported historical control datasets.

In addition, the incidence of both combined incidences in the low dose male group displayed statistically significant results compared with controls (56% vs 28% in controls). The result was higher than mean reported in the three HCD, but was within the range of the datasets reported by the NTP Carcinogenesis Program in 1983 (range=0-58%) and through 1990-1997 (range=4-60%). Besides, the incidence noted in the high male dose group (37%), was lower than observed in the low dose group, so a dose-related trend was not considered.

Moreover, the incidences of hepatocellular adenomas and carcinomas (combined) were significantly increased in female mice at high dose level (18% vs 4% in controls), and presented a statistically significant trend. These incidences were higher than overall historical mean incidence of the Southern Research Institute (mean= 7%) and NTP Carcinogenesis Program (mean=8%), reported until 1983. However, these incidences were lower than mean of the HCD recorded by NTP Carcinogenesis Program through 1990-1997 (mean: 25.7%) and within the overall historical range of NTP Carcinogenesis Program during both periods 1983 (range: 0-18%) and 1990-1997 (range: 0-50%), respectively.

Lesions diagnosed as hepatocellular adenomas consisted of solid nodules of well-differentiated hepatocytes and compressed adjacent hepatic parenchyma. Hepatocytes in these lesions were often larger, with cytoplasm that was more vacuolated and was often basophilic. On the other hand, hepatocellular carcinomas had a more disorderly arrangement of hepatocytes, usually with evidence of invasive growth into adjoining hepatic tissue. A key criterion for diagnosing hepatocellular carcinoma was the arrangement of hepatocytes into trabeculae. One male mouse in the low dose group had a liver tumor that had some areas characteristic of hepatocellular carcinoma, as well as areas consisting of a disorderly proliferation of structures resembling bile ducts. That tumor was classified as a mixed hepatocellular/cholangiocarcinoma.

Moreover, a slight increase incidence of follicular cell adenomas of the thyroid gland was observed in the high dose male mice. This incidence did not show statistically significant differences compared with controls, although a

statistically significant increased trend ($p < 0.05$) was noted (0%, 0% and 6% for control, low and high dose groups, respectively). The incidence of follicular cell adenomas in the high dose male group was higher than the mean incidence recorded by NTP Carcinogenesis program (1% and 1.5% for 1983 and 1990-1997 period, respectively), and slightly greater than the range (0-4%) of HCD recorded by the NTP Carcinogenesis Program during period 1990-1997.

No further differences were recorded in other neoplastic alterations between treated groups and controls.

Regarding non-neoplastic changes, increase incidences of focal inflammation of the kidney (16% vs 0% in controls), granulomatous inflammation (51% vs 36% in controls) and hyperplasia adenomatous (54% vs 44% in controls) of the lung were described in top dose males and females groups, respectively. Moreover, a slight increase of ovary follicular cysts was described in top dose female group (22%, 22% and 33% for control, low and high dose groups, respectively). This finding was not support by statistical analysis and historical control data were not provided, however, due to alterations were also noted in the uterus of rats, RMS deems that further data are needed to evaluate the potential endocrine disruption property of eugenol. No other relevant non-neoplastic lesions were detected in other organs or tissues in the low or high dose groups of both sexes.

NOAEL for carcinogenicity was considered to be **6000 ppm,** equivalent to 1250 mg/kg bw/day, based on no evidence of carcinogenicity. **NOAEL for toxicity** was considered to be **3000 ppm,** equivalent to 632 mg/kg bw/day, based on increase incidences of focal inflammation of the kidney in males, and focal granulomatous inflammation and adenomatous hyperplasia of lung, and ovary follicular cysts in females.

In the 18 month oncogenicity study in mice (B.6.5.3), four groups of 30 female CD-1 mice of 5 weeks of age were tested with vehicle (negative control), 0.5% eugenol (equivalent to 750 mg/kg bw/day), 0.5% eugenol + 0.05% phenobarbital, and 0.05% phenobarbital (positive control). Eugenol was administered for 12 months in the diet followed by 6-months recovery period, and phenobarbital was administered in the drinking-water for 18 months.

Survival at 18 months was comparable between eugenol-fed animals without phenobarbital and control animals. A reduction in survival (20%) at 18 months was detected between eugenol-fed animals with phenobarbital and control animals.

Bodyweight gain of treated mice measured at 1 month was comparable to those of controls. However, at 4 and 8 months, a decrease in bodyweight gain was detected in eugenol treated group (13% and 15%, respectively), and in eugenol plus phenobarbital treated group (20% and 23%, respectively) compared with controls.

Regarding neoplastic changes, and slight increase in thymic lymphoma (7%; 2/30 animals) and mammary adenocanthoma (3%; 1/30 animals) incidences, were recorded in eugenol treated group without phenobarbital. These findings did not display statistically significant results and were not reproduced in the further two-year study in mice, therefore, they were considered not relevant for overall long-term toxicity assessment.

A NOAEL cannot be established based on the increased incidences of thymic lymphomas and mammary gland adenocanthomas at the only dose level tested (750 mg/kg bw/day).

Therefore, based on the available data, the **overall carcinogenicity NOAEL** was 260/302 mg/kg bw/day for males/females (2-year study in rat).

2.6.5.2 Comparison with the CLP criteria regarding carcinogenicity

Comparison with criteria for Category 1A classification: In accordance with the criteria in the CLP regulation, classification for carcinogenicity Category 1A is reserved for substances known to have carcinogenic potential in humans. In the absence of human data for carcinogenicity, category 1A is not triggered.

Comparison with criteria for Category 1B classification: In accordance with the criteria in the CLP regulation, classification for carcinogenicity Category 1B is reserved for substances that are presumed to be carcinogenic in humans, and is largely based on data from animal studies where there is sufficient evidence to demonstrate animal carcinogenicity (presumed human carcinogen). This classification is not considered appropriate as no evidence of carcinogenicity can be derived from the assessed studies.

Comparison with criteria for Category 2 classification: In accordance with the criteria in the CLP regulation, classification for carcinogenicity Category 2 is reserved for substances where there is evidence obtained from human and/or animal studies but which is not sufficiently convincing to place the substance in Category 1. The available information does not also provide enough evidence to support a classification of eugenol in Category 2. The findings observed in the available studies could not be associated with the treatment with eugenol, mainly based on the absence of a dose-response, lack of statistical significance, and the high incidence of these tumors in overall historical controls.

Table 56: Compilation of factors to be taken into consideration in the hazard assessment

Tumour type and background incidence	**Rat:** *Lung* ♂: alveolar/bronchiolar adenomas/carcinomas (combined) in the low dose group. HCD NTP (1990-1997): ♂: mean: 3.3%; range: 0-14% ♀: mean: 1.9%; range: 0-10% NTP (1983): ♂: mean: 2.4%; range: - ♀: mean: 1.2%; range: - Southern Research Institute (1983) ♂: mean: 3%; range: - ♀: mean: -; range: - *Thyroid* ♀: Thyroid C-Cell adenoma in the low dose group. HCD NTP (1990-1997): ♂: mean: 13%; range: 2-35% ♀: mean: 11.7%; range: 4-22% NTP (1983): ♂: mean: 5.1%; range: - ♀: mean: 4.9%; range: - Southern Research Institute (1983) ♂: mean: -; range: - ♀: mean: -; range: - *Uterus* ♀: uterine endometrial stromal polyp or sarcoma at high dose tested. HCD NTP (1990-1997): ♀: mean: 14.7%; range: 0-30% NTP (1983): ♀: mean: 19.4%; range: - Southern Research Institute (1983) ♀: mean: 15%; range: - **Mouse:** *Thymus* ♀*:Thymic lymphoma (only one dose level tested).* HCD: Not available

	Mammary gland ♀*: mammary adenoacanthoma (only one dose level tested).* HCD: Not available *Liver* ♂: Hepatocellular adenoma in the low dose group. HCD NTP (1990-1997): ♂: mean: 29.4%; range: 4-60% ♀: mean: 17.3%; range: 2-50% NTP (1983): ♂: mean: 10%; range:0-22% ♀: mean: 4%; range:0-18% Southern Research Institute (1983) ♂: mean: 9%; range: - ♀: mean: 3%; range: - Hepatocellular carcinoma in the low dose group. HCD NTP (1990-1997): ♂: mean: 17.9%; range: 6-29% ♀: mean: 8.4%; range: 0-20% NTP (1983): ♂: mean: 21%; range:6-36% ♀: mean: 4%; range:0-15% Southern Research Institute (1983) ♂: mean: 23%; range: - ♀: mean: 4%; range: - ♀: Hepatocellular adenomas/ carcinomas (combined) in the high doe group. HCD NTP (1990-1997): ♂: mean: 47.3%; range: 4-60% ♀: mean: 25.7%; range: 0-50% NTP (1983): ♂: mean: 31%; range:0-58% ♀: mean: 8%; range:0-18% Southern Research Institute (1983) ♂: mean: 32%; range: - ♀: mean: 7%; range: - *Thyroid* ♂: Follicular cell adenoma in the high dose group. HCD NTP (1990-1997): ♂: mean: 1.5%; range:0-4% ♀: mean: 1.8%; range: 0-8% NTP (1983): ♂: mean: 1%; range:-

	♀: mean: 1.8%; range:- *See table 53 for more information regarding HCD.
Multi-site responses	Yes, the tumours appeared in thymus, mammary gland, liver, lung, thyroid and uterus.
Progression of lesions to malignancy	No, because although adenomas, and carcinomas have been observed, the most of them were adenomas, and the findings observed could not be associated with eugenol treatment, mainly based on low incidence rates, rather weak or absence dose-response relationship, lack of statistical significance and results being within the historical control range in most cases.
Reduced tumour latency	No, since the tumours were observed at study termination (105-106 month) (there were no interim sacrifice).
Whether response single or both sexes	Yes, liver adenomas and carcinomas were detected in both sexes in mice. Thyroid cell adenoma was observed in male mice and female rats, and alveolar/bronchiolar adenoma / carcinoma were detected mainly in male rats. Two thymic lymphomas and one mammary adenoacanthoma were found in female mice.
Routes of exposure	Only experimental studies by oral (dietary) route are available.
Possibility of a confounding effect of excessive toxicity at test doses	The available studies present several deficiencies, such as the testing of only one or two dose levels or the lack of important measurements (like haematology, clinical chemistry, urinalysis and organ weights), therefore, it does not allow a complete evaluation of long-term toxicity and carcinogenicity of eugenol.
Mode of action and its relevance for humans	Eugenol mode of action studies were not provided. The weight of the evidence is considered insufficient to justify a classification for carcinogenicity. Based on the comparison of the available carcinogenicity data with CLP classification criteria, eugenol need not be classified for carcinogenicity.

2.6.5.3 Conclusion on classification and labelling for carcinogenicity

Based on the data available, and according to the criteria under Regulation (EC) No 1272/2008, eugenol, does not require classification for carcinogenicity according to CLP Regulation.

2.6.6 Summary of reproductive toxicity [equivalent to section 10.10 of the CLH report template]

2.6.6.1 Adverse effects on sexual function and fertility – generational studies [equivalent to section 10.10.1 of the CLH report template]

No reproductive generational studies are available on the active substance eugenol. Below additional data is provided on isoeugenol for which a read across is not accepted by the RMS (see section 2.6.6.1.1).

Table 57: Summary table of animal studies on adverse effects on sexual function and fertility – generational studies

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL (for sexual function and fertility, parents) - target tissue/organ - critical effects at the LOAEL	Reference
Two-generation reproductive toxicity study in rats. GLP: Yes Method: None stated. Rat strain: Sprague-Dawley (CD® –Crl : (SD) BR) Sex: ♂ and ♀ No. animals: P: 20 rats/sex/dose. Deviations from current test guideline (OECD TG 416, 2001): -The length of P animals premating period was only one week, instead of 10 weeks (one complete spermatogenic cycle for males and several oestrus cycles for females). -Food consumption measurements were not performed weekly. -Food consumption measurements were not performed in the premating periods of F1 generation. -Pups bodyweights were not measured during lactation period. -No of implantations, corpora lutea and post-	Test substance: Isoeugenol Isoeugenol :Oral (gavage) Dose levels: ♂/♀:: 0, 70, 230, and 700 mg/kg bw/day Exposure: *Pre-mating treatment:* P: 7 days (1 weeks) F1: 9 weeks *Mating (P/F1):* 178 days (26 weeks; 9 weeks/litter) Three litters were obtained in F1 (F_{1a}, F_{1b} and F_{1c}) and F2 (F_{2a}, F_{2b} and F_{2c}) generations. Treatment continued in P and F_1 throughout gestation and lactation of each litter. Parameters observed *P/F_1:* Mortality, clinical signs, bw, and food consumption, organ weights, sperm analysis, oestrus cycles *F_1/F_2 offspring:* Anogenital distance, ratio AGD/pup wt., average	**PARENTAL TOXICITY (P)** Mortality: Mortality was observed in four males and ten females: ♂: -One 70 mg/kg bw/day male was killed moribund on starting day (SD) 138 because of severe clinical signs including dyspnoea, rales, few faeces, rough hair coat, and languid behaviour. -Three males (one of each dose group) were found dead on SD179, SD 10, and SD 154, respectively. Each animal appeared normal prior to death. ♀: -Two 230 mg/kg bw/day females were found dead, one on SD 114 and one on SD 133. One female appeared normal prior to death. The other female apparently died as a result of gavage error. - Eight 700 mg/kg/day females were found dead. Two deaths appear to be the result of gavage errors. Three deaths occurred due to parturition difficulties. Three females appeared normal prior to their deaths. Clinical signs: No relevant clinical signs were noted in the treated groups compared with controls. Only urine stains were increased in both sexes compared with controls (*males*: 20%, 10% and 25% for low, mid and high dose groups vs 5% in controls; *females*: 20%, 35% and 55% low, mid and high dose groups vs 15% in controls). **700 mg/kg bw/day** *Bodyweight* ▪ (↓) bw in ♂ throughout week 2-26 (10-18%). ▪ (↓) bw in ♀ throughout week 2-7, 9-14, and 17-26 (4-12%). ▪ (↓) abs terminal bw in ♂ (19%). ▪ (↓) abs terminal bw in ♀ (13%). *Food consumption* ▪ (↓) abs in ♂ at week 1 (27%) and 14 (12%). ▪ (↓) rel in ♂ at week 1 (25%) and (↑) at week 12 (9%, ndr). ▪ (↓) abs in ♀ at week 1 (20%). ▪ (↓) rel in ♀ at week 1 (20%). *Lactation* ▪ (↓) bw in ♀ at day 1 (13%), 4 (10%), 7 (7%) and 14 (7%). ▪ (↑) fc in ♀ through day 1-4 (23%, ndr). *Necropsy findings* *Organ weights* ▪ (↓) abs spleen wt in ♂ (26%, ndr). ▪ (↑) rel right epididymis wt in ♂ (14%). ▪ (↑) rel kidney wt in ♂ (14%). ▪ (↑) rel right testis wt in ♂ (19%). ▪ (↑) rel liver wt in ♀ (20%). ▪ (↑) rel kidney wt in ♀ (14%). *Histopathology (statistics not performed)* ▪ (↑) hyperkeratosis in non-glandular stomach in ♂/♀ (80/100%) vs 0% in controls. ▪ (↑) hyperplasia in non-glandular stomach in ♂/♀ (70/90%) vs 0% in controls. **Histopathological stomach findings in P animals**	NTP (2002) (CA) B.6.6.1.1

	Male				Female			
Group (mg/kg bw/day)	0	70	230	700	0	70	230	700
number examined	10	10	10	10	10	10	10	10

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL (for sexual function and fertility, parents) - target tissue/organ - critical effects at the LOAEL	Reference

Hyperkeratosis	0	4 (40%)	9 (90%)	8 (80%)	0	6 (60%)	7 (70%)	10 (100%)
Minimal	0	3 (30%)	1 (10%)	3 (30%)	0	3 (30%)	1 (10%)	4 (40%)
Mild	0	1 (10%)	6 (60%)	2 (20%)	0	3 (30%)	4 (40%)	0
M0derate	0	0	2 (20%)	3 (30%)	0	0	2 (20%)	6 (60%)
Hyperplasia	0	3 (30%)	9 (90%)	7 (70%)	0	5 (50%)	7 (70%)	9 (90%)
Minimal	0	2 (20%)	8 (80%)	2 (20%)	0	5 (50%)	5 (50%)	3 (30%)
Mild	0	1 (10%)	1 (10%)	3 (30%)	0	0	2 (20%)	5 (50%)
Moderate	0	0	0	2 (20%)	0	0	0	1 (10%)

implantation loss were not analysed.

-Brain, thyroid, pituitary and adrenal glands weights were not measured.

-Statistical analysis not performed in histopathological findings.

Study acceptable as supportive information.

dam weight, average sire weight, average days to litter.

Reproductive: Pregnancy index, litters per pair, live pups per litter, proportion of pups born alive, sex of pups born alive, live pup weight.

230 mg/kg bw/day

Bodyweight
- (↓) bw in ♂ throughout week 2-26 (3-8%).
- (↓) bw in ♀ throughout week 11, 12, and 20-26 (6-21%).
- (↓) abs terminal bw in ♀ (10%).

Food consumption
- (↓) abs in ♀ at week 1 (8%).
- (↓) rel in ♀ at week 1 (8%).

Necropsy findings
Organ weights
- (↓) rel ovary wt in ♀ (29%, ndr).
- (↑) rel liver wt in ♀ (14%).

Histopathology (statistics not performed)
- (↑) hyperkeratosis in non-glandular stomach in ♂/♀ (90/70%) vs 0% in controls.
- (↑) hyperplasia in non-glandular stomach in ♂/♀ (90/70%) vs 0% in controls.

70 mg/kg bw/day

Bodyweight
- (↓) bw in ♀ throughout week 21-26 (9-20%).
- (↓) abs terminal bw in ♀ (11%).

Necropsy findings
Organ weights
- (↑) rel liver wt in ♀ (10%).
- (↑) rel kidney wt in ♀ (10%).

Histopathology (statistics not performed)
- (↑) hyperkeratosis in non-glandular stomach in ♂/♀ (40/60%) vs 0% in controls.
- (↑) hyperplasia in non-glandular stomach in ♂/♀ (30/50%) vs 0% in controls.

REPRODUCTIVE PARAMETERS (P→F1)

700 mg/kg bw/day
- (↓) live male pups per litters (21%) in all F1 litters.
- (↓) live combined male and female pups per litter (15%) in all F1 litters.

OFFSPRING TOXICITY (F_1)

700 mg/kg bw/day

- (↑) ratio anogenital distance (AGD)/pup wt in ♀ in the F1a litter (10%, ndr).
- (↓) AGD in ♀ in the F1c litter (8%, ndr).

PARENTAL TOXICITY (F_1)

Mortality: Mortality was observed in six F1c adult males and five adult females:

♂:
- One 70 mg/kg/day male was killed moribund on PND 171 because of severe clinical signs including rough hair coat, thinness, hunched posture, diarrhoea, few faeces, red discharge from the mouth, and anorexia.
- One 230 mg/kg/day male was found dead on PND 50. Each animal appeared normal prior to its death.
- One 230 mg/kg/day male was killed moribund on PND 193 because of severe clinical signs including rales, dyspnoea, red discharge from the mouth, and hypoactivity. This death was a result of a gavage error.
- Two 700 mg/kg/day males were found dead on PND 66. Each animal appeared normal prior to its death.
- One 700 mg/kg/day male was found dead on PND 171. The animal appeared normal prior to its death.

♀:
- One 230 mg/kg/day female was found dead on PND 107. The death was a result of a gavage error.
- One 230 mg/kg/day female was found dead on PND 124. The animal appeared normal prior to its death.
- One 700 mg/kg1day female died ten minutes after dosing on PND 74. The time of death suggests a possible aspiration of compound as the cause of death.
-Two 700 mg/kg/day females were found dead on PND 109 and PND 153. The first animal died immediately after dosing of possible aspiration of compound. The second animal appeared normal prior to its death

Clinical signs: No relevant clinical signs were noted in the treated groups compared with controls. Urine stains were increased in both sexes compared with controls (*males*: 10%, 25% and 55% for low, mid and high dose groups vs 5% in controls; *females*: 15%, 25% and 45% low, mid and high dose groups vs 0% in controls). Abrasion was increased in low and mid dose male groups (40% and 20% respectively, vs 10% in controls), and alopecia was increased in low dose male group (45% vs 20% in controls).

700 mg/kg bw/day

Premating

Bodyweight
- (↓) bw in ♂ throughout PND 51 - 79 ± 10 (7-10%).

Mating

Bodyweight
- (↓) bw in ♂ throughout week 1-15 (12-22%).
- (↓) bw in ♀ throughout week 2-15 (9-14%).
- (↓) abs terminal bw in ♂ (22%).
- (↓) abs terminal bw in ♀ (12%).

Food consumption
- (↓) abs in ♂ at week 12 (15%) and 14 (23%).
- (↓) abs in ♂/♀ at week 3 (8%) and 6 (8%).
- (↑) rel in ♂/♀ at week 6 (9%, ncdr).

Necropsy findings
Organ weights

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL (for sexual function and fertility, parents) - target tissue/organ - critical effects at the LOAEL	Reference

- (↑) rel liver wt in ♂ (19%).
- (↑) rel cauda epididymis wt in ♂ (24%).
- (↑) rel right epididymis wt in ♂ (20%).
- (↑) rel kidney wt in ♂ (20%).
- (↑) rel spleen wt in ♂ (17%).
- (↑) rel right testis wt in ♂ (21%).
- (↑) rel liver wt in ♀ (24%).
- (↑) rel ovaries wt in ♀ (18%).
- (↑) rel kidney wt in ♀ (15%).

Histopathology (statistics not performed)
- (↑) hyperkeratosis in non-glandular stomach in ♂/♀ (100/100%) vs 0% in controls.
- (↑) hyperplasia in non-glandular stomach in ♂/♀ (90/100%) vs 0% in controls.

Histopathological stomach findings in F1 parental animals

	Male				Female			
Group (mg/kg bw/day)	**0**	**70**	**230**	**700**	**0**	**70**	**230**	**700**
number examined	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Hyperkeratosis	0	9 (90%)	10 (100%)	10 (100%)	0	6 (60%)	9 (90%)	10 (100%)
Minimal	0	4 (40%)	2 (20%)	0	0	2 (20%)	2 (20%)	0
Mild	0	4 (40%)	3 (30%)	1 (10%)	0	4 (40%)	4 (40%)	2 (20%)
Moderate	0	1 (10%)	5 (50%)	9 (90%)	0	0	3 (30%)	8 (80%)
Hyperplasia	0	9 (90%)	9 (90%)	9 (90%)	0	5 (50%)	9 (90%)	10 (100%)
Minimal	0	5 (50%)	4 (40%)	2 (20%)	0	5 (50%)	4 (40%)	1 (10%)
Mild	0	4 (40%)	5 (50%)	5 (50%)	0	0	4 (40%)	1 (10%)
Moderate	0	0	0	2 (20%)	0	0	1 (10%)	8 (80%)

230 mg/kg bw/day

Premating

Bodyweight
- (↓) bw in ♂ at PND 79 ± 10 (5%).

Mating

Bodyweight
- (↓) bw in ♂ throughout week 2-15 (6-12%).
- (↓) abs terminal bw in ♂ (9%).

Food consumption
- (↓) abs in ♂ at week 12 (14%) and 14 (15%).
- (↓) abs in ♂/♀ at week 3 (8%).

Necropsy findings
Organ weights
- (↑) rel liver wt in ♀ (6%).

Histopathology (statistics not performed)
- (↑) hyperkeratosis in non-glandular stomach in ♂/♀ (100/90%) vs 0% in controls.
- (↑) hyperplasia in non-glandular stomach in ♂/♀ (90/90%) vs 0% in controls.

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL (for sexual function and fertility, parents) - target tissue/organ - critical effects at the LOAEL	Reference

70 mg/kg bw/day

Premating

Bodyweight
- (↑) bw in ♂ at PND 30-44 ± 10 (10-12%, ndr).
- (↑) bw in ♀ at PND 37± 10 (9%, ndr).

Mating
Food consumption
- (↓) abs in ♂ at week 12 (12%).
- (↑) rel in ♀ at week 12 (16%, ndr).

Histopathology (statistics not performed)
- (↑) hyperkeratosis in non-glandular stomach in ♂/♀ (90/60%) vs 0% in controls.
- (↑) hyperplasia in non-glandular stomach in ♂/♀ (90/50%) vs 0% in controls.

REPRODUCTIVE PARAMETERS (F1→F2)

700 mg/kg bw/day
- (↑) number of females with regular cycles (29%, ncdr).
- (↑) pregnancy index (100%, ndr) during F2b litter vs 80% in controls.

230 mg/kg bw/day
- (↑) pregnancy index (83%, ndr) during F2c litter vs 45% in controls.

70 mg/kg bw/day
- (↑) pregnancy index (100%, ndr) during F2b litter vs 80% in controls.

SUMMARY OF PREGNANCY INDEX OF BREEDING PAIRS					
Litter	**0 mg/kg bw/day**	**70 mg/kg bw/day**	**230 mg/kg bw/day**	**700 mg/kg bw/day**	**p value**
F2a	20/20 (100)	19/20 (95)	18/20 (90)	20/20 (100)	P=0 487
F2b	16/20 (80)	***20/20 (100)**	17/19 (89)	***19/19 (100)**	p=0 114
F2c	9/20 (45)	14/20 (70)	***15/18 (83)**	14/19 (74)	p=0 129

OFFSPRING TOXICITY (F2)

700 mg/kg bw/day
- (↓) live pup weight in ♂ (6%) in all F2 litters.
- (↓) live pup weight in ♀ (4%) in all F2 litters.
- (↓) live pup weight in combined ♂/♀ (5%) in all F2 litters.
- (↑) ratio anogenital distance (AGD)/pup wt in ♀ in the F2a litter (12%, ncdr).

NOAEL offspring toxicity**:** 230 mg/kg bw/day based on decreased male and females pup weights in F2 generation.
LOAEL offspring toxicity**:** 700 mg/kg bw/day
Critical effect at the LOAEL: Decrease male and females pup weight

NOAEL reproductive performance**:** 230 mg/kg bw/day based on decreased number of male pups per litter in F1 generation**.**
LOAEL reproductive performance**:** 700 mg/kg bw/day
Critical effect at the LOAEL: Decreased number of male pups per litter

NOAEL parental toxicity: -

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL (for sexual function and fertility, parents) - target tissue/organ - critical effects at the LOAEL	Reference
		LOAEL parental toxicity**:** 70 mg/kg bw/day **Critical effect at the LOAEL:** Hyperkeratosis and hyperplasia in the non-glandular stomachs.	

Table 58: Summary table of human data on adverse effects on sexual function and fertility

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No data available				

Table 59: Summary table of other studies relevant for toxicity on sexual function and fertility

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
Anti-fertility effects of eugenol on rats. No guideline. <u>Rodent strain:</u> Wistar albino female rats. **Supportive only**.	Test substance: **Eugenol**	-Wistar albino female rats: 6 rats/group -Intramuscular injection <u>-Dose levels</u>: 0.4 ml/day for 15 days	<u>Oestrous cycle length</u> Proestrus: ↑29% Metestrus: ↑22% Diestrus: ↑16% Total duration: ↑17% <u>Sex hormones serum levels:</u> Testosterone: ↓78% Estradiol: ↑87% Progesterone: ↑48%	Poli., *et al.* (2019) (AS) B.6.8.3.6

2.6.6.1.1 Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility – generational studies

No reproductive generational studies were provided to support the renewal process of the active substance eugenol. However, a multi-generational study performed with isoeugenol was available in the public US EPA Health & Environmental Research Online (HERO)- National Technical Reports Library database. Study ID: 5923934. https://hero.epa.gov/hero/index.cfm/reference/details/reference_id/5923934. This study has been included by the applicant in the renewal dossier of the active substance clove oil, in order to be used in a read-across approach to eugenol (which is the main component of clove oil). Consequently, this study was included by RMS in order to completeness as for the renewal process of the active substance eugenol in case the read across between eugenol and isoeugenol is accepted. The RMS did not find enough justification fo the read across according to the following:

Isoeugenol and eugenol are isomeric compounds in which the structural difference relies on the position of the double bond in the propenyl substituent. The physicochemical properties, such as water solubility, of these compounds differ slightly. The Log P values, however, are within the same range. The metabolic pathway of both compounds may differ based on the susceptibility of the terminal double bond of eugenol to form oxidation products

as observed in the metabolism studies described in section 6.1. The percentage of allylic oxidation metabolites in humans detected in urine 3h after oral administration of eugenol accounts for 9 %, which indicates this metabolic pathway is relevant in eugenol. Furthermore, isoeugenol is not a relevant metabolite of eugenol (quantified at ~ 7% accounting for cis and trans isomerisation metabolites), and it has not been identified as a component in clove oil. Despite the lack of metabolism studies performed with isoeugenol, based on poor water solubility reported for this compound and the absence of a reactive terminal double bond, its ADME properties are expected to differ from eugenol. Overall, the RMS is of the opinion that this study could not be used as supporting substance for eugenol, and it is not the most approximate approach to address the sexual function and fertility of the active substance eugenol.

On the other hand, one study presented in endocrine disruption section (B.6.8.3.6) assessed the anti-fertility effects of eugenol in Wistar Albino rats, and therefore, it has been summarized and reported in this section.

-In the two-generation reproductive study in rats (B.6.6.1), isoeugenol was tested at 0, 70, 230 and 700 mg/kg bw/day dose levels for each sex, in which 20 pairs of males and females were mated from each test group.

The rationale for selection of dose levels arises from two previous range-finding studies. The first range-finding study consisted of a control group and four treated groups (8 pairs/group) in which isoeugenol was administered via feed at doses level of 0 (Control), 500, 3000, 5500, and 8000 ppm. The study consisted in a premating phase (1 week) and mating phase (6 weeks). Male and female bodyweights, feed consumption, water consumption, and reproductive data were comparable to the controls.

The second range-finding study used a control group and four treated groups (8 animals/sex/group), in which isoeugenol was tested at 0, 30, 100, 400, and 800 mg/kg bw/day dose levels via oral gavage during two weeks. Female body weights, feed consumption, and water consumption were comparable to controls. However, male bodyweights were decreased through weeks 2-3 (13%) in the high dose group. Moreover, male food consumption was decreased 12% and 31% at 400 and 800 mg/kg bw/day dose groups, respectively. Based on these data, gavage doses of 70, 230, and 700 mg/kg bw/day were selected for two-generation study.

In the main study, neither mortality, nor clinical signs related to treatment were observed in any dose group. Few sporadic animals were found dead, mostly due to gavage errors.

Bodyweights were statistically significant decreased in the mid (3-8%) and high (10-18%) male dose groups throughout weeks 2-26. In female dose groups, a statistically significant decrease was observed in the low dose group (9-20%) throughout weeks 21-26. In the mid dose female group, a significant decrease (6-21%) was recorded during weeks 11, 12, and 20-26, whereas in the high dose group, a significant decrease (4-12%) was observed throughout weeks 2-7, 9-14, and 17-26, respectively.

Absolute and relative food consumption was statistically significantly lower in high dose male group at week 1 (27% and 25%, respectively) and 14 (12%), however, an increase in relative food consumption was observed at week 12 (9%). On the other hand, both absolute and relative food consumption was decreased at week 1 in mid and high female dose groups (8% and 20%, respectively).

Regarding carcass and organ weights, statistically significant decrease in terminal bodyweight was noted in high dose parental (P) male group (19%). In addition, increases in relative organ weights of right epididymis (14%), right testis (19%), and kidneys (14%), were recorded in the high P male dose group**.** Histopathological examinations of these tissues did not reveal any morphological and cellular alteration.

In the other hand, female terminal bodyweights were decreased in all P dose groups (11%, 10%, and 13% for the low, mid and high dose groups, respectively). A decrease of absolute ovary weight (29%) was seen in the mid dose group. The relative liver weight was also increased (10%, 14%, and 20% in the 70, 230, and 700 mg/kg/day females, respectively), together with the relative kidney weight in the low (10%) and high (14%) dose female groups.

Incidental gross findings non-treatment related were described for both sexes in all dose groups.

Histopathological analysis of the P animals revealed dose-related changes in the non-glandular stomachs. These observations were described in all the dose level of both sexes, and their incidence and/or severity were related to dose. These alterations consisted of hyperkeratosis and hyperplasia of the squamous epithelium (acanthosis) and were characterized by an increased thickness of the non-keratinized (hyperplasia) and keratinized (hypkeratosis) layers of the epithelium. Hyperkeratosis in non-glandular stomach was observed in 40%, 90% and 80% of low, mid and high dose male groups, whereas 60%, 70% and 100% was observed in low, mid and high dose female groups, respectively, compared with controls (0%). Hyperplasia in non-glandular stomach was observed in 30%, 90% and

70% of low, mid and high dose male groups, whereas 50%, 70% and 90% was observed in low, mid and high dose female groups, respectively, compared with controls (0%). All other tissue microscopic changes were commonly detected in laboratory rats and were unrelated to treatment.

Reproductive parameters of P animals used Hamilton Thorne Integrated Visual Optic System for computer-assisted sperm motion analysis. The results revealed no changes in mean path velocity, progressive velocity, track speed, lateral amplitude, beat frequency, straightness, linearity, motile percentage, and progressive percentage of the treated groups as compared to the control. Also, no changes were seen in epididymal sperm density, percent abnormal sperm, number of spermatids per cauda epididymis, and the total number of spermatids per testis.

Vaginal cytology was performed on P dams for 14 days after weaning of the F1c litters. No changes were revealed in the number of females with regular cycles, cycle length, number of cycles, and in number of cycling females across the dose groups as compared to the control females.

Regarding reproductive parameters of offspring, statistically significant decrease in the number of live male pups (21%) were observed in all F1 litters (F1a, F1b and F1c) obtained from the high dose group. Considering a combination of both sexes, a decrease in the number of male and female live pups, was also noted in the whole high dose F1 litters. On the other hand, sporadic events not related to dose were observed in the high dose group such as an increase in the anogenital distance (AGD) to bodyweight ratio in the Fla litter (10%), or a decrease in absolute female anogenital distance in the F1c litter (8%). Importantly, dam bodyweights experiment a decrease (7-13%) during lactation of the F1c litter (PND 1, 4, 7, 14) and at delivery of all F1 litters (8-13%). In addition, increase in relative food consumption was recorded in high dose lactating dams during the F1c litter throughout PND 1-4 (23%).

Regarding F1 parental animals, neither mortality, nor clinical signs related to treatment were observed in any dose group. Few sporadic animals were found dead during study.

During the premating phase, increased bodyweights were recorded in low dose male group during the whole adolescent growing phase (PND 30±10 - PND 79±10), although data only were statistically significant throughout PND30-44 ± 10 (10-12%). However, a statistically significant decrease was noted in high dose male group throughout PND 51 - 79 ± 10 (7-10%), and in mid dose male group on PND 79 ± 10 (5%). On the other hand, no important differences were observed in mean female bodyweights during the adolescent growing phase compared with controls.

Sexual developmental parameters as testicular descent, preputial separation, and vaginal opening were comparable between dose groups and controls.

During the F1 mating phase, statistically significant decrease in bodyweight were detected in high dose male group (12-22%) throughout weeks 1-15, and in mid dose male group throughout weeks 2-15 (6-12%). On the other hand, a statistically significant decrease in bodyweight was detected in high dose female group (9-14%) during throughout weeks 2-15 (except week 11).

Statistically significant decrease in absolute food consumption was recorded in low (12%), mid (14%) and high (15%) dose male groups in week 12, whereas a decrease of 15% and 23% was noted through week 14 in mid and high dose male groups, respectively. On the other hand, a statistically significant increase in relative food consumption was observed in low dose female group at week 12 (16%). Take into account both sexes, a statistically significant decrease of 8% was described in absolute food consumption in mid and high dose groups during weeks 3 and 6, whereas an increase of 9% was described in relative food consumption in high dose groups at week 6.

Regarding carcass and organ weights, statistically significant decrease in terminal bodyweight was noted in the mid and high dose male groups (9% and 22% respectively). Besides, statistically significant increases in relative male organs weight were recorded in the high dose group for the following organs: liver (19%), cauda epididymis (24%), right epididymis (20%), kidneys (20%), spleen (17%) and right testis (21%). On the other hand, in the high female dose group, a statistically significant decrease in terminal bodyweight was recorded (12%), together with an increase in relative liver (24%), ovaries (18%) and kidneys (15%) weight. Moreover, an increase in relative liver weight (6%) was detected in female mid dose group. As occurred in P animals, histopathological examinations of these tissues did not revealed any morphological and cellular alteration.

Necropsy examination of F1 parental animals revealed incidental gross findings in male dose groups not associated to treatment.

Treatment-related changes were found in the non-glandular stomach and were comparable to changes observed in the P animals. These changes were found in all treated groups and their incidence and/or severity were dose related.

Hyperkeratosis in non-glandular stomach was observed in 90%, 100% and 100% of low, mid and high dose male groups, whereas 60%, 90% and 100% was observed in low, mid and high dose female groups, respectively, compared with controls (0%). Hyperplasia in non-glandular stomach was observed in 90%, 90% and 90% of low, mid and high dose male groups, whereas 50%, 90% and 100% was observed in low, mid and high dose female groups, respectively, compared with controls (0%). All other microscopic changes were those commonly found in laboratory rats and were unrelated to treatment.

Reproductive parameters of F1 parental animals such as sperm analysis and oestrous cycle of males and females treated groups were comparable to controls. Only, there was an increase in the number of females with regular cycles in the high dose female group (29%) compared to controls.

Regarding reproductive parameters of F2 parental animals, pregnancy index were increased for F2b generation (100%) in low and high dose groups, compared to control group (80%), and for F2c generation in mid dose group (83%), compared to control group (45%). During the F1 cohabitation, there was an unexplained decrease in pregnancy index in control animals. Twenty pairs were pregnant for the F2a litters, whereas in F2b and F2c litters, the pregnancy index decreased to 16/20 (80%), and to 9/20 (45%), respectively. Based on historical results in this laboratory, the pregnancy index would be expected to be between 15/20 and 20/20. This decrease is unexplained since there were no adverse effects noted in oestrous cyclicity or sperm endpoints.

In addition, decrease on the whole average live male, female and combined pup weights were described in F2 litters (6%, 4% and 5%, respectively) at high dose tested. Importantly, and also in the high dose group, dam bodyweights experiment a decrease at delivery of all F2 litters (13-16%).

Therefore, a **NOAEL** for **offspring toxicity** has been established at 230 mg/kg bw/day based on decreased male and females pup weights in F2 generation.

NOAEL for **reproductive performance** has been established at 230 mg/kg bw/day based on decreased number of male pups per litter in F1 generation.

NOAEL for **parental toxicity** could not be established due to parental toxicity has been observed in the low dose level (hyperkeratosis and hyperplasia in the non-glandular stomachs in both sexes at low dose level in P and F1 parental animals).

-In the supplementary study conducted by Poli *et al.* (B.6.8.3.6), Wistar Albino rats received eugenol via intramuscular injection at only one dosage level of 0.4 ml/day during 15 days. The authors evaluated the length of oestrus cycle according to the presence of cell types in the vaginal smear, and measured the serum levels of sexual hormones.

Eugenol enhanced the duration of proestrus, metestrus, diestrus phases, and total duration of cycles (29%, 22%, 16% and 17%, respectively). On the other hand, eugenol had no effect on hormones such as FSH, LH, and PRL. However, serum estradiol and progesterone levels were statistically significant increased (87% and 48%) compared with control, whereas testosterone levels were reduced (78%). These effects could be compatible with the potential anti-estrogenic role of eugenol observed in the "*in vitro*" assays (see *section 2.10* for further discussion).

2.6.6.1.2 Comparison with the CLP criteria regarding adverse effects on sexual function and fertility

No information in humans is available on the effects of eugenol on the reproductive system; and neither reproductive toxicity studies conducted in animals were provided. Consequently data lacking is proposed for sexual function and fertility for eugenol.

2.6.6.2 Adverse effects on development [equivalent to section 10.10.4 of the CLH report template]

Developmental toxicity studies are available both on the active substance eugenol and clove oil (minimal concentration, 80% w/w)

Table 60: Summary table of animal studies on adverse effects on development

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL (for parent, offspring and for developmental effects) - target tissue/organ - critical effects at the LOAEL	Reference
Teratology study in rats with clove oil. GLP: No Method: Non-stated. Rat strain: Wistar-rats Sex: 23-25 mated females/group. Deviations from current test guideline (OECD TG 414, 2018): -Eugenol technical was not tested. -Food consumption was not measured. -The following developmental endpoints were not measured: anogenital distance, and indication of incomplete testicular descent/cryptorchidism. -The degree of resorption should be described (early, late). -Thyroid weight and thyroid hormones (T3/T4/TSH) values from dams were not recorded. -Statistical analysis was not performed. -Dam necropsies were not reported. -Individual foetal data was not reported. - Historical control data were not provided. **Study acceptable as supportive information**	Test substance: **Clove oil** (eugenol 80% w/w Oral (gavage) Dose levels: ♀: 0, 2.8, 13, 60 and 280 mg/kg bw/day throughout day 6-15 of gestation. Parameters observed : *Maternal data:* Clinical signs, mortality and bw. *Reproductive data:* Number (no.) of pregnancy dams, no. implants and no. resorptions. *Foetal data:* Live and dead foetuses, foetus wt, foetus sex and foetus alterations (visceral and skeletal).	***Maternal toxicity (Statistical analysis was not performed)*** Survival: All pregnant dams survived at study termination. Clinical signs: No clinical signs were reported in the study. Bodyweights: No differences in bodyweights were observed between clove oil-treated groups and controls. ***Developmental toxicity (Statistical analysis was not performed)*** ***Reproductive data*** **280 mg/kg bw/day** ▪ ↓ implantations per litter (11%; ndr). ▪ ↓ live foetus per litter (12%; ndr). ▪ ↓ sex ratio (♂/♀) (33%, ndr). **60 mg/kg bw/day** ▪ ↑ resorptions per litter (79%, ndr). ***Foetal data*** **280 mg/kg bw/day** *Skeletal alterations:* ▪ ↑ ossification incidences in ribs (mostly wavy) (100%, ncdr) ▪ ↑ incomplete ossification incidences of vertebrae (50%, ndr). ▪ ↑ incomplete closure of skull (100%). **60 mg/kg bw/day** *Skeletal alterations:* ▪ ↑ incomplete closure of skull (82%). **13 mg/kg bw/day** *Skeletal alterations:* ▪ ↑ incomplete closure of skull (36%). *see annex B.6.6.2.1 for more information. **NOAEL** developmental toxicity: - **NOAEL** maternal toxicity: -	Morgareidge K., *et al.* (1973a) (AS) B.6.6.2.1
Teratology study in rabbits with clove oil. GLP: No Method: Non-stated. Rabbit strain: Dutch-belted rabbits Sex: 11-15 mated	Test substance: **Clove oil** (eugenol 80% w/w) Oral (gavage) Dose levels:	***Maternal toxicity (Statistical analysis was not performed)*** Survival:One pregnant dam from low clove oil dose group, and another from positive control-group aborted without evidence of adverse clinical signs. Clinical signs: No clinical signs were reported in the study. **37.1 mg/kg bw/day** Bodyweight.	Morgareidge K., *et al.* (1973b) (AS) B.6.6.2.2

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL (for parent, offspring and for developmental effects) - target tissue/organ - critical effects at the LOAEL	Reference
females/group. Deviations from current test guideline (OECD TG 414, 2018): - Eugenol technical was not tested. -Food consumption was not measured. -The following developmental endpoints were not measured: anogenital distance, and indication of incomplete testicular descent/cryptorchidism. -The degree of resorption should be described (early, late). -Thyroid weight and thyroid hormones (T3/T4/TSH) values from dams were not recorded. -Statistical analysis was not performed. -Dam necropsies were not reported. -Individual foetal data was not reported. - Historical control data were not provided. **Study acceptable as supportive information**	♀: 0, 1.72, 7.99. 37.1, and 172 mg/kg bw/day throughout day 6-18 of gestation. Parameters observed : *Maternal data:* Clinical signs, mortality and bw. *Reproductive data:* Number (no.) of pregnancy dams, no. corpora lutea. no. implants and no. resorptions. *Foetal data:* Live and dead foetuses, foetus wt, foetus sex and foetus alterations (visceral and skeletal).	▪ ↑ bwg throughout gestation day 0-29 (121-210%; ndr). **7.99 mg/kg bw/day** Bodyweight. ▪ ↓ bwg throughout gestation day 0-29 (8-90%; ndr). **1.72 mg/kg bw/day** Bodyweight. ▪ ↑ bwg throughout gestation day 0-29 (4-57%; ndr). ***Developmental toxicity (Statistical analysis was not performed)*** ***Reproductive data*** **172 mg/kg bw/day** ▪ ↓ resorptions per litter (27%; ndr). ▪ ↑ sex ratio (♂/♀) (26%, ndr). **37.1 mg/kg bw/day** ▪ ↓ resorptions per litter (65%; ndr). ▪ ↓ sex ratio (♂/♀) (25%, ndr). **7.99 mg/kg bw/day** ▪ ↑ resorptions per litter (55%; ndr). ▪ ↓ sex ratio (♂/♀) (21%, ndr). **1.72 mg/kg bw/day** ▪ ↓ resorptions per litter (27%; ndr). ▪ ↓ sex ratio (♂/♀) (45%, ndr). ***Foetal data*** **172 mg/kg bw/day** *Skeletal alterations:* ▪ ↑ ossification incidences in sternebrae (mostly incomplete ossification, bipartite, and extra) (560%, ncdr). **37.1 mg/kg bw/day** *Skeletal alterations:* ▪ ↑ ossification incidences in sternebrae (mostly incomplete ossification, bipartite, fused and extra) (560%, ncdr). **7.99 mg/kg bw/day** *Skeletal alterations:* ▪ ↑ ossification incidences in sternebrae (mostly incomplete ossification and bipartite) (450%, ncdr). **1.72 mg/kg bw/day** *Skeletal alterations:* ▪ ↑ ossification incidences in sternebrae (mostly incomplete ossification, fused and extra) (980%, ncdr). *see annex B.6.6.2.2 for more information. **NOAEL** developmental toxicity: - **NOAEL** maternal toxicity: -	

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL (for parent, offspring and for developmental effects) - target tissue/organ - critical effects at the LOAEL	Reference
Teratology study in mice with clove oil. GLP: No Method: Non-stated. Mice strain: Albino CD-1 Sex: 23-25 mated females/group. Deviations from current test guideline (OECD TG 414, 2018): - Eugenol technical was not tested. -Food consumption was not measured. -The following developmental endpoints were not measured: anogenital distance, and indication of incomplete testicular descent/cryptorchidism. -The degree of resorption should be described (early, late). -Thyroid weight and thyroid hormones (T3/T4/TSH) values from dams were not recorded. -Statistical analysis was not performed. -Dam necropsies were not reported. -Individual foetal data was not reported. - Historical control data were not provided. **Study acceptable as supportive information**	**Test substance:** **Clove oil** (eugenol 80% w/w) Oral (gavage) Dose levels: ♀: 0, 2.2, 10, 46 and 215 mg/kg bw/day throughout day 6-15 of gestation. Parameters observed : *Maternal data:* Clinical signs, mortality and bw. *Reproductive data:* Number (no.) of pregnancy dams, no. implants and no. resorptions. *Foetal data:* Live and dead foetuses, foetus wt, foetus sex and foetus alterations (visceral and skeletal).	***Maternal toxicity (Statistical analysis was not performed)*** Survival: All pregnant dams survived at study termination. Clinical signs: No clinical signs were reported in the study. **215 mg/kg bw/day** Bodyweights: ▪ ↓ bwg at gestation day 17 (10%) , 11 (13%) and 6 (21%) (ndr). **46 mg/kg bw/day** Bodyweights: ▪ ↓ bwg at gestation day 17 (13%) , 15 (14%), 11 (19%) and 6 (26%) (ndr). ***Developmental toxicity (Statistical analysis was not performed)*** ***Reproductive data*** **215 mg/kg bw/day** ▪ ↑ number of dead foetuses per litter (211%; 3 dead foetuses vs 1 dead foetus in controls, ncdr). **46 mg/kg bw/day** ▪ ↑ number of dead foetuses per litter (84%; 2 dead foetuses vs 1 dead foetus in controls, ncdr). **10 mg/kg bw/day** ▪ ↑ number of dead foetuses per litter (93%; 2 dead foetuses vs 1 dead foetus in controls, ncdr). ***Foetal data*** No treatment-related skeletal or visceral alterations were described. *see annex B.6.6.2.3 for more information. **NOAEL developmental toxicity: -** **NOAEL maternal toxicity: -**	Morgareidge K., *et al.* (1973c) (AS) B.6.6.2.3
Teratology study in hamsters with clove oil. GLP: No Method: Non-stated. Hamster strain: Golden hamsters Sex: 25-28 mated	Test substance: **Clove oil** (eugenol 80% w/w) Oral (gavage) Dose levels:	***Maternal toxicity (Statistical analysis was not performed)*** Survival: All pregnant dams survived at study termination. Clinical signs: No clinical signs were reported in the study. **38.2 mg/kg bw/day** Bodyweights No relevant differences in bodyweights were observed between clove oil-treated groups and negative controls.	Morgareidge K., *et al.* (1973d) (AS) B.6.6.2.4

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL (for parent, offspring and for developmental effects) - target tissue/organ - critical effects at the LOAEL	Reference
females/group. Deviations from current test guideline (OECD TG 414, 2018): -Eugenol technical was not tested. -Food consumption was not measured. -The following developmental endpoints were not measured: anogenital distance, and indication of incomplete testicular descent/cryptorchidism. -The degree of resorption should be described (early, late). -Thyroid weight and thyroid hormones (T3/T4/TSH) values from dams were not recorded. -Statistical analysis was not performed. -Dam necropsies were not reported. -Individual foetal data was not reported. - Historical control data were not provided. **Study acceptable as supportive information**	♀: 0, 1.8, 8.2, 38.2 and 177 mg/kg bw/day throughout day 6-15 of gestation. Parameters observed : *Maternal data:* Clinical signs, mortality and bw. *Reproductive data:* Number (no.) of pregnancy dams, no. implants and no. resorptions. *Foetal data:* Live and dead foetuses, foetus wt, foetus sex and foetus alterations (visceral and skeletal).	***Developmental toxicity (Statistical analysis was not performed)*** ***Reproductive data*** **177 mg/kg bw/day** ▪ ↓ resorptions per litter (30%; ndr). **38.2 mg/kg bw/day** ▪ ↑ resorptions per litter (250%; ndr). ▪ ↑ number of dead foetuses per litter (212%; 3 death foetuses vs 1 death foetus in controls, ndr). **8.2 mg/kg bw/day** ▪ ↑ resorptions per litter (115%; ndr). ▪ ↑ number of dead foetuses per litter (588%; 7 death foetuses vs 1 death foetus in controls, ndr). ***Foetal data*** **177 mg/kg bw/day** *Skeletal alterations:* ▪ ↑ ossification incidences in the sternebrae (31%, ncdr). ▪ ↑ incomplete ossification in the extremities (80%, ndr). ▪ ↑ hyoid missing or reduced (48%, ndr). **38.2 mg/kg bw/day** *Skeletal alterations:* ▪ ↑ ossification incidences in the sternebrae (40%, ncdr). ▪ ↓ incomplete ossification in the extremities (28%, ndr). ▪ ↓ hyoid missing or reduced (56%, ndr). **8.2 mg/kg bw/day** *Skeletal alterations:* ▪ ↑ ossification incidences in the sternebrae (27%, ncdr). ▪ ↓ incomplete ossification in the extremities (31%, ndr). ▪ ↓ hyoid missing or reduced (38%, ndr). **1.8 mg/kg bw/day** *Skeletal alterations:* ▪ ↑ ossification incidences in the sternebrae (20%, ncdr). ▪ ↑ incomplete ossification in the extremities (24%, ndr). ▪ ↓ hyoid missing or reduced (17%, ndr). *see annex B.6.6.2.4 for more information. **NOAEL** $_{\text{developmental toxicity}}$**: -** **NOAEL** $_{\text{maternal toxicity}}$**: -**	
Developmental toxicity study in rats with eugenol GLP: Yes Method: OECD TG 414 (2001).	Test substance: **Eugenol** Batch No.: 29424, purity not specified. Vehicle:	***Maternal toxicity*** Mortality: Only one female was found dead throughout the whole study. This was located in the high dose group and was found dead one hour after dosing on gestation day 5, probably caused by dosing trauma	[redacted] (2004) (AS) B.6.6.2.5

<table>
<tr><th>Method, guideline, deviations[1] if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
- NOAEL/LOAEL (for parent, offspring and for developmental effects)
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr>
<td>Rat strain: Sprague-Dawley CD rats

Sex: 25 mated females/group.

Deviations from current test guideline (OECD TG 414, 2018):

-Purity of test substance not stated.

-The following developmental endpoints were not measured: resorptions, anogenital distance, and indication of incomplete testicular descent/cryptorchidism.

- Thyroid weight and thyroid hormones (T3/T4/TSH) values from dams were not recorded.

- Historical control data sources are not detailed in the study report.

Study acceptable</td>
<td>1% carboxymethyl cellulose

Oral (gavage)

Dose levels: ♂/♀: 0, 100, 250 and 600 mg/kg bw/day throughout day 5-19 of gestation.

Parameters observed
Maternal data:

Clinical signs, mortality, bw and food consumption.

Reproductive data:

Number (no) of pregnancy dams, no of corpora lutea, no implants, no embryonic deaths, no implantation loss, placental wt and gravid uterus wt.

Foetal data:

Foetus wt, foetus sex and foetus alterations (external, visceral and skeletal)</td>
<td>and without evidence of systemic toxicity.

Clinical signs:

600 mg/kg bw/day

At high dose group, there was evidence of treatment-related clinical signs of toxicity 1h post dose, such as pilo-erection (72%), ataxia (12%), prostration (8%), increased lachrymation (8%), lethargy (4%), noisy respiration (4%) and increased salivation (4%). With the exception of one occasion, these findings were resolved within 24 hours. These clinical findings were seen in the majority of females at the highest dose level.

250 mg/kg bw/day

At mid dose group, there were also incidents of clinical signs 1h post dose, particularly pilo-erection (16%), hunched posture (4%) and increased salivation pre dose (8%)

Summary of clinical signs
<table>
<tr><th rowspan="2">Clinical Observations</th><th colspan="4">Number of Animals with Clinical observations</th></tr>
<tr><th>Group 1 (0 mg/kg/day)</th><th>Group 2 (100 mg/kg/day)</th><th>Group 3 (250 mg/kg/day)</th><th>Group 4 (600 mg/kg/day)</th></tr>
<tr><th></th><th>N=25</th><th>N=25</th><th>N=25</th><th>N=25</th></tr>
<tr><td>pilo erection pre dose</td><td>0</td><td>0</td><td>0</td><td>1</td></tr>
<tr><td>pilo erection 1h post dose</td><td>0</td><td>0</td><td>4 (16%)</td><td>18 (72%)</td></tr>
<tr><td>Hunched posture</td><td>0</td><td>0</td><td>1 (4%)</td><td>0</td></tr>
<tr><td>Increased salivation pre dose</td><td>0</td><td>0</td><td>2 (8%)</td><td>0</td></tr>
<tr><td>Ataxia</td><td>0</td><td>0</td><td>0</td><td>3 (12%)</td></tr>
<tr><td>Lethargy</td><td>0</td><td>0</td><td>0</td><td>1 (4%)</td></tr>
<tr><td>Prostrate</td><td>0</td><td>0</td><td>0</td><td>2 (8%)</td></tr>
<tr><td>Noisy respiration</td><td>0</td><td>0</td><td>0</td><td>1 (4%)</td></tr>
<tr><td>Increased lachrymation</td><td>0</td><td>0</td><td>0</td><td>2 (8%)</td></tr>
<tr><td>Found dead</td><td>0</td><td>0</td><td>0</td><td>1 (4%)</td></tr>
<tr><td>Increased salivation 1h post dose</td><td>0</td><td>0</td><td>0</td><td>1 (4%)</td></tr>
<tr><td>No animals with clinical signs</td><td>0</td><td>0</td><td>7 (28%)</td><td>19 (76%)</td></tr>
</table>
(% referred to total animals of each group)

600 mg/kg bw/day

Food consumption:
▪ (↓) through days 3-6 (12%), 6-9 (23%) and 9-12 (11%).

<u>Developmental toxicity</u>

Reproductive data

600 mg/kg bw/day
▪ ↓ foetal weight (6%).</td>
<td></td>
</tr>
</table>

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL (for parent, offspring and for developmental effects) - target tissue/organ - critical effects at the LOAEL	Reference
		Foetal data **600 mg/kg bw/day** *Skeletal alterations:* ▪ ↓ foetuses with >6 ossified metatarsals (9%). *see annex B.6.6.2.5 for more information. **NOAEL** developmental toxicity: 250 mg/kg bw/day based on decreased foetal weight and ossified metatarsals. **NOAEL** maternal toxicity: 100 mg/kg bw/day based on clinical toxicity signs.	
Developmental toxicity study in rabbits with eugenol. GLP: Yes Method: OECD TG 414 (2001). Rabbit strain: New Zealand White Sex: 24 mated females/group. Deviations from current test guideline (OECD TG 414, 2018): -Purity of test substance not stated. -The following developmental endpoints were not measured: resorptions, anogenital distance, and indication of incomplete testicular descent/cryptorchidism. - Thyroid weight and thyroid hormones (T3/T4/TSH) values from dams were not recorded. - Historical control data sources are not detailed in the study report. **Study acceptable**	Test substance: **Eugenol** Batch No.: 29424, purity not specified. Vehicle: 1% carboxymethyl cellulose Oral (gavage) Dose levels: ♂/♀: 0, 100, 250 and 500/350 mg/kg bw/day throughout day 5-28 of gestation. Parameters observed *Maternal data:* Clinical signs, mortality, bw and food consumption. *Reproductive data:* Number (no) of pregnancy dams, no of corpora lutea, no implants, no embryonic deaths, no implantation loss, placental wt and gravid uterus wt. *Foetal data:* Foetus wt, foetus sex and	***Maternal toxicity*** Mortality: *500/350 mg/kg bw/day dose group* -Two out five dead females were found dead after one and two doses respectively, and showed no clinical signs of morbidity or reaction to treatment. -Three out five dead females received between five and seven doses and showed clinical signs of reaction to treatment up to 1 h post dose, particularly ataxia and/or prostration, during the day(s) prior to death. No consistent post mortem macroscopic findings were observed for the decedent females but gastric changes were observed in 2/5 females. The findings included thickening with/without congestion and sloughing of the gastric mucosa. -One further female was killed *in extremis* following abortion of her offspring on day 25 of gestation. There were no clinical signs of reaction to treatment and no significant post mortem macroscopic changes. The abortion of the litter may have been a spontaneous event, unrelated to test material toxicity. *250 mg/kg bw/day dose group* -One female was found dead on day 12 of gestation. This female showed ataxia 10 min. post dosing on day 10 of gestation. Macroscopic post mortem examination showed colourless fluid around the mouth. With no significant findings, an association with test material toxicity could not be discounted. -One female was killed *in extremis* on day 8 of gestation due to laboured and noisy respiration. Macroscopic post mortem examination showed a portion of the dosing catheter in the oesophagus. The cause of death was therefore considered to be a dosing accident. -One female was killed *in extremis* following the abortion of offspring on day 23 of gestation. There were no clinical signs of reaction to treatment and no significant macroscopic post mortem findings. The abortion of offspring may have been a spontaneous event unrelated to test material toxicity. *100 mg/kg bw/day dose group* At 100 mg/kg bw/day dose group, there were no mortalities. *0 mg/kg bw/day dose group* -One female was killed *in extremis* following the abortion of offspring on day 25 of gestation. There were no clinical signs of	[redacted] (2004) (AS) B.6.6.2.6

<table>
<tr><th>Method, guideline, deviations[1] if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
- NOAEL/LOAEL (for parent, offspring and for developmental effects)
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr><td></td><td>foetus alterations (external, visceral and skeletal)</td><td>
morbidity and no significant macroscopic post mortem findings and it was concluded that the abortion of offspring was a spontaneous event.

Survival in dose groups

<table>
<tr><th></th><th colspan="4">Dose group (mg/kg bw/day)</th></tr>
<tr><th></th><th>0</th><th>100</th><th>250</th><th>500/350</th></tr>
<tr><td>no animals at initiation</td><td>24</td><td>24</td><td>24</td><td>24</td></tr>
<tr><td>Total Deaths/ killed in extremis</td><td>1</td><td>0</td><td>3</td><td>6</td></tr>
<tr><td>Deaths with clinical signs associated</td><td>0</td><td>0</td><td>1</td><td>3</td></tr>
<tr><td>Deaths without clinical signs associated</td><td>0</td><td>0</td><td>0</td><td>2</td></tr>
<tr><td>killed in extremis associated to abortions or dosing errors</td><td>1</td><td>0</td><td>2</td><td>1</td></tr>
<tr><td>Survival (gestation day 29)</td><td>23</td><td>24</td><td>21</td><td>18</td></tr>
<tr><td>% survival (gestation day 29)</td><td>96%</td><td>100%</td><td>88%</td><td>75%</td></tr>
</table>

Clinical signs: At 500 mg/kg bw/day dose level, relevant signs of toxicity were observed in females up to 1h post dose such as ataxia (17%), prostration (12.5%) or breathing difficulties (8%). As a result of this toxicity, four females dead and one female was killed *in extremis*. Consequently, the high dose level was reduced to 350 mg/kg bw/day.

The gestation day where there was a change in dose level is variable; between gestation day 10 and 13 for animals (numbers 73 to 84) within the first group used. Animals (numbers 85- 96) from group 2 were dosed at 350 mg/kg bw/day.

-At 250 mg/kg bw/day dose level, ataxia post dose (8%), abortions (4%), laboured respiration (4%) and noisy respiration (4%) were observed during treatment.

No relevant clinical signs were observed at low dose level.

Summary of clinical signs

<table>
<tr><th rowspan="2">Clinical Observations</th><th colspan="4">Number of Animals with clinical observations</th></tr>
<tr><th>Group 1 (0 mg/kg/d)</th><th>Group 2 (100 mg/kg/d)</th><th>Group 3 (250 mg/kg/d)</th><th>Group 4 (500/350) mg/kg/d)</th></tr>
<tr><td></td><td>n=24</td><td>n=24</td><td>n=24</td><td>n=24</td></tr>
<tr><td>Animal aborted</td><td>1(4%)</td><td>0</td><td>1 (4%)</td><td>1(4%)</td></tr>
<tr><td>Ataxia up to 1h post dose</td><td>0</td><td>0</td><td>2 (8%)</td><td>4 (17%)</td></tr>
</table>
</td><td></td></tr>
</table>

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL (for parent, offspring and for developmental effects) - target tissue/organ - critical effects at the LOAEL	Reference

Laboured respiration up to 1h post dose	0	0	1(4%)	1(4%)
Noisy respiration	0	0	1(4%)	0
Decreased respiration up to 1h post dose	0	0	0	1(4%)
Prostrate up to 1h post dose	0	0	0	3 (12.5%)
Pallor of the extremities up to 1h post dose	0	0	0	1(4%)
Eyes pale	0	0	0	1(4%)
Ears pale	0	0	0	1(4%)
No animals with clinical signs	**1 (4%)**	**0**	**4 (17%)**	**6 (25%)**

High dose levels adjustments

Animal number	Dose Level		
	500mg/kg	Off dose	350 mg/kg
	Gestation days	Gestation day	Gestation days
73	5-11	12	13-28
74*	5-11	-	-
75*	5-11	-	-
76*	5	-	-
77	5-10	11	12-28
78*	5-9	-	-
79	5-9	10	11-28
80	5-9	10	11-28
81*	5-6	-	-
82	5-8	9	10-28
83	5-8	9	10-28
84	5-8	9	10-28
85 to 96	-	-	5-28

500/350 mg/kg bw/day

Food consumption:

- (↓) through days 3-6 (16%, ns; ndr), 6-9 (40%), 9-12 (26%), 12-15 (18%, ns), 15-18 (22%, ns).

250 mg/kg bw/day

Food consumption:

- (↓) through days 3-6 (20%, ns; ndr), 6-9 (29%, ns), 9-12 (26%), 12-15 (16%, ns), 15-18 (20%, ns).

Developmental toxicity

Reproductive data

500/350 mg/kg bw/day

- (↑) postimplantation loss (275%, ns).

Foetal data

Method, guideline, deviations[1] if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL (for parent, offspring and for developmental effects) - target tissue/organ - critical effects at the LOAEL	Reference
		500/350 mg/kg bw/day *Skeletal alterations:* ▪ ↑ foetuses with at least sixteen forelimb phalanges ossified (14%, ndr). **250 mg/kg bw/day** *Skeletal alterations:* ▪ ↑ foetuses with at least sixteen forelimb phalanges ossified (6%, ns, ndr). **100 mg/kg bw/day** *Skeletal alterations:* ▪ ↑ foetuses with at least sixteen forelimb phalanges ossified (10%, ns, ndr). *see annex B.6.6.2.6 for more information. **NOAEL** $_{\text{developmental toxicity}}$: 250 mg/kg bw/day based on increased postimplantation loss. **NOAEL** $_{\text{maternal toxicity}}$: 100 mg/kg bw/day based on clinical signs and decreased food consumption.	

ns: no significative; ndr: no dose-response; ncdr: no clearly dose-response

Table 61: Summary table of human data on adverse effects on development

Type of data/report	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No data available				

Table 62: Summary table of other studies relevant for developmental toxicity

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
REACH DATA Developmental toxicity study in mouse. Reliability 3	Eugenol Purity: 99%	Mouse (NMRI) (♀) Vehicle: olive oil Dose: 100 mg/kg bw/day from day 5 to day 15 gestation (10 days)	Administration of Eugenol did not result in any teratogenicity (i.e., there were no effects on crown rump length and no defects of the craniofacial region, hands, feet, or tail) and there were no significant changes in number of live foetuses.	Unnamed (2003) (REACH registration dossier data not provided by applicant and not assessed by the RMS)

2.6.6.2.1 Short summary and overall relevance of the provided information on adverse effects on development

Six developmental toxicity studies have been presented to support the renewal assessment of the active substance

eugenol. In four of these studies, the test substance administered was the multi-component botanical active substance clove oil, and were conducted in rats (B.6.6.2.1), rabbits (B.6.6.2.2), mice (B.6.6.2.3) and hamsters (B.6.6.2.4), respectively. These studies predate the OECD TG 414 guidelines and GLP enforcement, and presented important methodological deficits, such as they were not conducted with the active substance eugenol, lack of the historical control data, and the absence of statistical analysis.

On the other hand, two additional studies conducted with eugenol, one in rats (B.6.6.2.5) and one in rabbits (B.6.6.2.6), were provided. These two studies were previously included in the eugenol peer review, and are considered adequate and relevant for the overall reproductive toxicity assessment of eugenol.

In the first developmental toxicity study conducted in rats with clove oil (B.6.6.2.1), botanical substance was tested via oral gavage from gestation day 6 to gestation day 15 at dose levels of 0, 2.8, 13, 60 or 280 mg/kg bw/day, in which 23-25 mated females were assigned to each test group. An additional group of 23 mated females received aspirin (250 mg/kg bw/day) as a positive control.

Neither mortality or morbidity signs, nor treatment related clinical signs were observed at any dose level.

No differences in bodyweights and bodyweight gain were observed between clove oil-treated groups and controls.

Regarding reproductive parameters, pregnancy index was similar in all treated groups, except in negative controls that were slightly lower (84%). The number of live foetuses per litter in the high dose group was lower than the controls (↓12%), but this could be accounted for the slightly lower number of implantations at this dose level (↓11%). Moreover, the sex ratio male/female pups was decreased (14-33%) in all treated groups compared with controls, however, a dose-related trend was not observed. On the other hand, an increase of resorptions was noted in the 60 mg/kg bw/day dose group (79%), but a dose-relationship was not observed in the treated groups, and no further effect on live foetuses mean was noted. Therefore, no dose-response relationship was observed in the clove oil-treated groups regarding these reproductive parameters, and importantly, no statistical analysis was performed in the study, so these results are not supported by statistical analysis. The positive control aspirin-treated group showed increased resorptions (and hence fewer live foetuses/litter) and reduced foetal weight (↓36%) compared with controls.

Increased incidences regarding ossification of ribs (mostly wavy), incomplete ossification of vertebrae and incomplete closure of skull were noted in treated groups, the latter being the only parameter displaying a dose-response pattern (36%, 82% and 100% for the 13, 60 and 280 mg/kg bw/day dose groups, respectively). However, without a proper statistical analysis, the whole findings are difficult to assess. On the other hand, no increase of visceral malformations was found in the treated groups compared with controls. The positive control aspirin-treated group showed high incidences of skeletal findings and head malformations.

NOAEL for developmental and maternal toxicity is not derived, due to the active substance eugenol was not tested, and the eugenol content in clove oil was not established.

In the second developmental toxicity study conducted in rabbits with clove oil (B.6.6.2.2), botanical substance was tested via oral gavage from gestation day 6 to gestation day 18 at dose levels of 0, 1.72, 7.99, 37.1 or 172 mg/kg bw/day, in which 11-15 females were artificially inseminated on each test group. An additional group of 12 rabbits received 6-aminonicotinamide (6-AN) at 2.5 mg/kg bw/day as positive control group.

No adverse clinical signs were recorded throughout the study. One pregnant dam from low clove oil dose group, and another from positive control-group aborted at gestation day 17 and 16, respectively, without evidence of adverse clinical signs.

No differences in bodyweights were observed between clove oil-treated groups and negative controls. Regarding bodyweight gain, the 7.99 mg/kg bw/day dose group showed lower bodyweight gain values throughout gestation days 0-29 (8-90%) compared with controls, but a dose-relationship was not observed.

Regarding reproductive performance, all treated groups showed a reduction in the male/female pups sex ratio (21-44%), except the high dose group, so a dose-response was not shown. Sex ratio data from historical controls (HCD) had been helpful to determine whether sex ratio of negative controls (1.37) observed in this study was higher or in the range of overall HCD. On the other hand, a non-dose-relationship increase of resorptions was noted in the 7.99 mg/kg/bw day dose group (55%), however, did not effect on the live foetuses mean.

Skeletal findings revealed and increased ossification incidences on sternebrae (mostly incomplete ossification, bipartite, fused and extra) in all treated groups compared with negative controls, however no clear dose-response

relationship was observed. Without HCD it is difficult to analyse whether control mean incidences was lower or in the range of overall HCD.

On the other hand, the incidence of another external, visceral and skeletal abnormalities was broadly similar between clove oil treated groups and controls.

NOAEL for developmental and maternal toxicity is not derived, due to the active substance eugenol was not tested, and the eugenol content in clove oil was not established.

In the third developmental toxicity study conducted in mice with clove oil (B.6.6.2.3), botanical substance was tested via oral gavage from gestation day 6 to gestation day 15 at dose levels of 0, 2.2, 10, 46 or 215 mg/kg bw/day, in which 23-25 mated females were assigned to each test group. An additional group of 24 mated females received aspirin (150 mg/kg bw/day) as a positive control.

Neither mortality or morbidity signs, nor treatment related clinical signs were observed at any dose level.

No differences in bodyweights were observed between clove oil-treated groups and controls Regarding bodyweight gain, an extensive decrease > 10% was recorded in all clove-oil treated groups throughout gestation days 0-11 (13-45%) compared with controls. During the last gestation week, bodyweight gain decrease was less marked (6-14%) in the clove oil treated groups than first 11 days of gestation, being >10% only in the 46 mg/kg bw/day dose group. Overall, no dose-response pattern was noted throughout measurements points.

Regarding reproductive parameters, the negative control and low clove oil dose group presented a lower pregnancy index (88%) compared with the rest of treated groups. No effect of treatment on resorptions and average foetus weight were recorded. A slight increase on total deaths and foetal deaths per litter were observed, however, no effect in live foetuses per litter was noted.

On the other hand, neither skeletal nor visceral incidences were recorded related to treatment.

NOAEL for developmental and maternal toxicity is not derived, due to the active substance eugenol was not tested, and the eugenol content in clove oil was not established.

In the fourth developmental toxicity study conducted in hamsters with clove oil (B.6.6.2.4), botanical substance was tested via oral gavage from gestation day 6 to gestation day 15 at dose levels of 0, 1.8, 8.2, 38.2 or 177 mg/kg bw/day, in which 25-28 mated females were assigned to each test group. An additional group of 25 mated females received aspirin (250 mg/kg bw/day) as a positive control.

Neither mortality or morbidity signs, nor treatment related clinical signs were observed at any dose level.

No relevant differences in bodyweights were observed between clove oil-treated groups and negative controls. Regarding bodyweight gain, loss of bodyweight was observed in the 1.8 and 8.2 mg/kg bw/day dose groups at gestation day 6. Moreover, in these two groups, bodyweight gain values were lower (>10%) than controls throughout gestation period, although the difference with controls was gradually reduced over time. On the other hand, in 38.2 and 177 mg/kg bw/day dose groups, the bodyweight gains was broadly similar between clove oil treated groups and negative controls, being slighlty higher in the gestation day 6. As described, a dose-relationship was not shown.

Regarding reproductive parameters, the negative control and clove oil treated groups displayed a pregnancy index between 71-88%. In the positive control group, a dam had a premature natural birth at gestation day 13 with alive foetuses. However, the authors of the study did not account this litter in the overall assessment.

No differences in live litters and mean of live foetuses per litter and pups bodyweights were described, compared with controls. An increased number of resorptions per litter were observed in the 1.8, 8.2 and 38.2 mg/kg bw/day (35, 115 and 250%, respectively) dose groups, but no at high dose group, so a dose-reponse was not noted. Moreover, the total death foetuses and it average per litter were increased in the 8.2 and 38.2 mg/kg bw/day dose group. These observations were primarily due to one dam of the 8.2 mg/kg bw/day dose group had 5 late resorptions and the whole 5 foetuses of the litter were still-born, and another dam of 38.2 mg/kg bw/day dose group that had 8 resorptions and consequently, no offspring was bred. However, neither the increase in the number of resorptions nor the increased number of death foetuses, affect the number and rate of live foetuses.

On the other hand, the sex ratio male/female was increased in all the treated groups, including positive control group treated with aspirin, compared with negative controls. These could be explained by the low sex ratio displayed by the group treated with vehicle (0.6). Sex ratio data from historical controls (HCD) had been helpful to determine

whether sex ratio of negative controls observed in this study was lower or in the range of overall HCD.

Increased no clearly dose-related ossification incidences in the sternebrae were described in the clove oil-treated groups. Moreover, increased incomplete ossification incidences of the extremities were described in the low and top dose groups (24 and 80%, respectively), whereas increased incidences of hyoid reduced or missing were noted only in the top dose group (48%). However, without a proper statistical analysis and the HCD incidence, the results are difficult to assess.

On the other hand, no visceral findings were described in the clove-oil treated groups.

NOAEL for developmental and maternal toxicity is not derived, due to the active substance eugenol was not tested, and the eugenol content in clove oil was not established.

The developmental study in rats conducted with eugenol (B.6.6.2.5) was designed to evaluate the potential teratogenicity effect of eugenol in Sprague-Dawley CD rats. Eugenol was tested at dose levels of 0, 100, 250, and 600 mg/kg bw/day, to each of which 25 copulated females were assigned dose level.

Only one female was found dead throughout the whole study. This was located in the high dose group and was found dead one hour after dosing on gestation day 5. There were no clinical signs of morbidity on the day of observation of death. Post mortem macroscopic findings included fluid within the thorax and haemorrhagic lungs. These findings are consistent with dosing trauma but are not evidence of systemic toxicity.

At high dose group, there was evidence of treatment-related clinical signs of toxicity 1h post dose, such as pilo-erection (72%), ataxia (12%), prostration (8%), increased lachrymation (8%), lethargy (4%), noisy respiration (4%) and increased salivation (4%). With the exception of one occasion, these findings were resolved within 24 hours. These clinical findings were seen in the majority of females at the highest dose level. In the mid dose group, there were also incidents of clinical signs 1h post dose, particularly pilo-erection (16%), hunched posture (4%) and increased salivation pre dose (8%). No clinical signs were described in low dose and control groups.

No statistically significant differences in bodyweight were observed in treated groups compared with controls. A slight decrease was noted in high dose group compared with controls throughout post coitum day 6-20 (3-5%).

Statistically significant reduction in food consumption was detected through gestation days 3-6 (12%), 6-9 (23%) and 9-12 (11%) in the high dose groups compared with controls. On the other hand, no differences were detected in food consumption in the mid and low dose groups.

Necropsy of dams did not show alterations compared with controls.

Regarding foetal data, at high dose group, there was a decreased in mean foetal weight compared with controls (6%). No other foetal endpoints revealed statistically significant differences compared with controls.

Skeletal and visceral examinations exhibited a reduced number of ossified metatarsals (9%) in the high dose group. At mid and low dose groups, treatment did not show effects on foetal data, nor in external malformations, visceral or skeletal anomalies.

Therefore, a **NOAEL for developmental toxicity** has been established at **250 mg/kg bw/day** on decreased mean foetal bodyweight and ossified metatarsals.

NOAEL for maternal toxicity has been established at **100 mg/kg bw/day** based on clinical signs observed.

The developmental study in rabbits conducted with eugenol (B.6.6.2.6) was designed to evaluate the potential teratogenicity effect of eugenol in New Zealand White Rabbits. Eugenol was tested at dose levels of 0, 100, 250, and 500 mg/kg bw/day, to each of which 24 copulated females were assigned dose level.

At high dose group, important signs of toxicity were observed up to 1h post dose, such as ataxia (17%) and prostration (12.5%). Low incidence (4%) pallor of the extremities, eyes and ears, together with respiratory difficulties were also noted. As a result of this toxicity, four females dead and one female was killed in extremis. Consequently, the high dose level was reduced to 350 mg/kg bw/day. The gestation day where there was a change in dose level varies between gestation day 10 and 13. At 350 mg/kg bw/day dose group, one dam aborted at gestation day 25 and was further killed *in extremis*.

On the other hand, at mid dose level, ataxia 10 min post dose (8%), abortions (4%), laboured respiration (4%) and noisy respiration (4%) were observed during treatment. No relevant clinical signs were observed at low dose level.

Mortality occurred in mid and high dose groups, as consequence of test substance toxicity described above. At high dose group, two out five dead females were found dead after one and two doses respectively, without showing any clinical sign of morbidity or reaction to treatment. Three out five dead females received between five and seven doses and showed clinical signs of reaction to treatment, particularly ataxia and/or prostration post dosing, during the day(s) prior to death. No consistent post mortem macroscopic findings were observed for the decedent females but gastric changes were observed in 2/5 females. The findings included thickening with/without congestion and sloughing of the gastric mucosa. One further female was killed *in extremis* following abortion of her offspring on day 25 of gestation, and there were no significant post mortem macroscopic changes.

At mid dose group, one female was found dead on day 12 of gestation. This female showed ataxia 10 min. post dosing on day 10 of gestation. Macroscopic post mortem examination showed colourless fluid around the mouth. With no significant findings, an association with test material toxicity could not be discounted. One further female was killed *in extremis* on day 8 of gestation due to laboured and noisy respiration. Macroscopic post mortem examination showed a portion of the dosing catheter in the oesophagus. The cause of death was therefore considered to be a dosing accident. A third female was killed *in extremis* following the abortion of offspring on day 23 of gestation. There were no significant macroscopic post mortem findings in this dam.

There were no significant differences in female mean bodyweights between eugenol dose groups and controls throughout gestation. A slight decrease was noted in high dose group compared with controls throughout post coitum day 6-29 (3-6%).

Regarding food consumption, a statistically significant reduction was detected in high dose group through gestation days 6-9 (40%) and 9-12 (26%), and a decreased trend was recorded throughout gestation day 3-18 (16-40%). At mid dose group, there was also a slight reduction in group mean female food consumption during gestation day 3-18 (16-29%), although only the difference displayed statistically significant result during gestation days 9-12 (26%).

Necropsy of gestation females revealed gastric changes associated with test material administration in two decedent high dose females. Afterwards, the reduction of dose level to 350 mg/kg bw/day resulted in no significant macroscopic findings.

Regarding foetal data, there was an increase in post implantation loss (275%) at high dose group compared to controls. This finding was non-statistically significant and presented a wide standard deviation. The data was limited primarily to two females with significant numbers of late embryonic deaths, and would suggest evidences of treatment-related toxicity. On the other hand, there were no statistically significant treatment-related effects on foetal bodyweight, placental or gravid uterine weight in the high or lower dose groups.

Skeletal and visceral alterations of foetuses showed an increase in percentage of foetuses with at least sixteen forelimb phalanges ossified (14%). This value was statistically significant compared to controls; however, this is not indicative of a treatment-related increase in bone ossification as there were no trends with the ossification of other bones of the skeleton.

At mid and low dose groups, treatment did not show effects on foetal data, nor in external malformations, visceral or skeletal anomalies.

Therefore, a **NOAEL for developmental toxicity** has been established at **250 mg/kg bw/day** based on increased postimplantation loss.

NOAEL for maternal toxicity has been established at **100 mg/kg bw/day** based on clinical signs and decreased food consumption.

Data from REACH registration dossier were included in the summary table of other studies relevant for developmental toxicity, since it is an ECHA requirement for the proposal of harmonised classification (CLH) according to Regulation (EC) no. 1272/2008 (CLP). However, it has to be underlined that these data were not available in the applicant submission dossier for the renewal and consequently they have not been evaluated by the RMS and not included in Volume 3 of this RAR. Only studywas found in the REACH dossier with low reliability, low number of tested animals and only one tested dose level.

2.6.6.2.2 Comparison with the CLP criteria regarding adverse effects on development

For the purpose of classification for reproductive toxicity according to the criteria of the CLP (Regulation (EC) No. 1272/2008), substances are allocated to one of two categories. Within each category, effects on sexual function, fertility, lactation and development, are considered separately.

The relevance of the developmental effects compared with CLP criteria are summarized below:

No human information is available on the effects of eugenol on development, hence a classification as Category 1A is not possible. There is information from two reliable developmental studies conducted in rat and rabbit with eugenol and, as additional information, four developmental toxicity studies conducted with clove oil in rat, rabbit, mouse and hamster.

Category 1B: The classification of a substance in Category 1B is largely based on data from animal studies. Such data shall provide clear evidence of an adverse effect on sexual function and fertility or on development in the absence of other toxic effects, or if occurring together with other toxic effects, the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate.

Category 2: Suspected human reproductive toxicant. Substances are classified in Category 2 for reproductive toxicity when there is some evidence from humans or experimental animals, possibly supplemented with other information, of an adverse effect on sexual function and fertility, or on development, and where the evidence is not sufficiently convincing to place the substance in Category 1. If deficiencies in the study make the quality of evidence less convincing, Category 2 could be the more appropriate classification. Such effects shall have been observed in the absence of other toxic effects, or if occurring together with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects.

Developmental toxicity study in rats:
Maternal toxicity: Relevant clinical signs were noted at mid (250 mg/kg bw/day) and high dose (600 mg/kg bw/day). Moreover, statistically significant reduction in food consumption was detected through gestation days 3-6 (12%), 6-9 (23%) and 9-12 (11%) in the high dose group compared with controls. On the other hand, no differences were detected in food consumption in the mid and low dose groups. No other signs of maternal toxicity were observed at doses tested.

Developmental effects
Relevant for classification:
Statistically significant reduction in the mean foetal bodyweight (6%) and in the number of ossified metatarsals (9%) were recorded at high dose. These data were not deemed for developmental toxicity classification due to the incidences were low and of minimal adversity. In addition, no other related-reproductive or developmental effects in ossification development were noted, and as previously pointed out, clear maternal toxicity was observed at high dose level.

Developmental toxicity study in rabbits:
Maternal toxicity: Relevant clinical signs were noted at mid (250 mg/kg bw/day) and high dose (500/350 mg/kg bw/day). Moreover, statistically significant reduction in food consumption was detected through gestation days 6-9 (40%) and 9-12 (26%) compared to controls. At 250 mg/kg bw/day dose group, there was also a slight reduction in group mean female food consumption throughout gestation days 3-18 (16-29%). However, the difference was only statistically significant through gestation days 9-12 (26%).

Developmental effects
Relevant for classification: An increase incidence of post implantation loss in high dose group was observed. This finding was not statistically significant and presented a wide standad desviation, mainly due to two females that showed an increased number of late embryonic deaths.. On the other hand, increased percentage of foetuses with at least sixteen forelimb phalanges ossified was noted (14%), however this is not indicative of a treatment-related increase in bone ossification as there were no trends with the ossification of other bones of the skeleton. Both developmental effects were observed in presence of clear maternal toxicity.
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Therefore, the developmental effects recorded in rat and rabbits were different in both species and were unequivocally associated with a marked maternal toxicity [relevant clinical signs and reduction in food consumption during the organogenis period (e.g. days 5-15 in the rodent, and days 6-18 in the rabbit)].

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Overall, according to the guidance on the application of the CLP criteria, eugenol does not require classification for developmental toxicity.

2.6.6.3 Adverse effects on or via lactation [equivalent to section 10.10.7 of the CLH report template]

Table 63: Summary table of animal studies on effects on or via lactation

Method, guideline, deviations[1] if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL**	**Reference**
No data available			

Table 64: Summary table of human data on effects on or via lactation

Type o data/report	**Test substance**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
No data available				

Table 65: Summary table of other studies relevant for effects on or via lactation

Type of study/data	**Test substance**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
No data available				

2.6.6.3.1 Short summary and overall relevance of the provided information on effects on or via lactation

No information on the potential of eugenol to cause adverse effects on the offspring via lactation has been provided.

2.6.6.3.2 Comparison with the CLP criteria regarding effects on or via lactation

There is no evidence in human or animal studies that eugenol is absorbed by women and has been shown to interfere with lactation, or which may be present (including metabolites) in breast milk in amounts sufficient to cause concern for the health of a breastfed child

2.6.6.4 Conclusion on classification and labelling for reproductive toxicity

Based on the available data, and according to CLP Regulation (EC) No 1272/2008, classification of **eugenol** as reproductive toxicant is not warranted.

2.6.7 Summary of neurotoxicity

Neurotoxicity studies with eugenol were not available in the submitted dossier.

According to Commission regulation (EU) No 283/2013, neurotoxicity studies in rodents shall be performed for active substances with structures that are similar or related to those capable of inducing neurotoxicity, and for active

substances which induce specific indications of potential neurotoxicity, neurological signs or neuropathological lesions in toxicity studies at dose levels not associated with marked general toxicity. Performance of such studies shall also be considered for substances with a neurotoxic mode of pesticidal action. Moreover, delayed polyneuropathy studies shall be performed for active substances of similar or related structures to those capable of inducing delayed polyneuropathy such as organophosphorus compounds.

Table 66: Summary table of animal studies on neurotoxicity

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results: - NOAEL/LOAEL - target tissue/organ -critical effect at LOAEL**	**Reference**
No data available			

Therefore, a review of the available information was carried out.

Table 2.6.7. Summary of neurotoxicity effects observed in the renewal eugenol dossier studies

Study type	**Dose**	**Neurological effects**	**Reference**
Acute toxicity effects			
Acute oral toxicity in Rat	Test substance: Eugenol (undiluted) Purity: Not stated Oral gavage Single doses (ml/kg): 1.5, 1.6, 1.75, 1.9, 2.0, 2.1 and 2.2, equivalent to 1597.5, 1704, 1863.75, 2023.5, 2130, 2236.5 and 2343 mg/kg bw/day 12 rats/group (male and female)	All animals experienced weakness of the hind legs after 5min post-dosing and by 15 min there was complete paralysis of the lower extremities. 86% of the animals (70 animals) experienced lower jaw relaxed that remained open for a period of time. A total of 70% (64 animals) finally became prostrate unable to move. A total of 54% animals went into a coma.	Sober, H.A. *et al.* (1950) (AS) B.6.2.1.2
Acute oral toxicity in dogs	Test substance: Eugenol (aqueous emulsion established with 5% gum acacia) Purity: Not stated Oral (instillation into stomach via Levin tube) 5 Female dogs for 20 experiments	Marked motor dysfunction (ataxia) primarily of the hind limbs observed in 3 dogs in the high dose group (500 mg/kg bw)	Lauber, F.U. and Hollander, F. (1950) (AS) B.6.2.1.5
Reproductive toxicity studies			
Developmental toxicity study in rats	Test substance: Eugenol Purity: Not stated Oral (gavage) Dose levels: ♂/♀: 0, 100, 250 and 600 mg/kg bw/day throughout day 5-19 of gestation. 25 mated females/group.	-At 600 mg/kg bw/day there was evidence of treatment-related clinical signs of toxicity 1h post dose, particularly pilo-erection, ataxia (12%), prostration (8%) and increased lachrymation (8%). With the exception of one occasion, these findings were resolved within 24 hours. These clinical findings were seen in the majority of females at the highest dose level. -At 250 mg/kg there were also incidents of clinical signs 1h post dose, particularly pilo-erection (16%) and hunched posture (4%). **Summary of neurotoxicity-related signs** **Clinical Observatio** / **Number of Animals with Clinical observations**: **Group 1** / **Group 2** / **Group 3** / **Group 4**	[redacted] (2004) (AS) B.6.6.2.5

<table>
<tr><th>Study type</th><th>Dose</th><th>Neurological effects</th><th>Reference</th></tr>
<tr><td></td><td></td><td>
<table>
<tr><th>ns</th><th>(0 mg/kg/day)</th><th>(100 mg/kg/day)</th><th>(250 mg/kg/day)</th><th>(600) mg/kg/day))</th></tr>
<tr><th></th><th>N=25</th><th>N=25</th><th>N=25</th><th>N=25</th></tr>
<tr><td>pilo erection pre dose</td><td>0</td><td>0</td><td>0</td><td>1</td></tr>
<tr><td>pilo erection 1h post dose</td><td>0</td><td>0</td><td>4 (16%)</td><td>18 (72%)</td></tr>
<tr><td>Hunched posture</td><td>0</td><td>0</td><td>1 (4%)</td><td>0</td></tr>
<tr><td>Increased salivation pre dose</td><td>0</td><td>0</td><td>2 (8%)</td><td>0</td></tr>
<tr><td>Ataxia</td><td>0</td><td>0</td><td>0</td><td>3 (12%)</td></tr>
<tr><td>Lethargy</td><td>0</td><td>0</td><td>0</td><td>1 (4%)</td></tr>
<tr><td>Prostrate</td><td>0</td><td>0</td><td>0</td><td>2 (8%)</td></tr>
<tr><td>Noisy respiration</td><td>0</td><td>0</td><td>0</td><td>1 (4%)</td></tr>
<tr><td>Increased lachrymation</td><td>0</td><td>0</td><td>0</td><td>2 (8%)</td></tr>
<tr><td>Found dead</td><td>0</td><td>0</td><td>0</td><td>1 (4%)</td></tr>
<tr><td>Increased salivation 1h post dose</td><td>0</td><td>0</td><td>0</td><td>1 (4%)</td></tr>
<tr><td>No. animals with clinical signs</td><td>0</td><td>0</td><td>7 (28%)</td><td>19 (76%)</td></tr>
</table>
</td><td></td></tr>
<tr><td>Developmental toxicity study in rabbits</td><td><u>Test substance:</u>
Eugenol

Purity: Not stated

Oral (gavage)

Dose levels:
♂/♀: 0, 100, 250 and 500/350 mg/kg bw/day throughout day 5-28 of gestation.

24 mated females/group.</td><td>-At 500 mg/kg bw/day dose level, relevant signs of toxicity were observed in females up to 1h post dose, such as ataxia (17%) and prostration (12.5%). Low incidence (4%) of prostrate, pallor of the extremities and respiratory difficulties were also noted. As a result of this toxicity, four females dead and one female was killed in extremis. Consequently, the high dose level was reduced to 350 mg/kg bw/day.

-At 250 mg/kg bw/day dose level, ataxia 10 min post dose (8%), abortions (4%), laboured respiration (4%) and noisy respiration (4%) were observed during treatment.

Summary neurotoxicity-related signs
<table>
<tr><th rowspan="2">Clinical Observations</th><th colspan="4">Number of Animals with clinical observations</th></tr>
<tr><th>Group 1 (0 mg/kg/day)</th><th>Group 2 (100 mg/kg/day)</th><th>Group 3 (250 mg/kg/day)</th><th>Group 4 (500/350) mg/kg/day))</th></tr>
<tr><th></th><th>n=24</th><th>n=24</th><th>n=24</th><th>n=24</th></tr>
<tr><td>Ataxia up to 1h post dose</td><td>0</td><td>0</td><td>2 (8%)</td><td>4 (17%)</td></tr>
<tr><td>Laboured respiration up to 1h post dose</td><td>0</td><td>0</td><td>1(4%)</td><td>1(4%)</td></tr>
<tr><td>Noisy respiration</td><td>0</td><td>0</td><td>1(4%)</td><td>0</td></tr>
<tr><td>Decreased respiration up to 1h post dose</td><td>0</td><td>0</td><td>0</td><td>1(4%)</td></tr>
<tr><td>Prostrate up to 1h post dose</td><td>0</td><td>0</td><td>0</td><td>3 (12.5%)</td></tr>
</table>
</td><td>[redacted] (2004) (AS) B.6.6.2.6</td></tr>
</table>

Study type	Dose	Neurological effects					Reference
		Pallor of the extremities up to 1h post dose	0	0	0	1(4%)	
		Eyes pale	0	0	0	1(4%)	
		Ears pale	0	0	0	1(4%)	
		No. animals with clinical signs	**0**	**0**	**3 (12.5%)**	**5 (21%)**	

Neurotoxicity findings in acute toxicity studies

In acute oral toxicity studies, impaired mobility, ataxia, prostration and paralysis were observed in rats at doses from 1597.5 mg/kg bw/day. In dogs, marked motor dysfunction (ataxia) was observed in dogs at high dose group (500 mg/kg bw/day). However, this study in dogs does not clearly specify the dosing regime and it uses 5 dogs for 20 experiments, hence the observed effects may result from using the same dogs, i.e. repeat dosing.

Neurotoxicity findings in developmental toxicity studies

In developmental toxicity study conducted with eugenol in rats, pilo-erection, ataxia, prostration, hunched posture, increased lachrymation and salivation were described in mid and high dose groups (250 and 600 mg/kg bw/day). In rabbits developmental toxicity study, ataxia, prostration, pallor of the extremities and respiratory difficulties were reported in mid and high dose groups (250 and 500/350 mg/kg bw/day). Most of these effects were resolved within 24 hours or reverted over time.

Conclusion on neurotoxicity

The available data show that eugenol may induce specific indications of potential neurotoxicity and neurological signs. The effects were mainly observed in the acute toxicity studies in rat and dogs, and in developmental toxicity studies conducted in rats and rabbits. These neurological findings were observed at high dose levels, and indeed the maternal NOAEL of developmental studies was based on these effects. Moreover, based on the reported effects in the acute toxicity studies, as part of this review process, a classification as STOT SE 3 for eugenol has been proposed.

In conclusion, based on the available data, the RMS is of the opinion that there is evidence to suggest the active substance eugenol might elicit neurotoxic effects. For that reason, the RMS concludes that neurotoxicity cannot be discarded for eugenol.

2.6.8 Summary of other toxicological studies

2.6.8.1 Toxicity studies of metabolites and impurities

Table 2.6.8.1/01. Summary table of toxicity studies of methyleugenol (impurity)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
Toxicity studies on the impurity Methyleugenol			
ADME			
Toxicokinetics in rats and mice Comparable to OECD TG 417 F344/N rats B6C3F1 mice	Methyleugenol Purity/Batch no.: not stated Single oral (gavage) doses: 37, 75 and 150 mg/kg bw	Plasma concentration Highest plasma concentrations were observed at the 2-min time point following IV administration in both sexes of rats and mice. The plasma concentration-versus-time profiles following gavage administration to rats demonstrated a rapid absorption phase that occurred within 15 min post dosing in both sexes.	Hong, S.P. *et al.* (2013) (AS) B.6.8.1.1.1-01

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
- NOAEL/LOAEL
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr><td>Blood samples collected at several time points.
Toxicokinetic parameters derived.

GLP: No

Supporting information</td><td>Vehicle (oral): corn oil

Single ip injection: 37 mg/kg

Vehicle (ip): Cremophor:ethanol: water (1/1/8; v/v/v)</td><td>In mice, the plasma concentration-versus-time profiles following gavage administration showed a rapid absorption phase that occurred within 30 min post dosing.

<u>Absorption</u>
Absorption following gavage administration was rapid with T_{max} values within 15 min in rats and 30 min in mice. AUC_T values increased with dose in both species but the magnitude of the increases was greater than proportional to dose

<u>Clearance</u>
Methyleugenol was cleared rapidly following both IV and gavage administration in both species and sexes.

<u>Bioavailability</u>
In rats, similar bioavailability values following a gavage dose of 37 mg/kg: 3.9 ± 0.9% for males and 4.1 ± 1.3% for females
In mice, sex differences in bioavailability values following a gavage dose of 37 mg/kg: 9.2 ± 6.4% for males and 7.3 ± 3.7% for females

The toxicokinetic data are consistent with extensive first-pass metabolism and saturation of metabolism at doses higher than 37 mg/kg bw</td><td></td></tr>
<tr><td>Metabolism in vitro

No guidance

Rat (male Wistar), bovine and human liver microsomes

Primary rat hepatocytes

Incubations with substrate at several time points.
Metabolites identified and quantified using HPLC.

GLP: No

Supporting information</td><td>Methyleugenol

Purity/Batch no.: not stated

Solvent: DMSO

Microsomes incubation: 0.1, 0.5 or 2.5mM substrate with NADPH-regenerating system at 37ºC for up to 24h

Metabolism Study: rat primary hepatocytes were treated with 200 µM of substrate for 5h (Phase I metabolism study) and for further 10h at 37ºC (Phase II metabolism study)</td><td>Highest yield of metabolites at 500 µM dose in ARLM.
Main metabolites of methyleugenol in various systems:

<table><tr><th>Metabolite</th><th>Identified in</th></tr><tr><td>1’-Hydroxymethyleugenol (2)</td><td>ARLM, RLM, BLM, HML</td></tr><tr><td>3’-Hydroxymethylisoeugenol (3)</td><td>ARLM, RLM, BLM, HML</td></tr><tr><td>6-Hydroxymethyleugenol (4)</td><td>ARLM, BLM, HML</td></tr><tr><td>3’-Oxomethylisoeugenol (6)</td><td>ARLM, RLM, BLM, HML</td></tr><tr><td>(RS)-2’,3’-dihydroxy-2’,3’-dihydromethyleugenol (7)</td><td>ARLM, RLM, BLM, HML</td></tr><tr><td>Eugenol (9)</td><td>ARLM, RLM, BLM, HML</td></tr><tr><td>Chavibetol (11)</td><td>ARLM, RLM, HML</td></tr></table>
The metabolic pathway of methyleugenol in liver microsomes was elucidated</td><td>Cartus, A.T. et al. (2012) (AS) B.6.8.1.1.1-02</td></tr>
<tr><td>Comparative in vitro metabolism

No specific testing regulations/guidelines

Human (mixed gender), mouse (male) and rat (male) microsomes and S9 fractions

GLP: No</td><td>Dosage: 20 µM (1.8 µCi) [^{14}C]-Methyleugenol, purity not stated

Methyleugenol, purity not stated

Incubation time: 180 min
NADPH generating system or UDPGA</td><td><u>Metabolism in vitro</u>:
Two metabolites identified in all three species: 1’-hydroxy glucuronide and phenoxy-glucuronide (only when incubated with NADPH + UDPGA)

<u>Covalent binding:</u>
Reduced covalent binding in comparison to eugenol. The addition of glutathione considerably reduces the covalent binding.

<u>Phase I metabolite – 1’hydroxylation (microsomes + NADPH)</u>:
- Human: 22.2 % ± 0.2
- Mouse: 52.4 % ± 13.38
- Rat: 24.4 % ± 5.7</td><td>Minet, E.F. et al. (2012) (AS) B.6.8.1.1.1-03</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
Study acceptable	Protein binding: GSH	Phase I covalent binding (microsomes + NADPH): - Human: 2.7 % ± 0.5 - Mouse: 7.8 % ± 3.1 - Rat: 2.5 % ± 0.5 Phase II glucuronidation (S9+UDPGA): - Human: no peak - Mouse: no peak - Rat: no peak Phase II glucuronidation (S9+NADPH+ UDPGA): - Human: 12.2 % ± 0.4 - Mouse: 4.0 % ± 0.4 - Rat: 3.9 % ± 0.8 Limited metabolism in lung compared to liver.	
Short term toxicity			
14-week study in rats GLP: Yes Method: Non-stated. Rat strain: F344/N rats Sex: 9-10 ♂/ 10 ♀/group. Deviations from current test guideline (OECD TG 408, 2018): -Food consumption was not measured. -Ophthalmological examination was not performed. -Thyroid gland, ovaries, uterus, brain, adrenals, prostate and pituitary gland were not weighted. - Circulating thyroid hormones (T4, T3, TSH) levels were not measured. -Complete histopathology was only performed on negative controls and top dose groups. **Study acceptable**	Test substance: **Methyleugenol** Batch No.: 8334801, Purity: 99% Vehicle: 0.5% methylcellulose Oral (gavage) **Doses:** Males/females: 0, 30, 100, 300 and 1000 mg/kg bw/day. 14-week exposure (5-day/week scheme administration).	Mortality: All rats survived until the end of the study. Clinical signs: Clinical findings possibly related to test substance administration included emaciation and urine staining in 100, 300, and 1000 mg/kg bw/day female dose rats. **1000 mg/kg bw/day** Bodyweights ▪ (↓) bw in ♂ at week 14 (30%). ▪ (↓) bwg in ♂ at week 14 (45%). ▪ (↓) bw in ♀ at week 14 (16%). ▪ (↓) bwg in ♀ at week 14 (30%). Haematology: ▪ (↓) Ht in ♂ (13%) and ♀ (6%) at week 14. ▪ (↓) Hb in ♂ (11%) and ♀ (7%) at week 14. ▪ (↑) Erythrocytes in ♂ (12%) and ♀ (12%) at week 4. ▪ (↑) Reticulocytes in ♀ at week 4 (150%; ncdr) and (↓) on day 5 (46%; ncdr). ▪ (↓) MCV in ♂ at week 14 (15%). and 4(16%), and in ♀ at week 14 (6%), 4(11%), and on day 5 (13%). ▪ (↓) MCHb in ♂ at week 14 (12%), 4 (15%), and on day 5 (2%), and in ♀ at week 14 (5%) and 4(12%). ▪ (↑) PLT in ♂ at week 14 (37%) and 4 (36%), and in ♀ at week 14 (28%), 4(27%), and on day 5 (26%). ▪ (↑) Leukocytes in ♀ at week 14 (60%). ▪ (↑) Lymphocytes in ♀ at week 14 (72%). Clinical biochemistry: ▪ (↓) urea nitrogen in ♂ at week 4 (12%, ndr) and in ♀ at week 14 (12%, ndr). ▪ (↑) creatinine in ♂ on day 5 (35%) and in ♀ at week 14 (23%), 4 (20%) and on day 5 (24%). ▪ (↓) total protein in ♂ at week 14 (12%) and on day 5 (12%), and in ♀ at week 14 (13%), 4 (7%) and on day 5 (14%). ▪ (↓) albumin in ♂ at week 14 (10%) and on day 5 (12%), and in ♀ at week 14 (14%), 4 (8%) and on day 5 (18%). ▪ (↑) alanine aminotransferase (ALT) in ♂ at week 14 (74%), 4 (22%) and on day 5 (92%), and in ♀ at week 14 (57%), 4 (82%) and on day 5 (58%). ▪ (↓) alkaline phosphatase (ALP) in ♂ at week 4 (12%), and in ♀ on day 5 (21%). ▪ (↑) Sorbitol dehydrogenase in ♂ at week 14 (50%, ncdr), and in ♀ at week 4 (60%) . ▪ (↑) bile acids in ♂ at week 14 (841%), 4 (312%) and on day 5 (120%), and in ♀ at week 14 (1105%) and 4 (370%).	NTP (2000) (AS) B.6.8.1.1.2-01

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
		Organ weights ▪ (↓) total bw in ♂ (31%) and in ♀ (18%). *Heart* ▪ (↓) abs wt (21%) and (↑) rel wt (15%) in ♂, and (↓) abs wt (11%) and (↑) rel wt (9%) in ♀. *Right kidney* ▪ (↑) rel wt in ♂ (41%) and in ♀ (26%). *Liver* ▪ (↑) rel wt in ♂ (57%) and abs wt (45%) and rel wt (77%) in ♀. *Lung* ▪ (↓) abs wt (16%) and (↑) rel wt (22%) in ♂, and (↑) rel wt (11%) in ♀. *Spleen* ▪ (↓) abs wt (28%) and (↑) rel wt (5%, ndr) in ♂; and (↑) abs wt (10%; ns, ndr) and (↑) rel wt (34%, ndr) in ♀. *Right testis* ▪ (↑) abs wt (64%) and (↑) rel wt (137%). *Thymus* ▪ (↓) abs wt (50%) and (↓) rel wt (28%) in ♂, and (↓) abs wt (38%) and (↓) rel wt (24%) in ♀. Histopathology *Liver* ▪ (↑) cytologic alteration in ♂ (100%) and ♀ (100%). ▪ (↑) cytomegaly in ♂ (100%) and ♀ (100%). ▪ (↑) pigment on Kupffer cell in ♂ (100%) and ♀ (90%). ▪ (↑) mixed cell focus in ♂ (90%) and ♀ (80%). ▪ (↑) hyperplasia on bile ducts in ♂ (100%) and ♀ (90%). *Glandular stomach* ▪ (↑) Atrophy in ♂ (100%) and ♀ (100%). ▪ (↑) Chronic inflammation in ♂ (100%) and ♀ (100%). *Adrenal cortex* ▪ (↑) Hypertrophy in ♂ (100%) and ♀ (100%). *Submaxillary salivary gland* ▪ (↑) cytoplasmic alteration in ♂ (100%) and ♀ (100%). *Testis* ▪ (↑) dilatation (100%) and degeneration (100%). *Uterus* ▪ (↑) atrophy (100%). **300 mg/kg bw/day** Bodyweights ▪ (↓) bw in ♂ at week 14 (8%). ▪ (↓) bwg in ♂ at week 14 (12%). ▪ (↓) bw in ♀ at week 14 (8%). ▪ (↓) bwg in ♀ at week 14 (16%). Haematology: ▪ (↓) Hb in ♀ (4%) at week 14. ▪ (↑) Reticulocytes in ♀ at week 4 (150%; ncdr). ▪ (↑) Erythrocytes in ♂ (6%) at week 4. ▪ (↓) MCV in ♂ at week 14 (1%). and 4 (12%). ▪ (↓) MCHb in ♀ at week 14 (2%). ▪ (↑) PLT in ♂ at week 14 (15%) and 4 (28%), and in ♀ at week 14 (11%), and on day 5 (20%). ▪ (↑) Leukocytes in ♀ at week 14 (40%). ▪ (↑) Lymphocytes in ♀ at week 14 (38%). Clinical biochemistry: ▪ (↑) creatinine in ♀ at week 14 (18%), 4 (14%) and on day 5 (16%).	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
		▪ (↓) total protein in ♂ at week 14 (5%) and on day 5 (4%), and in ♀ at week 14 (10%), and 4 (7%). ▪ (↓) albumin in ♂ on day 5 (12%), and in ♀ at week 14 (9%). ▪ (↑) alanine aminotransferase (ALT) in ♂ at week 14 (47%) and on day 5 (40%), and in ♀ at week 4 (32%) and on day 5 (42%). ▪ (↓) alkaline phosphatase (ALP) in ♂ at week 14 (22%), and in ♀ at week 14 (21%) and 4 (13%). ▪ (↑) Sorbitol dehydrogenase in ♂ at week 14 (50%, ncdr), and in ♀ at week 4 (20%). ▪ (↑) bile acids in ♂ at week 14 (107%), 4 (40%) and on day 5 (80%), and in ♀ at week 14 (122%) and 4 (135%). <u>Organ weights</u> ▪ (↓) total bw in ♂ (8%) and in ♀ (8%). *Right kidney* ▪ (↑) rel wt in ♂ (19%) and in ♀ (18%). *Liver* ▪ (↑) abs wt (17%) and rel wt in ♂ (28%) and abs wt (27%) and rel wt (38%) in ♀. *Lung* ▪ (↑) rel wt (10%) in ♂, and (↑) rel wt (10%) in ♀. *Spleen* ▪ (↑) rel wt (9%, ndr) in ♂; and (↑) abs wt (15%; ndr) in ♀. *Thymus* ▪ (↓) abs wt (26%) and (↓) rel wt (19%) in ♂. <u>Histopathology</u> *Liver* ▪ (↑) cytologic alteration in ♂ (100%). ▪ (↑) cytomegaly in ♂ (100%) and ♀ (90%). ▪ (↑) hyperplasia on bile ducts in ♂ (67%). *Glandular stomach* ▪ (↑) Atrophy in ♂ (78%) and ♀ (90%). ▪ (↑) Chronic inflammation in ♂ (100%) and ♀ (100%). *Adrenal cortex* ▪ (↑) Hypertrophy in ♂ (100%). *Submaxillary salivary gland* ▪ (↑) cytoplasmic alteration in ♂ (100%) and ♀ (100%). *Uterus* ▪ (↑) atrophy (40%). **100 mg/kg bw/day** <u>Bodyweights</u> ▪ (↓) bw in ♀ at week 14 (7%). ▪ (↓) bwg in ♀ at week 14 (13%). <u>Haematology:</u> ▪ (↑) Erythrocytes in ♂ (3%) at week 4. ▪ (↑) Reticulocytes in ♀ at week 4 (100%; ncdr). ▪ (↑) PLT in ♂ at week 14 (5%) and 4 (13%), and in ♀ at week 14 (8%), and on day 5 (9%). ▪ (↑) Leukocytes in ♂ at week 14 (31%, ndr). <u>Clinical biochemistry:</u> ▪ (↑) creatinine in ♀ at week 4 (8%) and on day 5 (12%). ▪ (↓) total protein in ♀ at week 14 (6%). ▪ (↑) alanine aminotransferase (ALT) in ♂ at week 14 (28%), and in ♀ at week 4 (9%) and on day 5 (14%). ▪ (↓) alkaline phosphatase (ALP) in ♂ at week 14 (10%). ▪ (↑) Sorbitol dehydrogenase in ♀ at week 4 (20%).	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
		Organ weights ▪ (↓) total BW in ♀ (9%). *Right kidney* ▪ (↑) rel wt in ♂ (8%) and in ♀ (13%). *Liver* ▪ (↑) abs wt (13%) and rel wt in ♂ (17%) and rel wt (12%) in ♀. *Thymus* ▪ (↓) abs wt (17%) and (↓) rel wt (15%) in ♂. Histopathology *Glandular stomach* ▪ (↑) Chronic inflammation in ♀ (60%). *Adrenal cortex* ▪ (↑) Hypertrophy in ♂ (40%). *Submaxillary salivary gland* ▪ (↑) cytoplasmic alteration in ♂ (100%) and ♀ (100%). **30 mg/kg bw/day** Bodyweights ▪ (↓) bw in ♀ at week 14 (5%). ▪ (↓) bwg in ♀ at week 14 (10%). Haematology: ▪ (↑) PLT in ♂ at week 4 (6%). ▪ (↑) Lymphocytes in ♂ at week 4 (38%). Organ weights ▪ (↓) total bw in ♀ (6%). *Right kidney* ▪ (↑) rel wt in ♂ (7%) and in ♀ (9%). *Liver* ▪ (↑) rel wt (9%) in ♂. *Thymus* ▪ (↓) abs wt (12%) and (↓) rel wt (12%) in ♂. Histopathology *Submaxillary salivary gland* ▪ (↑) cytoplasmic alteration in ♀ (70%). **10 mg/kg bw/day** Bodyweights ▪ (↓) bw in ♀ at week 14 (7%). ▪ (↓) bwg in ♀ at week 14 (10%). Haematology: ▪ (↑) PLT in ♀ on day 5 (9%; ncdr). Organ weights ▪ (↓) total bw in ♀ (9%). *Right kidney* ▪ (↑) rel wt in ♀ (7%). *Thymus* ▪ (↓) abs wt (16%, ncdr) and (↓) rel wt (16%, ncdr) in ♂. **-LOAEL**= 21.4 mg/kg bw/day (5-day/week dose scheme) **-$NOAEL_{toxicity}$**= 7.1 mg/kg bw/day (5-day/week dose scheme) -Critical effects at the LOAEL: ↑platelet concentration (thrombocytosis, males) and ↑ cytoplasmic alterations in	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
		submandibular salivary gland (females) Target tissue/organ: Liver, submaxillary gland, glandular stomach, uterus, testis	
14-week study in mice GLP: Yes Method: Non-stated. Mice strain: B6C3F1 Sex: 10 ♂/ 10♀/group. Deviations from current test guideline (OECD TG 408, 2018): -Food consumption was not measured. -Ophthalmological examination was not performed. - Haematology and clinical biochemistry analysis were not performed. -Thyroid gland, ovaries, uterus, brain, adrenals, prostate and pituitary gland were not weighted. - Circulating thyroid hormones (T4, T3, TSH) levels were not measured. - Animals died during study were not necropsied (organ weights). -Complete histopathology was only performed on negative controls, 300 and 1000 mg/ kg bw/day dose groups. **Study acceptable**	Test substance: **Methyleugenol** Batch No.: 8334801, Purity: 99% Vehicle: 0.5% methylcellulose Oral (gavage) **Doses:** Males/females: 0, 30, 100, 300 and 1000 mg/kg bw/day. 14-week exposure (5-day/week scheme administration).	Mortality: All mice of top dose group, except one male, died before the end of the study. Additionally, one male and one female of 300 mg/kg bw/day and 10 mg/kg bw/day dose groups died during week 3 and 12, respectively. Clinical signs: Generalized morbidity was observed in animals of top dose groups. **1000 mg/kg bw/day** Bodyweights ▪ (↓) bw in ♂ at week 14 (18%). ▪ (↓) bwg in ♂ at week 14 (44%). Histopathology *Liver* ▪ (↑) cytologic alteration in ♂ (50%) and ♀ (40%). ▪ (↑) necrosis in ♂ (50%) and ♀ (40%). ▪ (↑) hyperplasia on bile ducts in ♂ (50%) and ♀ (40%). ▪ (↑) inflammation in ♂ (40%). *Nose* ▪ (↑) Epithelial cell degeneration in ♂ (50%; ndr). **300 mg/kg bw/day** Bodyweights ▪ (↓) bwg in ♂ at week 14 (24%). ▪ (↓) bwg in ♀ at week 14 (24%). Organ weights ▪ (↓) total bw in ♀ (8%). *Heart* ▪ (↓) abs wt (15%) in ♀. *Liver* ▪ (↑) abs wt (20%) and rel wt (28%) in ♂, and abs wt (14%) and rel wt (23%) in ♀. *Thymus* ▪ (↓) abs wt (18%) in ♂, and (↓) abs wt (27%) and rel wt (21%) in ♀. Histopathology *Liver* ▪ (↑) cytologic alteration in ♀ (100%). ▪ (↑) necrosis in ♀ (100%). ▪ (↑) hyperplasia on bile ducts in ♀ (100%). ▪ (↑) inflammation in ♀ (100%). *Glandular stomach* ▪ (↑) atrophy in ♀ (100%). ▪ (↑) degeneration in ♂ (90%, ncdr) and ♀ (100%; ncdr). ▪ (↑) edema in ♀ (60%). ▪ (↑) mitotic alteration in ♂ (100%, ncdr) and ♀ (100%). ▪ (↑) cystic glands in ♀ (70%; ndr). *Nose* ▪ (↑) Epithelial cell degeneration in ♀ (50%; ndr). **100 mg/kg bw/day** Organ weights	NTP (2000) (AS) B.6.8.1.1.2-02

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
		Liver ▪ (↑) abs wt (11%) and rel wt (11%) in ♂. *Thymus* ▪ (↓) abs wt (18%) and rel wt (17%) in ♀. Histopathology *Glandular stomach* ▪ (↑) mitotic alteration in ♀ (50%). Reproductive parameters ▪ (↓) epididymal spermatozoal (34%). **30 mg/kg bw/day** Organ weights *Liver* ▪ (↑) abs wt (14%) and rel wt (15%) in ♂. *Testis* ▪ (↓) left cauda epididymis wt (38%; ndr). ▪ (↓) left epididymis wt (35%; ndr). ▪ (↓) left epididymis wt (13%; ndr). Histopathology *Glandular stomach* ▪ (↑) degeneration in ♀ (40%). ▪ (↑) mitotic alteration in ♀ (40%). **10 mg/kg bw/day** Organ weights *Liver* ▪ (↑) rel wt (9%) in ♂. *Testis* ▪ (↓) left cauda epididymis wt (30%; ndr). ▪ (↓) left epididymis wt (26%; ndr). ▪ (↓) left epididymis wt (12%; ndr). **-LOAEL**= 21.4 mg/kg bw/day (5-day/week dose scheme) **-NOAEL$_{toxicity}$**= 7.1 mg/kg bw/day (5-day/week dose scheme) -Critical effects at the LOAEL: ↑ histopathological alterations in glandular stomach. Target tissue/organ: Liver, glandular stomach	
14-week toxicity and cell proliferation study in rats and mice GLP: No Method: Non-stated. Rodents strain: F344/N rats B6C3F1 mice Sex: 10 ♀ rats/10 ♂ mice/group. Deviations from current test guideline	Test substance: **Methyleugenol** Oral (gavage) **Doses:** Rat females (30/90 days): 0, 37, 75, 150, 300 and 1000 mg/kg bw/day. Mice males (30/90 days): 0, 9, 1805, 37, 75, 150, and 300. 30 or 90 day	**Gastric pH and gastrin levels:** **1000 mg/kg bw/day** *Rats* 30 days ▪ (↑) serum gastrin (874%) and pH (191%). 90 days ▪ (↑) serum gastrin (1134%) and pH (73%). **300 mg/kg bw/day** *Rats* 90 days ▪ (↑) serum gastrin (898%) and pH (41%).	Abdo, K.M. *et al.* (2001) (AS) B.6.8.1.1.2-03

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
Not applicable, published study **Supportive only**	exposure	*Mice* 30 days ▪ (↑) serum gastrin (214%). **<u>Cell proliferation:</u>** **1000 mg/kg bw/day** *Rats* 30 days ▪ (↑) fundic glands (6000%), liver (1781%), pylorus (23%; ndr), forestomach (286%; ncdr), gastric pits (67%; ncdr). **300 mg/kg bw/day** *Rats* 30 days ▪ (↑) fundic glands (5300%), liver (1019%), forestomach (71%; ncdr), gastric pits (69%; ncdr). *Mice* 30 days ▪ (↑) fundic glands (500%; ndr) **150 mg/kg bw/day** *Rats* 30 days ▪ (↑) fundic glands (1000%), liver (195%), and gastric pits (63%; ncdr). *Rats* 90 days ▪ (↑) fundic glands (525%) and liver (376%). **75 mg/kg bw/day** *Rats* 30 days ▪ (↑) forestomach (64%). *Rats* 90 days ▪ (↑) fundic glands (100%). *Mice* 90 days ▪ (↑) fundic glands (1400%). **37 mg/kg bw/day** *Rats* 90 days ▪ (↑) fundic glands (100%). *Mice* 90 days ▪ (↑) fundic glands (500%). **18.5 mg/kg bw/day** *Mice* 90 days ▪ (↑) fundic glands (500%).	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
		9 mg/kg bw/day *Mice* 90 days (↑) fundic glands (200%).	
Genetic toxicity			
Bacterial gene mutation (Ames test) Comparable to OECD TG 471 (1983) Deviations from the current OECD TG 471 (2020): Only four strains tested, TA102 or *E.Coli* WP2 uvrA not tested, very limited level of reporting, additional indicator of S9 mix efficacy of 2-aminoanthracene not used and no historical control data. GLP: Yes **Supporting information**	Methyleugenol Purity 99% Batch No. 8334801 Solvent : not specified *S. typhimurium* TA1535, TA1537, TA98 and TA100 Rat/Hamster S9 Six concentrations tested (0, 3, 10, 33, 100, 333 and 666 µg/plate) Maximum dose 666 µg/plate	**Negative ± S9**	NTP (2000) (AS) B.6.8.1.1.3-01
***In vitro* Comet assay** No guidance GLP: No **Supporting information**	Methyleugenol Purity: not stated Batch no.: not stated Concentrations: 0. 5, 10, 25, 50, 75 and 100 µM Solvent: DMSO V79 chinese hamster lung fibroblasts Positive control: menadione Incubation time: 1h or 24 h	**Positive** Induction of DNA strand breaks at concentrations > 10 µM after 1h incubation	Groh, I.A.M. *et al.* (2012) (AS) B.6.8.1.1.3-03
Mammalian cell micronucleus assay Comparable to OECD TG 487	Methyleugenol Purity: not stated Batch no.: not stated Concentrations: 0. 5,	**Negative –S9**	Groh, I.A.M. *et al.* (2012) (AS) B.6.8.1.1.3-04

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
Deviations from the current OECD TG 487 (2016): Test item not characterised, dose selection not explained, no metabolic activation experiment, no individual data provided, very limited reporting, no historical data provided GLP: No **Supporting information**	10, 25, 50 and 100 µM V79 chinese hamster lung fibroblasts Treatment time: 1h or 24h Solvent: DMSO No metabolic activation Positive control: MMC		
HPRT forward mutation assay No guideline stated Deviations from the current OECD TG 476 (2016): Test item not characterised, dose selection not explained, longer treatment time, no metabolic activation experiment, no individual data provided, very limited reporting, no historical data provided GLP: No **Supporting information**	Methyleugenol Purity: not stated Batch no.: not stated Concentrations: 0. 5, 10, 25, 50 and 100 µM V79 chinese hamster lung fibroblasts Treatment time: 1h or 24h Solvent: DMSO No metabolic activation Positive control: MNNG	**Negative –S9**	Groh, I.A.M. *et al.* (2012) (AS) B.6.8.1.1.3-05
Mammalian cell chromosome aberration test Deviations from current OECD TG 473 (2016): Longer (-S9) and shorter (+S9) treatment times, only 200 metaphases scored, limited level of reporting (no individual data, only percentage of cells with aberrations), no	Methyleugenol Purity 99% Batch No. 8334801 Chinese hamster ovary cells (CHO) Solvent : DMSO Sprague-Dawley rat liver S9 Concentrations : 0, 50, 108, 233 and 500 µg/mL	**Negative - S9 (up to 233 µg/mL)** **Equivocal +S9 (up to 233 µg/mL)**	NTP (2000) (AS) B.6.8.1.1.3-06

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
historical control data GLP: Yes **Supporting information**	Treatment time : 14.5 h without metabolic activation, 2h with metabolic activation Positive controls : MMC (-S9), cyclophosphamide (+S9)		
Mammalian cell DNA damage (SCE) No comparable guideline **Supporting information**	Methyleugenol Purity 99% Batch No. 8334801 Chinese hamster ovary cells (CHO) Solvent : DMSO Sprague-Dawley rat liver S9 Concentrations : 0, 5, 17, 50 and 167 µg/mL (-S9) ; 0, 17, 50, 167, 250 and 500 µg/mL Positive controls : MMC (-S9), cyclophosphamide (+S9)	**Negative –S9** **Positive +S9**	NTP (2000) (AS) B.6.8.1.1.3-07
***In vitro* UDS assay** No comparable guideline GLP: No **Supporting information**	Methyleugenol Purity 99% F344 rats (♂) and B6C3F1 (♀) mice hepatocytes Incubation time : 18h Solvent : DMSO	**Positive**	Burkey, J.L. *et al.* (2000) (AS) B.6.8.1.1.3-08
***In vivo* Micronucleus assay** Comparable to OECD TG 474 (1997) Deviations from current OECD TG 474 (2016): no positive control group and no historical control data provided.	Methyleugenol Purity 99% Batch No. 8334801 B6C3F1 mice Groups of 10 ♀ and 10 ♂ Vehicle : 0.5 % methylcellulose	**Negative**	NTP (2000) (AS) B.6.8.1.1.3-09

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
- NOAEL/LOAEL
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr><td>GLP: Yes

Study acceptable</td><td>Route : oral gavage

Dose : 0, 10, 30, 100, 300, 1000 mg/kg bw

5 days per week for 14 wks treatment</td><td></td><td></td></tr>
<tr><td>Comet assay in vivo

Pre-guidance

Deviations from OECD TG 489 (2016): purity of test item not reported, lower number of animals used than recommended by guidance, limited level of reporting, number of total cells per organ is less than 150 and no historical data provided

GLP: No

Supporting information</td><td>Methyleugenol

Purity: not stated

Fischer 344 rats (♂)

Single oral (gavage) doses of 400 and 1000 mg/kg bw

Vehicle : 0.5% aqueous methylcellulose

Positive control: MMS

Sampling at 3h and 24h (100 cells) for the main experiment

Sampling at 1, 3, 6 and 8h (100 cells) for time-course experiment</td><td>Comet assay:
Negative (liver, lung, bone marrow, bladder and kidney)

Time-course experiment:
Negative in all tissues at 3 and 24h sampling time.

Positive in bone marrow at 8h sampling time.</td><td>Ding, W. et al. (2011) (AS) B.6.8.1.1.3-10</td></tr>
<tr><td colspan="4">Long-term/chronic toxicity</td></tr>
<tr><td>2-Year study in rats

GLP: Yes

<u>Method</u>: Non-stated.

<u>Rat strain:</u> F344/N rats

<u>Sex</u>: 50-60 ♂/♀/group.

<u>Deviations from current test guideline (OECD TG 453, 2018):</u>

-Food consumption was not measured.

-Ophthalmological examination was not performed.

-Organ weights were not presented.

-Circulating thyroid hormones (T4, T3, TSH) levels were</td><td><u>Test substance:</u>
Methyleugenol

Vehicle: 0.5% methylcellulose

Batch No.: 9224705, Purity: 99%

Oral (gavage)

Doses:
<u>Males/females</u>: 0, 37, 75, 150 and 300* mg/kg bw/day.

105-week exposure (5-day/week administration schedule).

*300 mg/kg bw/day dose group received test substance during 52 weeks followed by the 0.5%</td><td><u>Mortality:</u> All 150 and 300 mg/kg bw/day male rats died before study termination, the presence of moribund animals and the number of natural deaths was higher than controls.

Survival at study termination on female 150 and 300 mg/kg bw/day dose groups was slightly lower than controls (22% and 27%, respectively, vs 37% in controls).

Survival in 2-year rat study

<table>
<tr><th></th><th colspan="5">Dose (mg/kg bw/day)</th></tr>
<tr><th></th><th>Control (vehicle)</th><th>37</th><th>75</th><th>150</th><th>300</th></tr>
<tr><td>Males</td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>Animals initially in study</td><td>60</td><td>50</td><td>50</td><td>50</td><td>60</td></tr>
<tr><td>6-Month interim evaluation (a)</td><td>5</td><td>0</td><td>0</td><td>0</td><td>5</td></tr>
<tr><td>12-Month interim evaluation (a)</td><td>5</td><td>0</td><td>0</td><td>0</td><td>5</td></tr>
</table></td><td>NTP (2000) (AS) B.6.8.1.1.4-01</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference

not measured.

- No haematology, urinalysis and clinical chemistry were performed.

-Statistical analysis not performed in bodyweight measurements and in some non-neoplastic incidences.

Study acceptable

methylcellulose vehicle only for the remaining 53 weeks of the study (stop exposure group).

Accidental deaths	0	1	3	1	1
Moribund	15	18	15	20	23
Natural deaths	15	15	17	29	26
Animals surviving to study termination	**20 (33%)**	**16 (32%)**	**15 (30%)**	**0**	**0**
Percent probability of survival at end of study (b)	40	33	32	0	0
Mean survival (days) (c)	678	659	646	615	538
Survival analysis (d)	**P<0.001**	**P=0.389**	**P=0.294**	**P<0.001**	**P<0.001**
Females					
Animals initially in study	**60**	**50**	**50**	**50**	**60**
6-Month interim evaluation	5	0	0	0	5
12-Month interim evaluation	5	0	0	0	5
Accidental deaths	0	0	1	1	0
Moribund	17	16	14	26	25
Natural deaths	11	9	13	12	9
Animals surviving to study termination	**22 (37%)**	**25 (50%)**	**22 (44%)**	**11 (22%)**	**16 (27%)**
Percent probability of survival at end of study (b)	44	50	45	23	32
Mean survival (days) (c)	659	672	675	647	638
Survival analysis (d)	**P=0.015**	**P=0.640 N**	**P=0.807 N**	**P=0.053**	**P=0.343**

(a) Censored from survival analyses.
(b) Kaplan-Meier determinations.
(c) Mean of all deaths (uncensored, censored, and terminal sacrifice).
*(d) The result of the life table trend test (Tarone, 1975) is in the vehicle control column [the 300 mg/kg (stop-exposure) group was excluded from the trend test], and the results of the life table pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns. A lower mortality in a dose group is indicated by **N**.*

Clinical signs: Only moribund clinical signs were recorded and attributed to test substance administration.

300 mg/kg bw/day (stop exposure group)

Bodyweights
- (↓) bw in ♂ throughout week 37-101 (11-27%).

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
		▪ (↓) bw in ♀ throughout week 29-101 (13-26%). <u>Histopathology</u> Neoplastic changes: *Liver* ▪ (↑) hepatocellular adenoma in ♂ (64%; 32/50 animals vs 10%, 5/50 in controls). ▪ (↑) hepatocellular adenoma in ♀ (86%; 43/50 animals vs 2%, 1/50 in controls). ▪ (↑) hepatocellular carcinoma in ♂ (72%; 36/50 animals vs 4%, 2/50 in controls). ▪ (↑) hepatocellular carcinoma in ♀ (44%; 22/50 animals vs 0% in controls). ▪ (↑) hepatocholangioma in ♂ (12%; 6/50 animals vs 0% in controls). ▪ (↑) hepatocholangioma in ♀ (16%; 8/50 animals vs 0% in controls). ▪ (↑) hepatocholangiocarcinoma in ♂ (14%; 7/50 animals vs 0% in controls). ▪ (↑) hepatocholangiocarcinoma in ♀ (18%; 9/50 animals vs 0% in controls). *Glandular stomach* ▪ (↑) benign neuroendocrine tumour in ♀ (10%; 5/50 animals vs 0% in controls; ndr). ▪ (↑) malignant neuroendocrine tumour in ♀ (72%; 36/50 animals vs 0% in controls). *Forestomach* ▪ (↑) squamous cell carcinoma in ♀ (2%; 1/50 animals vs 0% in controls; ns). ▪ (↑) squamous cell carcinoma or papilloma (combined) in ♀ (2%; 1/50 animals vs 0% in controls; ndr; ns). *Kidney* ▪ (↑) renal tubule adenoma in ♂ (40%; 20/50 animals vs 8%, 4/50 in controls; ncdr). *Mesothelium* ▪ (↑) malignant mesothelioma in ♂ (10%; 5/50 animals vs 2%, 1/50 in controls; ndr). Non-neoplastic changes: *Liver* ▪ (↑) eosinophilic foci in ♂ (78%; 39/50 animals vs 22%, 11/50 in controls). ▪ (↑) eosinophilic foci in ♀ (74%; 37/50 animals vs 20%, 10/50 in controls). ▪ (↑) hepatocyte hypertrophy in ♂ (52%; 26/50 animals vs 0%, in controls). ▪ (↑) hepatocyte hypertrophy in ♀ (62%; 31/50 animals vs 2%, 1/50 in controls). ▪ (↑) oval cell hyperplasia in ♂ (54%; 27/50 animals vs 28%, 14/50, in controls). ▪ (↑) oval cell hyperplasia in ♀ (68%; 34/50 animals vs 2%, 1/50 in controls). ▪ (↑) cystic degeneration in ♂ (81%; 41/50 animals vs 8%, 4/50, in controls). ▪ (↑) cystic degeneration in ♀ (58%; 29/50 animals vs 0% in controls). ▪ (↑) bile duct hyperplasia in ♀ (60%; 30/50 animals vs 22%, 11/50 in controls). *Glandular stomach* ▪ (↑) atrophy in ♂ (58%; 29/50 animals vs 0% in controls). ▪ (↑) atrophy in ♀ (66%; 33/50 animals vs 0% in controls). ▪ (↑) neuroendocrine cell hyperplasia in ♂ (16%; 8/50 animals	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
		vs 0% in controls). *Kidney* ▪ (↑) renal tubule hyperplasia in ♂ (44%; 22/50 animals vs 26%, 13/50 in controls). ▪ (↑) nephropathy in ♀ (90%; 45/50 animals vs 66%, 33/50 in controls). *Bone marrow (statistics not performed)* ▪ (↑) bone marrow hyperplasia in ♀ (50%; 25/50 animals vs 8%, 4/50 in controls). *Salivary gland (statistics not performed)* ▪ (↑) cytoplasmic alteration in ♂ (100%; 48/48 animals vs 8%, 4/50 in controls). ▪ (↑) cytoplasmic alteration in ♀ (98%; 49/50 animals vs 2%, 1/50 in controls). *Spleen (statistics not performed)* ▪ (↑) splenic fibrosis in ♀ (30%; 15/50 animals vs 6%, 3/50 in controls). **150 mg/kg bw/day** <u>Bodyweights</u> ▪ (↓) bw in ♂ throughout week 53-101 (11-23%). ▪ (↓) bw in ♀ throughout week 33-101 (11-26%). <u>Histopathology</u> Neoplastic changes: *Liver* ▪ (↑) hepatocellular adenoma in ♂ (76%; 38/50 animals vs 10%, 5/50 in controls). ▪ (↑) hepatocellular adenoma in ♀ (67%; 33/49 animals vs 2%, 1/50 in controls). ▪ (↑) hepatocellular carcinoma in ♂ (50%; 25/50 animals vs 4%, 2/50 in controls). ▪ (↑) hepatocellular carcinoma in ♀ (16%; 8/49 animals vs 0% in controls). *Glandular stomach* ▪ (↑) malignant neuroendocrine tumour in ♂ (8%; 4/50 animals vs 0% in controls; ndr). ▪ (↑) malignant neuroendocrine tumour in ♀ (52%; 26/50 animals vs 0% in controls). *Forestomach* ▪ (↑) squamous cell carcinoma in ♀ (2%; 1/50 animals vs 0% in controls; ns). *Kidney* ▪ (↑) renal tubule adenoma in ♂ (26%; 13/50 animals vs 8%, 4/50 in controls; ncdr). *Skin* ▪ (↑) subcutaneous fibroma or fibrosarcoma in ♂ (16%; 8/50 animals vs 2%, 1/50 in controls; ndr). *Mammary gland* ▪ (↑) mammary gland fibroadenomas in ♂ (26%; 13/50 animals vs 10%, 5/50 in controls; ndr). Non-neoplastic changes: *Liver* ▪ (↑) eosinophilic foci in ♂ (94%; 47/50 animals vs 22%, 11/50 in controls). ▪ (↑) eosinophilic foci in ♀ (63%; 31/49 animals vs 20%, 10/50 in controls). ▪ (↑) hepatocyte hypertrophy in ♂ (60%; 30/50 animals vs 0%, in controls). ▪ (↑) hepatocyte hypertrophy in ♀ (53%; 26/49 animals vs 2%, 1/50 in controls).	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
		▪ (↑) oval cell hyperplasia in ♂ (68%; 34/50 animals vs 28%, 14/50, in controls). ▪ (↑) oval cell hyperplasia in ♀ (71%; 35/49 animals vs 2%, 1/50 in controls). ▪ (↑) cystic degeneration in ♂ (76%; 38/50 animals vs 8%, 4/50, in controls). ▪ (↑) bile duct hyperplasia in ♀ (45%; 22/49 animals vs 22%, 11/50 in controls). ▪ (↑) mixed cell foci in ♂ (16%; 8/50 animals vs 2%, 1/50 in controls). *Glandular stomach* ▪ (↑) atrophy in ♂ (74%; 37/50 animals vs 0% in controls). ▪ (↑) atrophy in ♀ (78%; 39/50 animals vs 0% in controls, ndr). ▪ (↑) neuroendocrine cell hyperplasia in ♂ (16%; 8/50 animals vs 0% in controls). ▪ (↑) neuroendocrine cell hyperplasia in ♀ (18%; 9/50 animals vs 0% in controls; ndr). *Kidney* ▪ (↑) renal tubule hyperplasia in ♂ (42%; 21/50 animals vs 26%, 13/50 in controls). *Bone marrow (statistics not performed)* ▪ (↑) bone marrow hyperplasia in ♀ (40%; 20/50 animals vs 8%, 4/50 in controls). *Salivary gland (statistics not performed)* ▪ (↑) cytoplasmic alteration in ♂ (100%; 48/48 animals vs 8%, 4/50 in controls). ▪ (↑) cytoplasmic alteration in ♀ (100%; 49/49 animals vs 2%, 1/50 in controls). *Spleen (statistics not performed)* ▪ (↑) splenic fibrosis in ♀ (24%; 12/49 animals vs 6%, 3/50 in controls). **75 mg/kg bw/day** <u>Bodyweights</u> ▪ (↓) bw in ♂ throughout week 93-97 (11-12%). ▪ (↓) bw in ♀ throughout week 45-101 (11-21%). <u>Histopathology</u> Neoplastic changes: *Liver* ▪ (↑) hepatocellular adenoma in ♂ (46%; 23/50 animals vs 10%, 5/50 in controls). ▪ (↑) hepatocellular adenoma in ♀ (22%; 11/49 animals vs 2%, 1/50 in controls). ▪ (↑) hepatocellular carcinoma in ♂ (28%; 14/50 animals vs 4%, 2/50 in controls). *Glandular stomach* ▪ (↑) malignant neuroendocrine tumour in ♀ (24%; 12/50 animals vs 0% in controls). *Kidney* ▪ (↑) renal tubule adenoma in ♂ (34%; 17/50 animals vs 8%, 4/50 in controls; ncdr). *Skin* ▪ (↑) subcutaneous fibroma in ♂ (16%; 8/50 animals vs 2%, 1/50 in controls; ndr). Non-neoplastic changes: *Liver* ▪ (↑) eosinophilic foci in ♂ (86%; 43/50 animals vs 22%, 11/50 in controls). ▪ (↑) eosinophilic foci in ♀ (55%; 27/49 animals vs 20%,	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
		10/50 in controls). ▪ (↑) hepatocyte hypertrophy in ♂ (50%; 25/50 animals vs 0%, in controls). ▪ (↑) hepatocyte hypertrophy in ♀ (33%; 16/49 animals vs 2%, 1/50 in controls). ▪ (↑) oval cell hyperplasia in ♂ (48%; 24/50 animals vs 28%, 14/50, in controls). ▪ (↑) oval cell hyperplasia in ♀ (39%; 19/49 animals vs 2%, 1/50 in controls). ▪ (↑) cystic degeneration in ♂ (50%; 25/50 animals vs 8%, 4/50, in controls). ▪ (↑) mixed cell foci in ♀ (39%; 19/49 animals vs 12%, 6/50 in controls). *Glandular stomach* ▪ (↑) atrophy in ♂ (64%; 32/50 animals vs 0% in controls). *Kidney* ▪ (↑) renal tubule hyperplasia in ♂ (40%; 20/50 animals vs 26%, 13/50 in controls). *Bone marrow (statistics not performed)* ▪ (↑) bone marrow hyperplasia in ♀ (22%; 11/49 animals vs 8%, 4/50 in controls; ncdr). *Salivary gland (statistics not performed)* ▪ (↑) cytoplasmic alteration in ♂ (98%; 49/50 animals vs 8%, 4/50 in controls). ▪ (↑) cytoplasmic alteration in ♀ (98%; 49/50 animals vs 2%, 1/50 in controls). **37 mg/kg bw/day** <u>Histopathology</u> Neoplastic changes: *Liver* ▪ (↑) hepatocellular adenoma in ♂ (24%; 12/50 animals vs 10%, 5/50 in controls). ▪ (↑) hepatocellular adenoma in ♀ (16%; 8/50 animals vs 2%, 1/50 in controls). Non-neoplastic changes: *Liver* ▪ (↑) eosinophilic foci in ♂ (56%; 28/50 animals vs 22%, 11/50 in controls). ▪ (↑) eosinophilic foci in ♀ (40%; 20/50 animals vs 20%, 10/50 in controls). ▪ (↑) hepatocyte hypertrophy in ♂ (26%; 13/50 animals vs 0%, in controls). ▪ (↑) hepatocyte hypertrophy in ♀ (26%; 13/50 animals vs 2%, 1/50 in controls). ▪ (↑) oval cell hyperplasia in ♀ (30%; 15/50 animals vs 2%, 1/50 in controls). *Glandular stomach* ▪ (↑) atrophy in ♂ (28%; 14/50 animals vs 0% in controls). *Bone marrow (statistics not performed)* ▪ (↑) bone marrow hyperplasia in ♀ (30%; 15/50 animals vs 8%, 4/50 in controls; ncdr). *Salivary gland (statistics not performed)* ▪ (↑) cytoplasmic alteration in ♂ (100%; 50/50 animals vs 8%, 4/50 in controls). ▪ (↑) cytoplasmic alteration in ♀ (96%; 48/50 animals vs 2%, 1/50 in controls). **-LOAEL**= 26.4 mg/kg bw/day (5-day/week dose scheme) **-NOAEL$_{toxicity}$**= -	

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
- NOAEL/LOAEL
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr><td></td><td></td><td>-Critical effects at the LOAEL: ↑ neoplastic and non-neoplastic incidences in liver, glandular stomach, kidney and spleen.

Target tissue/organ: Liver, glandular stomach and kidney.</td><td></td></tr>
<tr><td>2-Year study in mice
GLP: Yes
Method: Non-stated.
Mice strain: B6C3F1 mice
Sex: 50 ♂/♀/group.
Deviations from current test guideline (OECD TG 453, 2018):
-Food consumption was not measured.
-Ophthalmological examination was not performed.
-Organ weights were not presented.
-Circulating thyroid hormones (T4, T3, TSH) levels were not measured.
- No haematology, urinalysis and clinical chemistry were performed.
-Statistical analysis not performed in bodyweight measurements and in some non-neoplastic incidences.
Study acceptable</td><td>Test substance:
Methyleugenol

Vehicle: 0.5% methylcellulose

Batch No.: 9224705, Purity: 99%

Oral (gavage)

Doses:
Males/females: 0, 37, 75 and 150 mg/kg bw/day.

105-week exposure (5-day/week administration schedule).</td><td>Mortality: The survival rates (animals surviving at study termination and mean survival days) of male treated groups were similar to control group. By contrast, in female dosed groups, the animals surviving at study termination were reduced compared with controls, particularly at top dose group (36%, 36% and 4% for low, mid and high dose groups, respectively), compared with controls (62%). Additionally, an increase of the number of natural deaths, together with a decrease in the mean survival (days), were noted in female dose groups, compared with controls.

Survival in 2-year mice study
<table><tr><th></th><th colspan="4">Dose (mg/kg bw/day)</th></tr><tr><th></th><th>Control (vehicle)</th><th>37</th><th>75</th><th>150</th></tr><tr><td>Males</td><td></td><td></td><td></td><td></td></tr><tr><td>Animals initially in study</td><td>50</td><td>50</td><td>50</td><td>50</td></tr><tr><td>Accidental deaths (a)</td><td>0</td><td>0</td><td>1</td><td>2</td></tr><tr><td>Missing (a)</td><td>1</td><td>0</td><td>0</td><td>0</td></tr><tr><td>Moribund</td><td>5</td><td>6</td><td>5</td><td>9</td></tr><tr><td>Natural deaths</td><td>6</td><td>8</td><td>7</td><td>4</td></tr><tr><td>Animals surviving to study termination</td><td>38 (76%)</td><td>36 (72%)</td><td>37 (74%)</td><td>35 (70%)</td></tr><tr><td>Percent probability of survival at end of study (b)</td><td>78</td><td>72</td><td>76</td><td>73</td></tr><tr><td>Mean survival (days) (c)</td><td>690</td><td>705</td><td>679</td><td>686</td></tr><tr><td>Survival analysis (d)</td><td>P=0.815</td><td>P=0.825</td><td>P=1.000</td><td>P=0.829</td></tr><tr><td>Females</td><td></td><td></td><td></td><td></td></tr><tr><td>Animals initially in study</td><td>50</td><td>50</td><td>50</td><td>50</td></tr><tr><td>Accidental deaths (a)</td><td>5</td><td>0</td><td>0</td><td>0</td></tr><tr><td>Missing (a)</td><td>0</td><td>0</td><td>0</td><td>0</td></tr><tr><td>Moribund</td><td>5</td><td>7</td><td>8</td><td>13</td></tr><tr><td>Natural deaths</td><td>14</td><td>24</td><td>23</td><td>35</td></tr></table></td><td>NTP (2000) (AS) B.6.8.1.1.4-02</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference

Animals surviving to study termination	**31 (62%)**	**18 (36%)**	**18 (e) (36%)**	**2 (4%)**
Percent probability of survival at end of study (b)	62	38	37	4
Mean survival (days) (c)	696	662	664	637
Survival analysis (d)	**P<0.001**	**P=0.009**	**P=0..013**	**P<0.001**

(a) Censored from survival analyses.
(b) Kaplan-Meier determinations.
(c) Mean of all deaths (uncensored, censored, and terminal sacrifice).
(d) The result of the life table trend test (Tarone, 1975) is in the vehicle control column, and the results of the life table pairwise comparisons (Cox, 1972) with the vehicle controls are in the dosed group columns.

Clinical signs: Only moribund clinical signs were recorded and attributed to test substance administration.

150 mg/kg bw/day

Bodyweights
- (↓) bw in ♂ in week 101 (13%).
- (↓) bw in ♀ throughout week 17-101 (17-46%).

Histopathology
Neoplastic changes:
Liver
- (↑) hepatocellular adenoma in ♂ (78%; 39/50 animals vs 53%, 26/49 in controls, ncdr).
- (↑) hepatocellular adenoma in ♀ (82%; 41/50 animals vs 40%, 20/50 in controls).
- (↑) hepatocellular carcinoma in ♀ (94%; 47/50 animals vs 14%, 7/50 in controls).
- (↑) hepatoblastoma in ♂ (6%, 3/50 animals vs 0% in controls, ns)
- (↑) hepatoblastoma in ♀ (30%, 15/50 animals vs 0% in controls).
- (↑) hepatocholangiocarcinoma in ♀ (4%, 2/50 animals vs 0% in controls, ns).

Glandular stomach
- (↑) carcinoma in ♂ (2%; 1/50 animals vs 0% in controls; ns).
- (↑) malignant neuroendocrine tumour in ♂ (4%; 2/50 animals vs 0% in controls, ns).

Non-neoplastic changes:
Liver
- (↑) eosinophilic foci in ♂ (38%; 19/50 animals vs 20%, 10/49 in controls, ncdr).
- (↑) hepatocyte hypertrophy in ♂ (92%; 46/50 animals vs 0%, in controls).
- (↑) hepatocyte hypertrophy in ♀ (26%; 23/50 animals vs 0% in controls, ncdr).
- (↑) oval cell hyperplasia in ♂ (92%; 46/50 animals vs 0% in controls).
- (↑) oval cell hyperplasia in ♀ (76%; 38/50 animals vs 0% in

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
		controls, ncdr). ▪ (↑) chronic inflammation in ♂ (56%; 28/50 animals vs 39%, 19/49 in controls). ▪ (↑) hemosiderin pigmentation in ♀ (38%; 19/50 animals vs 0% in controls, ncdr). ▪ (↑) bile duct hyperplasia in ♀ (18%; 9/50 animals vs 2%, 1/50 in controls, ncdr). ▪ (↑) hepatocyte necrosis in ♀ (34%; 17/50 animals vs 10%, 5/50 in controls). ▪ (↑) hematopoietic cell proliferation in ♀ (48%; 24/50 animals vs 8%, 4/50 in controls). *Glandular stomach* ▪ (↑) atrophy in ♂ (90%; 45/50 animals vs 0% in controls). ▪ (↑) atrophy in ♀ (22%; 10/45 animals vs 0% in controls). ▪ (↑) ectasia in ♂ (98%; 49/50 animals vs 27%, 13/49 in controls). ▪ (↑) ectasia in ♀ (84%; 38/45 animals vs 31%, 14/45 in controls). ▪ (↑) hyperplasia in ♂ (40%; 20/50 animals vs 0% in controls). *Bone marrow (statistics not performed)* ▪ (↑) bone marrow hyperplasia in ♂ (70%; 35/50 animals vs 29%, 14/49 in controls). ▪ (↑) bone marrow hyperplasia in ♀ (100%; 50/50 animals vs 36%, 18/50 in controls). **75 mg/kg bw/day** <u>Bodyweights</u> ▪ (↓) bw in ♂ throughout week 97-101 (12-16%). ▪ (↓) bw in ♀ throughout week 37-101 (12-45%). <u>Histopathology</u> Neoplastic changes: *Liver* ▪ (↑) hepatocellular adenoma in ♂ (76%; 38/50 animals vs 53%, 26/49 in controls, ncdr). ▪ (↑) hepatocellular adenoma in ♀ (94%; 46/49 animals vs 40%, 20/50 in controls). ▪ (↑) hepatocellular carcinoma in ♀ (96%; 47/49 animals vs 14%, 7/50 in controls). ▪ (↑) hepatoblastoma in ♂ (2%, 1/50 animals vs 0% in controls, ns) ▪ (↑) hepatoblastoma in ♀ (22%, 11/50 animals vs 0% in controls). Non-neoplastic changes: *Liver* ▪ (↑) eosinophilic foci in ♂ (50%; 25/50 animals vs 20%, 10/49 in controls, ncdr). ▪ (↑) hepatocyte hypertrophy in ♂ (14%; 7/50 animals vs 0%, in controls). ▪ (↑) hepatocyte hypertrophy in ♀ (14%; 7/49 animals vs 0% in controls, ncdr). ▪ (↑) oval cell hyperplasia in ♂ (54%; 27/50 animals vs 0% in controls). ▪ (↑) oval cell hyperplasia in ♀ (73%; 36/49 animals vs 0% in controls, ncdr). ▪ (↑) chronic inflammation in ♂ (56%; 28/50 animals vs 39%, 19/49 in controls). ▪ (↑) hemosiderin pigmentation in ♀ (49%; 24/49 animals vs 0% in controls, ncdr).	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
		▪ (↑) bile duct hyperplasia in ♀ (22%; 11/49 animals vs 2%, 1/50 in controls, ncdr). ▪ (↑) hepatocyte necrosis in ♀ (33%; 16/49 animals vs 10%, 5/50 in controls). ▪ (↑) hematopoietic cell proliferation in ♀ (47%; 23/49 animals vs 8%, 4/50 in controls). *Glandular stomach* ▪ (↑) atrophy in ♂ (71%; 35/49 animals vs 0% in controls). ▪ (↑) atrophy in ♀ (22%; 10/46 animals vs 0% in controls). ▪ (↑) ectasia in ♂ (82%; 40/49 animals vs 27%, 13/49 in controls). ▪ (↑) ectasia in ♀ (67%; 31/46 animals vs 31%, 14/45 in controls). ▪ (↑) hyperplasia in ♂ (31%; 15/49 animals vs 0% in controls). *Bone marrow (statistics not performed)* ▪ (↑) bone marrow hyperplasia in ♂ (66%; 33/50 animals vs 29%, 14/49 in controls). ▪ (↑) bone marrow hyperplasia in ♀ (92%; 46/48 animals vs 36%, 18/50 in controls). **37 mg/kg bw/day** <u>Bodyweights</u> ▪ (↓) bw in ♀ throughout week 65-101 (11-65%). <u>Histopathology</u> Neoplastic changes: ▪ (↑) hepatocellular adenoma in ♂ (86%; 43/50 animals vs 53%, 26/49 in controls, ncdr). ▪ (↑) hepatocellular adenoma in ♀ (96%; 48/50 animals vs 40%, 20/50 in controls). ▪ (↑) hepatocellular carcinoma in ♀ (74%; 37/50 animals vs 14%, 7/50 in controls). ▪ (↑) hepatoblastoma in ♀ (6%, 12/50 animals vs 0% in controls). Non-neoplastic changes: *Liver* ▪ (↑) eosinophilic foci in ♂ (40%; 20/50 animals vs 20%, 10/49 in controls, ncdr). ▪ (↑) hepatocyte hypertrophy in ♂ (2%; 1/50 animals vs 0%, in controls, ns). ▪ (↑) hepatocyte hypertrophy in ♀ (20%; 10/50 animals vs 0% in controls, ncdr). ▪ (↑) oval cell hyperplasia in ♂ (16%; 8/50 animals vs 0% in controls). ▪ (↑) oval cell hyperplasia in ♀ (92%; 46/50 animals vs 0% in controls, ncdr). ▪ (↑) chronic inflammation in ♂ (42%; 21/50 animals vs 39%, 19/49 in controls, ns). ▪ (↑) hemosiderin pigmentation in ♀ (22%; 11/50 animals vs 0% in controls, ncdr). ▪ (↑) hepatocyte necrosis in ♀ (18%; 9/50 animals vs 10%, 5/50 in controls, ns). ▪ (↑) hematopoietic cell proliferation in ♀ (28%; 14/50 animals vs 8%, 4/50 in controls). *Glandular stomach* ▪ (↑) atrophy in ♂ (6%; 3/48 animals vs 0% in controls, ns). ▪ (↑) ectasia in ♂ (52%; 25/48 animals vs 27%, 13/49 in controls). ▪ (↑) ectasia in ♀ (67%; 33/49 animals vs 31%, 14/45 in	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
		controls). ▪ (↑) hyperplasia in ♂ (2%; 1/48 animals vs 0% in controls, ns). *Bone marrow (statistics not performed)* ▪ (↑) bone marrow hyperplasia in ♂ (53%; 26/49 animals vs 29%, 14/49 in controls). ▪ (↑) bone marrow hyperplasia in ♀ (94%; 47/50 animals vs 36%, 18/50 in controls). **-LOAEL**= 26.4 mg/kg bw/day (5-day/week dose scheme) **-NOAEL$_{toxicity}$**= - -Critical effects at the LOAEL: ↑ neoplastic and non-neoplastic incidences in liver and glandular stomach. Target tissue/organ: Liver and glandular stomach.	
Summary report of 2-year study in rat and mice GLP: No Method: Non-stated. Rodents strain: F344/N rats B6C3F1 mice Sex: 50 rats/mice/sex/group Deviations from current test guideline Not applicable, published study **Supportive only**	Test substance: **Methyleugenol** Oral (gavage) **Doses:** 0, 37, 75, and 150, mg/kg bw/day. 105 weeks exposure	This study summarised the oral 2-year studies in rats and mice conducted with methyleugenol and reported by NTP (B.6.8.1.1.4-01 and B.6.8.1.1.4-02). In order to avoid duplications, the results were not assessed again (see B.6.8.1.1.4-01 and B.6.8.1.1.4-02).	Johnson. *et al.* (2000). (AS) B.6.8.1.1.4-03
Other toxicological studies of impurities			
Identification of DNA adducts on human liver samples GLP: No Method: ULPC-MS/MS (ultra-performance liquid chromatography tandem mass spectrometry). 30 subjects (18 ♂/12 ♀) Deviations from current test guideline Not applicable, published study **Supportive only**	Test substance: **Methyleugenol dietary exposure** Dosage and source not stated	-N_6-(*trans*-methylisoeugenol-3-yl)-2-deoxyadenosine(N_6-IE-dA), and N_2-(*trans*-methylisoeugenol-3-yl)-2′-deoxyguanosine (N_2-MIE-dG) adducts were detected in human liver samples using ULPC-MS/MS (ultra-performance liquid chromatography tandem mass spectrometry). -The detected ratio for N_2-MIE-dG and N_6-MIE-dA was 60:1. -The maximal and median levels of these adducts (N_2-MIE-dG and N_6-MIE-dA combined) were 37 and 13 per 108 nucleosides, corresponding to 4700 and 1700, respectively, adducts per diploid genome (6.6×10^9 base pairs).	Herrmann, K. *et al.* (2013) (AS) B.6.8.1.1.5-01
Identification of DNA adducts on human liver HepG2	Test substance: **Methyleugenol**	-Methyleugenol induced DNA adducts with a chromatographic pattern of two spots. The structures of the two main adducts were identified as derivatives of **N$_2$-(trans-propenylbenzene-30-yl)**	Zhou *et al.* (2007). (AS)

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
cells GLP: No Method: radioactivity. Deviations from current test guideline Not applicable, published study **Supportive only**	Dose tested: 0. 50, 150 and 450 µM.	**deoxyguanosine** (spot 1, major adduct) and **N_2-(allylbenzene-10-yl) deoxyguanosine** (spot 2, minor adduct) after exposure of HepG2 cells at 150 µM of methyleugenol. -The highest induction of DNA-adducts was obtained at 50 µM.	B.6.8.1.1.5-02
Correlation between liver carcinomas and methyleugenol DNA-adducts Rodent strain: F344/N male rats Method: Statistics regression Deviations from current test guideline Not applicable, published study **Supportive only**	Test substance: **Methyleugenol**	Liver tumor generation is correlated with methyleugenol DNA-adducts formation. -Threshold dose for adduct formation = $10^{19\,3}$ molecules of methyleugenol/kg/day. -Threshold dose for tumor formation = $10^{20\,1}$ molecules of methyleugenol/kg/day and 30 adducts/10^8 nucleotides.	Waddell, W.J. *et al.* (2004) (AS) B.6.8.1.1.5-03
Hepatocellular cancer and methyleugenol induction of DNA-adducts GLP: No Rodent strain: F344/N male rats Method: Not stated Deviations from current test guideline Not applicable, published study **Supportive only**	Test substance: **Methyleugenol** **Methyleugenol (MEG)**: Oral (gavage) **Phenobarbital (PB)** Oral (diet) Dose tested: MEG: 0, 27, 54 and 107 mg/kg bw/day PB: 500 ppm 40 weeks: 16 w (initiation phase) + 24 w (Promotion/ maintenance phase w/ or w/o PB)	Mortality No compound-related deaths occurred in the study. Clinical signs: No clinical signs were attributed to test substance administration. **107 mg/kg bw/day** Bodyweights ▪ (↓) bw in at week 8 (5%). Liver weights: ▪ (↑) rel wt (15%) at week 8. Cell proliferation: ▪ (↑) at week 8 (180%) and week 16 (148%). DNA adducts: ▪ (↑) in PB+ at week 40 (148200%). ▪ (↑) in PB- at week 40 (58333%). ▪ (↑) at week 16 (63370%). ▪ (↑) at week 8 (121525%). Histopathology (statistical analysis was not performed) *Liver* ▪ (↑) hepatocellular altered foci in PB+ at 40 weeks (100%; 13/13 animals vs 7.1%; 1/14 animals in controls). ▪ (↑) hepatocellular adenomas in PB+ at 40 weeks (77%; 10/13 animals vs 0%; 0/14 animals in controls). ▪ (↑) hepatocellular altered foci in PB- at 40 weeks (100%; 12/12 animals vs 0%; 0/12 animals in controls). ▪ (↑) hepatocellular adenomas in PB- at 40 weeks (100%; 12/12 animals vs 0%; 0/12 animals in controls). ▪ (↑) hepatocellular altered foci at 16 weeks (100%; 3/3	Williams, G.M. *et al.* (2012) (AS) B.6.8.1.1.5-04

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results - NOAEL/LOAEL - target tissue/organ - critical effects at the LOAEL	Reference
		animals vs 0%; 0/3 animals in controls). ▪ (↑) hepatocellular altered foci at 8 weeks (100%; 3/3 animals vs 0%; 0/3 animals in controls). **54 mg/kg bw/day** <u>Bodyweights</u> ▪ (↓) bw in at week 8 (3%). <u>Liver weights:</u> ▪ (↑) rel wt (12%) at week 8. <u>Cell proliferation</u>: ▪ (↑) at week 8 (111%) and week 16 (83%). <u>DNA adducts:</u> ▪ (↑) in PB+ at week 40 (69400%). ▪ (↑) in PB- at week 40 (29200%). ▪ (↑) at week 16 (41450%). ▪ (↑) at week 8 (90325%). <u>Histopathology (statistical analysis was not performed)</u> *Liver* ▪ (↑) hepatocellular altered foci in PB+ at 40 weeks (85.7%; 12/14 animals vs 7.1%, 1/14 animals in controls). ▪ (↑) hepatocellular adenomas in PB+ at 40 weeks (21.4%; 3/14 animals vs 0%; 0/14 animals in controls). ▪ (↑) hepatocellular altered foci in PB- at 40 weeks (91.7%; 11/12 animals vs 0%; 0/12 animals in controls). ▪ (↑) hepatocellular adenomas in PB- at 40 weeks (8.3%; 1/12 animals vs 0%; 0/12 animals in controls). ▪ (↑) hepatocellular altered foci at 16 weeks (66.7%; 2/3 animals vs 0%; 0/3 animals in controls). **27 mg/kg bw/day** <u>Bodyweights</u> ▪ (↓) bw in at week 8 (3%). <u>Cell proliferation</u>: ▪ (↑) at week 8 (105%) and week 16 (74%). <u>DNA adducts:</u> ▪ (↑) in PB+ at week 40 (41600%). ▪ (↑) in PB- at week 40 (25200%). ▪ (↑) at week 16 (28040%). ▪ (↑) at week 8 (34150%). <u>Histopathology (statistical analysis was not performed)</u> *Liver* ▪ (↑) hepatocellular altered foci in PB+ at 40 weeks (15.4%; 2/13 animals vs 7.1%, 1/14 animals in controls). ▪ (↑) hepatocellular altered foci in PB- at 40 weeks (8.3%, 1/12 animals vs 0%; 0/12 animals in controls). ▪ (↑) hepatocellular altered foci at 16 weeks (66.7%, 2/3 animals vs 0%; 0/3 animals in controls). **-LOAEL**= 27 mg/kg bw/day **-$NOAEL_{toxicity}$**= - -Critical effects at the LOAEL: ↑ DNA adducts, ↑ Hepatocellular altered foci.	

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
- NOAEL/LOAEL
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr>
<td>Bioactivation of methyleugenol

GLP: No

<u>Rodent strain:</u>
F344/N male rats

<u>Method:</u> Not stated

<u>Deviations from current test guideline</u>
Not applicable, published study
Supportive only</td>
<td><u>Test substance:</u>
Methyleugenol

Methyleugenol (MEG): intraperitoneal injection (i.p.)

<u>Dose tested</u>: MEG: 0, 10, 30, 100 and 300 mg/kg bw/day

<u>MEG:</u> 5 days:

<u>P450 inducers:</u>
-Sodium phenobarbital: 100 mg/kg bw/day; 3 days.
-Isoniazid: 100 mg/kg bw/day; 10 days.
-Dexamethasone: 80 mg/kg bw/day; 3 days.
Isosafrole: 150 mg/kg bw/day: 3 days.

<u>P450 inhibitors:</u>
-Troleandomycin (200 µM).
-Furafylline (25 and 100 µM).
-Cimetidine (50 µM)
- p-nitrophenol (50 and 200 µM)
-Diallylsulfide (200 µM and 1 mM).
-α-naphthoflavone (1 and 10 µM).
-Quinine (1 and 10 µM).
-Tolbutamide (200 µM, 500 µM and 1mM).</td>
<td>- Kinetics of 1 '-hydroxylation of methyleugenol formation in F344 rat liver microsomes yielded a non-linear Eadie-Hofstee plot.

-P450 isozymes induction and 1´- hydroxymethyleugenol formation

<table>
<tr><th></th><th></th><th colspan="2">1 'hydroxymethyleugenol formation</th></tr>
<tr><th>Inducer</th><th>Cyt- P450 content (nmol/mg protein)</th><th>(nmol/min /mg protein)</th><th>(nmol/min/nmol P450)</th></tr>
<tr><td>Saline control</td><td>0.70 ± 0.05</td><td>0.36 ± 0.03</td><td>0.51 ± 0.04</td></tr>
<tr><td>Tricaprylin control</td><td>0.73 ± 0.09</td><td>0.31 ± 0.02</td><td>0.42 ± 0.03</td></tr>
<tr><td>Phenobarbi _tone</td><td>1.82 ± 0.09* (↑160%)</td><td>2.11 ± 0.14* (↑486%)</td><td>1.16 ± 0.08* (↑127%)</td></tr>
<tr><td>Isoniazid</td><td>0.86 ± 0.07* (↑22%)</td><td>0.73 ± 0.09* (↑102%)</td><td>0.85 ± 0.10* (↑66%)</td></tr>
<tr><td>Dexamethas _one</td><td>1.33 ± 0.15* (↑90%)</td><td>2.03 ± 0.10* (↑463%)</td><td>1.52 ± 0.08* (↑198%)</td></tr>
<tr><td>Isosafrole</td><td>1.16 ± 0.04* (↑65%)</td><td>1.91 ± 0.26* (↑430%)</td><td>1.65 ± 0.22* (↑223%)</td></tr>
</table>

-P450 isozymes inhibition and 1´- hydroxymethyleugenol formation

<table>
<tr><th>P450 inhibitors</th><th>1 '-hydroxymethyleugenol formation (percent of control activity)</th></tr>
<tr><td>p-Nitrophenol (50 µM)</td><td>78 ± 5* (↓22%)</td></tr>
<tr><td>p-Nitrophenol (200 µM)</td><td>46 ± 12* (↓54%)</td></tr>
<tr><td>Diallylsulfide (200 µM)</td><td>88 ± 10</td></tr>
<tr><td>Diallysulfide (1 mM)</td><td>63 ± 0* (↓37%)</td></tr>
<tr><td>Tolbutamide (200 µM)</td><td>83 ± 10</td></tr>
<tr><td>Tolbutamide (500 µM)</td><td>73 ± 7* (↓27%)</td></tr>
<tr><td>Tolbutamide (1 mM)</td><td>60 ± 7* (↓40%)</td></tr>
<tr><td>α-naphthoflavone (1 µM)</td><td>88 ± 7</td></tr>
<tr><td>α-naphthoflavone (10 µM)</td><td>76 ± 2.5* (↓24%)</td></tr>
<tr><td>Troleandomycin (50 µM)</td><td>111 l± 9</td></tr>
<tr><td>Troleandomycin (200 µM)</td><td>89 ± 10</td></tr>
<tr><td>Furafylline (25 µM)</td><td>100 ± 3</td></tr>
<tr><td>Furafylline (100 µM)</td><td>89 ± 5</td></tr>
<tr><td>Quinine (1 µM)</td><td>100 ± 0.2</td></tr>
<tr><td>Quinine (10 µM)</td><td>98 ± 1</td></tr>
<tr><td>Cimetidine (50 µM)</td><td>90 ± 9.8</td></tr>
</table>

-37-fold variation was observed in the rate of 1'-hydroxymethyleugenol formation in the 13 human liver microsomes preparations.</td>
<td>Gardner, I., et al. (1997) (AS) B.6.8.1.1.5-05</td>
</tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results
- NOAEL/LOAEL
- target tissue/organ
- critical effects at the LOAEL</th><th>Reference</th></tr>
<tr>
<td>Gene expression changes in liver mice
GLP: No

<u>Rodent strain:</u>
B6C3F1 female mice

<u>Method:</u> Not stated

<u>Deviations from current test guideline</u>
Not applicable, published study
Supportive only</td>
<td><u>Test substance:</u>
Methyleugenol

Oral (gavage)

Doses:
75 mg/kg bw/day.

2-week exposure.</td>
<td><u>Gene expression changes (±1.5 fold change)</u>

<table>
<tr><th>Cell process</th><th>Gene</th></tr>
<tr><td>Cell cycle</td><td>↑GADD45β
↑CDKN1A
↑CCNG1
↑EGR1
↓LZTS2</td></tr>
<tr><td>Apoptosis</td><td>↑TSC-22
↑EGR1
↑DNASEα
↓FHIT
↓WWOX</td></tr>
<tr><td>DNA repair</td><td>↑EST2
↑EGR1
↓FHIT
↓WWOX</td></tr>
<tr><td>Tumor suppressors</td><td>↑CDKN1A
↑TSC-22
↓FHIT
↓WWOX
↓LZTS2</td></tr>
<tr><td>Inflammation</td><td>↓CISH</td></tr>
</table>
</td>
<td>Iida, M., et al. (2005) (AS) B.6.8.1.1.5-06</td>
</tr>
</table>

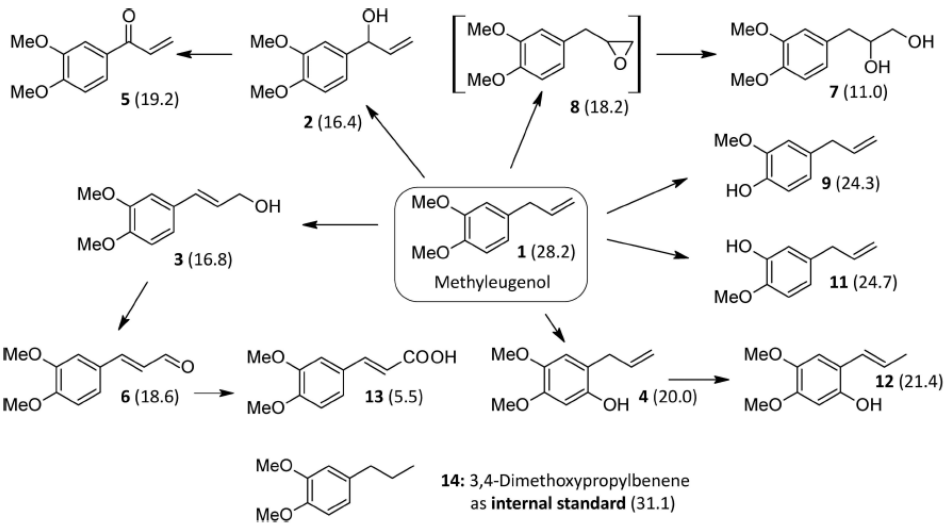
Toxicity studies on the impurity Methyleugenol:

ADME

Two studies have been submitted for this endpoint. An additional study (B.6.8.1.1.1-03) has also been included in this section.

In a toxicokinetic study in rats and mice [Hong S.P. *et al*. (2013); B.6.8.1.1.1-01] intravenous and oral doses of methyleugenol showed relatively rapid absorption and clearance in both species. No sex-differences were observed. In rats, absolute bioavailability following a gavage dose of 37 mg/kg was similar in both sexes with values of 3.9 ± 0.9% for males and 4.1 ± 1.3% for females. In mice, absolute bioavailability following gavage dose of 37 mg/kg, was 9.2 ± 6.4% for males and 7.3 ± 3.7% for females.

The outcome of a comparative *in vitro* metabolism study using rat, bovine and human liver microsomes identified 1'-hydroxymethyleugenol (compound no. 2) as the primary metabolite [Cartus A.T. *et al*. (2012); B.6.8.1.1.1-02] in all three species. The metabolite 6-hydroxymethyleugenol (compound no. 4) was the second major metabolite detectable in bovine but not human or rat liver microsomes. The metabolic pathway of methyleugenol in liver microsomes is displayed in the following figure:



In a separate *in vitro* metabolism study with methyleugenol [Minet E.F. *et al*. (2012); B.6.8.1.1.1-03] two metabolic routes (phase I - covalent binding and 1'-hydroxylation and phase II -glucuronidation) are investigated in both liver and lung in various species (rat, mouse and human). Based on the results of the study, methyleugenol is metabolised to 1'-OHMethyleugenol in the mouse and to a lesser extent in human and rat. No glucuronidation was observed in the absence of UDPGA. The extent of glucuronidation was higher in humans (12.2 %) in comparison to mouse (4.0 %) or rat (3.9 %). Covalent binding was detected, implying the formation of the quinone methide reactive intermediate, which was depleted by the addition of glutathione.

Short term toxicity studies

Three studies regarding the impurity methyleugenol were provided for complete short term toxicity. The main studies were conducted and compiled by NTP, and subsequently summarized and evaluated in one published study.

First of all, two 14-week dose range-finding studies conducted in rats and mice were performed to stablish a proper dosage for long-term and carcinogenicity toxicity studies.

In the first 14-week study conducted in rats [NTP (2000); B.6.8.1.1.2-01], methyleugenol was tested at of 0, 10, 30, 100, 300 or 1000 mg/kg bw/day, in which 9-10 animals/group/sex were subjected to chemical gavage administration.

All the animals survived at the end of study. Clinical findings possibly related to test substance administration included emaciation and urine staining in 100, 300, and 1000 mg/kg bw/day female dose rats.

Final mean bodyweights and bodyweight gains of 300 and 1000 mg/kg bw/day dose groups of males, and all dosed groups of females were statistically significantly lower than controls.

Data of haematology parameters showed a slight decrease in haemoglobin concentrations and haematocrit values in the 300 mg/kg bw/day female dose group and in top dose groups for both sexes at week 14. The haematocrit and haemoglobin decreases would be consistent with anaemia, but there was no corresponding decrease in erythrocyte counts. There was, however, an erythrocyte microcytosis demonstrated by decreased mean cell volumes in the 300 mg/kg bw/day male dose group and in top dose groups for both sexes in almost all measure points. Animals in the 300 and 1000 mg/kg bw/day dose groups also had decreased mean cell haemoglobin values; this would be consistent with the decreases in mean cell volumes. This suggests that, while there were equal numbers of circulating erythrocytes for animals in the 300 and 1000 mg/kg bw/day dose groups at week 14, the erythrocytes were smaller, resulting in lower haematocrit and haemoglobin values. Moreover, there was evidence of a thrombocytosis at all time points, demonstrated by increased platelet counts in the 100 mg/kg bw/day or greater dose groups at study termination.

Regarding clinical chemistry parameters, the serum activities of alanine aminotransferase; and sorbitol dehydrogenase were increased in the 100 mg/kg bw/day or greater male and female groups at various time points and would be consistent with hepatocellular injury or leakage. Additionally, bile acid concentrations were increased in the 300 and 1000 mg/kg bw/day males dose groups at all time points, and in 300 and 1000 mg/kg bw/day female dose groups at weeks 4 and 14, and these observations would be consistent with cholestasis or altered hepatic function. However, serum alkaline phosphatase activity, another marker of cholestasis, was either unaffected or

decreased in the same animals. A hypoproteinaemia and hypoalbuminemia were evidenced by decreased total protein and albumin concentrations in the 300 and 1000 mg/kg dose groups at the majority of time points. There were increases of creatinine concentrations in the 100 mg/kg or greater female groups at all time points. However, urea nitrogen, another marker of renal function, was either unaffected or decreased in the same animals.

Terminal bodyweights were statistically significant reduced in 300 and 1000 mg/kg bw/day male dose groups (8 and 31%) and in all female dose groups (6-18%). Absolute and/or relative liver weights of 100, 300, and 1000 mg/kg bw/day dose groups, were statistically significantly greater than controls. Testis weight (both absolute and relative) of top dose male group was also higher than controls. Moreover, absolute and relative thymus weights in all dosed males and in top dose female group were significantly lower than controls. Increased relative kidney weights were recorded in 30 and higher male groups (7-41%), and in all female dose groups (7-26%), however, no histological alterations were found in kidneys of rats tested. Non dose-related increased of spleen weights were noted in mid and top dose male groups, and in all female dose groups.

Histopathological examinations revealed increased incidences of liver lesions only in 300 and 1000 mg/kg bw/day males and females dose groups. The lesions were observed in almost all the animals tested and were generally more severe in males than in females. The hepatic lesions included cytologic alteration, cytomegaly, Kupffer cell pigmentation, bile duct hyperplasia, and foci of cellular alteration. Moreover, one hepatocellular adenoma was present in a male rat of top dose group.

On the other hand, incidences of cortical hypertrophy of the adrenal cortex were statistically significantly higher in 100, 300, and 1000 mg/kg bw/day males and top dose female groups, than controls. Cytoplasmic alteration of the submandibular salivary glands were also recorded and were statistically significant increased in rats administered 30 mg/kg or greater doses for both sexes. Cytoplasmic alteration of the submandibular salivary gland consisted of a loss of cytoplasmic zymogen granules with reduction in the size of serous cells and their ducts.

Alterations on glandular stomach were also noted, likely due to oral gavage administration. These incidences were mainly atrophy and chronic inflammation of the mucosa of the glandular stomach and were statistically significantly higher in 300 and 1000 mg/kg bw/day dose groups for both sexes. Lesions were generally of minimal to mild severity in the 300 mg/kg bw/day groups and mild to moderate in the top dose groups. Atrophy consisted of a decrease in the thickness of the gastric mucosa due to generalized loss of glandular epithelial parietal and chief cells accompanied by condensation of the lamina propria. Inflammation was of mild severity and consisted of fibrosis and a diffuse infiltration of the lamina propria by lymphocytes, neutrophils, and macrophages.

Regarding sexual organs, top dose male group showed increased incidences of moderate dilatation of the seminiferous tubules and testicular degeneration characterized by diffuse loss of spermatogenic cells within the seminiferous tubules. Spermatogonia remaining within the seminiferous and epididymal tubules were morphologically normal. On the other hand, the incidences of mild uterine atrophy were significantly increased in 300 and 1000 mg/kg bw/day dose groups. Reproductive parameters such as sperm motility or vaginal cytology were also analysed in the dose groups, however, no differences were observed compared with controls.

Therefore, **NOAEL for toxicity** could be stablished on 7.1 mg/kg bw/day (5-day/week dose scheme), based on increased platelet concentration (thrombocytosis, males), and cytoplasmic alterations in submandibular salivary gland (females)**.**

In the second 14-week study conducted in mice [NTP (2000); B.6.8.1.1.2-02], methyleugenol was tested at of 0, 10, 30, 100, 300 or 1000 mg/kg bw/day, in which 10 animals/group/sex were subjected to chemical gavage administration.

All mice of top dose group, except one male, died before the end of the study. Additionally, one male and one female of 300 mg/kg bw/day and 10 mg/kg bw/day dose groups died during week 3 and 12, respectively.

The only clinical sign described was morbidity associated to top dose animals.

No differences in bodyweights were found at study termination. Bodyweight gains were statistically significant reduced in the 300 mg/kg bw/day dose groups compared with controls.

Absolute and relative liver weights were statistically significant increased in 30 or greater male dose groups (11-28%) and in the top dose female groups (14-23%). On the other hand, absolute and relative thymus weights were significantly lower than controls in the 300 and 1000 mg/kg bw/day female dose groups, whereas in males, absolute thymus weight was only reduced in top dose group.

Liver weights alterations were further confirmed in necropsy and histopathological examinations. Thus, significant chemical-related gross lesions were observed in the liver of male and female mice in the top dose groups. One male and two females had enlarged livers, one male had a liver nodule, and one female had a pale liver.

Hepatic alterations such as cytologic alteration, necrosis, bile duct hyperplasia, and focal subacute inflammation were described in the top dose male group (40-50%) and in the 300 and 1000 mg/kg bw/day female groups (100% and 40%, respectively). Female top dose group had generally lower incidences of lesions due to early mortality. Cytologic alteration was observed primarily in periportal sites and was the term used to describe a variety of hepatocellular alterations that included mild nuclear and cytoplasmic enlargement (hypertrophy) and increased cytoplasmic eosinophilia. Necrosis of scattered individual hepatocytes occurred throughout the hepatic lobules. Bile duct hyperplasia consisted of proliferation of immature biliary cells within portal areas. Inflammation consisted of multiple small foci of primarily mononuclear inflammatory cells randomly scattered throughout the liver.

The incidences of atrophy, degeneration, edema, mitotic alteration, and cystic glands of the fundic region of the glandular stomach were increased in females mice administered 30 mg/kg or greater doses compared to controls. The incidences of atrophy, degeneration, edema, mitotic alteration, and cystic glands were statistically significantly increased in 300 mg/kg bw/day female dose group (100%, 100%, 60%, 100% and 70%, respectively). Lower incidences of degeneration, mitotic alteration and cystic gland were also recorded in the 30 and 100 mg/kg bw/day female groups. On the other hand, in males dose groups, the incidences of cystic glands (60%) in the 30 mg/kg bw/day group, and degeneration and mitotic alteration (90% and 100%, respectively) in the 300 mg/kg bw/day group were statistically significantly increased compared with controls, however, a clear dose-response relationship was not observed.

In the top dose mice groups, early death and subsequent autolysis significantly precluded adequate histopathologic evaluation of gastric lesions. Lesions were generally of minimal to mild severity in the 30, 100, and 300 mg/kg bw/day dose groups and of mild to marked severity in the 1000 mg/kg dose groups. Atrophy consisted of a generalized decrease in the thickness of the mucosal epithelium due to loss of parietal and chief cells and shortening of the mucosal glands. Necrosis in the glandular epithelium was characterized by necrosis of parietal cells primarily, and to a lesser extent, chief cells. Degeneration consisted of dilated glands lined by dysplastic atypical epithelial cells and cellular detritus. Cystic glands were dilated and lined by flattened epithelium. Increased numbers of morphologically normal mitotic figures were present in regenerative areas of the glandular epithelium. Edema was of minimal to mild severity and occurred in the lamina propria.

Statistically significant increase of minimal to mild focal degeneration of the olfactory epithelium of the nose were observed in the 30 and 1000 mg/kg bw/day dose males group, and in 30-300 mg/kg females dose groups. The incidences and severities of these lesions in both sexes were not dose-related.

Therefore, **NOAEL for toxicity** is stablished at 7.1 mg/kg bw/day (5-day/week dose scheme) based on increased histopathological alterations in glandular stomach**.**

-The 14-week studies conducted in rat and mice were further summarised by [Abdo K.M. *et al.* (2001); B.6.8.1.1.2-03], together with new experiments in which gastric pH, gastrin and cell proliferation in glandular stomach and liver were assayed. Thus, in order to avoid duplications, only new experiments mentioned had been evaluated and described by RMS.

Gastric pH showed a statistically significant increase in female rats that received 1000 mg/kg bw/day dose at the end of 30 or 90 days of treatment (191% and 73%, respectively), and in female rats given 300 mg/kg bw/day for 90 days (41%), compared with controls. The increase in pH was accompanied by a statistically significant increase of serum gastrin levels in these groups (874% and 1134% for the 30 and 90 days treatments at top dose groups, and 898% in the 300 mg/kg bw/day 90-day dose group, respectively). On the other hand, gastric pH was statistically significant higher in 75 mg/kg bw/day male mice dose group for 30 days (29%), whereas serum gastrin was in 150 and 300 mg/kg bw/day dose groups for 30 days (329% and 214%, respectively). No dose-relationship was observed in these mice recorded parameters, and the increase of serum gastrin was not correlated with a pH increase. In 90-day treatment design, no differences were noted.

Regarding cell proliferation assays, statistically significant increases in cell proliferation was observed in the fundic glands of the glandular stomach and in the liver in most of the dose female rats groups given methyleugenol for 30 or 90 days. None of the statistically significant increases observed in the gastric pits or the pylorus were greater than two-fold the values observed in controls. Thus, the increases at these two sites were not considered biologically significant by authors. In forestomach, only 30-day top dose group showed greater two fold values, however a non-clear dose-response was noted.

On the other hand, statistically significant increases in cell proliferation occurred in the fundic glands of the glandular stomach of male mice given 150 or 300 mg/kg bw/day methyleugenol for 30 days (1800% and 500%, respectively), and in all dose groups that received methyleugenol for 90 days (200-1400%). None of the statistically significant decreases observed in the forestomach, gastric pits or the pylorus of 30-day treatment schedule was greater than two-fold the values observed in controls, and no dose-relationship was noted. Consequently, the changes at forestomach, gastric pics and pylorus were not considered biologically significant.

Serum gastrin concentrations increased in response to high pH values due to the absence of acid feedback repression mechanism and were observed to be significantly elevated at doses as low as 150 mg/kg bw/day. Mice appeared to be less sensitive than rats to this effect. Methyleugenol increased cell proliferation of the fundic glands. This effect is probably a result of a feedback reaction to mucosal atrophy and the trophic effect of gastrin. Methyleugenol cause the loss of parietal and chief cells of the glandular stomach. Acid conditions and production of protein digesting enzymes are the function of these two types of cells, respectively. Loss of these cells could inhibit proper protein utilization and essentially lead to protein deficiency.

The magnitude of any possible risk to humans, however, has not been estimated. It would appear unlikely that humans would be exposed to sufficient amounts of methyleugenol to produce the gastric lesions resulting in increased stomach pH and elevated serum gastrin.

Therefore, based on the available data, the **overall oral short-term NOAEL** was **7.1 mg/kg bw/day** (14-week dietary study in rat and mouse).

Genotoxicity

A total of 10 genotoxicity studies *in vitro/in vivo* with methyleugenol have been submitted to assess this endpoint.

***In vitro studies*:**
Methyleugenol showed negative results *S.typhimurium* strains TA98, TA100, TA1535 and TA1537 in the presence and absence of metabolic activation [NTP (2000); B.6.8.1.1.3-01]. This study did not include a strain to test for cross-linking mutagens. Based on the available information, methyleugenol is not mutagenic in a bacteria gene mutation assay.

Methyleugenol did not induce an increase in mutant frequencies in male Chinese Hamster V79 lung fibroblasts without metabolic activation [Groh I.A.M. *et al.* (2012), B.6.8.1.1.3-05]. The study was not carried out in the presence of metabolic activation, hence mutagenicity in mammalian cells is incomplete.

In cytogenicity tests *in vitro*, methyleugenol did not induce micronuclei in male Chinese hamster V79 lung fibroblasts in the absence of metabolic activation [Groh I.A.M. *et al.* (2012), B.6.8.1.1.3-04]. Methyleugenol failed to induce chromosome aberrations in CHO cells [NTP (2000); B.6.8.1.1.3-06] both in the presence and absence of metabolic activation. However, a positive trend was observed in the activation assay and a longer treatment time would have allowed to confirm/disregard a positive outcome. For this reason, the outcome result is deemed as equivocal in the presence of metabolic activatin. Methyleugenol also produced sister chromatid exchanges in the presence of metabolic activation [NTP (2000); B.6.8.1.1.3-07].

Significant induction of DNA strand breaks in V79 cells was obtained with methyleugenol tested in the Comet assay *in vitro* [Groh I.A.M. *et al.* (2012); B.6.8.1.1.3-03].

Evidence of an impact of methyleugenol on DNA damage and repair was obtained in an *in vitro* UDS assay in rat and mouse hepatocytes [Burkey J.L. *et al.* (2000); B.6.8.1.1.3-08].

***In vivo*:**
Methyleugenol did not induce micronuclei in peripheral blood derived erythrocytes up to 1000 mg/kg bw in male and female mice following a 14-week exposure [NTP (2000); B.6.8.1.1.3-09].

Methyleugenol did not induce DNA damage in rat liver, lung, bone marrow, bladder and kidney cells when dosed orally at 400 or 1000 mg/kg bw at both 3h and 24h [Ding W. *et al.* (2011); B.6.8.1.1.3-10]. However, positive results in the bone marrow were obtained when sampling was done at 8h following an oral dose of 2000 mg/kg bw methyleugenol, although this is a non-standard assay.

In conclusion, methyleugenol failed to produce positive results in the standard battery of genotoxicity assays. However, some of those studies did not include metabolic activation and none were GLP with the exception of the

in vivo micronucleus assay (B.6.8.1.1.3-09). Positive results were obtained in the UDS assay *in vitro* and also a positive result was obtained in a non-standard *in vitro* comet assay. Taking into consideration non-standard DNA reactivity assays (please refer to the section 'Other studies' B.6.8.1.1.5-01 to B.6.8.1.1.5-06), using a weight of evidence approach and a conservatory assessment, it may be concluded that methyleugenol is mutagenic and according to criteria of Regulation (EC) No. 1272/2008 classification of methyleugenol as **Mutagenic Category 2 (Muta.2; H341)** is proposed.

Long term/carcinogenicity toxicity studies

Three studies regarding the impurity methyleugenol were provided for complete long-term toxicity. The main studies were conducted and compiled by NTP, and subsequently summarized and evaluated in one published study.

-In the 2-year carcinogenicity study in rats [NTP (2000), B.6.8.1.1.4-01], methyleugenol was tested at dose levels of 0, 37, 75, 150 and 300 mg/kg bw/day for males and females. The top dose group received test substance during 52 weeks followed by the 0.5% methylcellulose vehicle only for the remaining 53 weeks of the study (stop exposure group).
All 150 and 300 mg/kg bw/day male rats died before study termination. The presence of moribund animals and the number of natural deaths was higher than controls. On the other hand, the survival rates (animals surviving at study termination and mean survival days) of the 37 and 75 mg/kg bw/day male dose groups were slightly lower than controls. Survival at study termination on female 150 and 300 mg/kg bw/day dose groups was slightly lower than controls (22% and 27%, respectively, vs 37% in controls), and no statistical significance was displayed. It is only remarkable that the number of moribund animals was increased in these top dose groups (42% and 52% for 150 and 300 mg/kg bw/day dose groups vs 28% in controls). On the other hand, 37 and 75 mg/kg bw/day dose groups showed survival rates (animals surviving at study termination and mean survival days) slightly higher than controls. The other parameters were similar between study groups.

The only clinical sign recorded was moribund and it was attributed to test substance administration.

Mean bodyweights of all dosed males and females were less than those of the vehicle controls throughout most of the 2-year study. There were no signs of recovery in mean bodyweights in the stop-exposure 300 mg/kg bw/day group in the remaining 53 weeks of the study without methyleugenol. The reductions of bodyweights were >10% from week 37 in top dose male group (11-27%) and from week 29 in top dose female group (13-26%) to study termination. In 150 mg/kg bw/day dose group, reductions of bodyweights were >10% from week 53 in males (11-23%) and from week 33 in females (11-26%) groups to the end of the study. In low dose groups, only females of 75 mg/kg bw/day groups showed a continuous reduction of bodyweight from week 45 to study termination (11-21%).

Necropsy examinations showed gross lesions in the liver and stomach of males and females. Focal areas of discoloration, nodules (raised lesions less than 5 mm in diameter), and masses (raised lesions greater than 5 mm in diameter) were observed in the livers of dosed rats. The incidences and multiplicity of focal areas of discoloration, nodules, and masses increased with increasing dose. Grossly, there was diffuse thickening of the entire glandular portion of the stomach with or without the presence of one or more nodules and masses.

Regarding histopathology examinations, important lesions were mainly described in liver, glandular stomach and kidney. In liver, hepatocellular adenomas were increased in all dose-groups for both sexes compared with controls. The incidences ranging from 24-76% in males, and from 16-86% in females groups. Incidences of hepatocellular carcinomas were increased compared with controls in 75, 150 and 300 mg/kg bw/day male dose groups (28%, 50% and 72%, respectively), and in 150 and 300 mg/kg bw/day female dose groups (16% and 44%, respectively).

Both incidences of hepatocellular adenomas and carcinomas were dose related and exceed the overall mean and the range of HCD from Battelle Columbus Laboratories and NTP Carcinogenicity studies from 1990-1997 (Haseman *et al., Toxicol Pathol.*, 1998, 26(3):428-41) (see table below).

Moreover, incidences of hepatocholangioma and hepatocholangiocarcinoma were increased only in top dose male and female dose groups (12% and 14% for males, and 16% and 18% for females, respectively). The incidences of cholangiocarcinomas were higher than mean and out of range of NTP Carcinogenicity studies (1990-1997).

Non-neoplastic lesions in liver included eosinophilic, hepatocellular hypertrophy, oval cell hyperplasia, cystic degeneration, and bile duct hyperplasia (only females). These incidences were found in all the animals of the 6 and 12-months interim evaluation, and in all the dose groups of the 2-year study**.** These incidences were increased with dosage and were statistically significant compared with controls.

Lesions in glandular stomach were also relevant. Occurrence of malignant neuroendocrine tumours was statistically

significant in 75 and higher female dose groups (24%, 52% and 72% for 75, 150 and 300 mg/kg bw/day, respectively) compared with controls (0%). The incidence of this type of tumour was present only in 150 mg/kg bw/day male dose groups (8%). Benign neuroendocrine tumours were also found in 75 and higher female dose group, but no in males, without showing a dose-relation pattern (26%, 18% and 10% for 75, 150 and 300 mg/kg bw/day, respectively). Incidences of benign or malignant neuroendocrine tumors have not been described in the HCD from Battelle Columbus Laboratories or in the Haseman *et al.* review (NTP 1990-1997 period update, 2-year studies).

Non-neoplastic lesions in this organ include atrophy in all female groups treated with methyleugenol. The incidence rate was very high in all female dose groups (66-90%) compared with controls (0%). In males, these incidents were, however, increase with dosage level. On the other hand, incidences of neuroendocrine cell hyperplasia were described in all female dose groups, and no in controls, however the incidence in top dose group was not statistically significant and a dose-related trend was not clearly noted. In rat males, same rate of neuroendocrine cell hyperplasia was recorded in the 150 and 300 mg/kg bw/day dose groups (16% vs 0% in controls).

Kidney was one of the mostly affected organs. Increased incidences of renal tubule adenoma were described in 75, 150 and 300 mg/kg bw/day male dose groups (34%, 26% and 40%, respectively) compared with controls (8%). The incidences in all groups exceeded the historical control range from Battelle Columbus Laboratories and NTP Carcinogenicity studies from 1990-1997 (see table below). On the other hand, no renal tubule adenomas or carcinomas were described in females.

Increased incidences of renal tubule hyperplasia were recorded in 75 and higher male dose groups compared with controls (40%, 42% and 44%, respectively vs 26% in controls). The only statistically significant non-neoplastic lesion found in females was nephropathy in top dose group (90% vs 70% in controls).

On the other hand, the incidences of fibroma in skin in 37 and 75 mg/kg bw/day males dose groups (18% and 16%), and combined incidences of fibroma or fibrosarcoma in 37, 75, and 150 mg/kg males (24%, 16% and 16%) were significantly increased compared with controls (2%); however, the incidences did not increase with increasing dose. The incidences of these lesions in these groups exceeded the HCD mean and range from Battelle Columbus Laboratories and NTP Carcinogenicity studies (1990-1997). Fibromas were well-demarcated, solid, expansive masses composed of well-differentiated fibrous connective tissue. Fibrosarcomas were expansive, locally invasive masses composed of anaplastic spindle cells.

Statistically significant incidences of malignant mesothelioma were also described in 150 and 300 mg/kg bw/day male dose groups (24% and 10%, respectively), compared with controls (2%). Mesotheliomas were disseminated along the peritoneal surface of several organs in the abdominal cavity and/or the serosa of the testis and epididymis. The incidence was higher and out of the range of HCD from Battelle Columbus Laboratories.

Additionally, and only in males, statistically significant incidences of mammary gland fibroadenomas were recorded in 75 and 150 mg/kg bw/day male dose groups (30% and 26%, respectively), compared with controls (10%). The incidence was higher and out of the range of HCD from the NTP Carcinogenicity (1990-1997) (see table below). However, no increased occurrence of mammary gland fibroadenomas was recorded in top dose male group and in any of female dose groups.

Increased incidences of bone marrow hyperplasia were recorded in all dosed female groups (30%, 22%, 40% and 50% for 37, 75, 150 and 300 mg/kg bw/day dose groups, respectively) compared with controls (8%). Bone marrow hyperplasia consisted of a marked increase in the density of erythroid or myeloid cells or a mixture of both, frequently accompanied by proliferation of megakaryocytes. Bone marrow hyperplasia was considered to be secondary to the increased incidences of necrosis and inflammation associated with the large and multiple liver neoplasms in dosed animals.

Moreover, most of the rats of all dose groups treated with methyleugenol during 2-year study and all animals from 6 and 12-month interim examinations showed cytoplasmic alteration of the submandibular salivary gland. Cytoplasmic alteration consisted of a loss of the eosinophilic granules within the striated ducts of the submandibular salivary glands. The increased eosinophilic granularity (cytoplasmic alteration) in the submandibular salivary gland in dosed rats is considered secondary to the toxic effects of methyleugenol on the glandular stomach. Dietary factors such as protein starvation are known to cause loss of zymogen granules granules (McBride *et al*., *J. Dent. Res*., 1987, 66: 1445-1448). Methyleugenol administration in rats may have created such a condition due to loss of parietal and chief cells of the glandular stomach. Acid conditions and production of protein digesting enzymes are the functions of these two types of cells, respectively. Loss of these cells could inhibit proper protein utilization and essentially lead to protein deficiency.

Another non-neoplastic finding was recorded in spleen, in which splenic fibrosis was increased in 150 and 300 mg/kg bw/day female dose groups (24% and 30% vs 6% in controls).

The following table summarized the overall mean values and range of historical neoplasm incidences of Battelle Columbus Laboratories, laboratory that conducted the 2-year studies, and NTP Carcinogenicity studies during 1990-1997 period.

Historical neoplasm incidences in rats published by Battelle Columbus Laboratories and NTP Carcinogenicity studies during 1990-1997 period.

	Historical Incidence at Battelle Columbus Laboratories			Historical Incidence at NTP 1990-1997 (Haseman *et al.*)		
	Total	**Mean ± SD**	**Range**	**Total**	**Rate**	**Range**
Liver						
Males						
Adenomas	12/400	3.0 ± 3.0	0-8%	31/1352	2.3	0-10%
Carcinomas	4/400	1.0 ± 1.8	0-4%	9/1352	0.7	0-6%
Adenoma or carcinoma	16/400	4.0 ± 3.5	0-10%	38/1352	2.8	0-10%
Hepatocholangiocarcinoma	n.a.	n.a.	n.a.	0	0	0
Females						
Adenomas	1/401	0.3 ± 0.7	0-2%	8/1351	0.6	0-6%
Carcinomas	0/401	0	0	1/1351	0.1	0-2%
Adenoma or carcinoma	1/401	0.3 ± 0.7	0-2%	9/1351	0.7	0-6%
Hepatocholangiocarcinoma	n.a.	n.a.	n.a.	1/1351	0.1	0-2%
Kidney						
Males						
Renal tubule adenoma	3/400	0.8 ± 1.0	0-2%	10/1352	0.7	0-6%
Renal tubule carcinoma	2/400	0.5 ± 1.4	0-4%	3/1352	0.2	0-2%
Adenoma or carcinoma	5/400	1.3 ± 1.5	0-4%	n.a.	n.a.	n.a.
Males						
Renal tubule adenoma	n.a.	n.a.	n.a.	0/1348	0	0
Renal tubule carcinoma	n.a.	n.a.	n.a.	1/1348	0.1	0-2%
Adenoma or carcinoma	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Skin						
Males						
Fibroma	17/402	4.3 ± 3.6	0-12%	69/1354	5.1	0-12%
Fibrosarcoma	3/402	0.8 ± 1.0	0-2%	17/1354	1.3	0-4%
Fibroma or fibrosarcoma	20/402	5.0 ± 3.4	0-12%	n.a.	n.a.	n.a.
Females						
Renal tubule adenoma	n.a.	n.a.	n.a.	22/1351	1.6	0-4%
Renal tubule carcinoma	n.a.	n.a.	n.a.	8/1351	0.6	0-4%
Adenoma or carcinoma	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Malignant mesothelioma						
Males						
Malignant mesothelioma	7/402	1.7 ± 2.2	0-6%	n.a.	n.a.	n.a.
Mammary gland fibroadenoma						
Males						
fibroadenoma	n.a.	n.a.	n.a.	58/1354	4.3	0-12%
Females						
fibroadenoma	n.a.	n.a.	n.a.	556/1351	41.2	8-60%

Therefore, after an overall assessment of the effects of methyleugenol in long-term exposure in rats, there is an unequivocal evidence of carcinogenicity signs in liver and glandular stomach, together with other neoplastic effects in kidney, skin, mesothelium and mammary gland. Thus, due to adverse effects (neoplastic and non-neoplastic multisite changes) were observed at all dose levels, a NOAEL cannot be established.

-In the 2-year carcinogenicity study in mice [NTP (2000); B.6.8.1.1.4-02], methyleugenol was tested at dose levels of 0, 37, 75 and 150 mg/kg bw/day for males and females.

The survival rates (animals surviving at study termination and mean survival days) of male treated groups were similar to control group. By contrast, in female dosed groups, the animals surviving at study termination were reduced compared with controls, particularly at top dose group (36%, 36% and 4% for low, mid and high dose groups, respectively), compared with controls (62%). Additionally, an increase of the number of natural deaths, together with a decrease in the mean survival (days), were noted in female dose groups.

The only clinical sign recorded was moribund and it was attributed to test substance administration.

Mean bodyweights of 75 and 150 mg/kg bw/day male dose groups were slightly lower than controls. It was only >10% at last two weeks (97 and 101) in the mid dose group, and at last week (101) in high dose group.

On the other hand, all the females dose groups showed reduced bodyweights compared with controls throughout study. The reductions of bodyweights were >10% from week 17 in top dose group (11-46%), from week 37 in the mid dose female group (12-45%), and from week 65 in the low dose group (11-39%) to study termination.

Histopathology examinations showed an increase of liver hepatocellular adenomas in all dose males (86%, 76% and 78% for low, mid and high dose groups, respectively) and females (96%, 94% and 82% for low, mid and high dose groups, respectively) compared with controls (53% and 40% for male and female control groups).

Hepatocellular carcinomas were also increased in all female dose groups (74%, 96% and 94% for low, mid and high dose groups, respectively) compared with controls (14%). However, only low (40%) and mid (38%) dose male groups showed a statistically significant increase compared with controls (20%). The top male dose groups displayed an incidence similar to control group (18% vs 20% in controls), so a dose-relationship was not observed.

In addition, occurrences of hepatoblastoma were statistically significant increase in all female dose groups (12%, 22% and 30% for low, mid and high dose groups, vs 0% in controls), and a slightly increase was noted in top dose male group (6%) without showing statistical significance. Moreover, 2 females of top dose group developed hepatocholangiocarcinoma, whereas this type of tumour was not recorded in mice males.

The incidences of hepatocellular adenomas in both sexes, hepatoblastoma in females and in top dose male group, and hepatocholangiocarcinoma, exceed the overall mean and the range of HCD from Battelle Columbus Laboratories and NTP Carcinogenicity studies from 1990-1997 (Haseman *et al., Toxicol Pathol.*, 1998, 26(3):428-41). On the other hand, incidences of hepatocarcinomas in low (40%) and mid (38%) male dose groups were higher than historical control mean of the Battelle laboratories (20%) and NTP Carcinogenesis studies (17.9%), and exceed the range of NTP (6-29%) report 1990-1997. However, the occurrence in the low and mid dose groups was slightly out and in the limit of historical range revealed by Battelle laboratories (8-38%), respectively. By contrast, the percentage of hepatocarcinoma in all female dose groups exceeds the overall mean and the range of both HCD sources.

Non-neoplastic incidences in liver included several alterations. Presence of eosinophilic foci was statistically significant increased in male dosed groups (40%, 50% and 38% for low, mid and high dose group vs 20% in controls). In all dosed groups of both sexes, the incidences of oval cell hyperplasia were significantly increased; in males, these incidences were increased with the dose (16%, 54% and 92% for low, mid and high dose, respectively, vs 0% in controls), whereas in females, most of animals of low dose group developed oval cell hyperplasia (92%), and the incidence was slightly reduced in the mid and high dose groups (73% and 76%, respectively). The incidences of periportal hypertrophy were significantly greater in mid and top dose male groups (14% and 92%), and in all female dose groups (20%, 14% and 26% in low, mid and high dose groups, respectively), than controls of both sexes (0%).

The incidences of bile duct hyperplasia in 75 and 150 mg/kg female mice (22% and 18%, respectively), and of hemosiderin pigmentation (22%, 49% and 38% for low, mid and high dose groups, respectively) in all female dose groups were significantly increased, compared with controls. Moreover, a dose-related trend was recorded in hepatocyte necrosis incidences for mid and high female dose (33% and 34%), and in hematopoietic cell proliferation incidences in all female dose groups (28%, 47% and 48% for low, mid and high dose groups, respectively).

As well as in rats, glandular stomach was also affected. Isolated incidences of carcinoma (2%) and malignant neuroendocrine tumours (4%) were noted in top dose males, without showing statistical significance compared with controls. The incidences of carcinoma in males were higher than mean and exceed the range reported by NTP carcinogenesis studies during 1990-1997. The HCD occurrences in glandular stomachs from Battelle laboratories were not reported. No differences in neoplastic alterations were found between female dose groups and controls.

Non-neoplastic features in glandular stomach included dose-related increases of atrophy, ectasia, and hyperplasia incidences in all male dose groups, and the differences were statistically significant compared with controls. In female dose groups, ectasia occurrences were statistically significantly higher in dosed groups (67%, 67% and 84% for low, mid and high dose groups, respectively), compared with controls (31%); whereas same atrophy incidence was noted in mid and high dose group (22% vs 0% in controls).

One lesion observed in mice but not in rats consisted of multifocal hyperplasia of the columnar foveolar epithelium of the gastric pits. This hyperplasia often extended toward the base of the mucosa with displacement of parietal and chief cells of the mucosal glands and was often accompanied by minimal to mild chief cell hyperplasia.

Atrophy of the glandular mucosa was histologically similar to that observed in rats. It was characterized by loss of glandular epithelial cells (chief and parietal cells) in the fundic region of the stomach with concomitant reduction in the height of the fundic mucosa.

No additional differences regarding neoplastic incidences were recorded in other organs compared with controls. It is remarkable the dose-related increase incidences of bone marrow hyperplasia in all dose groups of both sexes, and it was considered such as secondary event to the changes in hepatic and gastric pathology.

The following table summarized the overall mean values and range of historical neoplasm incidences of Battelle Columbus Laboratories, laboratory that conducted the 2-year studies, and NTP Carcinogenicity studies during 1990-1997 period.

Historical neoplasm incidences in mice published by Battelle Columbus Laboratories and NTP Carcinogenicity studies during 1990-1997 period.

	Historical Incidence at Battelle Columbus Laboratories			**Historical Incidence at NTP 1990-1997 (Haseman *et al.*)**		
	Total	**Mean ± SD**	**Range (%)**	**Total**	**Rate (%)**	**Range (%)**
Liver						
Males						
Adenomas	201/514	39.2 ± 11.0	21-58%	397/1350	29.4	4-60%
Carcinomas	102/514	20.0 ± 8.1	8-38%	241/1350	17.9	6-29%
Adenoma or carcinoma	267/514	52.1 ± 14.9	25-72%	570/1350	42.2	10-68%
Hepatoblastoma	2/514	0.3 ± 1	0-3%	0	0	0
Hepatocholangiocarcinoma	n.a.	n.a.	n.a.	1/1350	0.1	0-2%
Females						
Adenomas	108/511	21.0 ± 9.5	6-40%	234/1350	17.3	2-50%
Carcinomas	37/511	7.2 ± 6.7	0-22%	113/1350	8.4	0-20%
Adenoma or carcinoma	138/511	26.8 ± 14.1	8-58%	319/1350	23.6	6-56%
Hepatoblastoma	0/511	0	0	1/1350	0.1	0-2%
Hepatocholangiocarcinoma	1/511	0.2 ± 0.6	0-2%	2/1350	0.1	0-2%
Glandular stomach						
Males						
Carcinomas	n.a.	n.a.	n.a.	0	0	0
Females						
Carcinomas	n.a.	n.a.	n.a.	1/1353	0.1	0-2%

Therefore, after an overall assessment of the effects of methyleugenol in long-term exposure in mice, there were unequivocal evidence of carcinogenicity signs in the liver of all dosed females, and weak carcinogenicity signs in the glandular stomach of high dose males. Thus, due to adverse effects (neoplastic and non-neoplastic multisite changes) were observed at all dose levels, a NOAEL cannot be established.

Based on the results of the long term/carcinogenicity studies, and according to the criteria of Regulation (EC) No 1272/2008, classification of the impurity methyleugenol as Carcinogen, Category 2 (Carc. 2; H351) is proposed.

Other toxicological studies

Methyleugenol DNA adducts were detected in human liver samples using UPLC-MS/MS (ultra-performance liquid chromatography tandem mass spectrometry) [Herrmann K. *et al.* (2013); B.6.8.1.1.5-01]. These adducts were found in form of N_6-(*trans*-methylisoeugenol-3-yl)-2-deoxyadenosine (N_6-MIE-dA) and N_2-(*trans*-methylisoeugenol-3-yl)-2′-deoxyguanosine (N_2-MIE-dG). The detected ratio for N_2-MIE-dG and N_6-MIE-dA was 60:1. The maximal and median levels of these adducts (N_2-MIE-dG and N_6-MIE-dA combined) were 37 and 13 per 108 nucleosides, corresponding to 4700 and 1700, respectively, adducts per diploid genome (6.6×10^9 base pairs).

This study showed the evidence of the genotoxic potential of methyleugenol in humans and it plausible role in liver tumorigenesis. However, the study lacks of important information for a proper assessment and conclusion, such a as methyleugenol dietary exposure data, the source, and the clinical information of the subjects used in the study.

-In the study conducted by Zhou *et al.* (B.6.8.1.1.5-02), HepG2 cells were treated during 24h at 0, 50, 150 and 450 µM of methyleugenol. DNA adducts were further detected using ^{32}P-postlabeled and were separated by thin-layer chromatography on polyethyleneimine (PEI)-cellulose sheets. Radioactivity of fractions from individual cell cultures was determined by scintillation counting.

The treatment of methyleugenol for 24h at 150 µM showed a chromatographic pattern of two spots. The major adduct was identified as N_2-(trans-propenylbenzene-30-yl) deoxyguanosine, and a second minor adduct was identified as N_2-(allylbenzene-10-yl) deoxyguanosine. Moreover, methyleugenol displayed the highest DNA binding activity at 50 µM.

-In the study conducted by Waddell *et al.* (B.6.8.1.1.5-03), it was exposed a correlation between the development of hepatocellular carcinomas and methyleugenol-DNA adducts formation with increasing dose in F344/N male rats.

A method based on statistic showed a linear response for both adduct formation and tumour incidence. The threshold dose of administered methyleugenol for adduct formation (zero adducts) was $10^{19\,3}$ molecules of methyleugenol/kg/day, whereas a threshold of $10^{20\,1}$ molecules of methyleugenol/kg/day was calculated for tumour formation. Moreover, the data also revealed that tumor formation did not begin until there were about 30 adducts/10^8 nucleotides. This threshold for tumor formation is lower than obtained for B6C3F1 mice of 30 adducts/10^6 nucleotides reported by Phillips *et al. (Carcinogenesis,* 1984, 5, 1623–1628). This analysis supports the findings that link the genotoxic potential of methyleugenol with liver neoplasm development.

-A chronic toxicity study was conducted with methyleugenol by Williams *et al.* (B.6.8.1.1.5-04) in F344/N male rats, in which the test chemical was administered *via* oral gavage at 0, 27, 54 and 107 mg/kg bw/day during 16 weeks (initiation phase), following 24 weeks treatment with (promotion) or without (maintenance) phenobarbital (PB) at 500 ppm. Neither compound-related deaths nor clinical signs were found in the study.

A reduction in bodyweight was observed in all methyleugenol treated groups compared to controls during the initiation phase, displaying statistical significance only at 8 weeks (3%, 3% and 5% for low, mid and high dose group, respectively). During the 24-week post-initiation, no differences in bodyweights were found between treated groups and controls.

A dose-related increase in relative liver weights in both mid and high dose groups compared to control was observed at 8 weeks (12% and 15%, respectively). In the 24 weeks promotion/maintenance phase, no differences in both absolute and relative liver weights with or without phenobarbital (PB) were detected between treated groups and controls.

No changes were present between methyleugenol treated groups and controls in serum levels of any of the five liver enzymes monitored at the 16 week time point (alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transpeptidase, sorbitol dehydrogenase, and alkaline phosphatase).

The hepatocellular replicating fraction values, expressed in percent of PCNA-positive cells over total hepatocytes counted, were statistically significantly higher in all methyleugenol treated groups at week 8 (105%, 111% and 180% for low, mid and high dose group, respectively) and 16 (74%, 83% and 148% for low, mid and high dose group,

respectively), than controls.

The mean adduct values in all methyleugenol-treated groups of all measure points were statistically significantly higher than controls. Two-thin-layer chromatography (TLC) analysis revealed the presence of two major adducts and one minor DNA adduct in the livers of all methyleugenol treated groups at 8, 16 and 40 week time points, whereas one major adduct was detected in control group. During the promotion/maintenance phase without methyleugenol treatment (but with or without PB), a reduction of liver DNA adducts compared to levels at 8 and 16 weeks were observed in all treated groups, including controls. The DNA adducts formation after methyleugenol administration seems to be reversible as noted at the end of promotion phase (40 week termination), in which rats did not receive methyleugenol. According to the authors, the reduction in adduct levels is probably due to DNA repair mechanisms in hepatic cells.

Regarding liver histopathology, hepatocellular altered foci (HAF) and neoplasms development was assessed. During the initiation phase, methyleugenol treated groups showed dose and time related increases in liver HAF. At week 8, only top dose group showed HAF (100%), whereas at week 16, all dose groups developed HAF (66.7% for low and mid dose groups, and 100% for top dose group). At maintenance phase without PB (PB-), an incidence of 8.3%, 91.7% and 100% was recorded for low, mid and high dose groups, respectively vs 0% in controls. On the other hand, at promotion phase with PB (PB+), and incidence of 15.4%, 85.7% and 100% was noted for low, mid and high dose groups, respectively vs 7.1% in controls. Hepatocellular adenomas were developed in mid and top dose PB- (8.3% and 100%, respectively), and PB+ groups (21.4% and 77%, respectively) during the last 24 weeks of treatment. However, no liver carcinomas were noted in any of treated groups during the whole study.

This study showed how methyleugenol promotes liver neoplasm and histopathology alterations through DNA-adduct formation as first impairment event. The authors reported in same in vivo study the relation between DNA alteration and the further development of liver neoplasm. Therefore, these results support previous findings that link the genotoxic potential of methyleugenol with liver neoplasm development.

Therefore, due to adverse effects (mainly DNA-adducts and altered liver foci), were observed at all dose levels, a NOAEL cannot be established.

-The study conducted by Gardner I. *et al.* (B.6.8.1.1.5-05), showed how P450 isozymes are involved in the first steps of methyleugenol metabolism. This study used liver microsomes from treated F344/N rats and from human donors to assess the hydroxylation of methyleugenol. Thus, assays conducted with P450 inducers and inhibitors revealed that hydroxylation of methyleugenol is catalysed predominantly by **CYP 2E1** and **CYP 2C6** isozymes.

P450 inducers phenobarbitone, isoniazid, dexamethasone and isofrole statistically significant induced P450 protein expression and higher formation of 1-hydroxymethyleugenol than controls. Phenobarbital has been shown to induce CYP 2B1/2, **CYP 2C** and CYP 3A isozymes, dexamethasone is an inducer of CYP 3A and 2B isozymes, isosafrole induces CYP 1A1 and CYP 1A2 isozymes, whereas isoniazid is a selective **CYP 2E1** inducer.

On the other hand, formation of 1´hydroxymethyleugenol was inhibited by P450 inhibitors p-Nitrophenol **(**PNP), diallylsulfide, tolbutamide and α-naphthoflavone, but not by troleandomycin, furafylline, quinine or cimetidine. PNP was the most potent inhibitor, causing 54% inhibition, followed by tolbutamide (40%), diallylsulfide (37%) and a-naphthoflavone (24%).

Involvement of **CYP 2E1** in 1'-hydroxymethyleugenol formation is consistent with the inhibition of the reaction seen in the presence of diallylsulfide and PNP (37% and 54% inhibition, respectively), which selectively inhibit this isozyme. This interpretation is further supported by the observation that methyleugenol competitively inhibited hydroxylation of PNP to 4-nitrocatechol, which is a reaction catalysed by CYP 2E1. Moreover, YP 2El has been implicated previously in the bioactivation and metabolism of a number of other hepatotoxins and carcinogens, such as paracetamol, benzene, nitrosamines and carbon tetrachloride (Guengerich, F.P., *et al.*, *Chem. Res. Toxicol.,* 1991*,* 4, 168-179; Patten, C.J., *et al., Chem. Res. Toxicol.,* 1993*,* 6, 511-518).

Involvement of **CYP 2C6** in 1'-hydroxylation of methyleugenol seems likely in view of the significant (40%) inhibition of the reaction by tolbutamide, which has been shown to inhibit CYP 2C6 mediated reactions and by α-naphthoflavone (24% inhibition). α-naphthoflavone has been shown to inhibit tolbutamide hydroxylation (Veronese, M., *et al.*, *Drug. Met. Disp.,* 1990, 18, 356-361), and to inhibit the N_4- hydroxylation of sulfamethoxazole, which is catalysed by CYP 2C6 (Cribb, A.E., *et al.*, *Drug. Met. Disp.,* 1995, 23, 406-414). Although α-naphthoflavone is known to be a potent inhibitor of CYP 1A isozymes (Shimada, T. *et al.*, *Biochem. Pharmacol.,* 1988, 37, 459-464), 1'-hydroxylation of methyleugenol was not inhibited significantly by the potent CYP 1A2 inhibitor furafylline (Kunze, K.L. *et al.*, *Chem. Res. Toxicol.,* 1992, 6, 649-656; Sesardic, B. *et al.*, *Br. J. Clin. Pharmacol.,* 1990, 29, 651-653). This excludes the possibility that CYP 1A2 makes a significant contribution to catalysis of the high affinity

component of 1'–hydroxymethyleugenol formation in livers of untreated rats.

In addition, hydroxylation of methyleugenol was also evaluated in 13 human liver microsome fractions obtained from deceased donors. These assays showed a 37-fold variation in 1`hydroxymethyleugenol formation (nmol/min-1/mg protein^{-1}) in the samples tested. Therefore, different metabolic activities and enzymatic expression levels could explain the variable hydroxylation levels of methyleugenol found between individuals.

-A section regarding methyleugenol-induced changes in genes expression was evaluated in the study conducted by Iida *et al.* (B.6.8.1.1.5-06), and consequently, an overview has been included in this chapter. The authors analysed the genes expression changes in liver mice after 2-week treatments with the chemical (75 mg/kg bw/day). First of all, it was examined the changes in a group of 12 genes found previously to be altered in liver after 2 weeks treatment with oxazepam and Wyeth-14643 (Iida, M., *et al., Carcinogenesis*, 2003, 24: 757-770). Then, expression of 20842 genes was assessed by oligonucleotide microarray. In summary, relevant genes involved in DNA repair (*EST2* and *EGR1*), cell cycle regulation (*CDKN1A*, *CCNG1, EGR1* and *GADD45β)* and apoptosis (*TSC-22*, *EGR1* and *DNASEα*) were up-regulated. These findings are signs that cell presents DNA damage, and the DNA repair mechanisms are thereupon activated. Consequently, due to this potential DNA damage, cell activates cycle arrest and apoptosis pathway. A down-regulated expression was found in *CISH,* revealing that an inflammatory process is occurring in liver tissue. Moreover, relevant tumor suppressors such as *FHIT*, *WWOX* and *LZTS2* are down-regulated, and these are common features in tumorigenesis.

2.6.8.2 Supplementary studies on the active substance

Four supplementary published studies have been provided to support the renewal of the active substance eugenol. Three of these studies were included in the original DAR (2011) in support of the inclusion of eugenol in Annex I of Directive 91/414/EEC (Yokota *et al.*, 1988, B.6.8.2.1, Rompelberg *et al.*, 1993, B.6.8.2.2, and Thompson *et al.*, 1998, B.6.8.2.3). Thus, one new study (Iida *et al.*, 2005; B.6.8.2.4), which analyses gene expression changes induced after eugenol treatment, has been submitted in the renewal of active substance.

Table 2.6.8.2/01. Summary table of supplementary studies

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels, route of exposure duration of exposure**	**Observations**	**Reference**
Liver enzymatic activity assessment. No guideline. **Supportive only**. Male Fisher F344/N rats 3 rats /group.	Eugenol Oral (diet) 0, 1000, and 3000 mg/kg bw/day, for 23 days.	Eugenol increases the enzymatic activity of liver drug-detoxifying enzymes UDP-Glucuronyltransferase (GT), UDP-glucose dehydrogenase (DH) and glutathione S-transferase (GST).	Yokota *et al.* (1988) (AS) B.6.8.2.1
Enzymatic activity of phase I and II enzymes in rats. No guideline. **Supportive only**. Male Wistar rats 3 rats /group.	Eugenol Oral (diet) 0, 250, 500 and 1000 mg/kg bw/day, for 10 days.	Eugenol increases the levels and activity of phase I and II liver enzymes and alters some clinical chemistry parameters: Clinical biochemistry ▪ (↓) WBC at 500 (13%; ns; ndr.) and 1000 mg/kg bw/day (12%, ns; ndr.). ▪ (↑) PLT at 1000 mg/kg bw/day (12%; ns). ▪ (↑) ASAT at 1000 mg/kg bw/day (13%; ns). ▪ (↑) ALAT at 250 (22%; ns), 500 (38%, ns) and 1000 mg/kg bw/day (60%, ns).	Rompelberg *et al.* (1993) (AS) B.6.8.2.2
Toxicity of eugenol and its quinone methide metabolite GLP: No Method: Rat liver cells (Clone 9, ATCC) Method: Not stated Deviations from current test guideline Not applicable, published study **Supportive only**	Test substance: **Eugenol** **Eugenol Quinone Methide (EQM)** Dose: Eugenol: 1, 10, 100 y 1000 µM EQM: : 1, 10, 100 y 1000 µM	Eugenol toxicity: -↓GSH at 10µM. -↓ gap junction-mediated intercellular communication (GJIC) at 10 and 100 µM. -↓ reactive oxygen species (ROS) at 10 and 100 µM. - ↓ pH at 1000 µM. -↓ MMP at 100 µM. -↓ $[Ca^{2+}]$ at 1000 µM. - ↑ plasma membrane potential (PMP) at 1000 µM. Eugenol Quinone Methide toxicity (EQM): -↓GSH at 1, 10 and 100 µM. -↓ gap junction-mediated intercellular communication (GJIC) at 1, 10 and 100 µM. -↑ reactive oxygen species (ROS) at 10 µM. - ↓pH at 100 and 1000 µM. -↓ MMP at 100 and 1000 µM. -↑ $[Ca^{2+}]$ at 100 µM. - ↓ plasma membrane potential (PMP) at 1000 µM.	Thompson, D.C. *et al.* (1998) (AS) B.6.8.2.3
Gene expression changes in liver mice No guideline. **Supportive only**. Female B6C3F1 mice 4 mice /group.	Eugenol Oral (gavage) 75 mg/kg bw/day, for 2 weeks.	Eugenol treatment displayed expression changes in the following genes (±1.5 fold change): **QRT-PCR**: ▪ ↑*GADD45β* and *EST2*. ▪ ↓*IGFBP5* **Microarray** ▪ ↑ *DNASE2a*.	Iida *et al.* (2005) (AS) B.6.8.2.4

		▪ ↓ *INBHA* and *CISH.*	

The enzymatic activity studies (Yokota *et al.*, B.6.8.2.1 and Rompelberg *et al.*, B.6.8.2.2) showed that eugenol increased the enzymatic activity and levels of phase I enzyme P-450 and phase II enzymes, UDP-Glucuronyltransferase (GT), UDP-glucose dehydrogenase (DH) and glutathione S-transferase (GST). Both published studies suggest that eugenol, in certain concentrations, might induce the activity of important detoxifying enzymes and exerts a protective role in drugs detoxification, controlling antioxidant defences and cell survival.

The toxicity of eugenol and its quinone methide metabolite (EQM) was assessed in cultured liver cells [Thompson D.C. *et al.* (1998) B.6.8.2.3]. This study investigated parameters such as intracellular glutathione and calcium levels, mitochondrial and plasma membrane potentials (MMP and PMP) intracellular pH, reactive oxygen species generation (ROS) and gap junction-mediated intercellular communication (GJIC) in cells after exposure to various concentrations of the test compounds.

Eugenol depleted intracellular GSH, inhibited GJIC and generation of ROS, and had a modest effect on MMP at concentration of 10 to 100 µM. At high concentrations (1000 µM), eugenol also decreases [Ca^{2+}], PMP, and pH.

Effects of EQM were seen at lower concentrations (1 and 10 µM). The earliest and most potent consists in GSH depletion at lower concentrations than eugenol. Moreover, EQM enhanced ROS at low concentrations, whereas eugenol decreased ROS. This is most likely due to the more extensive depletion of GSH by EQM than by eugenol. GSH is used in the cellular defense system against ROS (as a substrate for glutathione peroxidase) and, if GSH is lowered beyond a certain point, basal ROS will increase due to the inability to remove hydroperoxides through this peroxidase pathway. EQM caused a sustained elevation in [Ca^{2+}] at 100 µM, whereas eugenol treatment decreased [Ca^{2+}] at 1000 µM. These observations correspond to reports that [Ca^{2+}] increases following exposure to hepatotoxicants such as acetaminophen and its reactive metabolite N-acetyl- *p*-benzoquinone imine (Moore, M., *et al., J. Biol. Chem.,* 1985*,* 260: 13035–13040; Orrenius, S., *et al., TIPS* 1989, 10: 281–285; Boobis, A.R., *et al., Biochem. Pharmacol.,* 1990, 39: 1277–1281).

These results suggest that eugenol mediates its hepatotoxic effects primarily through depletion of cytoprotective thiols-dependent processes such as GJIC. Furthermore, these results support the hypothesis that the toxic effects of eugenol are mediated through its quinone methide metabolite. The effects of EQM closely mimicked those of eugenol, with the major difference being that EQM elicited the same effects at lower concentrations. EQM is more potent than eugenol, and this is consistent with the hypothesis that EQM is the toxic metabolite of eugenol and it is directly responsible for the hepatotoxic effects of the parent compound.

The study conducted by Iida *et al.* (B.6.8.2.4) analysed the expression changes in the liver of B6C3F1 mice of 20842 genes after eugenol treatment at 75 mg/kg bw/day. However, in contrast to expression changes observed as a result of methyleugenol treatment (B.6.8.1.1.5-06), no relevant changes compared with control livers were found after eugenol dosage; no differences were noted in expression genes involved in key cellular processes such as DNA repair, cell cycle, apoptosis or tumor suppression.

Immunotoxicity

No supplementary studies on the active substance regarding immunotoxicity were provided.

With the aim of analysing the effect of eugenol on the normal function of the immune system, it has been carried out a detailed review of the existing studies from other human and animal health sections. Immune-related parameters were reviewed from short-term studies, chronic studies, and reproductive studies in order to evaluate the immunotoxic potential of eugenol.

Furthermore, ADME studies and medical data were also evaluated for this purpose. Only one ADME study carried out in humans was included in the summary, whereas the other ADME studies were *in vitro* studies performed in rodents that did not present information regarding immune-related parameters. The distribution of the active substance and its metabolites in tissues may be indicative of its immunotoxic potential, whilst medical data can provide useful information about effects related to inability to fight infection or excessive, poorly controlled responses (as anaphylaxis or autoimmunity).

Therefore, the effects of eugenol on each endpoint were collected from the available studies and evaluation was performed according to the respective OECD guidelines.

The most relevant endpoints for this task were selected based on the *Retrospective analysis of the immunotoxic effects of plant protection products as reported in the Draft Assessment Reports for their peer review at EU level* (Dewhurst, I. *et al.* 2015, EFSA supporting publication 2015: EN-782) and *Guidance for Immunotoxicity risk assessment for chemicals* (World Health Organization & International Programme on Chemical Safety (2012). Guidance for immunotoxicity risk assessment for chemicals. World Health Organization. IPCS harmonization project document; no. 10).

These endpoints were the following:
- Survival and infections, as indicators of potential immunotoxicity, considering that laboratory animals do not use to be exposed to infections.
- From the haematology parameters, total white blood cell counts (WBC) and/or differential counts, as key cell types involved in immune functioning/response.
- Globulin levels in serum, as an indicator of antibody synthesis. If globulin levels were not presented, A:G ratio or serum protein changes were used as surrogates.
- Lymph nodes, as integral parts of the immune system. At least one site of lymph nodes should be included for histopathological examination in most study protocols, but also the finding of lymph nodes with increased size from the clinical observations was included in the analysis.
- Gut associated lymphoid tissue or Peyer's patches, as important tissue in antigen presentation.
- Spleen (weight and histopathology), as organ involved in the maturation of lymphocytes. Altered weights can indicate atrophy or abnormal stimulation. Pathology can indicate altered immune function but can be secondary to other functions of the spleen.
- Thymus (weight and histopathology), as primary organ for T-lymphocyte maturation. Changes in young animals are reported to be more likely to indicate immunotoxicity, as thymus weight varies with the age of the animal, due to its normal age-associated shrinking or involution.
- Bone marrow smear, as reduced cellularity could indicate reduced potential to produce WBC (& RBC).
- Adrenal glands are an organ target of cytokines, and indeed, ACTH and adrenal steroids regulate the cytokine synthesis. Besides, deposits of immunoglobulins could be observed in adrenal glands. Some autoimmune syndromes as Addison´s disease are characterised by adrenal cortex damage.

Table 2.6.8.2/02. Analysis of immune parameters in studies with eugenol

Study	Immune parameters analysed	Not analysed*
ADME studies		
ADME studies about the distribution of eugenol in immune system tissues were not provided.		
Short-term studies		
Substance: Eugenol -National Toxicology Program (1983) -Carcinogenesis studies of eugenol in F344/N rats an B6C3F$_1$ mice (feed studies) -Species: rat -14-day study (AS) B.6.3.1.1 Supportive study	- Mortality: Only at very high doses, at 100000 ppm in male and females. - Infections: not reported.	- Organs weight -Macroscopic findings - Histopathology - Haematology - Biochemistry - Globulin levels
Substance: Eugenol -National Toxicology Program (1983) -Carcinogenesis studies of eugenol in F344/N rats an B6C3F$_1$ mice (feed studies) -Species: mice -14-day study (AS)	- Mortality: Only at very high doses, at 100000 ppm in male and females and at 50000 only males. - Infections: not reported.	- Organs weight -Macroscopic findings. - Histopathology - Haematology - Biochemistry - Globulin levels

Study	Immune parameters analysed	Not analysed*
B.6.3.1.2 Supportive study		
Substance: Eugenol -Lauber, F.U. (1950). -Toxicity of the Mucigogue, Eugenol, Administered by Stomach Tube to Dogs. -Species: dog -22-day study (AS) B.6.3.1.3 Supportive study	- Mortality: not reported. - Infections: not reported.	- Organs weight -Macroscopic findings - Histopathology - Haematology - Biochemistry - Globulin levels
Substance: Eugenol - WHO/FAO Joint Expert Committee on Food Additives (JECFA). WHO Food additive series No. 56, 155-200. (2006) (review from Hirose *et al.*, 1987). - Induction of forestomach lesions in rats by oral administrations of naturally occurring antioxidants for 4 weeks. -Species: rat -28-day study (AS) B.6.3.2.1 Supportive study	- Mortality: not reported. - Infections: not reported.	- Organs weight -Macroscopic findings - Histopathology - Haematology - Biochemistry - Globulin levels
Substance: Eugenol - WHO/FAO Joint Expert Committee on Food Additives (JECFA). WHO Food additive series No. 56, 155-200. (2006) (review from Hagan *et al.*, 1965) - Toxic properties of compounds related to safrole -Species: rat -34-day study (AS) B.6.3.3.1 Supportive study	- Mortality: few deaths were reported at 2000 mg/kg bw/day and the number increased with the dose (no more data specified). - Infections: not reported. -Adrenal macroscopic observation: slight adrenal enlargement, with marked yellow discolouration. -Adrenal histopathology: unaffected.	- Organs weight -Macroscopic findings. - Histopathology. - Biochemistry - Globulin levels
Substance: Eugenol -National Toxicology Program (1983) -Carcinogenesis studies of eugenol in F344/N rats an B6C3F$_1$ mice (feed studies) -Species: rat -90-day study (AS)	- Mortality: not reported - Infections: not reported. --Adrenal histopathology: unaffected - Mesenteric and submaxillary lymph nodes histopathology: unaffected. - Spleen histopathology: unaffected. - Thymus histopathology: unaffected. - Femur bone marrow: unaffected.	-Organ weights Haematology - Biochemistry - Globulin levels

Study	Immune parameters analysed	Not analysed*
B.6.3.4.1 Supportive study		
Substance: Eugenol -National Toxicology Program (1983) -Carcinogenesis studies of eugenol in F344/N rats an B6C3F$_1$ mice (feed studies) -Species: mice -90-day study (AS) B.6.3.4.2 Supportive study	- Mortality: not reported - Infections: not reported. -Adrenal histopathology: unaffected - Mesenteric and submaxillary lymph nodes histopathology: unaffected. - Spleen histopathology: unaffected. - Thymus histopathology: unaffected. - Femur bone marrow: unaffected.	-Organ weights - Haematology - Biochemistry - Globulin levels
Substance: Eugenol -Hagan, E.C. (1967). - Toxic properties of compounds related to safrole -Species: rat -133-day study (AS) B.6.3.5.1 Supportive study	- Mortality: not reported - Infections: not reported. - White blood cell counts (WBC): unaffected. - Spleen weight: unaffected. - Spleen histopathology: unaffected. - Femur bone marrow: unaffected.	-Organ weights -Macroscopic findings - Histopathology - Biochemistry - Globulin levels
Long-term and carcinogenesis studies		
Substance: Eugenol -National Toxicology Program (1983) -Carcinogenesis studies of eugenol in F344/N rats an B6C3F$_1$ mice (feed studies) -Species: rat -2-year study (AS) B.6.5.1 Supportive study	- Mortality: compared to controls - Infections: not reported. -Adrenal histopathology: unaffected - Mesenteric and submaxillary lymph nodes histopathology: unaffected. - Spleen histopathology: (↑) incidence of spleen haemosiderosis (14%) in high dose female group (12500 ppm). - Thymus histopathology: unaffected. - Femur bone marrow: unaffected. -Peyer patches histopathology: unaffected	- Organs weight - Haematology - Biochemistry - Globulin levels.
Substance: Eugenol -National Toxicology Program (1983) -Carcinogenesis studies of eugenol in F344/N rats an B6C3F$_1$ mice (feed studies) -Species: mice -2-year study (AS) B.6.5.2 Supportive study	- Mortality: A reduction in survival was detected in 3000 ppm and 6000 ppm male groups (12%), and in 3000 ppm female group (6%), compared with controls at termination of the study - Infections: not reported. -Adrenal histopathology: unaffected - Mesenteric and submaxillary lymph nodes histopathology: unaffected. - Spleen histopathology: unaffected. - Thymus histopathology: unaffected. - Femur bone marrow: unaffected. -Peyer patches histopathology: unaffected	- Organs weight - Haematology - Biochemistry - Globulin levels.

Study	Immune parameters analysed	Not analysed*
Substance: Eugenol -Miller, E.C. (1983). - Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. -Species: mice -18-months study (AS) B.6.5.3 Supportive study	- Mortality: similar to controls - Infections: not reported. - Thymus: (↑) Thymic lymphoma (7%) at the single dose tested (750 mg/kg bw/day) compared with controls.	- Organs weight - Haematology - Biochemistry - Globulin levels. - Histopathology.
Reproduction		
Substance: Eugenol -[redacted] (2004). -Eugenol: Oral gavage pre-natal developmental toxicity study in the rat .-Species: rat -Developmental study (AS) B.6.6.2.5 Acceptable study	- Mortality: One female death at high dose group (600 mg/kg bw/day), but not was associated to treatment. - Infections: not reported.	- Organs weight
Substance: Eugenol -[redacted] (2004). - Eugenol: Oral gavage pre-natal developmental toxicity study in the rabbit. .-Species: rabbit -Developmental study (AS) B.6.6.2.6 Acceptable study	- Mortality: Three females (12.5%) at high dose group (500/300 mg/kg bw/day) and one female (4%) in the mid dose group (250 mg/kg bw/day) died. The deaths were associated to treatment. - Infections: not reported.	- Organs weight
Medical Data		
Substance: Eugenol -Volpe, M.J. (2021) (AS) B.6.9.1	The manufacturing plant conducts regular medical monitoring of workers involved in the production, packaging or handling of eugenol. There have been no reports of reactions or ill health in any workers.	-n/a
Substance: Eugenol - Greif, N. (1967) -Cutaneous safety of fragrance material as measured by the Maximization test (AS) B.6.9.2.1	This study classified eugenol as non-sensitizer in a maximization test performed in human volunteers following Kligman methodology.	-n/a
Substance: Eugenol	No cases of delayed hypersensitivity that were directly attributable to eugenol or clove leaf oil were induced in the	-n/a

Study	Immune parameters analysed	Not analysed*
- Rothenstein, A.S. (1987). - Eugenol and clove leaf oil: A survey of consumer patch sensitisation. (AS) B.6.9.2.2	patch tests on consumer products and fragrance blends containing these ingredients.	
Substance: Eugenol - De Frutos, F.J.O. (2004). - Eczema alérgico de contacto profesional en auxiliar de odontología (Occupational allergic contact eczema in a dental assistant). (AS) B.6.9.2.3	This is a clinical case report of a 46-year-old woman employed as dental clinic assistant. She developed pruritic, flaky, erythematous-edematous lesions, with indistinct borders, on the backs and sides of the fingers, which had been developing for several months. The woman was subjected to skin tests to ascertain the compound that causes the skin sensitization and allergy process. Positivity was observed in the compound test with eugenol 2%, endodontics liquid, fragrance mix and colophony. The eugenol concentration and source of the endodontics liquid, fragrance mix and colophony was unknown.	-n/a
Substance: Eugenol - Sanchez-Perez, J (1999). - Occupational allergic contact dermatitis from eugenol, oil of cinnamon and oil of cloves in a physiotherapist. (AS) B.6.9.2.4	This is a clinical case report of a 32-year-old man employed as physiotherapist. He performed patient massaged with topical products, including ‘‘Balsam from ash extract’’ cream. The man was subjected to skin tests to ascertain the compound that causes the skin sensitization and allergy process. Positivity was observed in the compound test with eugenol and clove oil 1%. However, the eugenol and clove oil concentration in massage products such as balsam and creams was unknown.	-n/a
Substance: Clove oil - Hartnoll, G. (1993). - Near fatal ingestion of oil of cloves. B.6.9.3.1	This is a clinical case report of an accidental clove oil ingestion in a 2-year-old male. He fell into a coma within 3 hours post ingestion. Twenty-four hours after ingestion the patient was still unconscious, clinical tests showed evidence of liver toxicity, and disseminated intravascular coagulation was diagnosed. Patient was treated with fresh frozen plasma, heparin, antithrombin III, protein C and factor VII. Full recovery was observed on day 6.	-n/a

** Parameters not analysed, despite they should be reported according to the respective OECD protocol.*

The collected data permit to build an overview on the immunotoxic potential of eugenol, considering the following groups of parameters:

General health condition

Within the limitations of the experiments performed under laboratory conditions, no particular concern about the immune system functioning arises from the analysis of mortality and infections in this dataset.

Haematology parameters

White blood cells counts were not altered.

Biochemical parameters

Globulins levels and other immunological-related parameters were not measured in the studies provided.

Organs and tissues

Lymph nodes: No alteration in lymph nodes was detected in the 90-day and two-year studies in rodents.

Peyer’s patches: Peyer’s patches examination was only performed in 2-year carcinogenicity studies in rodents. No abnormalities were described.

Spleen: Spleen weights were unaffected in the studies assessed. Spleen haemosiderosis was observed in the two-year carcinogenicity study in rats. However, neither clinical biochemistry nor haematology were evaluated in this study to support this observation. Besides, organ macroscopic evaluation did not show additional spleen changes. No more alterations in spleen were found in the other studies. Thus, this effect was not considered as sign of immunotoxicity.

Thymus: Increased incidence of thymic lymphomas was found in the 18-month chronic toxicity study in mice. This incidence was low (7%), and was considered a single event due to no other alterations were found in thymus in the short-term, chronic/long- term and reproductive toxicity studies.

Bone marrow: No alteration in bone marrow was detected in the short-term and long-term studies.

Adrenal gland: Adrenal gland weights were unaffected in the studies assessed. There was a slight enlargement of adrenals glands in rats after 34-day eugenol treatment. This observation was considered a single event due to no more changes were reported in adrenals glands in short-term, chronic/long- term and reproductive toxicity studies.

Human data

Regarding to the available medical data, one manufacturing plant involved in the production, packaging or handling of eugenol, no reported reactions or ill health in any workers.

Data collected on humans involved skin sensitization tests and accidental oral ingestion of variable quantities of clove oil. Regarding skin sensitization, tests performed on human volunteers or individuals who usually used eugenol-containing products displayed diverse results. Wide-battery tests carried out in human volunteers did not show relevant results regarding skin sensitization for eugenol. On the other hand, two clinical cases from individuals that managed eugenol-containing products such as endodontics liquids and fragrance mixtures displayed positive results in skin sensitization tests.

Regarding oral toxicity, several accidental oral ingestion cases of clove oil (>80% eugenol) had been described in the literature. Clinical effects usually course with coagulation and hepatic impairment (Hartnoll *et al*., B.6.9.3.1). The currently routine clinical practice uses N-acetyl-cysteine (NAC) as primary curative therapy based on Thompson *et al.* studies (1998, B.6.8.1.2.1; and 1991, B.6.1.1.6), that revealed that NAC administration prevents eugenol hepatotoxicity

Conclusion on immunotoxicity

Based on the available toxicology data, no treatment related changes in the immunotoxic sensitive parameters were observed. In addition, eugenol does not belong to a class of chemicals (e.g., the organotins, heavy metals, or halogenated aromatic hydrocarbons) that would be expected to be immunotoxic. Within the scope of this brief analysis, **it can be concluded that eugenol is devoid of immunotoxic potential**.

2.6.9 Summary of medical data and information

The manufacturing plant involved in the production, packaging or handling of eugenol, no reported reactions or ill health in any workers.

Data collected on humans involved skin sensitization tests and accidental oral ingestion of variable quantities of clove oil. Regarding skin sensitization, tests performed on human volunteers or individuals who usually used eugenol-containing products displayed diverse results. Wide-battery tests carried out in human volunteers did not show relevant results regarding skin sensitization for eugenol. On the other hand, two clinical cases from individuals that managed eugenol-containing products such as endodontics liquids and fragrance mixtures displayed positive results in skin sensitization tests.

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2.6.10 Toxicological end points for risk assessment (reference values)

Table 67: Overview of relevant studies for derivation of reference values for risk assessment

Species	Study (method/type, length, route of exposure)	Test substance	Critical effect	NOAEL	LOAEL	Cross reference
Acute oral toxicity						
Rat strain: Albino rats ♂ and ♀ No. animals: 12 rats/sex/ dose	Single doses Eugenol :Oral (gavage) 10-day observation period	Eugenol	Clinical signs: Weakness of hind legs, paralysis of lower extremities, lethargy, coma.		LOAEL= 1597.5 mg/kg bw/day	B.6.2.1.2
Short-term toxicity						
Rat strain: F344/N rats: ♂ and ♀ No. animals: 10 rats/sex/ dose	13-week oral study in rats Eugenol :Oral (diet)	Eugenol	↓bodyweight and bodyweight gain.	-NOAEL= 6000 ppm (~540 mg/kg bw/day)	12500 ppm (~1125 mg/kg bw/day).	B.6.3.4.1
Mice strain: $B6C3F_1$: ♂ and ♀ No. animals: 10 mice/sex/ dose	13-week oral study in mice Eugenol :Oral (diet)	Eugenol	↓ bodyweight gain.	-NOAEL= 3000 ppm (~600 mg/kg bw/day)	-LOAEL= 6000 ppm (~1200 mg/kg bw/day).	B.6.3.4.2
Long-term toxicity						
Rat strain: F344/N rats: ♂ and ♀ No. animals: 50 mice/sex/ dose 40 mice in control group	2-year carcinogenicity study in rats Eugenol :Oral (diet)	Eugenol	↑ incidence of uterine endometrial stromal polyp/sarcoma and cystic hyperplasia in the uterus, ↑ spleen haemosiderosis, ↓ bodyweight in ♀.	-NOAEL= 6000 ppm (~260 mg/kg bw/day)	-LOAEL = 12500 ppm (~648 mg/kg bw/day)	B.6.5.1
Mice strain: $B6C3F_1$: ♂ and ♀ No. animals: 50 mice/sex/ dose	2-year carcinogenicity study in mice Eugenol :Oral (diet)	Eugenol	↑ increase incidences of focal inflammation of the kidney in ♂, focal granulomatous inflammation and adenomatous hyperplasia of lung, and ovary follicular cyst in ♀).	-NOAEL= 3000 ppm (~ 632 mg/kg bw/day)	-LOAEL= 6000 ppm (~ 1250 mg/kg bw/day)	B.6.5.2
Developmental toxicity						
Rat strain: Sprague-Dawley CD rats	Developmental toxicity study in rat.	Eugenol	↑ incidence of clinical signs	-NOAEL= 100 mg/kg/ bw/day	LOAEL= 250 mg/kg/ bw/day	B.6.6.2.5

Species	Study (method/type, length, route of exposure)	Test substance	Critical effect	NOAEL	LOAEL	Cross reference
No. animals: 25 mated females/group.	Eugenol :Oral (gavage)					
Rabbit strain: New Zealand White rabbits No. animals: 24 mated females/group.	Developmental toxicity study in rabbit. Eugenol :Oral (gavage)	Eugenol	↑incidence of clinical signs; ↓food consumption	-NOAEL= 100 mg/kg/ bw/day	LOAEL= 250 mg/kg/ bw/day	B.6.6.2.6

2.6.10.1 Toxicological end point for assessment of risk following long-term dietary exposure – ADI (acceptable daily intake)

The acceptable daily intake (ADI) for humans is normally derived from the NOAEL in the most susceptible species in long-term toxicity studies and applying an appropriate safety factor.

The most sensitive effects in the most sensitive species were found in a developmental toxicity study in rat and rabbit (Vol 3 AS-B.6.6.2.5 and B.6.6.2.6). In both studies, at same dose tested, several clinical signs compatible with neurotoxicity were observed in dams (piloerection in rats, and ataxia and breathing difficulties in rabbits were the most relevant). Therefore, an **ADI of 0.17 mg/kg bw/day** is proposed, based on the NOAEL of 100 mg/kg bw/day for maternal toxicity applying a 100 fold assessment factor (derived from 10-fold factor for inter-species variability and 10-fold factor for inter-individuals variability), and an additional factor of 6 that accounts the extrapolation for the subacute to chronic exposure. The latter factor is mentioned in the EFSA Guidance document on uncertainty factors (EFSA Journal 2012; 10(3):2579).

There was a disagreement between the RMS and the co-RMS in this point. The co-RMS agreed on the basis for ADI setting but considered that an additional uncertainty factor on 10 should be used because there is no information on the sensitivity of the developing nervous system to eugenol neurotoxicity (e.g. DNT data) and also due to the overall quality of the database (e.g. lacking of a generational reproductive toxicity study with eugenol). In the opinion of the RMS, this strategy could be applied, but only should be considered at the end of the process in case the data lacking remains.

2.6.10.2 Toxicological end point for assessment of risk following acute dietary exposure - ARfD (acute reference dose)

Potential neurotoxic effects were observed in acute and developmental toxicity studies. Impaired mobility, ataxia, prostration and paralysis were observed in acute toxicity study in rats at high doses (from 1597.5 mg/kg bw/day). As noted, the doses used in this study were close to acute oral LD_{50} in rats (1930 mg/kg bw/days) and disappeared over the fifth day. On the other hand, two developmental toxicity studies were conducted with eugenol via oral gavage in rat and rabbit. Pilo-erection in rats, and ataxia and breathing difficulties in rabbits were the most relevant neurotoxicity signs observed in these developmental toxicity studies. None of these clinical signs described in rats and rabbit appeared before the fifth day, hence these animals showed the effects after several days of repeated doses. In addition, most of these effects were resolved within 24 hours or reverted over time.

Therefore, based on the low evidence of acute toxic effects of the eugenol, and in agreement with EFSA conclusion 2012 [EFSA Journal 2012; 10(11):2914], and ARfD is not required.

There was a disagreement between the RMS and the co-RMS in this point. According to the co-RMS: "*The Co-RMS does not agree that an ARfD should not be set. As described in the RAR, potential neurotoxic effects were observed in acute and developmental toxicity studies. A proposal has been made to classify eugenol as STOT SE 3 for narcotic effects. In the developmental toxicity study in rats, piloerection was observed 1h post dose in 16% and 72% of the animals at 250 and 600 mg/kg bw (see Section 2.6.7, page 126). The co-RMS proposes to use the NOAEL = 100 mg/kg bw/day as the basis for ARfD setting. There is no information on the sensitivity of the developing*

nervous system to eugenol neurotoxicity (e.g. DNT data). In order to account for this uncertainty an additional factor of 10 should be considered in ARfD setting. Co-RMS proposes that this issue is discussed in a peer review meeting of experts". In the opinion of the RMS, the additional uncertainty factor could be applied, but only should be considered at the end of the process in case the data lacking remains.

2.6.10.3 Toxicological end point for assessment of occupational, bystander and residents risks – AOEL (acceptable operator exposure level)

The acceptable operator exposure level (AOEL) is defined on the basis of short-term toxicity studies in the most sensitive species and with the application of an appropriate safety factor.

The most sensitive effects in the most sensitive species were found in a developmental toxicity studies in rat and rabbit (Vol 3 AS-B.6.6.2.5 and B.6.6.2.6). In both studies, at same dose tested, several clinical signs compatible with neurotoxicity were observed in dams (pilo-erection in rats, and ataxia and breathing difficulties in rabbits were the most relevant). Therefore, a systemic **AOEL of 1.0 mg/kg bw/day** is proposed based on the NOAEL of 100 mg/kg bw/day for maternal toxicity applying a 100 fold assessment factor (derived from 10-fold factor for inter-species variability and 10-fold factor for inter-individuals variability).

There was a disagreement between the RMS and the co-RMS in this point. The co-RMS agreed on the basis for AOEL setting but considered that an additional uncertainty factor on 10 should be used because there is no information on the sensitivity of the developing nervous system to eugenol neurotoxicity (e.g. DNT data) and also due to the overall quality of the database (e.g. lacking of a generational reproductive toxicity study with eugenol). In the opinion of the RMS, this strategy could be applied, but only should be considered at the end of the process in case the data lacking remains.

2.6.10.4 Toxicological end point for assessment of occupational, bystander and residents risks – AAOEL (acute acceptable operator exposure level)

No ARfD has been derived and hence, no value of acute acceptable operator exposure (AAOEL) is proposed.
There was a disagreement between the RMS and the co-RMS in this point, as in the case of the ARfD setting.

2.6.11 Summary of product exposure and risk assessment

The product Mevalone is a capsule suspension (CS) formulation containing 33.3 g/L eugenol, 67.3 g/L geraniol and 66.7 g/L thymol (technical) which is applied to grapes and pome fruit as a fungicide.

No dermal absorption studies have been performed with Mevalone. Following the EFSA Guidance on dermal absorption (2017) and the SANTE/2018/10591 rev. 1 (Guidance on dermal absorption) document, default dermal absorption values have been defined for thymol, geraniol and eugenol.

Considering that the preparation Mevalone is formulated as a capsule suspension (CS), and taking into account the concentration of the active substances in the formulation (66 g/L for thymol and geraniol, and 33 g/L for eugenol), the following values are established for these active substances:

- Concentrate: 25% for thymol and geraniol, and 70% for eugenol.
- Dilution: 70% for thymol, geraniol and eugenol.

To perform the assessment, EFSA model has been applied and the following conclusions have been rised:

- **OPERATOR:**

According EFSA model, operator exposure to MEVALONE (4 L/ha) from tractor mounted air assisted sprayer application outdoor to high crops is below the AOEL with the use of workwear (arms, body and legs covered) and chemical protective gloves during mixing/loading and application.

However, a safe use for the operator to thymol from manual application is not obtained.

In the opinion of the RMS, as according EFSA Guidance, 2014, the penetration factor of the workwear is 10 %, a type 6 protective coverall (or the equivalent according EN-ISO 27065 :2017/A1 :2019) should be used.

Besides, and due to the toxicological classification of the product as skin sensitiser, face shield (according EN 166:2002) is advisable during mixing/loading of the product.

In case of tractor spraying, the specific chemical protective gloves will be used only to handle the application equipment or contaminated surfaces.

Moreover, during cleaning and handling of the equipment, the same PPE as mixing/loading should be used.

- **WORKER:**

For worker, the results of the exposure risk assessment indicate that the risk to residues of eugenol, geraniol and thymol is acceptable with the following conditions:

- For pome fruit (4 L fp/ha), the exposure of workers is acceptable when they wear work clothing (arms, body and legs covered) and chemical protective gloves and the numbers of applications are reduced to two.
- For vineyard, (4 L fp/ha) workers need work clothing and chemical protective gloves and the numbers of applications are reduced to a single one.

Treated crops should not be re-entered before spray deposits on leaf surfaces have completely dried.

- **RESIDENTS/BYSTANDERS**

For residents/bystanders, according EFSA model, and considering the SVC approach for vapour pathway, exposure to MEVALONE (4 L/ha) is below the AOEL of Eugenol, Geraniol, and Thymol, and AAOEL of Thymol.

2.7 RESIDUE

2.7.1 Summary of storage stability of residues

An interim study was evaluated in the original EU Review of eugenol, but only the one-month interim report was submitted. Now the final 1-year report has been submitted. Whole grapes were fortified at a rate of 0.5 mg/kg for eugenol. Whole grape samples were placed directly into frozen storage at -18°C immediately after fortification. At intervals of 0, 1, 3, 7, 14 and 27 days, 3, 6 and 12 months, stored samples were analysed for residues of eugenol. Under these conditions, residues of eugenol on whole grapes were stable for at least 12 months, with 71% of fortified eugenol recovered at 1 year. % mean recovery (% of nominal spike) at 12 months of storage was acceptable (71%), but almost at 30% of decline. In this sense, it should be reminded that mean procedural recovery conducted at 12 months was just 70%.

A new study has been submitted in order to determine the storage stability of eugenol and methyl-eugenol in grapes under frozen conditions for up to 278 days. Homogenised grapes were fortified with eugenol and methyleugenol at a rate of 0.1 mg/kg. Samples were placed directly into frozen storage at ≤-18°C immediately after fortification. At intervals of 0, 43, 111, 154 and 278 days stored samples were analysed for residues of eugenol and methyleugenol. Under these conditions, residues of methyleugenol on homogenised grapes were stable at 278 days, with 82 % of the nominal fortified amount. Residues of eugenol on homogenised grapes were stable for at least 154 days, with 74 % of the nominal fortified amount recovered. However, although eugenol was stable at 154 days after fortification, only 67% of the nominal fortified amount was obtained 111 days after fortification (with 105% procedural recovery at 111 days). It should be recognized that value at 111 days (67% of nominal fortified amount) is close to the limit of 30% of maximum acceptable decline value. Since the former storage stability study showed stability for eugenol of 12 months, we think that at least 154 days of storage stability for eugenol in grapes could be considered.

Moreover, a study was submitted in the context of first inclusion in order to determine the storage stability of eugenol in grapes surface residues under frozen conditions for up to 6 months. The results showed that 1 month after fortification, surface residues of eugenol on grapes were not stable, with 15% of fortified eugenol . However from a consumer point of view, relevant information should be referred to the entire fruits and not to the surface residues.

Regarding apples, a new study has been submitted in order to determine the storage stability of eugenol and methyl-eugenol in apples under frozen conditions for up to 287 days. Homogenised apples were fortified with eugenol and methyleugenol at a rate of 0.1 mg/kg. Samples were placed directly into frozen storage at ≤-18°C immediately after fortification. At intervals of 0, 30, 112, 161, 202 (eugenol only), 285 and 287 (eugenol only) days stored samples were analysed for residues of eugenol and methyleugenol. For eugenol, stability was demonstrated for 112 days, with 89 % of the nominal fortified amount of eugenol recovered at 112 days. For methyleugenol, stability was demonstrated for 285 days, with 79 % of the nominal fortified amount of methyleugenol recovered at 285 days.

Apples are high water content whilst grapes are considered high acid content according to OECD Guideline No. 506. Therefore, high acid and high water content commodities are represented.

Regarding analytical methods, previously agreed and new storage studies are fully supported by relevant method validations. For eugenol and methyl eugenol, an LOQ of 0.01 mg/kg was set in apples. For grapes, lowest LOQ achieved was also of 0.01 mg/kg for eugenol and methyl eugenol.

Extract stability

Animal commodities

Not any storage stability study for animal commodities has been submitted in order to support the intended uses. However, since not any animal feeding studies were required according to the intended uses, those storage stability studies are not considered necessary.

2.7.2 Summary of metabolism, distribution and expression of residues in plants, poultry, lactating ruminants, pigs and fish

Plants

During Annex I inclusion of eugenol (EFSA Journal 2012;10(11):2914), plant metabolism studies were not submitted. According to that EFSA conclusion, eugenol occurs naturally in plants and very limited information from the published literature was reported in the former DAR. Data on the natural background levels of eugenol in grapes from retail samples were also submitted and gave indication of residue levels far below 0.05 mg/kg (validated LOQ of the method). EFSA was of the opinion that no metabolism data was required to conduct a reliable consumer risk assessment with regard to the eugenol residues if the submitted residue trials are considered as acceptable. However in 2017 EFSA indicated that the occurrence of methyl eugenol as a residue in grapes when eugenol is applied as a pesticide active substance according to the representative use on grapes was identified as a data gap during the initial peer review (EFSA, 2012) and was part of the confirmatory data to compare the natural dietary background exposures of methyl eugenol to exposure to methyl eugenol from the use of eugenol as a plant protection product. EFSA considered that the assessment of the confirmatory data could not be finalised without these data (EFSA Supporting publication 2017:EN-1165).

The Notifier (Eden Research) presented an additional metabolism study which has been submitted by Xeda in the the context of clove oil dossier. The study considers the metabolism of eugenol in apples. Apples are a member of the fruit crops metabolism group, and therefore this study is relevant to both grapes and apple intended uses. This metabolism study should be considered as bringing additional information and was submitted for completeness purposes to address concerns in relation to the potential formation of methyleugenol

According to OECD 501, a foliar study can substitute for a post-harvest study if the mature commodity is present and exposed at application. Notifier suggest extrapolating from the available post-harvest metabolism study to the intended uses, assuming Mevalone to be applied to the mature crop commodity. However, grape uses are intended at BBCHs from 60 (beginning of flowering) to BBCH 89 (end of ripening). Unfortunately only in the later intended growth stages the mature fruits are present and exposed at application. Therefore, at least for BBCH 60-79, clearly extrapolation is not possible for grapes. Regarding apples, uses are intended at BBCHs from 75 (fruits about half final size) to BBCH 87 (fruit ripe for picking). Unfortunately only in the later intended growth stages the mature fruits are present and exposed at application. Therefore, at least for BBCH 75-79, clearly extrapolation is not possible for apples.
Summarizing, the available post-harvest metabolism study is only valid when the fruits are mature at application, i.e BBCH 81-89 for grapes and BBCH 81-87 for apples.

Regarding N rate, it must be recognized that metabolism study is a post-harvest study, whilst intended uses are foliar pre-harvest uses. It has already indicated that a post-havest study can substitute a foliar study if the mature commodity is present and exposed at application. Actually the intended use rates are not comparable. However, an estimation can be drawn up. The metabolim study was conducted with 0.7% of bioxeda (0.7 l product/hL water x

203.8 g clove oil/l product = 142.66 g clove oil/hL water, equivalent 114,13 g eugenol/hL water). Taking into account the concentration for the intended foliar treatments (13.3 g eugenol/hL water), a theoretical N rate could be calculated as 8.59NX). Obviously, this comparation is not completely reliable since fruits are wetted depending on the dose rate in kg as/sa (or l water/ha). However the estimated N rate can be considered as a worst case, since the final concentration over the fruits in the foliar application never should be higher than 13.3 g eugenol. Therefore, the real N rate should be 8.59X or higher.

According to the submitted study, it seems that the metabolism of eugenol in stored apples proceeds *via* glucose conjugation to the eugenol core and the subsequent reduction of the propenyl chain to the dihydroeugenol glucose conjugate. Moreover, the compound methyl-eugenol was not observed in the samples analysed

It has already been indicated that metabolism study is only valid when the fruits are mature at application. However it should be emphasized that the potential formation of methyleugenol due to the earlier application growth stages, could be addressed considering the results of the available residue trials (see 2.7.4). In this sense, methyleugenol was not detected in the trials when the application were conducted before or after the beginning of the fruit maturity (BBCH 81).

According to thestudy, eugenol was converted completely to the glucose conjugate after two weeks of storage,. In fact, as indicated in Table B.7.2.1-2, [14C]-Eugenol-glucose conjugate already reached 79.1% of TRR at 0 weeks (i.e., directly after completion of the application procedure). It should be recognized that metabolism study was conducted with a heated formulation in the range 45-50 °C, which could make the metabolim faster. On the other hand, residue trials were not conducted with a heated formulation.
% TRR at final timepoint, i.e. following storage for 24 weeks after application of eugenol, is 64.1% TRR attributable to [14C]-Eugenol-glucose conjugate and 35.9% TRR attributable to [14C]-Dihydro Eugenol-glucose conjugate.

Regarding analytical methods and according to the summary provided, the method seems to fulfil SANCO 7028/VI/95 rev.3 criteria. Methods of analysis were adequately chosen for the development of the study: LSC for analysis on radioactivity, HPLC-UV for radiochemical purity and LC-MS for identification of the metabolites formed during the study. However there are two methods –Method 1 and Method 2- for the LC-MS analysis used for identification of eugenol and/or glucose-conjugate residues.

Livestock
The only relevant feed commodity for the intended uses of eugenol is the apple wet pomace. According to OECD Series on Pesticides No. 73 (ENV/JM/MONO(2013)8, apple wet pomace is not a feed item relevant to poultry. Therefore, metabolism studies on poultry are not required.

For ruminants, Notifier has compared this safe value of 25 mg/kg complete feed with the calculated values coming from using the *pesticides_mrl_guidelines-animal-model-2017.xls* and the proposed STMR of the residue figures.

The currently calculated STMR is 0.01 mg eugenol (free and conjugated)/kg. A processing factor (PF) from apple to wet pomace should be used too. Default PF (5) is used in the calculation. According to the output of the animal model, the trigger values would be exceeded for not any group of animals. Moreover the values of mg eugenol/kg diet (dry matter) for all the relevant groups are negligible in comparison with the indicated safe value of 25 mg/kg complete feed (EFSA Journal 2011;9(12):2440). Therefore, the intended use for grapevines and apple trees can be considered safe regarding the residues of eugenol which are ingested by the ruminants, and metabolism studies on lactating ruminants are not required.

The only relevant feed commodity for the intended uses of eugenol is the apple wet pomace. According to OECD Series on Pesticides No. 73 (ENV/JM/MONO(2013)8, apple wet pomace is not a feed item relevant to pigs. Therefore, metabolism studies on pigs are not required. Even so, Notifier has submitted a published paper which can be used as only supporting information for metabolism in pigs.

A metabolism study in fish is not required as the log Pow of eugenol, is not ≥ 3 (SANCO/11187/2013 rev.3). According to SANCO 11187/2013 rev.3, grapes, pome fruits and their processing products are not considered as commodities commonly used for the formulation of aquaculture diets (see Annex 2. Feedingstuffs table). Therefore, the use of eugenol according to the intended uses is not foreseen to affect fishes feeding. Therefore, metabolism studies for fishes are nor necessary.

Chemical formulations containing eugenol are used in water as an anaesthetic for fish. Severalpapers were identified in the literature search as being potentially relevant to the metabolism of eugenol in fish. Eugenol is used as a

sedative for fish and the paper by Meinertz *et al*. (2014) looks at residues of eugenol in fish following exposure. The paper by Ke *et al*. (2018) was identified in the literature search as being potentially relevant to the metabolism of eugenol and methyleugenol in fish. The paper looks at residue levels of eugenol and methyleugenol found in fish following its use as an anaesthetic for fish. It does not discuss any metabolism, but it does demonstrate that residues may be found in fish from non-plant protection product uses. Besides, it was demonstrated that, after the exposure, the depletion of eugenol residues in fish meat is rapid, when exposed to flesh flowing water.

2.7.3 Definition of the residue

Based on the outcome of the metabolism study and considering that conjugated eugenol could be potentially bioavailable, the residue definition for risk assessment in plant products should theoretically change to: eugenol (sum of free and conjugated). Conjugated forms of eugenol may be representative of the total eugenol content when no parent eugenol is present after 2 weeks (T2) according to the metabolism study. Since the analytical method in the metabolism study measures residue components separately and there is a potential cleavage of the conjugates into eugenol it is reasonable that the residue definition is expressed additively as "sum of free and conjugated eugenol expressed as eugenol. Therefore this residue definition should theoretically be applicable for, risk assessment, since free eugenol was not the only analyte monitored.

On the other hand, methyleugenol is a relevant impurity present in eugenol and due to its classification for potential genotoxicity and carcinogenicity, in the previous EU Review, it was requested to consider methyleugenol in the consumer risk assessment. For this reason, the residue trials considered residues of the parent eugenol and also the relevant impurity, methyleugenol in order to to confirm the lack of this impurity in the fruits.The residue trials demonstrate that no residues of eugenol or methyleugenol were present at harvest, arising from the plant protection product use. Eugenol and methyleugenol are naturally occurring in a wide variety of fruits, vegetables, herbs and spices. In this submission, use is supported on grapes and pome fruits. Residue trials have demonstrated eugenol residues to be <LOQ (<0.01 mg/kg) in both grapes and apples at harvest and no residues of methyleugenol were detected in any sample at any timepoint (see 2.7.4). It should be emphasized that eugenol conjugates have not been analysed in the residue trials.

Eugenol and methyleugenol are naturally occurring in grapes. In a study to measure natural background concentrations in grapes (Jones, 2012), in 20 different grape samples eugenol was found to be present in the range ND to 0.0042 mg/kg. Fenoll *et al*. (2009) detected eugenol in Muscat grapes at concentrations of 0.00043 to 0.00462 ~~µ~~mg/kg, depending on the stage of ripening.
Thus, the levels of eugenol and methyleugenol found in the residue trials are comparable to levels reported to be naturally occurring in some varieties of grapes. Indeed, no residues of methyleugenol were detected at any timepoint and residues of eugenol were not detectable at harvest.

Eugenol and methyleugenol are also both naturally occurring in apples (Yauk *et al*. 2017). Levels of eugenol in the range 0.0161-0.0346 mg/kg have been reported by Thedy *et al*. (2005). Concentrations of eugenol and methyleugenol in Royal Gala apples were found to be in the range 0.0412 and 0.0144 mg/kg respectively (Yauk *et al*. (2015)).
Thus, the levels of eugenol and methyleugenol found in the residue trials are comparable to levels reported to be naturally occurring in some varieties of apples. Indeed, no residues of methyleugenol were detected at any timepoint and residues of eugenol were <LOQ at harvest.

According to the available information, eugenol seems to fit the Criterion 4 to SANCO/11188/2013: The consumer exposure to residues of eugenol linked to use as plant protection product is considered as negligible compared to other uses in the food chain and/or natural background. Therefore it could be proposed that a monitoring residue definition for plant matrices is not required, since it is proposed to include eugenol into Annex IV to Regulation (EC) No. 396/2005. However a risk assessment residue definition is proposed as eugenol (sum of free and conjugated), useful in order to evaluate the possible risk for consumers.

Separate residue definitions for rotational crops and processed commodities are not required. Specific studies to cover these areas were not triggered.

A residue definition in animal matrices is not required. Only apple pomace is fed to livestock in the EU. Residue levels in apple commodities are all <LOQ (<0.01 mg/kg). Consequently, the livestock dietary intake of eugenol is predicted to be low and below the trigger of 0.004 mg/kg bw/d. Taking into account the likely dietary exposure of livestock by consumption of treated apple products, it is expected that there will be negligible exposure. Based on

this information, it is considered not necessary to change the existing residue situation; therefore enforcement and risk assessment residue definitions are not proposed for livestock products.

2.7.4 Summary of residue trials in plants and identification of critical GAP

The proposed GAP for grapevines is a up to 4 applications at 13.2 g eugenol/hL, 26.4 g geraniol/hL and 26.4 g thymol/hL with a 7 day interval and 7 day PHI. It is noted that the critical GAP supported for renewal is the same as the one supported for the first inclusion in the EU.

Residues of eugenol in grapes were determined in 3 studies. A total of 8 valid trials in grapes was conducted in Northern EU (Austria, Germany and Northern France) and in Southern EU countries (Spain, Portugal and Italy) in 2006 and 2020.

Regarding study S20-06337, six residue trials were conducted according to the critical GAP for the renewal and are therefore relevant to support the use of Mevalone in the EU. In addition in these 2020 trials, methyleugenol was considered.

Regarding study AF/11125/ED only surface extractable residues were considered. Since the storage stability study on surface extractable residues did not demonstrate stability of surface extractable residues when stored at -18°C, this residue trial is considered not to be valid and provides supporting information only. Moreover from a consumer point of view, relevant information should be referred to the entire fruits and not to the surface residues.

Regarding study AF/10728/ED, Notifier explained that four residue decline trials for eugenol on grapes were conducted in Spain (2 trials) and Italy (2 trials), during the 2006 growing season. However, it should be emphasized that trials conducted in Spain were carried out less than 20 km far from one another, and therefore different geographical sites could not be demonstrated. Dates of treatment are different but not more than 30 days apart. The same situation was seen in the two Italian trials. Therefore, only 2 residue trials (one in Spain and one in Italy) can be considered, and the other two residue trials must be considered as replicates.

In all the 2006 considered residue trials, residues were less than the LOQ (<0.05 mg/kg) in the season trials. For the 2020 season trials, the LOQ was set at <0.01 mg/kg. No residues of eugenol or methyleugenol were detected in any of the untreated samples. Residues of eugenol were not detected to 0.02 mg/kg in the treated samples on the day of application. All residues had declined to not detectable (ND) by one day after the last application. No residues of methyleugenol were detected in any of the treated samples.

The determined residue values have been used to calculate the highest residue (HR), the supervised trial median residue (STMR) and theoretical calculated MRL_{EU} (although no MRL is proposed). LOQ (0.01 mg/kg for the new trials) is taken as the STMR and also the HR and calculated MRL. The two old trials showing residues of <0.05 mg/kg (<LOQ) (Bailey, 2007) are supportive of the results of the new trials showing no detectable residues. The old studies did not differentiate between ND and <LOQ.

The proposed GAP for pome fruits is up to 4 applications at 13.2 g eugenol/hL with a 7 day interval and 1 day PHI for the Northern residue zone. A 3 day PHI is being supported for the southern residue zone in the MRL application that accompanies this submission, but it is not a representative use for the EU Review.

Residues of eugenol in apples were determined in one study. A total of 6 trials in apples was conducted in Northern EU (Austria, Germany and Northern France) and in Southern EU countries (Spain, Italy and Sothern France) in 2020. Residues of eugenol and methyleugenol were all <LOQ, even on the day of application of Mevalone according to the cGAP. Taking into account its natural occurrence and residues below the LOQ after application, it is considered that 6 trials over the two zones are sufficient.

Apple is the representative crop for pome fruit and the results can be extrapolated to the entire pome fruit group. However it should be emphasized that black chokeberry (*Aronia melanocarpa*) and mountain ash (*Sorbus* sp.) have been applied for the Notifier, but they are not included in the pome fruits group (see Commission Regulation 2018/62). Therefore they cannot be supported.

It should be indicated that application in most of the valid residue trials were conducted after beginning the fruit maturity (BBCH 81). However in two S-EU residue trials in apples (S20-06361-04 and S20-06361-06), the first applications were conducted before fruit maturity.

The determined residue values have been used to calculate the highest residue (HR), the supervised trial median residue (STMR) and theoretical calculated MRL_{EU} (although no MRL is proposed). LOQ (0.01 mg/kg for the new trials) is taken as the STMR and also the HR and calculated MRL.

No MRL is required for eugenol as it is listed in Annex IV to Commission Regulation (EC) No 839/2008. According to Commission Regulation (EC) No 839/2008 it was temporarily included in Annex IV,

It is proposed that eugenol is included into Annex IV to Regulation (EC) No. 396/2005, meaning that MRLs are not required. Eugenol and methyleugenol are naturally occurring in grapes and apples, and no detectable residues are found following application of Mevalone according to the cGAP for grapes and apples. Taking this into account, it is considered that the available trials over the two zones are sufficient for grapevines and apple trees.

Table. **Summary of the available residues trials data for RAC (whole fruit) for eugenol (sum of free and conjugated)**

Crop	**Region/ Indoor**	**Residue levels of eugenol(mg/kg) observed in the residue trials representative for the intended GAPs**	**Recommendations/comments** (OECD calculations)	**HR** (mg/kg)	**STMR** (mg/kg)
Grapevine	Outdoor S-EU	Fruit: 3x <0.01 (LOQ); 2x <0.05 (LOQ)	MRL†: 0.01 mg/kg	0.01†	0.01†
Grapevine	Outdoor N-EU	Fruit: 3x <0.01 (LOQ)	MRL†: 0.01 mg/kg	0.01†	0.01†
Apple tree††	Outdoor S-EU	Fruit: 3x <0.01 (LOQ)	MRL: 0.01 mg/kg	0.01	0.01
Apple tree	Outdoor N-EU	Fruit: 3x <0.01 (LOQ)	MRL: 0.01 mg/kg	0.01	0.01

† LOQ (0.01 mg/kg for the new trials) is taken as the STMR and also the HR and calculated MRL. The two old trials showing residues of <0.05 mg/kg (<LOQ) (Bailey, 2007) are supportive of the results of the new trials showing no detectable residues. The old studies did not differentiate between ND and <LOQ.
†† PHI of 1 day for the Northern residue zone. A 3 day PHI is being supported for the Southern residue zone in the MRL application that accompanies the re-approval Dossier, but it is not a representative use for the EU Review.

It should be taken in to account that no acidification after extraction was performed in the methods to transform glucose conjugates into eugenol. The quantification of the conjugates was not performed because the standards used were eugenol and not the conjugates. Since for risk assessment residue definition not only eugenol also conjugates have been considered, the methods may not be considered fully validated according to the residue definition for risk assessment.

2.7.5 Summary of feeding studies in poultry, ruminants, pigs and fish

The only relevant feed commodity for the intended uses of eugenol is the apple wet pomace (grape pomace is not fed to livestock in the EU).
According to OECD Series on Pesticides No. 73 (ENV/JM/MONO(2013)8, apple wet pomace is not a feed item relevant to poultry. Therefore, feeding studies on poultry are not required.

Notifier´s rationale is based in the EFSA Scientific Opinion on the safety and efficacy of allylhydroxybenzenes (chemical group 18) when used as flavourings for all animal species (EFSA Journal 2011;9(12):2440). This EFSA Scientific Opinion states that eugenol can be used safely in all animal species (other than fish) at levels up to 25 mg/kg complete feed. It is further stated that the use of eugenol as a flavouring for all mammal species up to the highest use levels proposed in feed is safe for the consumer.

Notifier has compared this safe value of 25 mg/kg complete feed with the calculated values coming from using the *pesticides_mrl_guidelines-animal-model-2017.xls* and the proposed STMR of the apple residue figures at the cGAP

A calculation can be carried out using the *pesticides_mrl_guidelines-animal-model-2017.xls.*

The currently calculated STMR is 0.01 mg eugenol (free and conjugated)/kg. A processing factor (PF) from apple to wet pomace should be used too. Default PF (5) is used in the calculation.

Relevant groups	Dietary burden expressed in				Most critical diet (a)	Most critical commodity (b)		Trigger exceeded (Yes/No)
	mg/kg bw per day		mg/kg DM					0.004
	Median	Maximum	Median	Maximum				mg/kg bw
Cattle (all diets)	0,001	0,001	0,03	0,03	Beef cattle	Apple	pomace, wet	No
Cattle (dairy only)	0,000	0,000	0,01	0,01	Dairy cattle	Apple	pomace, wet	No
Sheep (all diets)	0,001	0,001	0,01	0,01	Lamb	Apple	pomace, wet	No
Sheep (ewe only)	0,000	0,000	0,01	0,01	Ram/Ewe	Apple	pomace, wet	No
Swine (all diets)								No
Poultry (all diets)								No
Poultry (layer only)								No
(a): When several diets are relevant (e.g. cattle, sheep and poultry "all diets"), the most critical diet is identified from the maximum dietary burdens expressed a								
(b): The most critical commodity is the major contributor identified from the maximum dietary burden expressed as "mg/kg bw per day".								

According to the output of the animal model, the trigger values would be exceeded for not any group of animals. Moreover the values of mg eugenol/kg diet (dry matter) for all the relevant groups are negligible in comparison with the indicated safe value of 25 mg/kg complete feed (EFSA Journal 2011;9(12):2440).
Therefore, the intended use for grapevines and apple trees can be considered safe regarding the residues of eugenol which are ingested by the ruminants, pigs and poultry.

The only relevant feed commodity for the intended uses of eugenol is the apple wet pomace (grape pomace is not fed to livestock in the EU). According to OECD Series on Pesticides No. 73 (ENV/JM/MONO(2013)8, apple wet pomace is not a feed item relevant to pigs. Therefore, feeding studies on pigs are not required.

According to SANCO 11187/2013 rev.3, grapes, pome fruits and their processing products are not considered as commodities commonly used for the formulation of aquaculture diets (see Annex 2. Feedingstuffs table). Therefore, the use of eugenol according to the intended uses is not foreseen to affect fishes feeding.

Therefore, feeding studies for fishes are not necessary.

2.7.6 Summary of effects of processing

A study on the nature of the residue is not required. No residues at or above the LOQ (0.01 mg/kg) were found in grapes or apples (representative crop for pome fruit). According to Commission Regulation (EU) No 283/2013 a study on the nature of the residue is only required if residues above 0.01 mg/kg are found in the raw commodity to be processed. Only two old trials in grapevines (from a total of 14 trials results in grapes and apples) showed residues of <0.05 mg/kg (<LOQ) (Bailey, 2007). These 2 old trials did not differentiate between ND and <LOQ.

Regarding fruit peeling, no data are required as the representative crops (grapes and pome fruits) do not have an inedible peel.

According to Commission Regulation (EU) No 283/2013, studies are only required if residues in the part of the plant to be processed are >0.1 mg/kg. If this trigger is not exceeded, then a study is necessary if the TMDI is greater than 10% of the ADI or the dietary intake is greater than 10% of the ARfD. Processing or household studies are not required, since residues in the parts of the plant to be processed are lower than the trigger value of 0.1 mg/kg. No residues at or above the LOQ were found in grapes or apples (representative crop for pome fruit). Furthermore, the TMDI is less than 10% of the ADI for grapes and pome fruit. An ARfD is not required. Therefore, a magnitude of the residue processing study is not triggered.

2.7.7 Summary of residues in rotational crops

A metabolism study in rotational crops is not triggered. Grapes and pome fruit are perennial crops and therefore not grown in rotation.
Furthermore, according to Regulation (EU) 283/2013 a study is only required if the parent or its metabolites are persistent or significant concentrations of metabolites will occur in soil. In a soil metabolism study (Jones, A. 2015) the rate of degradation in 4 laboratory soils was studied. The DT_{90} for eugenol was clearly <3 days in all soils. A DT_{90} of 100 days is generally accepted as the trigger for persistence in soil. The DT_{90} for eugenol in soil is clearly below the trigger and therefore a study is not required.
No metabolites were formed in the soil metabolism study and therefore no metabolite uptake needs to be considered with respect to rotational crop studies.

2.7.8 Summary of other studies

2.7.8.1. Effect on the residue level in pollen and bee products

According to Appendix II to SANTE/11956/2016, grapes and pome fruit may be foraged by bees. For grapes the intended timing of application (BBCH 60-89) includes flowering stages (i.e. BBCH 60-69). However, the timing of application for pome fruit (BBCH 75-87) is after the flowering stage (BBCH 60-69) and therefore the bees will not forage blossoms following application.

It should be reminded that for grapes, intended use is for BBCH 60-89. However as indicated in the metabolism conclusion, the available post harvest metabolism study is only valid when the fruits are mature at application, i.e BBCH 81-89 for grapes and BBCH 81-87 for apples. Depending on the conclusion about validity of metabolism data for earlier applications, flowers could not be present at treatment for grapevine too (see B.7.2.1).

Available residue trials have showed that eugenol and methyleugenol levels in treated crops are below the trigger of 0.05 mg/kg on the day of the last application and after this date. The only available figures are for fruits, but it seems probable that the level of residues in the grapevine blossoms would be <0.05 mg/kg too.

Eugenol and methyleugenol are naturally occurring in a wide variety of plants upon which bees may forage and indeed naturally occurring residues of eugenol in honey have been reported in the literature.

Therefore studies on residues in bee products are not considered necessary.

2.7.8.2. Natural background levels

Eugenol and methyleugenol are naturally occurring in grapes. In a study to measure natural background concentrations in grapes, in 20 different grape samples eugenol was found to be present in the range ND to 0.0042 mg/kg. In the literature, eugenol concentrations in grapes between 0.00043 to 0.00462 µmg/kg have been reported. Eugenol concentrations have also been studied in wine and values between 5 and 68 mg/L wine are reported in the literature. Eugenol and methyleugenol are also both naturally occurring in apples (Yauk *et al*. 2017). Levels of eugenol in the range 0.0161-0.0346 mg/kg have been reported by Thedy *et al*. (2005). Concentrations of eugenol and methyleugenol in Royal Gala apples were found to be in the range 0.0412 and 0.0144 mg/kg respectively (Yauk *et al*. (2015)).
Concentrations found in the residue trials are comparable to those reported in the literature.
Eugenol is also approved for use as a food additive at higher concentrations than any residues reported in the residue trials. The estimated daily intake by the WHO of eugenol as a food additive in the EU is 0.018 mg/kg bw/day based on a 60 kg adult. This is 10 – 100 times greater than calculated exposures due to plant protection product use. The estimated daily intake by the WHO of methyleugenol is 0.00001 – 0.00065 mg/kg bw/day, mean 0.00016 mg/kg bw/day, based on a 60 kg adult in the EU and in the USA the intake is notably higher at 0.00004 – 0.0071 mg/kg bw/day, mean 0.00134 mg/kg bw/day, based on a 60 kg adult. This is in the range of very worst-case calculations made for exposures due to plants protection product use.

2.7.9 Estimation of the potential and actual exposure through diet and other sources

Assessments of the potential chronic dietary consumer risk due to exposure to residues of eugenol were performed using the EFSA model for chronic risk assessment (PRIMo rev. 3.1). An ARfD is not proposed for eugenol and therefore it is not necessary to perform an acute risk assessment.
It is proposed that MRLs are not required for eugenol, due to its natural occurrence in a wide variety of crops. Residue trials have demonstrated that the plant protection product use of eugenol does not result in any detectable residues of eugenol or methyleugenol in grapes and in pome fruit.

Nevertheless, a risk assessment has been performed for eugenol. It should be emphasized that the risk assessment residue definition include eugenol (sum of free and conjugated), and therefore conjugates should be considered for the risk assessment; since available data do not include the conjugates, the chronic risk is subestimated in this provisional approach.
For each commodity, default residue values of 0.01 mg/kg for eugenol have been used for the risk assessment, as indicated within Table 2.7.9-01. For grapes there were no detectable residues of eugenol and methyleugenol. For apples, all residues of eugenol were <LOQ and methyleugenol was not detected; so again an input at LOQ is considered. For all other commodities this is a default value based on the default levels at which MRLs would be set, if they were required.

Table 2.7.9-01: Eugenol and methyleugenol input values for the consumer risk assessment

Commodity	Chronic risk assessment		Acute risk assessment	
	Input (mg/kg)	**Comment**	**Input** (mg/kg)	**Comment**
Eugenol				
Products of plant origin				
Grapes	0.01	Default value*	-	Not required
Apples	0.01	Default value†	-	Not required
All other commodities listed within Regulation (EU) 2018/62	0.01	Default value	-	Not required
Products of animal origin				
All products of livestock origin (as listed within Regulation (EU) 2018/62)	0.01	Default value	-	Not required
Methyleugenol				
Products of plant origin				
Grapes	0.01	Default value**	-	Not required
Apples	0.01	Default value**	-	Not required
All other commodities listed within Regulation (EU) 2018/62	0.01	Default value	-	Not required
Products of animal origin				
All products of livestock origin (as listed within Regulation (EU) 2018/62)	0.01	Default value	-	Not required

† Residues in all residue trials were <LOQ (0.01 mg/kg) for eugenol. No quantification of conjugates performed.
* There were no detectable residues (LOD = 0.003 mg/kg) for eugenol. No quantification of conjugates performed.
** There were no detectable residues (LOD = 0.003 mg/kg)

Acceptable Daily Intake (ADI) and Dietary Exposure Calculation:

Eugenol
The ADI for eugenol is proposed at 0.17mg/kg bw/day (please refer to Vol.3.B6 for further details).,
The calculation of the TMDI was performed using the EFSA PRIMo model rev. 3.1, and using a default value of 0.01 mg/kg for all commodities (see Table 2.7.9-3).
With the current EFSA model the chronic risk assessment constitutes 0.7 % of the ADI. The diet with the highest TMDI was the NL toddler diet. For this diet the highest contributor is milk, followed by apples.
No refinement with use of the STMR is required.
When only the intended uses were considered, TMDI was performed too using the EFSA PRIMo model rev. 3.1 (see Table 2.7.9-4~~5~~).

It should be indicated that the chronic risk is subestimated in this provisional approach, since available data do not include the conjugates. However, it can be concluded that since there is a broad security margin for the calculated chronic risk assessment (only 0.7% of ADI), a chronic risk situation for the consumer is not foreseen to be reached.

Methyleugenol

An ADI is not proposed for methyleugenol (please refer to Vol.3.B6 for further details). A risk assessment was not conducted. However methyeugenol was not found (ND) in any of the available residue trials.

Acute Reference Dose (ARfD) and Dietary Exposure Calculation:

ARfDs are not proposed for eugenol or methyleugenol and therefore it is not necessary to perform acute risk assessments.

Comparison to background exposure

Eugenol and methyleugenol are naturally occurring in a wide variety of fruits, vegetables, herbs and spices (refer to Introduction to Vol.3CA-B.7). In this submission, use is supported on grapes and pome fruit. Residue trials have demonstrated no detectable residues in grapes at harvest. In apples, residues of eugenol were <LOQ and methyleugenol was not detected.
Eugenol and methyleugenol are also naturally occurring in grapes. In a study to measure natural background concentrations in grapes (Jones, 2012), in 20 different grape samples eugenol was found to be present in the range ND to 0.0042 mg/kg. Fenoll *et al*. (2009) detected eugenol in Muscat grapes at concentrations of 0.00043 to 0.00462 mg/kg, depending on the stage of ripening.
Eugenol concentrations in wine are dependent upon the concentrations in the grapes and also the wood used in the barrels or wood chips during the aging process. Ferreira *et al*. (2000) demonstrated eugenol to be present at up to 0.016 mg/L in wine following the analysis of 52 young single variety red wines. Ortega-Heras *et al*. (2007) found that the concentrations of eugenol in wine aged in American oak barrels varied between ca. 5 to 0.08 µmg/L, depending upon the duration of aging and the time the wine spent in the oak barrels. Rodriguez-Bencoma *et al*. (2009) demonstrated that the concentration of eugenol in wine was also affected by the origin of oak chips (France, Spain or USA), ranging from 0.008 µmg/L to 0.033 mg/L.
Thus, the levels of eugenol and methyleugenol found in the residue trials are comparable to levels reported to be naturally occurring in some varieties of grapes.
The per capita wine consumption within the EU varies between Member States, with per capita consumption for each MS summarised in Table 2.7.9-02[1]. The potential exposure to eugenol based on 0.005-0.068 mg/L wine is also presented in Table 2.7.9-02.

Table 2.7.9-02: Per capita wine consumption in EU in 2014 and potential exposure to eugenol based on 5-68 µg a.s./L

Country	Wine consumed (Litres per capita)	Potential eugenol exposure (µg/person/year) based on 5 µg a.s./L	Potential eugenol exposure (µg/person/year) based on 68 µg a.s./L
Luxembourg	10	50	680
France	43	215	2924
Slovenia	44	220	2992
Portugal	42	210	2856
Italy	33	165	2244
Denmark	14	70	952
Austria	31	155	2108
Belgium	23	115	1564
Hungary	24	120	1632
Greece	28	140	1904
Romania	24	120	1632
Germany	25	125	1700
Malta	33	165	2244
Sweden	26	130	1768
Spain	21	105	1428
Czech Republic	20	100	1360
Netherlands	18	90	1224
Estonia	3	15	204
Slovakia	16	80	1088
Cyprus	28	140	1904
Ireland	5	25	340

[1] Wine Institute: Wine consumption in Europe by country per year per capita (jakubmarian.com)

Country	Wine consumed (Litres per capita)	Potential eugenol exposure (µg/person/year) based on 5 µg a.s./L	Potential eugenol exposure (µg/person/year) based on 68 µg a.s./L
Finland	4	20	272
Bulgaria	21	105	1428
Lithuania	1	5	68
Latvia	3	15	204
Poland	4	20	272

The potential per capita exposure of humans to eugenol in wine varies from 0.005 to 2.992 mg a.s./person/year, equivalent to 0.000013 to 0.00820 mg a.s./person/day if it is presumed that the annual consumption is spread uniformly over 365 days.

Eugenol and methyleugenol are both naturally occurring in apples (Yauk *et al*. 2017). Levels of eugenol in the range 0.0161-0.0346 mg/kg have been reported by Thedy *et al*. (2005). Concentrations of eugenol and methyleugenol in Royal Gala apples of 0.0412 and 0.0144 mg/kg respectively, have been reported by Yauk *et al*. (2015).

Thus, the levels of eugenol and methyleugenol found in the residue trials are comparable to levels reported to be naturally occurring in some varieties of apples.

It is clear from the information presented that eugenol and methyleugenol are present in many plant species. Human dietary exposure can therefore occur by either consuming the 'raw' source of eugenol or methyleugenol (e.g. strawberries, basil, pimento pepper) or processed foods that contain sources of eugenol or methyleugenol (e.g. pesto sauce, processed meat). Consumption of eugenol and methyleugenol in beverages, such as coffee and wine, is also a likely exposure route for humans. It is clear that the use of eugenol on grapes and pome fruit will not increase exposure of humans to eugenol and methyleugenol above background levels. Residue trials demonstrated residues at harvest were all <LOQ and there is significant human exposure from other routes.

Nevertheless, since an ADI is set for eugenol, a conventional risk assessment is possible (see above) and the comparison to background is not necessary for the demonstration of safe use in eugenol.

As part of the confirmatory data submission for the first EU Review, an assessment of the exposure to eugenol was made based on naturally occurring concentrations reported in the literature. Although the assessment was questioned by EFSA, the calculation demonstrates the potential for significant, natural exposure to eugenol. It is important to note that:

1. This assessment is not necessary in terms of risk, because a conventional risk assessment using an ADI and the results of residue trials indicates an acceptable risk.

2. Literature data and proprietary studies show that eugenol is naturally occurring in grapes and apples in levels comparable to those observed in the residue trials. Natural levels vary widely.

3. Methyleugenol occurs in nature alongside eugenol and thus natural exposure to both is expected.

4. A GLP metabolism study does not indicate formation of methyleugenol from eugenol following application to fruit.

5. The residue trials showed no residues at the or above the LOQ and so there is no appreciable increase in concentrations of eugenol or methyleugenol following application.

6. Eugenol is approved for use as a food additive at higher concentrations than any residues reported in the residue trials (<LOQ <0.01 mg/kg).

7. Eugenol is approved for use in animal feed and is also used as a fish anaesthetic, resulting in a potential for exposure via non-plant protection product use.

Table 2.7.9-3: Eugenol TMDI calculations (EFSA PRIMo 3.1) (using a default value of 0.01 mg/kg)

European Food Safety Authority

EFSA PRIMo revision 3.1 2019/03/19

Eugenol

LOQs (mg/kg) range from: 0,01 to: 0,01

Toxicological reference values

ADI (mg/kg bw/day):	0,17	ARfD (mg/kg bw):	not necessary
Source of ADI:		Source of ARfD:	
Year of evaluation:		Year of evaluation:	

Input values

Details - chronic risk assessment | Supplementary results - chronic risk assessment

Details - acute risk assessment/children | Details - acute risk assessment/adults

Comments:

Normal mode

Chronic risk assessment JMPR methodology (IEDI/TMDI)

No of diets exceeding the ADI : ---

	Calculated exposure (% of ADI)	MS Diet	Exposure (µg/kg bw per day)	Highest contributor to MS diet (in % of ADI)	Commodity / group of commodities	2nd contributor to MS diet (in % of ADI)	Commodity / group of commodities	3rd contributor to MS diet (in % of ADI)	Commodity / group of commodities	Exposure resulting from: MRLs set at the LOQ (in % of ADI)	Exposure resulting from: commodities not under assessment (in % of ADI)
TMDI/NEDI/IEDI calculation (based on average food consumption)	0,7%	NL toddler	1,24	0,4%	Milk: Cattle	0,1%	Apples	0,0%	Maize/corn	0,7%	
	0,4%	NL child	0,65	0,1%	Milk: Cattle	0,0%	Sugar beet roots	0,0%	Apples	0,4%	
	0,4%	DE child	0,61	0,1%	Milk: Cattle	0,1%	Apples	0,0%	Wheat	0,4%	
	0,4%	UK infant	0,61	0,2%	Milk: Cattle	0,0%	Potatoes	0,0%	Wheat	0,4%	
	0,3%	FR toddler 2 3 yr	0,54	0,2%	Milk: Cattle	0,0%	Apples	0,0%	Wheat	0,3%	
	0,3%	FR child 3 15 yr	0,53	0,1%	Milk: Cattle	0,0%	Wheat	0,0%	Sugar beet roots	0,3%	
	0,3%	UK toddler	0,45	0,1%	Milk: Cattle	0,0%	Wheat	0,0%	Potatoes	0,3%	
	0,2%	DK child	0,41	0,1%	Milk: Cattle	0,0%	Rye	0,0%	Wheat	0,2%	
	0,2%	GEMS/Food G11	0,39	0,0%	Milk: Cattle	0,0%	Potatoes	0,0%	Soyabeans	0,2%	
	0,2%	RO general	0,38	0,1%	Milk: Cattle	0,0%	Wheat	0,0%	Potatoes	0,2%	
	0,2%	GEMS/Food G06	0,38	0,0%	Wheat	0,0%	Tomatoes	0,0%	Milk: Cattle	0,2%	
	0,2%	SE general	0,37	0,1%	Milk: Cattle	0,0%	Bovine: Muscle/meat	0,0%	Potatoes	0,2%	
	0,2%	GEMS/Food G07	0,37	0,0%	Milk: Cattle	0,0%	Wheat	0,0%	Potatoes	0,2%	
	0,2%	GEMS/Food G15	0,37	0,0%	Milk: Cattle	0,0%	Wheat	0,0%	Potatoes	0,2%	
	0,2%	GEMS/Food G08	0,36	0,0%	Milk: Cattle	0,0%	Wheat	0,0%	Potatoes	0,2%	
	0,2%	GEMS/Food G10	0,36	0,0%	Milk: Cattle	0,0%	Wheat	0,0%	Soyabeans	0,2%	
	0,2%	ES child	0,35	0,1%	Milk: Cattle	0,0%	Wheat	0,0%	Oranges	0,2%	
	0,2%	DE women 14-50 yr	0,34	0,1%	Milk: Cattle	0,0%	Sugar beet roots	0,0%	Apples	0,2%	
	0,2%	DE general	0,34	0,1%	Milk: Cattle	0,0%	Sugar beet roots	0,0%	Apples	0,2%	
	0,2%	IE adult	0,32	0,0%	Milk: Cattle	0,0%	Sweet potatoes	0,0%	Wheat	0,2%	
	0,2%	FR infant	0,29	0,1%	Milk: Cattle	0,0%	Potatoes	0,0%	Apples	0,2%	
	0,2%	NL general	0,28	0,0%	Milk: Cattle	0,0%	Sugar beet roots	0,0%	Potatoes	0,2%	
	0,1%	PT general	0,21	0,0%	Potatoes	0,0%	Wheat	0,0%	Wine grapes	0,1%	
	0,1%	ES adult	0,20	0,0%	Milk: Cattle	0,0%	Wheat	0,0%	Oranges	0,1%	
	0,1%	FR adult	0,19	0,0%	Milk: Cattle	0,0%	Wine grapes	0,0%	Wheat	0,1%	
	0,1%	FI 3 yr	0,17	0,0%	Potatoes	0,0%	Bananas	0,0%	Wheat	0,1%	
	0,1%	IT toddler	0,16	0,0%	Wheat	0,0%	Other cereals	0,0%	Tomatoes	0,1%	
	0,1%	DK adult	0,16	0,0%	Milk: Cattle	0,0%	Potatoes	0,0%	Wheat	0,1%	
	0,1%	LT adult	0,16	0,0%	Milk: Cattle	0,0%	Potatoes	0,0%	Apples	0,1%	
	0,1%	UK vegetarian	0,14	0,0%	Milk: Cattle	0,0%	Wheat	0,0%	Potatoes	0,1%	
	0,1%	UK adult	0,13	0,0%	Milk: Cattle	0,0%	Wheat	0,0%	Potatoes	0,1%	
	0,1%	FI 6 yr	0,13	0,0%	Potatoes	0,0%	Wheat	0,0%	Bananas	0,1%	
	0,1%	FI adult	0,13	0,0%	Coffee beans	0,0%	Potatoes	0,0%	Rye	0,1%	
	0,1%	IT adult	0,12	0,0%	Wheat	0,0%	Tomatoes	0,0%	Apples	0,1%	
	0,1%	PL general	0,10	0,0%	Potatoes	0,0%	Apples	0,0%	Tomatoes	0,1%	
	0,0%	IE child	0,08	0,0%	Milk: Cattle	0,0%	Wheat	0,0%	Potatoes	0,0%	

Conclusion:
The estimated long-term dietary intake (TMDI/NEDI/IEDI) was below the ADI.
The long-term intake of residues of Eugenol is unlikely to present a public health concern.

Table 2.7.9-4: Eugenol TMDI calculations (EFSA PRIMo 3.1) (using only values for the intended uses)

European Food Safety Authority

EFSA PRIMo revision 3.1 2019/03/19

Eugenol

LOQs (mg/kg) range from: 0,01 to: 0,01

Toxicological reference values

ADI (mg/kg bw/day):	0,17	ARfD (mg/kg bw):	not necessary
Source of ADI:		Source of ARfD:	
Year of evaluation:		Year of evaluation:	

Input values

Details - chronic risk assessment | Supplementary results - chronic risk assessment | Details - acute risk assessment/children | Details - acute risk assessment/adults

Comments:

Refined calculation mode

Chronic risk assessment JMPR methodology (IEDI/TMDI)

No of diets exceeding the ADI : ---

TMDI/NEDI/IEDI calculation (based on average food consumption)

Calculated exposure (% of ADI)	MS Diet	Expsoure (µg/kg bw per day)	Highest contributor to MS diet (in % of ADI)	Commodity / group of commodities	2nd contributor to MS diet (in % of ADI)	Commodity / group of commodities	3rd contributor to MS diet (in % of ADI)	Commodity / group of commodities	Exposure resulting from: MRLs set at the LOQ (in % of ADI)	Exposure resulting from: commodities not under assessment (in % of ADI)
0,1%	NL toddler	0,17	0,1%	Apples	0,0%	Pears	0,0%	Table grapes	0,1%	0,1%
0,1%	DE child	0,15	0,1%	Apples	0,0%	Table grapes	0,0%	Pears	0,1%	0,1%
0,0%	NL child	0,08	0,0%	Apples	0,0%	Pears	0,0%	Table grapes	0,0%	0,0%
0,0%	PT general	0,04	0,0%	Wine grapes	0,0%	Apples	0,0%	Pears	0,0%	0,0%
0,0%	DE women 14-50 yr	0,04	0,0%	Apples	0,0%	Wine grapes	0,0%	Table grapes	0,0%	0,0%
0,0%	FR toddler 2 3 yr	0,04	0,0%	Apples	0,0%	Pears	0,0%	Wine grapes	0,0%	0,0%
0,0%	DE general	0,04	0,0%	Apples	0,0%	Wine grapes	0,0%	Table grapes	0,0%	0,0%
0,0%	RO general	0,03	0,0%	Wine grapes	0,0%	Apples	0,0%	Table grapes	0,0%	0,0%
0,0%	FR adult	0,03	0,0%	Wine grapes	0,0%	Apples	0,0%	Pears	0,0%	0,0%
0,0%	DK child	0,03	0,0%	Apples	0,0%	Pears	0,0%	Table grapes	0,0%	0,0%
0,0%	GEMS/Food G11	0,03	0,0%	Apples	0,0%	Wine grapes	0,0%	Table grapes	0,0%	0,0%
0,0%	GEMS/Food G07	0,03	0,0%	Wine grapes	0,0%	Apples	0,0%	Table grapes	0,0%	0,0%
0,0%	GEMS/Food G08	0,03	0,0%	Apples	0,0%	Wine grapes	0,0%	Table grapes	0,0%	0,0%
0,0%	GEMS/Food G15	0,03	0,0%	Apples	0,0%	Wine grapes	0,0%	Table grapes	0,0%	0,0%
0,0%	FR child 3 15 yr	0,03	0,0%	Apples	0,0%	Wine grapes	0,0%	Table grapes	0,0%	0,0%
0,0%	IE adult	0,03	0,0%	Wine grapes	0,0%	Apples	0,0%	Pears	0,0%	0,0%
0,0%	PL general	0,03	0,0%	Apples	0,0%	Table grapes	0,0%	Pears	0,0%	0,0%
0,0%	NL general	0,02	0,0%	Apples	0,0%	Wine grapes	0,0%	Table grapes	0,0%	0,0%
0,0%	DK adult	0,02	0,0%	Apples	0,0%	Wine grapes	0,0%	Pears	0,0%	0,0%
0,0%	GEMS/Food G06	0,02	0,0%	Table grapes	0,0%	Apples	0,0%	Pears	0,0%	0,0%
0,0%	UK toddler	0,02	0,0%	Apples	0,0%	Table grapes	0,0%	Pears	0,0%	0,0%
0,0%	LT adult	0,02	0,0%	Apples	0,0%	Pears	0,0%	Table grapes	0,0%	0,0%
0,0%	FR infant	0,02	0,0%	Apples	0,0%	Pears	0,0%	Wine grapes	0,0%	0,0%
0,0%	UK infant	0,02	0,0%	Apples	0,0%	Pears	0,0%	Table grapes	0,0%	0,0%
0,0%	GEMS/Food G10	0,02	0,0%	Apples	0,0%	Wine grapes	0,0%	Table grapes	0,0%	0,0%
0,0%	ES child	0,02	0,0%	Apples	0,0%	Pears	0,0%	Table grapes	0,0%	0,0%
0,0%	UK adult	0,02	0,0%	Wine grapes	0,0%	Apples	0,0%	Pears	0,0%	0,0%
0,0%	ES adult	0,02	0,0%	Apples	0,0%	Wine grapes	0,0%	Pears	0,0%	0,0%
0,0%	UK vegetarian	0,02	0,0%	Wine grapes	0,0%	Apples	0,0%	Pears	0,0%	0,0%
0,0%	SE general	0,01	0,0%	Apples	0,0%	Pears	0,0%	Quinces	0,0%	0,0%
0,0%	FI 3 yr	0,01	0,0%	Apples	0,0%	Table grapes	0,0%	Pears	0,0%	0,0%
0,0%	IT toddler	0,01	0,0%	Apples	0,0%	Pears	0,0%	Table grapes	0,0%	0,0%
0,0%	IT adult	0,01	0,0%	Apples	0,0%	Pears	0,0%	Table grapes	0,0%	0,0%
0,0%	FI adult	0,01	0,0%	Apples	0,0%	Wine grapes	0,0%	Table grapes	0,0%	0,0%
0,0%	FI 6 yr	0,01	0,0%	Apples	0,0%	Pears	0,0%	Table grapes	0,0%	0,0%
0,0%	IE child	0,00	0,0%	Apples	0,0%	Table grapes	0,0%	Pears	0,0%	0,0%

Conclusion:

The estimated long-term dietary intake (TMDI/NEDI/IEDI) was below the ADI.

The long-term intake of residues of Eugenol is unlikely to present a public health concern.

2.7.10 Proposed MRLs and compliance with existing MRLs

No MRL is required for eugenol as it is listed in Annex IV to Commission Regulation (EC) No 839/2008. According to Commission Regulation (EC) No 839/2008 it was temporarily included in Annex IV, Eugenol fit the Criterion 4 to SANCO/11188/2013: The consumer exposure to residues of eugenol linked to use as plant protection product is considered as negligible compared to other uses in the food chain and/or natural background

In residue trials, for grapes, no detectable residues were found at harvest. No residues at or above the LOQ (0.01 mg/kg) were found in apples at harvest (representative crop for pome fruit). Eugenol is naturally occurring in grapes and apples, so that the exposure due to plant protection product use is not higher than natural exposure to eugenol and methyleugenol. Furthermore, eugenol and methyleugenol are both naturally found in a wide variety of plants in addition to grapes and apples, and therefore natural background exposure is further increased via consumption of other foodstuffs containing eugenol and methyleugenol.

It is proposed that eugenol should be fully included into Annex IV to Regulation (EC) No. 396/2005. MRLs are not required for eugenol.

Methyleugenol is a relevant impurity that is present naturally in eugenol. It is naturally occurring in a wide variety of fruits and vegetables. It is not formed as a metabolite following application and was not detected (LOD = 0.003 mg/kg) in any of the residue trials at any timepoint. Therefore, it does not need to be considered in MRL setting.

Proposed MRLs for commodities relevant to this submission are detailed in the table below.

Table CA 6.7.2-01: Proposed MRLs for eugenol for commodities in this submission

Code	Commodity	Proposed EU MRL	Current MRL*
0151010	Table grapes	No MRL required	No MRL required
0151020	Wine grapes	No MRL required	No MRL required
0130000	Pome fruit	No MRL required	No MRL required
1000000	Products of animal origin	No MRL required	No MRL required

* Temporary inclusion into Annex IV

2.7.11 Proposed import tolerances and compliance with existing import tolerances

Not relevant.

2.8 FATE AND BEHAVIOUR IN THE ENVIRONMENT

2.8.1 Summary of fate and behaviour in soil

2.8.1.1 Route of degradation in soil

2.8.1.1.1 Aerobic degradation in soil

The route and rate of degradation of eugenol were studied in four soils in the laboratory under aerobic conditions (Jones, A., 2015a). The soils were selected to represent a range of textural characteristics, pH (5.0 - 7.7) and organic carbon contents (1.6 – 3.6 %). Soil samples were set up and allowed to acclimatise before being treated with [^{14}C]-eugenol at a nominal concentration of 0.52 mg/kg, approximately equivalent to a use rate of 520 g ai/ha. The samples were incubated under aerobic conditions in the dark at about 20°C at a moisture content equivalent to pF 2 for periods of up to 120 days after application. Combined soil extracts were analysed by HPLC with fraction collection. The overall recoveries of applied radioactivity (AR) were in the range 81.2 – 116.1% AR. The variable recoveries of radioactivity may be due to the inhomogeneous nature of the non-extractable residue, and the difficulties in accurately quantifying this fraction.
The proportion of radioactivity extracted from soil decreased with time with a corresponding increase in the levels of non-extractable radioactivity and $^{14}CO_2$ evolution. Extractable radioactivity declined with time from mean values of 46.2 – 74.4% AR in each soil at zero-time to mean values of 7.6 – 14.2% AR after 3 days incubation before plateau through the remainder of the incubation period. Non-extractable radioactivity accounted for mean values of 65.6 – 70.4% AR after 120 days. Volatile radioactivity, characterised as $^{14}CO_2$, represented 19.1 – 20.2% AR after 120 days. Eugenol declined rapidly in each soil type. The DT_{50} and DT_{90} values were <1 day and <2 days respectively.
Eugenol degraded to multiple minor unidentified degradates (none of which individually accounted for >10% AR at a single timepoint or >5% AR at two consecutive timepoints), bound residues and carbon dioxide. The compound of concern Methyleugenol was not identified in this study.

2.8.1.1.2 Anaerobic degradation in soil

No studies on anaerobic degradation have been performed. Based on the fast aerobic degradation and the application timing of Mevalone, it is unlikely that eugenol residues would be found in anaerobic conditions.

2.8.1.1.3 Photodegradation in soil

The photodegradation of [^{14}C]-eugenol was studied on a sandy loam soil (USDA) from Rheinland-Pfalz, Germany at 132 g ai/ha soil for 30 days at 20 ± 2°C and under dry conditions (D. Kelly, 2021a). Samples were continuously irradiated using a Suntest accelerated exposure machine (xenon lamp fitted with a filter to remove radiation below 290 nm). Test vessels were connected to two 2M NaOH traps for the collection of liberated CO_2.
The mean recovery of radioactivity ranged from 88.9 to 93.8% applied radioactivity (AR) in both irradiated and dark control samples throughout the incubation period. Levels of extractable radioactivity dropped sharply following 0 DAT analysis (86.6% AR) to between 54.5 and 72.5% AR. Radioactivity remaining unextracted from soil increased sharply following 0 DAT analysis with maximum levels of 28.0 and 30.8% AR observed in 30 DAT samples for irradiated and dark control samples, respectively. In volatile traps, up to 8.7% AR was recovered from irradiated samples and 0.5% from dark control samples and was confirmed to be carbon dioxide by barium chloride precipitation.
Degradation of eugenol was rapid in both irradiated and dark control conditions, with a DT_{50} of < 1 hour. There were no significant differences in the rate of degradation of eugenol between irradiated and dark control samples. No conversion to equivalent summer sunlight days was therefore calculated.

In irradiated samples, volatile radioactivity reached a maximum of 8.7% AR at the end of the test, compared to 0.5% AR from dark control samples, and was confirmed to be carbon dioxide.
The increased carbon dioxide in irradiated samples suggests that the route of degradation for irradiated samples differs from dark controls.

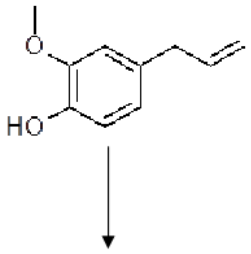
Eugenol degraded to multiple unknowns, the largest of which reached 22.8% AR in irradiated samples (6 hours) and 27.6% AR in dark control samples (2 hours). The total unknown radioactivity reached a maximum value of 45.9%AR at 14 days in irradiated samples, with largest unknown above 10%AR.
Non-extractable radioactivity increased throughout the test with maximum levels of 28.0 and 30.8% AR observed in irradiated and dark control samples, respectively, at the end of the test.

The characterization of the photoproducts was not carried out in the experiment since reference compounds were not included. Therefore, the route of degradation in soil under irradiated conditions is not considered fully addressed.

2.8.1.1.4 Overall route of degradation in soil

In studies designed to investigate the route and rate of aerobic soil degradation of eugenol, no major soil metabolites or degradation products were observed at levels exceeding 10% of the applied amount, nor at above 5% of the applied amount for two sequential measurements, nor above 5% of the applied amount at the final measurement.

Eugenol degraded rapidly to multiple minor unidentified degradates, bound residues and carbon dioxide.
The proposed degradation pathway is shown below in Figure 1.



Minor metabolites,
bound residues
and carbon dioxide.

Figure 1

2.8.1.2 Rate of degradation in soil

2.8.1.2.1 Laboratory conditions

Eugenol declined rapidly in each soil type. The DT50 and DT90 values were <1 day and <3 days respectively. The decline of eugenol in aerobic soil with time was well described by the single first order (SFO) kinetic model. The persistence and modelling endpoints are summarized in the table below.

Table 68: Persistence and Modelling endpoints for eugenol in soil

Soil	OC (%)	pH*	T. °C / % moisture	DT_{50} /DT_{90} (d)	DT_{50} (d) 20 °C pF2/10kPa[b)]	Kinetic model	Visual goodness-of-fit
Brierlow (Jones 2015)	3.6	5.3	20°C / pF2	<1 / <3	<1	NA	-
Calke (Jones, 2015)	2.8	5.1	20°C / pF2	0.5 / 1.8	0.5	SFO	Good
Ingleby (Jones, 2015)	1.6	4.0	20°C / pF2	0.6 / 1.9	0.6	SFO	Good
Empingham (Jones, 2015)	3.3	7.4	20°C / pF2	<3 / <3	<3	NA	-

For modelling purposes, a conservative DT 50 of 1 day has been proposed

2.8.1.2.2 Field dissipation studies

Based on the laboratory DT90 of < 3 days, field studies are not triggered for eugenol and therefore no data is submitted.

2.8.1.2.3 Soil accumulation studies

Not required.

2.8.1.2.4 Assessment of Persistence (P) in soil

Based on the rapid degradation rates of eugenol in soil, it is not expected to be persistent in a viable soil

environment.

2.8.1.3 Mobility in soil

2.8.1.3.1 Adsorption/Desorption studies

Two adsorption/desorption studies have been submitted. The first study by Jones, 2015b was previously peer reviewed in the context of the confirmatory data. Several deviations were found with respect to the OECD 106 guideline and it was considered that the study by Jones, 2015b was not robust enough to be used to derive the sorption endpoint for eugenol.
The Annex I inclusion RMS (UK) suggested that due to the rapid degradation of eugenol, it's stability during a batch equilibrium adsorption/desorption study could not be relied upon. Additionally, the indirect method was employed, while the OECD106 specifically recommends as not appropriate for this type of substance. Therefore, it was concluded that the adsorption calculated in this study using the indirect method, was not representative of eugenol alone. Instead, the values calculated were mean characteristics for the range of compounds shown on the radiochromatograms.

The second study has been submitted for the renewal process since the previous one was not considered valid. Kelly, 2021 studied the adsorption/desorption characteristics of [^{14}C]-eugenol in four soils using a batch equilibrium method, in the dark at 20 ± 2°C.
For all soils, low adsorption to soil was noted. Significant degradation of [^{14}C]-eugenol was seen in soil extracts, indicating chemical instability in the sterile soils tested, therefore the direct method was employed.

The result from this study indicated that the Freundlich adsorption coefficients, K_{FOC}, were in the range 48.2 to 123.3 L/kg. Using the McCall Classification scale to assess the potential mobility of a chemical in soil (based on K_{OC}), eugenol can be classified as having high mobility in soil (K_{OC} 50-150 L/kg) for each of the soils tested.
Desorption values were generally higher than corresponding adsorption values indicating that the adsorption was not fully reversible.
In all four soils, low adsorption to the soil was noted. Significant degradation of [^{14}C]-eugenol was seen in soil extracts, indicating chemical instability in sterile soils tested.
The percent adsorbed ranged from 2.1 to 6.7. It was calculated as 'calculated as % Concentration on soil/Initial concentration x 100', since parameter *delta* which is the decrease in concentration in aqueous phase, would not be equivalent to the percentage adsorbed in the direct method. The adsorbed percentages was considered too low outside the acceptable range being <20%. According to the OECD 106 guideline where low adsorption occurs, a 1:1 soil/solution ratio is recommended, although for some very organic soil types smaller ratios may be necessary to obtain a slurry. Additionally, it is pointed out that care must be taken with the analytical methodology to measure small changes in solution concentration; otherwise, the adsorption measurement will be inaccurate. For eugenol, the instability of the test substance must be added to the low adsorbed percentages. Therefore, the reliability of the calculated parameters should be questioned.

According to the guideline, in the absence of reliable endpoints needed to perform an exposure assessment the use of conservative default values may be appropriate, especially when adsorption was too low to reliably measure.

Therefore, considering the difficulties to achieve a precise adsorption endpoint for eugenol due to the low adsorption and the instability of this substance, default values will be used for risk assessment purposes as it was previously proposed in the confirmatory data: KFoc of 10 mg/L and 1/n of 0.9.

2.8.2 Summary of fate and behaviour in water and sediment [equivalent to section 11.1 of the CLH report template]

Available environmental fate and ecotoxicology studies have been considered and summarised in the Eugenol Monograph (Volume 3, Annex B8 and Annex B9, May 2011) and in the renewal of approval dossier (dRAR, Volume 3, Annex B8 and Annex B9). There have been also some studies that are included in the REACH dossier of Eugenol.

The key information pertinent to determine the environmental hazard classification for Eugenol is presented below. Unless otherwise stated, these studies were conducted in accordance with GLP and the validity criteria of the representative test guideline, if applicable.

2.8.2.1 Rapid degradability of organic substances

Table 69: Summary of relevant information on rapid degradability

Method	Results*	Key or Supportive study[1]	Remarks	Reference
Ready biodegradability. OECD 301F, C.4-D Manometric Respirometry Test, 1992	84% degradation after 28 days.	Key The study is considered acceptable.	Test substance: Eugenol Purity: 98.8% Readily biodegradable	CA 7.2.2.1/01 Seyfried, B (2008) Study number B65160
Aerobic mineralisation in surface water. OECD 309	DT50 = 1.8 -2.0 days with carbon dioxide reaching a maximum of 59.4% at 62 days.	Key The study is considered acceptable.	Test substance: Eugenol Purity: 99.7% Eugenol degrades under non-sterile conditions.	Anonymous, (2021).
Hydrolysis OECD 111	Stable at 50 ºC and pH 4 and pH 7. $DegT_{50}$ 120 days at 25ºC, pH 9.	Key The study is considered acceptable.	Test substance: Eugenol Purity: 99.7% Hydrolytically stable at pH 4 and 7. Hydrolytically unstable at pH 9.	Anonymous, (2021).
Photolysis in water. OECD 316	DT50 = 9.2 days at 25ºC, pH 7.	Key The study is considered acceptable.	Test substance: Eugenol Purity: 99.7% Photolytically unstable at pH 7.	Anonymous, (2021).

2.8.2.1.1 Ready biodegradability

The ready biodegradability of eugenol has been previously evaluated, and the study remains valid. Eugenol is readily biodegradable.

Seyfried, B. (2008).
The ready biodegradability of eugenol was determined in a manometric respirometry test over 28 days in accordance with OECD test guideline 301 F and to GLP. The percent biodegradation of the test item was calculated based on the theoretical oxygen demand (ThOD) of 2.34 mg O2/mg test item.

Aqueous test solutions of eugenol (purity 98.8%) at a concentration of 100 mg/L were inoculated with aerobic activated sludge from a wastewater treatment plant treating predominantly domestic wastewater. Samples were incubated in airtight flasks under aerobic conditions in the dark at 22°C, and oxygen consumption over the 28-day test period was measured using an electrode-type manometer. Procedure controls containing a readily biodegradable reference compound, sodium benzoate, were tested simultaneously under the same conditions, and controls containing inoculum (but without test substance) were run to determine oxygen blanks. A toxicity control containing both eugenol and the reference item sodium benzoate was also tested to determine the effect of eugenol on the activity of the activated sludge micro-organisms.

At the end of the 28-day incubation period, the mean biodegradation of eugenol was 84% and the pass level for ready biodegradability (biodegradation ≥60% of the theoretical oxygen demand [ThOD] of the test item in a 10-day window within the 28-day test period) was reached.

Eugenol can be classified as 'readily biodegradable' under the test conditions. The study is considered acceptable.

2.8.2.1.2 BOD5/COD

No data available.

2.8.2.2 Other convincing scientific evidence

No data available.

2.8.2.2.1 Aquatic simulation tests

This new study was provided in support of the assessment for the renewal of Eugenol according to Regulation (EC) No 1107/2009.

Anonymous (2021)

The mineralisation and degradation rate of eugenol (purity : 99.7%) in a surface water system (Fountains Abbey) was studied at 20 ± 2°C in the dark according to OECD 309 and GLP The test was performed using two concentration levels (95 and 10 µg/L). Sterilised samples were also tested at the higher concentration.

Non-sterile samples were taken for analysis at 0, 0.25, 0.75, 1, 5, 14, 30 and 62 days after treatment (DAT) at the low application rate and at 0, 1, 1.25, 2, 7, 14, 30 and 62 DAT. The sterile vessels were sampled at 62 DAT.

The total recovery (mean values) at each sampling interval ranged from 85.7 % to 96.0 % and 87.7 % to 98.8 % at the non-sterile low and high application rates, respectively. The overall total radioactive recovery (mean value) was > 90 % at both application rates (non-sterile and sterile vessels).
Volatile radioactivity in alkaline hydroxide traps was confirmed to be carbon dioxide by precipitation with barium chloride.

Eugenol decreased from 92.8% at 0 DAT to 6.4% at 5 DAT at the low application rate and from 95.7% at 0 DAT to < 1% by 7 DAT at the high application rate. Single first-order kinetics provided a good fit for the data. Eugenol degraded quickly in natural surface water under non-sterile conditions, with DT_{50} values of 1.8 and 2.0 days at the low and high application rate, respectively. No detectable levels of eugenol were observed by 14 DAT at both application rates. Degradation under sterile conditions was slower, with eugenol present at 9.6% at the end of the 62 day incubation period.
Degradation resulted in the formation of two major degradation products (> 10% AR). Unknown 2, (low and high application rate) and Unknown 3 (high application rate). Although attempts were made, it was not possible to characterise and identify the unknown degradation products by LC-MS/MS due to the nature of the metabolites, the matrix and compound ionisability.
Mineralisation was observed as the major route of degradation under non-sterile conditions, reaching maximum levels of ≥ 59.4% AR at 62 DAT. Under sterile conditions, mineralisation was a minor route of degradation (1.6 % AR at 62 DAT).

The study is considered acceptable.

2.8.2.2.2 Field investigations and monitoring data (if relevant for C&L)

No data available.

2.8.2.2.3 Inherent and enhanced ready biodegradability tests

No studies provided.

2.8.2.2.4 Soil and sediment degradation data

No studies available.
According to section 7.2.2.3 of Regulation (EU) No. 283/2013, *the water/sediment study shall be reported unless the applicant shows that contamination of surface water will not occur*. **Consequently, RMS has identified a data gap for a water/sediment study.**

Based on Ready Biodegradability, DT_{50}'s from ReACH guidance of 15 days in water and 300 days in sediment have been used for the risk assessments.

2.8.2.2.5 Hydrolysis

This study has not been previously evaluated and it was provided in support of the assessment for the renewal of Eugenol according to Regulation (EC) No 1107/2009.

Anonymous (2021).

The hydrolysis of [^{14}C]-Eugenol (purity: 99.7%) was studied in sterile aqueous buffered solutions at pH 4, pH 7 and pH 9 according to OECD guideline 111 and GLP

In the preliminary test at 50 ºC, the mean total recovery of applied radioactivity (AR) in buffer solutions was in the range 98.9 to 101.3% AR. Eugenol was shown to be stable in pH 4 and 7 buffers, accounted for 92.1- 92.9 % AR initially and at the end of 5-day incubation accounted for 88.7 – 89.2 % AR. In pH 9 buffer, eugenol was unstable, dropped from 92.5% AR initially to 63.7% AR at the end of the 5-day incubation. At pH 9, several unknowns were observed, the largest reaching a maximum of 15.9% AR, after the 5-day incubation period.

At Tier II test, concentration of 1.0 mg [^{14}C]-Eugenol/L was treated in pH 9 buffer at 25, 35 and 50ºC for up to 30 days. The mean total recovery of AR for all samples was 94.6 – 100.7 % AR. [^{14}C]-Eugenol decreased from mean values of 94.1 – 95.0% AR at 0 DAT to between 24.2% AR at 50°C and 81.1% AR at 25°C, at the end of incubation. The DegT50 value were calculated using non-linear regression and first-order kinetics (SFO) according to FOCUS kinetic modelling and, at 25ºC it was estimated as 120 days at pH 9, showed eugenol was not hydrolytically stable at environmental relevant temperatures under basic conditions (DegT50 < 1 year).

There were two major regions, which accounted percentages of applied radioactivity above 10%. The Unknown 1 was described as a polar peak observed at ca 3 minutes in the HPLC. The Unknown 2 was observed after the elution of eugenol, at ca 39 minutes with maximum of 22.3% AR (50°C, 21 DAT). Unknown 2 increased throughout the incubations at 25 and 35°C, and at 50°C reached a maximum at 21 days before decreasing at the end of the incubation at 50ºC. This decrease could not be observed at the other two lower temperatures.
Finally, there were many other minor degradation products formed (<10% AR), where, according to the study report, the largest individual peak reached a maximum of 9.3% AR (50°C, 21 DAT).

According to OECD 111, any mayor hydrolysis products at least those representing ≥ 10% of the applied dose should be identify by appropriate analytical methods. In the study, no reference standards other than eugenol were used and therefore the identification of any degradation product was not possible. Therefore, applicant is called to complete the identification of the unknowns 1 and 2.

Finally, there were many other minor degradation products formed (<10% AR), where, according to the study report, the largest individual peak reached a maximum of 9.3% AR (50°C, 21 DAT). This could not be checked by RMS, since the percentages reached by other any individual compound were not submitted. Therefore, more information is needed regarding to the percentages of AR reached by 'other unknowns.

Eugenol is hydrolytically stable in buffered aqueous solution at 50ºC, under sterile conditions, at pH 4 and pH 7 but shows hydrolysis at pH 9 with a DT50 of 120 days (DegT50 < 1 year).

The study is considered acceptable.

2.8.2.2.6 Photochemical degradation

This study has not been previously evaluated and it was provided in support of the assessment for the renewal of Eugenol according to Regulation (EC) No 1107/2009.

Anonymous (**2021**).

The photolysis of [Ring-^{14}C(U)]-Eugenol (purity : 99.7%) was investigated in a sterile aqueous buffered solutions at pH 7 maintained at 25 ± 2ºC over a period of 30 days according to OECD 316. [Ring-^{14}C(U)]-Eugenol was applied, at a rate of 0.9 µg/mL, to sterile pH 7 buffer solutions in individual photolysis vessels and the treated irradiated buffer solutions were continuously irradiated using light from a xenon arc lamp. Duplicate samples were taken from the dark vessels for analysis immediately after treatment and from both dark and light vessels at six sampling intervals during irradiation (2, 4, 8, 14, 21 and 30 days after treatment (DAT)). The pH values were within 7 ± 0.2 during the test period. All analysed samples were found to be sterile.
The mean recovery of applied radioactivity (mass balance) was 83.7-98.3% for irradiated samples and 98.9-100.1 for non-irradiated samples.

Volatile radioactivity recovered in sodium hydroxide traps (carbon dioxide) was low (≤ 0.2% AR) during the definitive test. In the preliminary test, up to 10.5 %AR was recovered in sodium hydroxide traps after 29 days of irradiation. Despite attempts being made in the definitive test to recover carbon dioxide, ca 10% AR is suggested to have been lost during incubation. Low levels (< 2% AR) of radioactivity were also present in the foam bung traps. Radioactivity present in the vessel and tubing washes was ≤ 1% AR.

Eugenol degraded extensively under irradiated conditions to numerous, mostly polar, unidentified components. Only one component (retained for *ca* 25 minutes on HPLC) showed a clear maximum (8.1% AR at 14 DAT) followed by a decrease to the end of the incubation (30 days, 4.2% AR). After 30 days incubation under dark conditions, 89.5% AR (mean) was present as Eugenol.
Two unknown degradation products with HPLC retention times of 2.5 and 6 minutes exceeded 10% AR.
Degradation in non-irradiated samples was minor and primarily produced a non-polar product present at the start of the incubation. This hydrolysis product was not seen in irradiated samples after 8 days.

Single first-order kinetics provided a good fit for the data The DT_{50} value for Eugenol applicable to Europe and North America (latitudes 30°, 40° and 50°) for irradiated samples was 9.2 days. In Japanese spring sunlight, the DT_{50} value for Eugenol in irradiated samples was the equivalent of 29.8 days.
The major degradation products did not decline during the incubation and were not, therefore, kinetically modelled. The identity of the unknown peaks should be resolved to identify any possible photoproduct susceptible to be included in the residue definition for risk assessment.

The study is considered acceptable to establish the rate of degradation under irradiated conditions but not the route.

2.8.2.2.7 Other / Weight of evidence

No other data available.

2.8.3 Summary of fate and behaviour in air

The vapour pressure of eugenol is 2.7 Pa at 20°C (CA 2.2) and the water solubility at 20°C is 1.85 mg/l (EFSA conclusion 2012). A Henry's Law constant of 0.24 Pa $mol^{-1}.m^3$ was derived (CA 2.2).
An estimation of the photochemical oxidative degradation rate (using the Atkinson equation) has estimated that the expected half-life in air to be 1.975 hours. This indicates that any volatilised eugenol will be extremely short-lived in the atmosphere, therefore there will be no local or global effects.

2.8.3.1 Hazardous to the ozone layer

Table 70: Summary table of studies on hazards to the ozone layer

Method	Results	Remarks	Reference
No data	-	-	-

2.8.3.1.1 Short summary and overall relevance of the provided information on hazards to the ozone layer

Based on the vapor pressure (2.7 Pa at 20°C) eugenol is highly volatile and losses due to volatilization would not be excluded. An estimation of the photochemical oxidative degradation rate (using the Atkinson equation) has estimated that the expected half-life in air to be 1.975 hours (0.165 days). This indicates that any volatilised eugenol will be extremely short-lived in the atmosphere. Therefore, global warming potential, ozone depleting potential, photochemical ozone creation potential and accumulation in the troposphere are all unlikely to occur

following use of eugenol according to good agricultural practice.

There are no data provided regarding the hazard of Thymol to the ozone layer, the Ozone Depleting Potential (ODP) of Thymol has not been measured.

2.8.3.1.2 Comparison with the CLP criteria

A substance is considered hazardous to the ozone layer if the available evidence concerning its properties and its predicted or observed environmental fate and behaviour indicate that it may present a danger to the structure and/or the functioning of the stratospheric ozone layer.
Any substances having an ODP of greater than or equal to the lowest ODP (i.e., 0.005) of the substances currently listed in Annex I to Regulation EC No 1005/2009 should be classified as hazardous to the ozone layer (category 1).
Although no specific data have been provided for this hazard, considering the chemical structure and other available information on the physicochemical properties, eugenol is not expected to be hazardous to stratospheric ozone.

2.8.3.1.3 Conclusion on classification and labelling for hazardous to the ozone layer

Not Classified.

2.8.4 Summary of monitoring data concerning fate and behaviour of the active substance, metabolites, degradation and reaction products

No monitoring data is available and based on the short half-life of eugenol is not required.

2.8.5 Definition of the residues in the environment requiring further assessment

The residue definition for the risk assessment:

Soil:	Eugenol
Groundwater:	Eugenol
Surface water:	Eugenol
Sediment:	Eugenol
Air:	Eugenol

Definition of the residue for monitoring:

Soil:	Eugenol
Groundwater:	Eugenol
Surface water:	Eugenol
Sediment:	Eugenol
Air:	Eugenol

2.8.6 Summary of exposure calculations and product assessment

2.8.6.1 Predicted environmental concentration in soil

The predicted environmental concentrations in soil (PEC_{soil}) have been calculated according to the guidance in SANCO/9188/VI/97. The worst-case laboratory DT50 of 1 day was set to 1 day for eugenol. All PECsoil calculations were performed assuming a soil bulk density of 1.5 g/cm³ and an equal distribution in the top 5 cm. The crop interception value was set to 60% for grapes and 65% for pome fruits. The following results were obtained:

Table 71: PECsoil values for eugenol on grapes

PEC_{soil} (mg/kg)	Grapes			
	Single application		Multiple Application	
	Actual	TWA	Actual	TWA
Initial	0.070	-	0.071	-

Table 72: PECsoil values for eugenol on apples

PEC_{soil} (mg/kg)	Pome Fruit			
	Single application		Multiple Application	
	Actual	TWA	Actual	TWA
Initial	0.062	-	0.062	-

2.8.6.2 Predicted environmental concentration in groundwater

The predicted environmental concentrations in groundwater (PECgw) of eugenol were calculated using the environmental fate model FOCUS-MACRO (v5.5.4), FOCUS-PEARL (v5.5.5) and FOCUS-PELMO (v6.6.4) and in line with the current FOCUS guidelines.
The following PECgw values were obtained:

Table 73: Summary of PEARL and PELMO PECgw values after application to vines

Crop	Scenario	80th Percentile PEC_{gw} at 1 m Soil Depth (µg/L)	
		PEARL (v5.5.5)	PELMO (v6.6.4)
		Eugenol	Eugenol
Vines	Châteaudun	0.000000	<0.0005
	Hamburg	0.000014	<0.0005
	Kremsmünster	0.000000	<0.0005
	Piacenza	0.000001	<0.0005
	Porto	0.000001	<0.0005
	Sevilla	0.000025	<0.0005
	Thiva	0.000002	<0.0005

Table 74: Summary of PEARL and PELMO PECgw values after application to apples

Crop	Scenario	80th Percentile PEC_{gw} at 1 m Soil Depth (µg/L)	
		PEARL (v5.5.5)	PELMO (v6.6.4)
		Eugenol	Eugenol
Apples	Châteaudun	0.000000	<0.0005
	Hamburg	0.001115	<0.0005
	Jokioinen	0.000612	<0.0005
	Kremsmünster	0.000000	<0.0005
	Okehampton	0.000264	<0.0005

	Piacenza	0.000003	<0.0005
	Porto	0.000001	<0.0005
	Sevilla	0.000005	<0.0005
	Thiva	0.000001	<0.0005

Table 75: Summary of MACRO PECgw values after application to vines and apples

Crop	**Scenario**	**80th Percentile PEC_{gw} at 1 m Soil Depth (µg/L)**
		MACRO (v5.5.4)
		Eugenol
Vines	**Châteaudun**	0.0
Apples	**Châteaudun**	0.0

The 80th percentile PEC_{gw} of eugenol is lower than the 0.1 µg/L regulatory threshold in groundwater at 1 m depth after simulations with the models PEARL, PELMO and MACRO, for both crops (vines and apples).
The risk to groundwater was determined to be acceptable for all uses of Mevalone containing eugenol.

2.8.6.3 Predicted environmental concentration in surface water and sediment

The predicted environmental concentrations in surface water (PECsw) and sediment (PECsed) of eugenol, was assessed through simulations using STEPS 1-2 calculator (version 3.2), FOCUS-SWASH (version 5.3) and followed the recommendations of the FOCUS Working Group on Surface Water Scenarios (SANCO/4802/2001).

The worst-case PEC values are summarised below. The maximum PEC_{sw} values are provided, plus the 21-day time weighted average and the maximum PEC_{sed}.

Table 76: Summary of Worst-Case PEC Values for eugenol at STEPS 1-3

FOCUS STEP / Crop	**Scenario**	**Max PEC_{sw} (µg/L)**	**Dominant entry route**	**21 d- $PEC_{sw,twa}$ (µg/L)**	**Max PEC_{sed} (µg/kg)**
STEP 1					
Vines	-	187.81	-	178.84	18.72
Apples	-	201.36	-	191.48	20.5
STEP 2					
Vines	Southern Europe Mar-May	7.64	-	3.11	0.67
Apples	Southern Europe Mar-May	11.67	-	4.61	1.00
STEP 3					
Vines	R3 stream	2.318	Drift	0.05026*	0.08748*
Apples	R3 stream	5.205	Drift	0.1834	0.3117

*R1 stream

2.8.6.4 Predicted environmental concentration from airborne transport

For information on local in-field or edge-of-field exposure for operators or bystanders, please refer to data submitted under datapoint Vol 3 CP B.6.2.

The vapors pressures 2.7 Pa at 20ºC of eugenol, are above the triggers for volatilization of 1 x 10 4 Pa for soil and 1 x 10 5 Pa for plants. Therefore, the short-range transport needs to be addressed.

Based on the DT50 on air of eugenol, it can be concluded that the substance is not persistent in the atmosphere and would not be subject to significant concerns related to long-range atmospheric transport and atmospheric accumulation.

2.9 EFFECTS ON NON-TARGET SPECIES

2.9.1 Summary of effects on birds and other terrestrial vertebrates

The previous EU-agreed acute oral avian LD_{50} value of > 10000 mg Mevalone/kg bw (corresponding to >320 mg eugenol/kg bw) for Northern bobwhite quail *Colinus virginianus* (EFSA Journal 2012; 10(11):2914) is considered acceptable to support renewal of eugenol. The data on the representative formulation, Mevalone, are sufficient to address the active substance data requirement. No data are available for the long-term avian toxicity of eugenol.

A data gap for « further information to address the short-term and long-term risk to birds to insectivorous birds » was identified during the first EU review of eugenol (EFSA Journal 2012; 10(11):2914). Since short-term study is no longer required for the risk assessment according to the current EFSA Guidance Document on Risk Assessment for Birds and Mammals (EFSA Journal 2009; 7(12): 1438), no further data are necessary to address short-term risk to birds.

The applicant has requested a waiver for long-term reproductive toxicity data to mammals based on a weight of evidence. The weight of evidence included data of residues of eugenol in a range of plants other than grapes or pomes from "Dr Duke's phytochemical and ethnobotanical databases" (https://phytochem.nal.usda.gov/phytochem/search), residue data of eugenol on fruits (grapes and pomes) from residue trials of the residue section (Vol. 3 CA B.7) and in the short persistence of the compound, its natural occurrence and volatility, and known low acute oral avian toxicity. Furthermore,the appicant proposed to conduct long-term avian risk assessment using the acute toxicity value LD_{50}/10 as a surrogate.

However, the residue data database showed deficiencies that question their reliability. Furthermore, the range of estimated residues indicates that the dietary exposure, assuming 100% consumption of the food items, would be between 0.02 – 180000 mg/kg for eugenol, thus, in most cases this would result in background exposure greater the predicted exposure following the proposed use. Moreover, the residue trials data from the residue section are relevant specifically to consideration of frugiovorus birds.

The applicant states that due to the high volatilisation (Vp = 2.7 Pa at 20º C) and the rapid degradation (DT_{50} soil 1 < day) initial environmental exposure of eugenol will decline rapidly. However, reliable data with regards to the background level of geraniol in the environment has not been provided.

Moreover, the applicant proposed the use of LD_{50}/10 as surrogate of the long-term endpoint for risk assessment since no avian reproduction study is avaiable and a low acute toxicity was observed (LD50 > 320 mg eugenol/kg bw). Birds and Mammals EFSA Guidance (2009) considers the use of the lowest acute LD_{50}/10 and the NOAEL from avian reproduction studies for long-term risk assessment. However, no long-term endpoint on birds for eugenol is available for comparison. Therefore, the weight of evidence submitted by the applicant is not sufficient to address the avian long term risk. Consequently, **further information should be submitted to address reproductive risk to avians.**

The previous EU-agreed mammalian (rat) acute oral LD_{50} value of 1930 mg eugenol/kg bw (EFSA Journal 2012; 10(11):2914) is considered acceptable to support renewal of eugenol. The previous EU-agreed mammalian (rat) NOAEL value of 250 mg eugenol/kg bw based on a developmental study (EFSA Journal 2012; 10(11):2914) is considered acceptable to support renewal of eugenol in the absence of a multi-generation study. No new studies are considered to be necessary.

2.9.2 Summary of effects on aquatic organisms [section 11.5 of the CLH report]

2.9.2.1 Bioaccumulation [equivalent to section 11.4 of the CLH report template]

Table 77: Summary of relevant information on bioaccumulation

Method	Species	Results	Key or Supportive study[1]	Remarks	Reference
Partition co-efficient n-octanol/water EEC A8, OECD 107	-	log P_{ow} value for eugenol = 2.47 (at pH 4) log P_{ow} value for eugenol =	Key The study is considered acceptable	Eugenol purity 99.2% w/w	Anonymous

		2.49 (at pH 7) log P_{ow} value for eugenol = 2.44 (at pH 9)			(2020)

2.9.2.1.1 Estimated bioaccumulation

Not relevant. Please refer to section 2.9.2.1.2 below.

2.9.2.1.2 Measured partition coefficient and bioaccumulation test data

The log P_{ow} value for eugenol is 2.47, 2.49 and 2.44 at pH values of 4, 7 and 9 respectively (please see Vol. 3 CA B.2 for details). The pH value has no impact on the octanol/water partition coefficient of eugenol. In line with Annex I, Section 4.1.2.8.1 of the CLP Regulation[2], these log P_{ow} values are less than the CLP cut-off criteria of 4, indicating eugenol does not show potential to bioaccumulate. Studies on active substance bioconcentration in organisms are therefore not required, as previously agreed during the EU review for the EU inclusion of eugenol (EFSA Journal 2012;10(11):2914).

However, eugenol is a surface-active substance and the log Kow is not a valid descriptor for assessing the bioaccumulation potential alone. Still in the absence of other data and taking into account the information from studies of ready biodegradability and the aerobic mineralization in surface water indicating that eugenol can be considered rapid degradable, its potential for bioconcentration in aquatic organisms would be expected to be low.

2.9.2.2 Acute aquatic hazard [equivalent to section 11.5 of the CLH report template]

Table 78: Summary of relevant information on acute aquatic toxicity

Method	Species	Test material	Results[1]	Key or Supportive study[1]	Remarks	Reference
Acute toxicity to fish OECD 203 (1992)	*Oncorhynchus mykiss* (rainbow trout)	Eugenol technical (98.8% w/w)	96-hour LC_{50} >10 mg eugenol/L nom (semi-static)	Key The study is considered acceptable	-	Anonymous (2008a).
Acute toxicity to fish OECD 203 (1992)	*Danio rerio* (zebra fish)	Eugenol technical (98.8% w/w)	96-hour LC_{50} = 11.9 mg eugenol/L nom (semi-static)	Key The study is considered acceptable	-	Anonymous (2008b).
Acute toxicity to fish OECD 203 (1992)	*Danio rerio* (Zebra fish)	Eugenol technical (99.98% w/w)	96-hour LC_{50} = 13 mg eugenol/L (nom) (semi-static)	Key The study is considered acceptable.		Anonymous, 2013. included in REACH Registration dossier
Acute toxicity to *Daphnia* OECD 202 (2004)	*Daphnia magna*	Eugenol technical (98.8% w/w)	48-hour EC_{50} = 1.11 mg eugenol/L nom (static)	Key The study is considered acceptable	-	CA 8.2.4.1/01 Pavić, B., Wydra, V. 2008 Report No. 37982220

[2] Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006

Acute toxicity to *Daphnia* OECD 202 (2004)	*Daphnia magna*	Eugenol technical (99.9% w/w)	48-hour EC_{50} = 1.13 mg eugenol/L (nom) (static)	The study is considered acceptable.	-	Bayer 1999 included in REACH Registration dossier
Growth inhibition to green algae OECD 201 (2006)	*Pseudokirchneriella subcapitata*	Eugenol technical (98.8% w/w)	<u>Growth rate</u> 72-hour E_rC_{50} = 15.4 mg eugenol/L (mm) <u>Biomass</u> 72-hour EbC50 = 10.0 mg eugenol/L (mm) <u>Yield</u> 72-hour EyC50 = 10.8 mg eugenol/L (mm)	Key. The study is considered acceptable.		Meister-Werner, A., Wydra, V., 2008.
Growth inhibition to green algae OECD 201 (2006)	*Desmodesmus subspicatus*	Eugenol technical (99.98% w/w)	<u>Growth rate</u> 72-hour E_rC_{50} = 24 mg eugenol/L (nom) <u>Biomass</u> 72-hour EbC50 = 36 mg eugenol/L (nom)	Key. The study is considered acceptable.		LAUS GmbH, 2013 included in REACH Registration dossier
Please refer to Section 2.9.2.3 where both acute (short-term) and chronic toxicity to algae are discussed						

nom: based on nominal concentrations

2.9.2.2.1 Acute (short-term) toxicity to fish

Two acute fish toxicity studies with eugenol (Anonymous, 2008a and Anonymous, 2008b) were previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.2.1.1). These studies are considered appropriate for the current assessment to support renewal of eugenol; full summaries are provided in Vol. 3 CA B.9 (CA 8.2.1/01 and CA 8.2.1/02).

And another acute fish toxicity study (Anonymous 2013) with eugenol was included in REACH Registration dossier and it is summarised below.

The EU-agreed 96-hour LC_{50} value of >10 mg eugenol/L for rainbow trout (EFSA Journal 2012; 10(11):2914) (from CA 8.2.1/01) is considered the appropriate critical endpoint for acute toxicity to fish.

Anonymous (2008a).

The 96-hour acute toxicity of eugenol to *Oncorhynchus mykiss* was studied under semi-static conditions in accordance with OECD 203. Groups of seven rainbow trout (*Oncorhynchus mykiss*) were exposed to eugenol at nominal concentrations of 1, 1.8, 3.2, 5.6 and 10 mg/L for 96 hours in a semi-static system under 16 hours light and 8 hours dark per day. There was also a control of test medium only. The test media were renewed after 48 hours. The temperature, which was measured daily in all test units, ranged between 14 and 15°C and the dissolved oxygen, which was measured in each vessel every 24 hours, was 95% – 101%. Test concentrations were analysed at the start and the end of each renewal period. Mortality and other observations were made after 1, 24, 48, 72 and 96 hours.

The mean recovery in the fortified samples was 100% (n = 8, RSD 6%). At the start of the test, 105 % of the nominal test concentration was found (average for the test concentrations). In the aged test media (48 hours old), 92 % of the nominal concentration was determined (average for the test concentrations). Thus, during the test period the fish were exposed to an overall mean measured concentration of 96 % of nominal (average for the test concentrations). As the active ingredient was found in concentrations close to the nominal concentrations at the start and the end of the test all reported results are expressed in terms of the nominal concentrations of the test item.

No mortality or sublethal symptoms were observed in the control or at test concentrations up to 1.8 mg/L. One fish died in the solvent control but this was not statistically significantly different from mortality in the control. Sublethal symptoms were observed at test concentrations of 3.2 mg/L and above. Mortality was observed only at the highest treatment level, 10 mg/L (43%).

The 96-hour LC_{50} value for eugenol to *Oncorhynchus mykiss* was estimated to be >10 mg/L and the NOEC was 1.8 mg/L, based on nominal concentrations.

The study is considered acceptable and the EU-agreed 96-hour LC_{50} value of >10 mg eugenol/L for rainbow trout (EFSA Journal 2012; 10(11):2914) is considered the appropriate critical endpoint for acute toxicity to fish.

Anonymous (2008b).

The 96-hour acute toxicity of eugenol to *Danio rerio* was studied under semi-static conditions in accordance with OECD 203. Groups of seven zebra fish (*Danio rerio*) were exposed to eugenol at nominal concentrations of 1.8, 3.2, 5.6, 10.0 and 18.0 mg/L for 96 hours in a semi-static system under 16 hours light and 8 hours dark per day. There was also a control of test medium only. The test media were renewed after 48 hours. The temperature, which was measured daily in all test units, ranged between 22°C and 23°C, and the dissolved oxygen, which was measured in each vessel every 24 hours, was 94 – 107 %. Test concentrations were analysed at the start and end of each renewal period. Mortality and other observations were made after 1, 24, 48, 72 and 96 hours.

Measured mean concentrations in the fortified samples were 97% of nominal, and the average measured concentrations of the test concentrations were 105% and 106% of nominal at the start and end of each renewal period, respectively. Hence, results are expressed based on nominal concentrations.

No mortality or sublethal effects were observed in the controls or at concentrations up to 3.2 mg/L. Sublethal symptoms were observed at 5.6 and 10 mg/L. Slight mortality occurred at 5.6mg/L but not at 10 mg/L, whereas all fish died at 18 mg/L. The 96-hour LC_{50} value for eugenol to *Danio rerio* was estimated to be 11.9 mg/L and the NOEC was 3.2 mg/L, based on nominal concentrations.

The study is considered acceptable.

Anonymous (2013).

The 96-hour acute toxicity of eugenol to *Danio rerio* was studied under semi-static conditions in accordance with OECD 203. The study was conducted under GLP. Fish were exposed to the substance at a range of nominal concentrations of 0, 10, 18, 32, 56 and 100 mg/L in freshwater. The test was performed with 7 fish per vessel and concentration. For each concentration, the percentage of mortality at 24, 48, 72, and 96 hours was recorded. The following OECD 203 validity criteria were met: mortality in the control did not exceed 10% at the end of the test; the dissolved oxygen concentration did not drop below 60% throughout the test; constant conditions (temperature between 21°C and 22.1°C and pH between 7.7 and 8) were within specified deviations; and the concentration of

the substance was at least 80% of the nominal concentration throughout the test. Under the conditions of the test, the 96-hour LC50 was 13 mg/L.

The study is considered acceptable.

2.9.2.2.2 Acute (short-term) toxicity to aquatic invertebrates

One acute *Daphnia* toxicity study with eugenol (Pavić, B., Wydra, V., 2008) was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.2.1.2). This study is considered appropriate for the current assessment to support renewal of eugenol; a full summary is provided Vol. 3 CA B.8 (CA 8.2.4.1/01).

Moreover, another acute Daphnia toxicity study (Bayer, 1999) with eugenol was included in REACH Registration dossier and it is summarised below.

The EU-agreed 48-hour EC_{50} value of 1.11 mg eugenol/L for *Daphnia magna* (EFSA Journal 2012; 10(11):2914) (from CA 8.2.4.1/01) is considered the appropriate critical endpoint for acute toxicity to aquatic invertebrates.

Pavić, B., Wydra, V., 2008.

The acute toxicity of eugenol to *Daphnia magna* was determined over a 48-hour exposure period under static conditions in accordance with OECD Guideline 202 (2004). *Daphnids* were exposed to five nominal concentrations of 0.31, 0.63, 1.25, 2.5 and 5 mg Eugenol /L and a treated control and solvent control for 48 hours. The pH of the test water was 7.8 at the start of the study, and ranged between 6.8 and 7.0 at the end.

No food was given during the exposure period. The photoperiod was 16 hours light and 8 hours dark. Observations for immobilised daphnids were recorded at 0, 24 and 48 hours of exposure. Analysis of the test media was performed at the start and end of the exposure period using a gas chromatography system with mass spectrometry detection.

Mean recovery in the fortified samples was 100% of nominal and the LOQ was established to be 1 mg/L.

At the start of the test, immediately before introduction of the daphnids, 100% of the nominal test concentrations were found (average for all concentrations). After 48 hours, 100% of the nominal values were again determined (average for all concentrations). Thus, during the test period of 48 hours the daphnids were exposed to an overall mean measured concentration of 100% of the nominal concentration (range, 93 to 120%). Therefore, all reported results are expressed in terms of nominal concentrations of the test substance.

No immobilisation was observed in the control, the solvent control and at test concentrations of up to 0.63 mg/L. Immobilisation values of 70%, 100% and 100 % were observed at 1.25, 2.5 and 5.0 mg/L, respectively. Based on nominal concentrations, the 48-hour EC_{50} value for eugenol to *Daphnia magna* was 1.11 mg/L, and the corresponding NOEC was estimated to be 0.63 mg/L

The study is considered acceptable and the EU-agreed 48-hour EC50 value of 1.11 mg eugenol/L for Daphnia magna (EFSA Journal 2012; 10(11):2914) is considered the appropriate critical endpoint for acute toxicity to aquatic invertebrates.

Bayer, 1999.

The acute toxicity of eugenol to *Daphnia magna* was determined over a 48-hour exposure period under static conditions in accordance with OECD Guideline 202 (2004). *Daphnids* were exposed to five nominal concentrations of 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 mg Eugenol /L. The photoperiod was 16 hours light and 8 hours dark. The test was performed with 10 daphnia per vessel and for each concentration; the percentage of immobility at 24 and 48 hours was recorded and test concentrations were confirmed by HPLC analysis.

No immobilisation was observed in the control, and at test concentrations of up to 0.8 mg/L. Immobilisation values of 95%, 100% and 100 % were observed at 1.6, 3.2 and 6.4 mg/L, respectively after 48 hours. Based on nominal concentrations, the 48-hour EC_{50} value for eugenol to *Daphnia magna* was 1.13 mg/L.

2.9.2.2.3 Acute (short-term) toxicity to algae or aquatic plants

One green algal study with eugenol was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.2.1.3). Another algae toxicity study (LAUS GmbH, 2013) with eugenol was included in REACH Registration dossier and it is summarised below.

Meister-Werner, A., Wydra, V., 2008.

The toxicity of eugenol to *Pseudokirchneriella subcapitata* was tested in an algal growth inhibition test in accordance with OECD 201. Test species were exposed to control, solvent control, and test chemical at nominal concentrations of 4, 8, 16, 32 and 64 mg Eugenol/L, a control containing culture medium only, and a solvent control. Three replicates of each test concentration were used and exponentially growing cultures of *P. subcapitata* were inoculated at 5×10^3 cells/mL, and cultured for 96 hours at temperatures of 21°C to 24°C.

Measured concentrations of eugenol were determined at 0 and 96 hours. Algal cell numbers were determined spectrophotometrically after approximately 24, 48, 72 and 96 hours.

At the start of the test immediately before introduction of the algae, 105% of the nominal test concentrations were found (average for all test concentrations), verifying the dosing of the test systems. At the end of the test, the mean measured test item concentrations were 55% of the nominal values (average for the test concentrations of 16, 32 and 64 mg/L), indicating degradation of the test substance under the test conditions. At lower test concentrations, the measured values were below the LOQ at the end of the exposure. Thus, the algae were exposed to a mean concentration of 76% of nominal concentrations. Test results for EC50s are given based on mean measured test concentrations.

The test was considered valid because the cell density in the control cultures increased by a factor of 167 over 72 hours, with coefficients of variation for the sectional daily growth rates and average growth of 34.1% and 2.7%, respectively.

The 72-hours results were:
72-hour E_rC_{50} (growth rate) = 15.4 mg eugenol/L (mean measured).
72-hour E_bC_{50} (biomass) = 10.0 mg eugenol/L (mean measured).
72-hour E_yC_{50} (yield) =14.2 mg eugenol/L (mean measured).
72-h EC_{10} (growth rate) = 11.1 mg eugenol/L (mean measured).
72-h EC_{10} (biomass) = 4.9 mg eugenol/L (mean measured).
72-h EC_{10} (yield) = 6.1 mg eugenol/L (mean measured).

The study is considered acceptable.

LAUS GmbH, 2013.

The toxicity of eugenol to *Desmodesmus subspicatus* was tested in an algal growth inhibition test in accordance with OECD 201. The study was conducted in a static system and algae were exposed to the substance at a range of concentrations of 0, 10, 18, 32, 56 and 100 mg/L in freshwater for 72 hours. The test was performed with 3 replicates per test concentration and 6 replicates for the control. The following OECD 201 validity criteria were met: cell concentration in the control increased by a factor of 38 within 72h; the mean coefficient of variation of the daily growth rates were 33% and the coefficient of variation of average growth rate during the whole test period was 4%. However, the pH of the control changed by 1.9 units due to carbon dioxide decrease in the headspace, as the carbon dioxide was absorbed during photosynthesis of the algae due to the closed test design; growth in the controls was normal.

Under the conditions of the test, the 72-hours results were:
72-hour E_rC_{50} (growth rate) = 24 mg eugenol/L (nominal).
72-hour E_bC_{50} (biomass) = 36 mg eugenol/L (nominal).
72-h EC_{10} = 23 mg eugenol/L (nominal).
72-h NOEC = 23 mg eugenol/L (nominal)

The study is considered acceptable.

2.9.2.2.4 Acute (short-term) toxicity to other aquatic organisms

There are no further studies on other aquatic organisms that are considered relevant to the classification of eugenol.

2.9.2.3 Long-term aquatic hazard [equivalent to section 11.6 of the CLH report template]

Table 79: Summary of relevant information on chronic aquatic toxicity

Method	Species	Test material	Results[1]	Relevant study	Remarks	Reference
Chronic toxicity to *Daphnia* OECD 211 (2004)	*Daphnia magna*	Eugenol technical (99.74% w/w)	21-day reproduction NOEC = 0.0959 mg eugenol/L mm, semi-static (highest concentration tested) Reliable EC_{10} could not be calculated	Key The study is considered acceptable.	-	Anonymous (2021)
Growth inhibition to green algae OECD 201 (2006)	*Pseudokirchneriella subcapitata*	Eugenol technical (98.8% w/w)	<u>Growth rate</u> 72-hour E_rC_{50} = 15.4 mg eugenol/L mm 72-hour E_rC_{10} = 11.1 mg eugenol/L mm 72-hour NOE_rC = 12.2 mg eugenol/L mm <u>Biomass</u> 72-hour E_bC_{50} = 10.0 mg eugenol/L mm 72-hour E_bC_{10} = 4.9 mg eugenol/L mm 72-hour NOE_bC = 3.0 mg eugenol/L mm <u>Yield</u> 72-hour E_yC_{50} = 10.8 mg eugenol/L mm 72-hour E_yC_{10} = 6.1 mg eugenol/L mm 72-hour NOE_yC = 6.1 mg eugenol/L mm	Key The study is considered acceptable.	-	CA 8.2.6.1/01 Meister-Werner, A., Wydra, V., 2008 Report No. 37981210
Growth inhibition to green algae OECD 201 (2006)	*Desmodesmus subspicatus*	Eugenol technical (99.98% w/w)	72-hour E_rC_{10} = 23 mg eugenol/L (nom) 72-hour	The study is considered acceptable.	-	LAUS GmbH, 2013

			NOE_rC = 23 mg eugenol/L (nom)			

nom: based on nominal concentrations; mm: based on mean measured concentrations

2.9.2.3.1 Chronic toxicity to fish

No data are available on the chronic toxicity to fish. According to the acute toxicity endpoints obtained from EFSA Journal 2012; 10(11):2914 (see also Section 2.9.2.2 above), it is observed that *Daphnia magna* is more sensitive (up to 10 times more acutely toxic) than fish. However, according to the Regulation 283/2013 the long-term and chronic toxicity study on fish is a data requirement. The study should be provided unless it is proved that the substance is stable in water, that is to say there is less than 90% loss of the original substance over 24 hours via hydrolisis. Since, eugenol was considered hydrolytically stable at pH 4 and 7 (Vol. 3 CA Study B.8.2.1.1/01; Anonymous, 2021) the data gap for further information to address the chronic risk to aquatic organisms has not been addressed. Thus, **a data gap** has been identified **to submit a chronic toxicity study on fish**.

Considering the suggestion of the Co-RMS, an ELS test (OECD TG 210) could be performed to study the chronic risk to fish and EAS-adversity of the active substance eugenol.

2.9.2.3.2 Chronic toxicity to aquatic invertebrates

A new chronic toxicity study with *Daphnia magna* has been provided to support the renewal of eugenol; a full summary is provided in Vol. 3 CA B.9 (CA 8.2.5.1/01), a summary is provided below.

Anonymous (2021).

The chronic toxicity of eugenol to *Daphnia magna* was studied under semi-static conditions in accordance with OECD Test Guideline 211 (2012). *Daphnids* were exposed to nominal concentrations of 0.00781, 0.0156, 0.0313, 0.0625 and 0.125 mg eugenol/L (corresponding to mean measured concentrations of 0.0045, 0.0108, 0.0210, 0.0471 and 0.0959 mg eugenol/L) and an untreated control (Elendt medium M4) for 21 days.

Adult daphnids were observed daily for immobility, presence of eggs and mortality. They were transferred to fresh media 3 times per week. Any juveniles produced were counted and removed daily.

Analytical verification of the test item concentrations indicated they were unstable in the test solutions, decreasing to <80% of the nominal concentrations. Therefore, biological endpoints are based on time-weighted mean of the measured concentrations.

The validity criteria according to OECD guideline 2011 were met.

The endpoints evaluated were mortality (F0) and fecundity (total number of living offspring (F1) per parental animal (F0)). No statistically significant differences were observed in mortality (immobility) or reproduction. Furthermore, no concentration-response relationship could be established for the measured endpoints, thus no reliable EC_x values could be calculated.

Therefore, the 21-day NOEC value for *Daphnia magna*, based on the total number of living offspring per surviving *Daphnia magna* parental daphnids, was the highest tested concentration, 0.0959 mg Eugenol/L and the corresponding 21-day LOEC value was estimated to be > 0.0959 mg Eugenol/L (time-weighted mean of measured concentration).

This study is considered acceptable and the 21-day NOEC (reproduction) value of 0.0959 mg eugenol/L, the highest concentration tested, (from CA 8.2.5.1/01) is considered the appropriate critical endpoint for chronic toxicity to aquatic invertebrates. It should be noted that the NOEC value of 0.0959 mg eugenol/L was the highest concentration (mean measured) tested in the study (nominally 0.125 mg eugenol/L), at which there was 0% adult immobility and no significant effects on reproduction compared to the control (reduction of 1.7% and increase of 4.6% compared to control when based on number of offspring per introduced parent and number of offspring per living parent at test end, respectively).

2.9.2.3.3 Chronic toxicity to algae or aquatic plants

One green algal study with eugenol was previously evaluated as part of the EU review for the EU inclusion of eugenol (DAR, Volume 3, Annex B.9, 2011, B.9.2.1.3). This study is considered appropriate for the current assessment to support renewal of eugenol; a full summary is provided in Vol. 3 CA B.9 (CA 8.2.6.1/01).

The EU-agreed E_rC_{50} value of 15.4 mg eugenol/L for *Pseudokirchneriella subcapitata* (EFSA Journal 2012; 10(11):2914) (from CA 8.2.6.1/01) is considered the appropriate critical endpoint for acute/short-term effects on growth of green algae.

Please refer to Section 2.9.3.2.3 'Acute (short-term toxicity to algae or aquatic plants' where the data for both acute (short-term) and chronic toxicity to algae are discussed. For the chronic aquatic hazard assessment, the EU-agreed 72-hour ErC10 value of 11.1 mg eugenol/L for Pseudokirchneriella subcapitata (EFSA Journal 2012; 10(11):2914) is considered the appropriate critical endpoint for chronic effects on growth of green algae.

2.9.2.3.4 Chronic toxicity to other aquatic organisms

There are no further studies on other aquatic organisms that are considered relevant to the classification of eugenol.

2.9.2.4 Comparison with the CLP criteria

2.9.2.4.1 Acute aquatic hazard

Table 80: Summary of information on acute aquatic toxicity relevant for classification

Method	Species	Test material	Results[1]	Remarks	Reference
Acute toxicity to fish OECD 203 (1992)	*Oncorhynchus mykiss* (rainbow trout)	Eugenol technical (98.8% w/w)	96-hour LC_{50} >10 mg eugenol/L nom (semi-static)	Accepted	Anonymous (2008a)
Acute toxicity to *Daphnia* OECD 202 (2004)	*Daphnia magna*	Eugenol technical (98.8% w/w)	48-hour EC_{50} = 1.11 mg eugenol/L nom (static)	Accepted	CA 8.2.4.1/01 Pavić, B., Wydra, V. 2008 Report No. 37982220
Growth inhibition to green algae OECD 201 (2006)	*Pseudokirchneriella subcapitata*	Eugenol technical (98.8% w/w)	72-hour E_rC_{50} = 15.4 mg eugenol/L mm	Accepted	CA 8.2.6.1/01 Meister-Werner, A., Wydra, V., 2008 Report No. 37981210

[1] nom: based on nominal concentrations; mm: based on mean measured concentrations

Full acute set was available for Eugenol as there were acute studies on fish, aquatic invertebrates and algae and aquatic plants, covering the three trophic levels (see Table 80). The acute toxicity (LC_{50} / EC_{50}) values for all three trophic levels are >1 mg eugenol/L, and invertebrates are the most sensitive trophic level with the 48h-EC_{50} of 1.11 mg/L.

For classification of a substance in relation to acute aquatic hazard, table 4.1.0 (a) of Annex I of Regulation (EC) No. 1272/2008 should be used. The acute endpoint selected has to be compared with the cut-off value (acute toxicity values ≤ 1 mg/l). The 48- EC50 of 1.11 mg/L is > 1 mg/L. Therefore, *Eugenol* should be classified as **Not classified** for acute aquatic hazard.

2.9.2.4.2 Long-term aquatic hazard (including bioaccumulation potential and degradation)

Table 81: Summary of information on long-term aquatic toxicity relevant for classification

Method	Species	Test material	Results[1]	Remarks	Reference
Chronic toxicity to *Daphnia* OECD 211 (2004)	*Daphnia magna*	Eugenol technical (99.74% w/w)	21-day reproduction NOEC = 0.0959 mg eugenol/L mm, semi-static (highest concentration tested) Reliable EC_{10} could not be calculated	Accepted	Anonymous, (2021)
Growth inhibition to green algae OECD 201 (2006)	*Pseudokirchneriella subcapitata*	Eugenol technical (98.8% w/w)	Growth rate 72-hour E_rC_{50} = 15.4 mg eugenol/L mm 72-hour E_rC_{10} = 11.1 mg eugenol/L mm 72-hour NOE_rC = 12.2 mg eugenol/L mm	Accepted	CA 8.2.6.1/01 Meister-Werner, A., Wydra, V., 2008 Report No. 37981210

mm: based on mean measured concentrations

Degradability

Eugenol can be considered to be readily biodegradable since there was a ready biodegradation study available which demonstrated a high level of degradation within the 10-d window (biodegradation ≥60% of the theoretical oxygen demand [ThOD] of the test item in a 10-day window within the 28-day test period) and at the end of the 28-day incubation period, the mean biodegradation of eugenol was 84%. Therefore, Eugenol can also be considered as rapidly degradable substance.

Bioaccumulation

The log K_{OW} of Eugenol is 2.49 at pH 7 (2.47 at pH 4 and 2.44 at pH 9), thus it is below the threshold of ≥ 4 of potentially bioaccumulative substances. Therefore, Eugenol could be considered as having low potential for bioaccumulation.

Chronic aquatic hazard

A full set of chronic data for three trophic levels is not available. Chronic toxicity data with eugenol are available for two trophic levels (invertebrates and algae). Therefore, in line with Annex I, Section 4.1 (Figure 4.1.1) of the CLP Regulation, the long-term aquatic hazard classification is assessed according to the following two methods and the substance classifies according to the most stringent outcome:

(a) using the chronic toxicity data for invertebrates and algae according to the criteria given in Table 4.1.0(b)(ii) (for rapidly degradable substances)

The E_rC_{10} and NOE_rC values of 11.1 and 12.2 mg eugenol/L for green algae are well above 1 mg/L and therefore do not trigger a long-term aquatic classification.

The chronic toxicity (NOEC) value for invertebrates (crustacea) is 0.0959 mg eugenol/L. Since the substance is rapidly degradable and there is adequate chronic data for crustaceans, the chronic NOEC should be compared to the threshold values based on chronic data (table 4.1.0 (b)(ii)). The 21-d NOEC of 0.0959 mg/L is <0.1 mg/L. Thus *ortho*-Phenylphenol should be classified as Chronic 2.

(b) using the acute toxicity data according to the criteria given in Table 4.1.0(b) (iii) Substances for which adequate chronic toxicity data are not available.

To classify according to these criteria, substances should be classified as not rapidly degradable and/or the experimentally determined BCF ≥ 500 (or, is absent, the logKow ≥ 4). Taking into account these values, eugenol could not be classified according to these criteria since it is rapidly degradable and the logKow < 4.

Therefore, in line with Annex I, Section 4.1 (Figure 4.1.1) of the CLP Regulation it is proposed that eugenol is classified for long-term as aquatic chronic 2, the most stringent outcome.

2.9.2.5 Conclusion on classification and labelling for environmental hazards

Taking into account all the information and the assessment summarized in the previous sections 2.9.3.4, the following classification class and category can be concluded for this active substance Eugenol, in accordance with Regulation (EC) 1272/2008:

CLP Annex ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification[1]	Reason for no clasification[2]
4.1	Hazardous to the aquatic environment	Aquatic Chronic 2 H411	-	-	-
5.1	Hazardous to the ozone layer	-	-	-	Chemical structure and physicochemical properties warrant no classification.

Labelling: Signal word: -

Hazard statements: Toxic to aquatic life with long lasting effects (H411)

Precautionary statements:

P273: Avoid release to the environment
P391: Collect spillage
P501: Dispose of contents/container in accordance with national hazardous waste regulations

Pictogram: GHS09

The following additional statements are recommended.

- EUH401: To avoid risks to human health and the environment, comply with the instructions for use.

2.9.3 Summary of effects on arthropods

The data on the representative formulation, Mevalone, are considered sufficient to address the active substance data requirements. The previous EU-agreed (EFSA Journal 2012; 10(11):2914) acute oral LD_{50} value of > 224.6 µg Mevalone/bee and acute contact LD_{50} value of > 200 µg Mevalone/bee are considered the appropriate critical endpoints for acute and contact toxicity to honey bees. New chronic oral adult and repeated exposure larval honey bee (*Apis mellifera*) toxicity tests are submitted to support renewal of eugenol with a 10-day (adult) LDD_{50} value of 123.53 µg Mevalone/bee/day and a 22-day (larva) NOED value of 1300 µg Mevalone/larva/developmental period.

The previous EU-agreed (EFSA Journal 2012; 10(11):2914) LR_{50} values of > 12420 g Mevalone/ha for both *Aphidius rhopalosiphi* and *Typhlodromus pyri* are considered the appropriate critical mortality endpoints based on glass plate studies for non-target arthropods other than bees. No further studies are considered necessary.

2.9.4 Summary of effects on non-target soil meso- and macrofauna

The data on the representative formulation, Mevalone, are considered sufficient to address the active substance data requirements. The requirement for acute toxicity data for earthworms is now obsolete under Regulation (EU) No 283/2013, but the previous EU-agreed acute on *Eisenia fetida* (EFSA Journal 2012; 10(11):2914) is presented in Vol. 3 CP (Lührs, 2007 : Study B.9.7.1.1/01) as supporting information to support renewal of eugenol. A new chronic reproduction toxicity study for earthworm with formulation Mevalone, including eugenol, geraniol and thymol, has been provided to meet new data requirements under Regulation (EU) No 284/2013. The new chronic reproduction toxicity endpoint to earthworm (*Eisenia andrei)* 56-day NOEC (reproduction) value of 52.9 mg Mevalone/kg dry soil ($NOEC_{corr}$ = 26.5 mg Mevalone/kg dry soil) ($NOEC_{corr}$ corresponding to 0.85 mg eugenol/kg dry soil) is considered to be the appropriate critical reproduction endpoint.

In addition, one new chronic reproduction toxicity test with the soil macro-organisms *Folsomia candida* and the representative product, Mevalone, has been submitted to support renewal of eugenol. It is noted that *Hypoaspis acuelifer* and *Folsomia candida* studies are not formally required as there is no direct application to soil and a low risk is concluded at Tier 1 with *T. pyri* and *A. rhopalosiphi*, but a new *Folsomia candida* study is provided for completeness. The new *Folsomia candida* 28-day EC_{10} value of 37.3 mg Mevalone/kg dry soil ($EC_{10\ corr}$ = 18.65 mg Mevalone/kg dry soil) ($EC_{10\ corr}$ corresponding to 0.6 mg eugenol/kg dry soil) is considered the appropriate critical reproduction endpoint to be used in the risk assessment.

No further studies are considered necessary.

2.9.5 Summary of effects on soil nitrogen transformation

The data on the representative formulation, Mevalone, are considered sufficient to address the active substance data requirements. The previous EU-agreed conclusion (EFSA Journal 2012;10(11):2914) is considered appropriate for effects on nitrogen transformation: No significant effects (<25% relative to the control) on nitrogen transformation were observed after 56 days at 54.4 mg Mevalone/kg dry soil (corresponding to 1.74 mg eugenol/kg dry soil). No further studies are considered necessary.

2.9.6 Summary of effects on terrestrial non-target higher plants

Eugenol does not exhibit herbicidal activity or a plant growth regulator mode of action and the available screening data on the representative formulation, Mevalone are expected to be sufficient to address the active substance data requirement. The previous information on plant screening was provided in the DAR (eugenol, geraniol and thymol DAR, Volume 3, Annex B.9, 2011, B.9.9.1) and is considered sufficient to demonstrate a lack of effects on non-target plants. Limit tests at rates including 4 x 4 L Mevalone/ha (equivalent to 4 x 4120 g Mevalone/ha based on a density of 1029 g/L) and higher were conducted with Mevalone. Effects were below the critical threshold as defined by the "Guidance Document on Terrestrial Ecotoxicology", (SANCO/10329/2002 rev.2 final, 2002). No further studies are considered necessary.

2.9.7 Summary of effects on other terrestrial organisms (flora and fauna)

No other groups of terrestrial organisms are considered to be at risk and no concerns were raised from a review of the open literature. No further data are considered necessary.

2.9.8 Summary of effects on biological methods for sewage treatment

The data on the representative formulation, Mevalone, are considered sufficient to address the active substance data requirements. One new study based on an activated sludge respiration inhibition test with Mevalone, is submitted to support the renewal of eugenol. The critical (lowest) 3-hour EC_{50} value, based on nitrification respiration, was calculated to be 204.9 mg product/L (CI: 115.5 – 361.7 mg product/L), corresponding to 6.5 mg eugenol/L. No further studies are considered necessary.

2.9.9 Summary of product exposure and risk assessment

The representative product, Mevalone, contains three active substances at nominally 6.4% w/w thymol, 6.4% w/w geraniol and 3.2% w/w eugenol. Mevalone is the common representative product of all three active substances (thymol, geraniol and eugenol), which are intended to be renewed at the same time. The three active substances share the same Vol. 3 CP B.9 and the same studies for Mevalone.

The risk assessments have been carried out considering the representative GAP of four applications (7-day interval) of 4.12 kg Mevalone/ha (based on a density of 1.029 g/L), which corresponds to 4 x 0.264 kg thymol/ha, 4 x 0.264 kg geraniol/ha and 4 x 0.132 kg eugenol/ha, in vineyards (BBCH 60-89) and pome fruit (BBCH 75-87).

Birds (Vol. 3 CP B.9.2.1)

The risk assessment was carried out according to the EFSA Guidance Document on Risk assessment for birds and mammals (EFSA Journal 2009; 7 (12): 1438). Taking into account that the shortcut values for vineyards are higher than orchards, the risk envelope approach has been applied, and therefore the calculations with vineyards also cover the application in orchards.

All acute TER values for birds were above the relevant trigger values at the screening step. Screening step for the acute oral risk to birds due to the use of Mevalone in vineyards: DDD_{90} = 707; TER_a >14. In the case of the active substance eugenol, a DDD_{90} of 22.6 mg/kg bw/d and a TER_a >14 were found.

However, no avian reproductive toxicity data for the active substance eugenol or formulation Mevalone are available, **birds reproductive risk assessment cannot be finalised**. Therefore, further information should submitted.

The acute risk to birds is considered to be acceptable without the need for any mitigation. The acute risk to birds *via* consumption of contaminated water and the risk *via* secondary poisoning is considered to be acceptable.

Terrestrial vertebrates other than birds (Vol. 3 CP B.9.2.2)

The risk assessment was carried out according to the EFSA Guidance Document on Risk assessment for birds and mammals (EFSA Journal 2009; 7 (12): 1438) and considering the EU agreed mammalian endpoints (LD_{50} > 2000 mg product/kg bw, LD_{50} =1930 mg eugenol/kg bw, NOAEL = 250 mg eugenol/kg bw).

The acute TER values for mammals were above the relevant trigger value at the screening step when considering the available acute toxicity data for the substance:

Screening step for the acute oral risk to mammals due to the use of eugenol in orchard/vineyards: DDD_{90} = 32.4; TER_a = 60.

When considering the acute mammalian toxicity value of >2000 mg Mevalone/kg bw for the representative product, the Tier 1 TER values were below the trigger of 10 for the two generic focal species, 'vole' and 'dormouse':

First tier assessment for the acute oral risk to mammals due to the use of Mevalone (LD_{50} >2000 mg Mevalone/kg bw):

Orchards/vineyards BBCH ≥40 'vole' DDD_{90} = 303.3; TER_a >6.6;

Orchards BBCH 71-79 'dormouse' DDD_{90} = 355.2; TER_a >5.6.

However, taking into account that the acute endpoint for Mevalone is a "greater than" value, with no mortalities observed at 2000 mg Mevalone/kg bw, an acute risk to herbivorous mammals to the formulation is unlikely. In addition, it is considered that mammals are unlikely to be exposed to Mevalone in their diet because following application the formulation will rapidly breakdown into its component active substances, thymol, geraniol and eugenol, which are all highly volatile. Since the acute formulation toxicity study did not derive an actual value for use as an endpoint (i.e. LD_{50} >2000 mg Mevalone/kg bw), the combined toxicity of the three active substance components was calculated using the Finney (1942) equation. Using the predicted mixture toxicity endpoint of 10033.7 mg Mevalone/kg bw, the resulting Tier 1 TER values clearly indicate a low acute risk to mammals:

First tier assessment for the acute oral risk to mammals due to the use of Mevalone (LD_{50} = 10033.7 mg Mevalone/kg bw):

Orchards/vineyards BBCH ≥40 'vole' DDD_{90} = 303.3; TER_a = 33;

Orchards BBCH 71-79 'dormouse' DDD_{90} = 355.2; TER_a = 28.

For the long-term risk to mammals, TER values were above the relevant trigger value at the screening step for eugenol and geraniol, and at Tier 1 for thymol:

Screening step for the long-term risk to mammals due to the use of eugenol in orchard/vineyards: DDD_m = 11.12; TER_{lt} = 22.

Screening step for the long-term risk to mammals due to the use of geraniol in orchard/vineyards: DDD_m = 22.25; TER_{lt} = 16.

First tier assessment for the long-term risk to mammals due to the use of thymol in orchard/vineyards: worst-case DDD_m = 6.987; TER_{lt} = 14.

Therefore, the acute and long-term risk to mammals is considered to be acceptable without the need for any mitigation. The risk to mammals *via* consumption of contaminated water and *via* secondary poisoning is considered to be acceptable.

Aquatic organisms (Vol. 3 CP B.9.4)

The evaluation of the risk for aquatic organisms was performed in accordance with the recommendations of the EFSA Journal 2013, 11(7):3290.

The relevant global maximum FOCUS PEC_{sw} values for risk assessments covering the proposed use pattern are calculated in Vol. 3 CP Mevalone B.8. The worst case PECsw values for vines and apples have been used in the risk assessment.

An acceptable risk to Mevalone is concluded at the first-tier based on instantaneous formulation PEC_{sw} calculations (spray drift only) for aquatic organisms (PEC/RAC values greater than the trigger of 1).

Table 2.9.9-1: Acceptability of risk (PEC/RAC < 1) for each organism group based on instantaneous formulation PEC_{sw} calculations (spray drift only) for the use of Mevalone –Vineyards and apples 4 applications at 4120 g product/ha

Group		**Fish acute**	**Inverteb. acute**
Test species		***O. mykiss***	***D. magna***
Endpoint (µg/L)		LC_{50} 31100	EC_{50} 35400
AF		100	100
RAC (µg/L)		311	354
Crop	**PEC $_{gl\text{-}max}$ (µg/L)**	PEC/RAC	PEC/RAC
Vines (late)	110.034	0.354	0.311
Apples (late)	215.816	0.694	0.610

For the intended uses of Mevalone in vines and apples, calculated PEC/RAC ratios for the formulated product indicate an acceptable risk for the most sensitive group of aquatic organisms (acute risk for fish) based on instantaneous formulation PEC_{sw} calculations (spray drift only). Therefore, no further assessment is necessary for Mevalone.

Table 2.9.9-2: Aquatic organisms: acceptability of risk (PEC/RAC < 1) for each organism group based on FOCUS STEP 1, 2 and 3 calculations for the use of eugenol – Vineyards and apples 4 applications at 132 g eugenol/ha

Group		**Fish acute**	**Inverteb. acute**	**Inverteb. chronic**	**Algae**
Test species		***O. mykiss***	***D. magna***	***D. magna***	***P. subcapitata***
Endpoint		LC_{50}	EC_{50}	NOEC	E_rC_{50}
(µg/L)		>10000	1110	95.9	15400
AF		100	100	10	10
RAC (µg/L)		>100	11.1	9.59	1540
FOCUS Scenario	**PEC $_{gl\text{-}max}$ (µg/L)**	PEC/RAC	PEC/RAC	PEC/RAC	PEC/RAC
STEP 1					
Vines	187.81	**1.88**	**16.92**	**19.58**	0.122
Apples	201.36	**2.01**	**18.14**	**21.00**	0.131
STEP 2					
Vines S-Europe	7.64	0.08	0.69	0.80	0.005
Apples S-Europe	11.67	0.12	**1.05**	**1.22**	0.008
STEP 3 -					
Apples R3 stream	5.205	-	0.47	0.54	-

Values in **bold** are above the trigger value

For the intended uses of Mevalone in vines and apples, calculated PEC/RAC ratios for the active substance eugenol indicate an acceptable risk for the most sensitive group of aquatic organisms (chronic risk for *Daphnia*) at FOCUS STEP 2 for vines, and in all FOCUS STEP 3 scenarios for apples. Therefore, no further assessment is necessary for eugenol.

Bees (Vol. 3 CP B.9.6.1)

The acute risk to honey bees from the use of Mevalone was first assessed using the maximum single application rate and the respective LD_{50} values to calculate hazard quotients (HQ) in accordance with the recommendations of the "Guidance Document on Terrestrial Ecotoxicology", as provided by the Commission Services (SANCO/10329/2002 rev.2 (final), October 17, 2002) as follows. The hazard quotients (HQ) are well below the trigger of 50, indicating an acceptable acute oral and contact risk to bees following the proposed use of Mevalone in vineyards and orchards:

First tier assessment for the acute oral risk to adult honey bees due to the use of Mevalone in orchard/vineyards: maximum single application rate = 4120 g product/ha; HQ <18.3.

First tier assessment for the acute contact risk to adult honey bees due to the use of Mevalone in orchard/vineyards: maximum single application rate = 4120 g product/ha; HQ <20.6.

The potential for inhalation exposure is at least partly covered by the available acute oral and contact toxicity tests. In any case it is noted that as a consequence of the high volatility of all three active substances, a low residence time in the treated field is expected after each application. Taking into account the HQ values for acute oral and contact toxicity, there is a wide margin of safety which is expected to also cover the potential risk to bees *via* the inhalation route of exposure.

The EFSA Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera, Bombus* spp. and solitary bees) (EFSA Journal 2013;11(7):3295) has not yet been noted at the EU level. Nevertheless, the current SANCO/10329/2002 guidance document does not cover the risk assessment for honey bee larvae and chronic adults, while endpoints for these are available according to the current data requirements. In the absence of alternative approaches, it was agreed in a general ecotoxicology meeting (EFSA Supporting publication 2015:EN-924) that the first-tier risk assessment to honey bees should be performed according to the EFSA Guidance Document on the risk assessment of plant protection products on bees (*Apis mellifera, Bombus* spp. and solitary bees) (EFSA Journal 2013;11(7):3295, hereafter referred to as EFSA/2013/3295).

In accordance with EFSA/2013/3295, the hazard quotient (HQ) is well below the trigger of 85 (for sideward sprays), indicating an acceptable acute contact risk to bees following the proposed use of Mevalone in vineyards and orchards:

Screening assessment for the acute contact risk to adult honey bees due to the use of Mevalone in orchard/vineyards: maximum single application rate = 4120 g product/ha; HQ <20.6.

In accordance with EFSA/2013/3295, the ETR_{oral} values for the acute oral risk to adult honey bees and chronic risk to larval honey bees are below the relevant trigger of 0.2 at the screening step indicating acceptable risk following the proposed use of Mevalone in vineyards and orchards:

Screening assessment for the acute oral risk to adult honey bees due to the use of Mevalone in orchard/vineyards: maximum single application rate = 4120 g product/ha; SV = 10.6; ETR_{oral} = 0.194.

Screening assessment for the chronic risk to larval honey bees due to the use of Mevalone in orchard/vineyards: maximum single application rate = 4120 g product/ha; SV = 6.1; ETR_{oral} = 0.019.

Tier 1 assessments were conducted in Vol. 3 CP B.9.6.1, to assess the chronic oral risk to adult honey bees in accordance with EFSA/2013/3295. Acceptable risk (ETR_{oral} >0.03) was demonstrated for all relevant scenarios in pome fruit (BBCH 75-87) and vineyards (BBCH ≥70). For the proposed uses in vineyards at BBCH 60-69, the Tier 1 chronic oral adult ETR_{oral} is above the trigger value of 0.03 only for the treated crop scenario:

Worst-case scenario in the first-tier assessment for the chronic oral risk to adult honey bees due to the use of Mevalone. Vineyards BBCH 60-69 treated crop scenario: maximum single application rate = 4120 g product/ha; EF = 1; SV = 8.2; TWA = 0.72; ETR_{oral} = 0.197.

According to the Appendix D of EFSA Journal 2013;11(7):3295, grapevines are of low attractiveness to bees for collection of nectar. It is known that grapevines are wind-pollinated so although they produce nectar they are rarely visited by bees for collection of pollen. The same conclusions were found in two open literature papers (Attractiveness of Agriculture Crops to Pollinating Bees- USDA Report 2017[3], Insect Pollination of Crops - FAO[4]) which indicates that vineyards are not attractive for nectar.. Therefore, vineyards can be considered attractive only for collection of pollen and the risk assessment for treated crop scenario can be conducted with the short-cut value indicated in Table J_x of EFSA Journal 2013;11(7):3295. EF = 1, SV = 0.06, TWA = 0.72, ETR_{oral} = 0.0014.

Overall, the risks to bees are considered acceptable following the proposed representative uses of Mevalone in vineyards and pome fruit without the need for mitigation,

Non-target arthropods other than bees (Vol. 3 CP B.9.6.2)

The evaluation of the risk for non-target arthropods was performed in accordance with the recommendations of the "Guidance Document on Terrestrial Ecotoxicology", as provided by the Commission Services (SANCO/10329/2002 rev.2 (final), October 17, 2002), and in consideration of the recommendations of the guidance document ESCORT 2. According to the drift values obtained from ESCORT 2, orchards represent the worst-case drift values. Therefore, the risk assessment of orchards also covers the risk assessment for vineyards applying the risk envelope approach.

All $HQ_{in\text{-}field}$ and $HQ_{off\text{-}field}$ values for both indicator species are below the relevant trigger value at the first tier, indicating acceptable in-field and off-field risk to non-target arthropods:
First tier assessment for the in-field risk to NTAs due to the use of Mevalone in orchards: $PER_{in\text{-}field}$ (foliar) = 11124 g product/ha; $HQ_{in\text{-}field}$ (foliar) <0.9; $PER_{in\text{-}field}$ (soil) = 14008 g product/ha; $HQ_{in\text{-}field}$ (soil) <1.1.
First tier assessment for the off-field risk to NTAs due to the use of Mevalone in orchards: $PER_{off\text{-}field}$ (foliar) = 2626.3 g product/ha; $HQ_{off\text{-}field}$ (foliar) <0.21; $PER_{off\text{-}field}$ (soil) = 3307.3 g product/ha; $HQ_{off\text{-}field}$ (soil) <0.27.

Overall, acceptable in-field and off-field risk for non-target arthropods is concluded following the representative uses of Mevalone in vineyards and orchards without the need for mitigation measures.

Earthworms (Vol. 3 CP B.9.8.1)

The evaluation of the risk for earthworms was performed in accordance with the recommendations of the "Guidance Document on Terrestrial Ecotoxicology", as provided by the Commission Services (SANCO/10329/2002 rev.2 (final), October 17, 2002).

[3] https://www.ars.usda.gov/ARSUserFiles/OPMP/Attractiveness%20of%20Agriculture%20Crops%20to%20Pollinating%20Bees%20Report-FINAL_Web%20Version_Jan%203_2018.pdf

[4] FAO, Insect Pollination of Crops. Academic Press, London, UK (1993)

The relevant initial PEC_{soil} values for risk assessments covering the proposed use pattern are calculated Vol. 3 CP B.8.

Acceptable chronic risk to earthworms is concluded at the first-tier based on a worst-case initial PEC_{soil} value (TER value greater than the trigger of 5):

First-tier assessment of the chronic risk for earthworms due to the use of Mevalone in vineyards: PEC_{soil} = 2.195 mg Mevalone/kg dw; TER_{lt} = 12.1.

Therefore, the chronic risk to earthworms is considered to be acceptable without the need for mitigation measures.

Non-target soil meso- and macro- fauna other than earthworms (Vol. 3 CP B.9.8.2)

The evaluation of the risk for other non-target soil meso- and macrofauna (other than earthworms) was performed in accordance with the recommendations of the "Guidance Document on Terrestrial Ecotoxicology", as provided by the Commission Services (SANCO/10329/2002 rev.2 (final), October 17, 2002).

The relevant initial PEC_{soil} values for risk assessments covering the proposed use pattern are calculated in Vol. 3 CP B.8.

Acceptable chronic risk to non-target soil meso- and macrofauna is concluded at the first-tier based on a worst-case initial PEC_{soil} value (TER value greater than the trigger of 5):

First-tier assessment of the chronic risk for *Folsomia candida* due to the use of Mevalone in vineyards: PEC_{soil} = 2.195 mg Mevalone/kg dw; TER_{lt} = 8.5

Soil nitrogen transformation (Vol. 3 CP B.9.10)

The evaluation of the risk for soil micro-organisms was performed in accordance with the recommendations of the "Guidance Document on Terrestrial Ecotoxicology", as provided by the Commission Services (SANCO/10329/2002 rev 2 (final), October 17, 2002).

The relevant initial PEC_{soil} values for risk assessments covering the proposed use pattern are calculated in the relevant Document MCP 9 for each active substance.

The relevant PEC_{soil} value for Mevalone of 2.195 mg Mevalone/kg dry soil is well below the appropriate critical endpoint of 54.4 mg Mevalone/kg dry soil. Therefore, the risk for soil nitrogen transformation is considered to be acceptable without the need for mitigation.

Terrestrial non-target plants (Vol. 3 CP B.9.12)

The risk assessment is based on the "Guidance Document on Terrestrial Ecotoxicology", (SANCO/10329/2002 rev.2 final, 2002). Limit tests at rates including 4 x 4 L product/ha (equivalent to 4 x 4120 g product/ha based on a density of 1029 g/L) and higher were conducted with Mevalone. Effects were below the critical threshold as defined by the "Guidance Document on Terrestrial Ecotoxicology", (SANCO/10329/2002 rev.2 final, 2002). The limit test rates equal/exceed the highest field application rate in vineyards and orchards of 4 x 4120 g Mevalone/ha and are thus considered an indicator for an acceptable risk without the need for mitigation. No further risk assessment is considered necessary.

2.10 ENDOCRINE DISRUPTING PROPERTIES

Introduction to this chapter
The scientific criteria for the determination of endocrine disrupting properties are set out in Commission Regulation (EU) 2018/605. The European Chemicals Agency (ECHA) and European Food Safety Authority (EFSA) provide guidance for the identification of endocrine disruption (ED) in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 (2018). Based on the available information, data on eugenol for potential ED in mammalian are summarized and assessed in this chapter according to the EFSA/ECHA guidance.

2.10.1 Gather all relevant information

The applicant has provided updated information on the endocrine disrupting properties of the active substance eugenol: an assessment of short-term toxicity studies, long-term/carcinogenicity studies, developmental toxicity studies, data from the scientific peer-reviewed open literature, and other sources of data relevant to endocrine disruptors (e.g. the US EPA CompTox Chemicals Dashboard). The endocrine disruption assessment itself carried out by the RMS can be found on this section, while relevant data has been compiled using the Excel Spreadsheet in Appendix E, where each study was given an identification number (Study ID Matrix) that is important for its identification in the data-matrix of the Excel.

2.10.1.1 Non-test information

In accordance with the OECD Conceptual Framework and the ED Guidance, Level 1 information was gathered by the RMS for eugenol:

- Qualitative structural activity relationship (QSAR) data was obtained for eugenol from the Danish QSAR database and the OECD QSAR Toolbox v.4.5. The Danish (Q)SAR Database was released in November 2015 and has since then been expanded and updated a number of times. It currently holds many predictions for around 650000 substances and is a tool that allows industry, research, authorities and others to search for hazard information on chemical substances, especially those with little or no testing data. While the Danish (Q)SAR Database contains around 650000 substances, there may still be substances of interest to some users which are not contained in the database. Or even when contained, there may in some cases be interest to obtain more detailed predictions in the QSAR Prediction Reporting Format, which includes information on prediction probability, possible alerts, nearest analogs in the training set etc. The Danish (Q)SAR Models website offers users to make on-the-fly predictions for user-defined chemical structures by use of >30 models developed by DTU in the Leadscope software. The use of the commercial Leadscope® Enterprise Server software as a back-end to this website was made possible based on a collaboration agreement between Leadscope Inc. and the National Food Institute at the Technical University of Denmark.
- Further information predicting the potential for eugenol to demonstrate estrogenic and androgenic activity was sourced from the United States Environmental Protection Agency (US EPA) ToxCast modelling data presented in the CompTox Dashboard.

  The ToxCast Model tab in the CompTox Dashboard includes predictions of the estrogen receptor activity of eugenol, based on the Collaborative Estrogen Receptor Activity Prediction Project (CERAPP; Mansouri *et al.*, 2016[5]). The CERAPP is a large-scale modelling project which has investigated the efficacy of using predictive computational models trained on high-throughput screening data (e.g. from the EDSP21 initiative) to evaluate the ER -related activity of thousands of chemicals, and identify priorities for further testing.

  The ToxCast Model tab in the CompTox Dashboard includes predictions of the androgen receptor activity of eugenol based on the COMPARA project. COMPARA is a large-scale collaboration between 35 international groups using QSAR models to predict androgen receptor activity using a common training set of 1746 compounds provided by the US EPA. The result is a consensus model of AR agonist activity that is run against the DSSTox chemical library, and aims to identify priorities for further testing.

[5] Mansouri K, Abdelaziz A, Rybacka A, Roncaglioni A, Tropsha A, Varnek A, Zakharov A, Worth A, Richard AM, Grulke CM, Trisciuzzi D, Fourches D, Horvath D, Benfenati E, Muratov E, Wedebye EB, Grisoni F, Mangiatordi GF, Incisivo GM, Hong H, Ng HW, Tetko IV, Balabin I, Kancherla J, Shen J, Burton J, Nicklaus M, Cassotti M, Nikolov NG, Nicolotti O, Andersson PL, Zang Q, Politi R, Beger RD, Todeschini R, Huang R, Farag S, Rosenberg SA, Slavov S, Hu X, Judson RS. CERAPP: Collaborative Estrogen Receptor Activity Prediction Project. Environ Health Perspect. 2016 Jul;124(7):1023-33.

The results of the QSAR analysis and predictive computational models are presented and discussed on sections 2.10.2.1 and 2.10.2.2. This information is not included in the ED Excel spreadsheet since *in vitro* an *in vivo* Level 2, 4 and 5 data are available.

2.10.1.2 In vitro mechanistic data – US EPA CompTox Chemicals Dashboard

In accordance with the OECD Conceptual Framework and the ECHA/EFSA GD on ED, available Level 2 data was gathered for eugenol. Level 2 data includes *in vitro* assays that provide data on selected endocrine mechanisms and pathways.

In vitro mechanistic data for eugenol was sourced from the US EPA Endocrine Disruptors Screening Program (EDSP). The EPA's EDSP is designed to detect the intrinsic ability of chemicals to interact with the endocrine system, specifically for chemicals that can interact with the estrogen, androgen or thyroid pathways. The US EPA initially developed high throughput data for approximately 1800 chemicals by using a series of 700 high throughput screens. These data were available for certain chemicals on the US EPA Aggregated Computational Toxicology Resource (ACToR) website. To further refine the activity specifically related to the EDSP Program, and to aid in screening the large number of chemicals subject to regulation in the US, the EPA developed the EDSP21 Dashboard to provide access to new chemical data on over 1800 chemicals of interest, many of which are the same chemicals evaluated through the ToxCast program. In August 2019, the EDPS21 and ToxCast Dashboards have been discontinued, and the updated the EDSP21, ToxCast/Tox21 bioassay data has been updated and integrated into US EPA CompTox Chemicals Dashboard Version 4. The bioassay data can be accessed at https://comptox.epa.gov/dashboard and https://www.epa.gov/chemical-research/toxicity-forecaster-toxcasttm-data. Published ER (PMID 26272952) and AR (PMID 27933809) model results are available for citation. ED bioassay data can be accessed using the CompTox Chemicals Dashboard via a bioactivity tabs: EDSP21 and TOXCAST/TOX21.

The data from the EDSP21 and ToxCast/Tox21 tabs provide information regarding the biological activity of a chemical in an *in vitro* system. The results summarised below (data also included in the ED Excel spreadsheet in appendix E) indicate whether a chemical has the potential to interact with a particular signalling cascade, and do not indicate with certainty whether a substance would cause adverse effects via that signalling pathway in experimental animals and/or humans.

2.10.1.3 Summary of all studies considered for the assessment of endocrine disrupting properties of eugenol

Study type (results, source)		**Reference**	**Study ID Matrix**
***In vitro* ToxCast mechanistic - OECD framework Level 2**			
Thyroid			
ATG_THRa1_TRANS_up	Toxcast T-Bioactivity Model		1
ATG_THRa1_TRANS_dn	Toxcast T-Bioactivity Model		2
LTEA_HepaRG_THRSP_dn	Toxcast T-Bioactivity Model		3
LTEA_HepaRG_THRSP_up	Toxcast T-Bioactivity Model		4
NCCT_TPO_AUR_dn	Toxcast T-Bioactivity Model		5
NCCT_HEK293T_CellTiterGLO	Toxcast T-Bioactivity Model		6

Study type (results, source)		Reference	Study ID Matrix
NCCT_QuantiLum_inhib_2_dn	Toxcast T-Bioactivity Model		7
Tox21_TSHR_Agonist_ratio	Toxcast T-Bioactivity Model		8
Tox21_TSHR_Antagonist ratio	Toxcast T-Bioactivity Model		9
Tox21_TSHR_wt_ratio	Toxcast T-Bioactivity Model		10
Tox21_TR_LUC_GH3_Agonist	Toxcast T-Bioactivity Model		11
Tox21_TR_LUC_GH3_Antagonist	Toxcast T-Bioactivity Model		12
Tox21_TR_LUC_GH3_Antagonist_viability	Toxcast T-Bioactivity Model		13
Estrogen			
ACEA_ER_80hr	Toxcast ER Bioactivity Model		14
ACEA_ER_AUC_viability	Toxcast ER Bioactivity Model		15
ATG_ERa_TRANS_up	Toxcast ER Bioactivity Model		16
ATG_ERE_CIS_up	Toxcast ER Bioactivity Model		17
NVS_NR_hER	Toxcast ER Bioactivity Model		18
OT_ER_ERaERa_0480	Toxcast ER Bioactivity Model		19
OT_ER_ERaERa_1440	Toxcast ER Bioactivity Model		20
OT_ER_ERaERb_0480	Toxcast ER Bioactivity Model		21
OT_ER_ERaERb_1440	Toxcast ER Bioactivity Model		22
OT_ER_ERbERb_0480	Toxcast ER Bioactivity Model		23
OT_ER_ERbERb_1440	Toxcast ER Bioactivity Model		24

Study type (results, source)		Reference	Study ID Matrix
OT_ERa_EREGFP_0120	Toxcast ER Bioactivity Model		25
OT_ERa_EREGFP_0480	Toxcast ER Bioactivity Model		26
TOX21_ERa_BLA_Agonist_ratio	Toxcast ER Bioactivity Model		27
TOX21_ERa_BLA_Antagonist_ratio	Toxcast ER Bioactivity Model		28
TOX21_ERa_BLA_Antagonist_viability	Toxcast ER Bioactivity Model		29
TOX21_ERa_LUC_VM7_Agonist	Toxcast ER Bioactivity Model		30
TOX21_ERa_LUC_VM7_Antagonist_specificity	Toxcast ER Bioactivity Model		31
TOX21_ERa_LUC_VM7_Antagonist_specificity_viability	Toxcast ER Bioactivity Model		32
Androgen			
ATG_AR_TRANS_up	Toxcast AR Bioactivity Model		33
NVS_NR_cAR	Toxcast AR Bioactivity Model		34
OT_AR_ARELUC_AG_1440	Toxcast AR Bioactivity Model		35
OT_AR_ARSRC1_0480	Toxcast AR Bioactivity Model		36
OT_AR_ARSRC1_0960	Toxcast AR Bioactivity Model		37
TOX21_AR_BLA_Agonist_ratio	Toxcast AR Bioactivity Model		38
TOX21_AR_BLA_Antagonist_ratio	Toxcast AR Bioactivity Model		39
TOX21_AR_BLA_Antagonist_viability	Toxcast AR Bioactivity Model		40
TOX21_AR_LUC_MDAKB2_Agonist	Toxcast AR Bioactivity Model		41
TOX21_AR_LUC_MDAKB2_Antagonist	Toxcast AR Bioactivity Model		42

Study type (results, source)		Reference	Study ID Matrix
TOX21_AR_LUC_MDAKB2_Antagonist_viability	Toxcast AR Bioactivity Model		43
TOX21_AR_LUC_MDAKB2_Antagonist_Specificity	Toxcast AR Bioactivity Model		44
TOX21_AR_LUC_MDAKB2_Antagonist_Specificity_viability	Toxcast AR Bioactivity Model		45
UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Agonist	Toxcast AR Bioactivity Model		46
UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Antagonist	Toxcast AR Bioactivity Model		47
Steroidogenesis			
CEETOX_H295R_11DCORT_dn	Toxcast S-Bioactivity Model		48
CEETOX_H295R_11DCORT_up	Toxcast S-Bioactivity Model		49
CEETOX_H295R_OHPREG_dn	Toxcast S-Bioactivity Model		50
CEETOX_H295R_OHPREG_up	Toxcast S-Bioactivity Model		51
CEETOX_H295R_OHPROG_dn	Toxcast S-Bioactivity Model		52
CEETOX_H295R_OHPROG_up	Toxcast S-Bioactivity Model		53
CEETOX_H295R_ANDR_dn	Toxcast S-Bioactivity Model		54
CEETOX_H295R_ANDR_up	Toxcast S-Bioactivity Model		55
CEETOX_H295R_CORTIC_dn	Toxcast S-Bioactivity Model		56
CEETOX_H295R_CORTIC_up	Toxcast S-Bioactivity Model		57
CEETOX_H295R_CORTISOL_dn	Toxcast S-Bioactivity Model		58
CEETOX_H295R_CORTISOL_up	Toxcast S-Bioactivity Model		59
CEETOX_H295R_DOC_dn	Toxcast S-Bioactivity Model		60

Study type (results, source)		Reference	Study ID Matrix
CEETOX_H295R_DOC_up	Toxcast S-Bioactivity Model		61
CEETOX_H295R_ESTRADIOL_dn	Toxcast S-Bioactivity Model		62
CEETOX_H295R_ESTRADIOL_up	Toxcast S-Bioactivity Model		63
CEETOX_H295R_ESTRONE_dn	Toxcast S-Bioactivity Model		64
CEETOX_H295R_ESTRONE_up	Toxcast S-Bioactivity Model		65
CEETOX_H295R_PROG_dn	Toxcast S-Bioactivity Model		66
CEETOX_H295R_PROG_up	Toxcast S-Bioactivity Model		67
CEETOX_H295R_TESTO_dn	Toxcast S-Bioactivity Model		68
CEETOX_H295R_TESTO_up	Toxcast S-Bioactivity Model		69
TOX21_Aromatase_Inhibition	Toxcast S-Bioactivity Model		70
CEETOX_H295R_MTT_cell_viability_up	Toxcast S-Bioactivity Model		71
CEETOX_H295R_MTT_cell_viability_dn	Toxcast S-Bioactivity Model		72
TOX21_Aromatase_inhibition_viability	Toxcast S-Bioactivity Model		73
In vitro mechanistic study - OECD framework Level 2			
Estrogen and androgen receptor binding screening, Howes *et al.* (2002)		B.6.8.3.5	74
Mammalian toxicologic studies			
Other studies			
Antifertility effect of eugenol in rats, Poli *et al.* (2019)		B.6.8.3.6	75
Short-term studies - OECD framework Level 4			
14-day range-finding studies on rats, NTP (1983)		B.6.3.1.1	76
14-day range-finding studies on mice, NTP (1983)		B.6.3.1.2	77
Subacute oral study in dog, Lauber *et al*. (1950)		B.6.2.1.5 B.6.3.1.3	78
28-day study on rats (Sipes 2006)		B.6.3.2.1	79
34-day study on rats (Sipes 2006)		B.6.3.3.1	80
Repeated dose toxicity studies - OECD framework Level 4			

Study type (results, source)	Reference	Study ID Matrix
13-week range-finding study on rats, NTP (1983)	B.6.3.4.1	81
13-week range-finding study on mice, NTP (1983)	B.6.3.4.2	82
19-week range-finding study on rats, Hagan *et al.* (1967)	B.6.3.5.1	83
Long term study on rats, NTP (1983)	B.6.5.1	84
Long term study on mice, NTP (1983)	B.6.5.2	85
12-month (6 month recovery) carcinogenicity study in rodents (mice), Miller, 1983	B.6.5.3	86
Developmental studies - OECD framework Level 4		
Pre-natal developmental oral study in rats, Morgareidge (1973)	B.6.6.2.1	87
Pre-natal developmental oral study in rabbits, Morgareidge (1973)	B.6.6.2.2	88
Pre-natal developmental oral study in mice, Morgareidge (1973)	B.6.6.2.3	89
Pre-natal developmental oral study in hamsters, Morgareidge (1973)	B.6.6.2.4	90
Pre-natal developmental oral study in rats, [redacted] (2004)	B.6.6.2.5	91
Pre-natal developmental oral study in rabbits, [redacted] (2004)	B.6.6.2.6	92

2.10.2 ED assessment for humans

2.10.2.1 ED assessment for T-modality

2.10.2.1.1 Analysis of non-experimental data

In accordance with the OECD Conceptual Framework and the ECHA/EFSA GD on ED, Level 1, T-related non-test information was gathered for eugenol. Qualitative structural activity relationship (QSAR) data was obtained for eugenol from the Danish QSAR database.

The results of the Danish QSAR database for eugenol in respect of T-mediated endocrine endpoints are summarised below.

Table 2.10.2.1.1: The results of Danish QSAR database for eugenol regarding T-modality

	Exp	Battery	CASE Ultra	Leadscope	SciQSAR
Thyroperoxidase (TPO) inhibition QSAR1 (Rat in vitro)	POS	NA	NA	POS_IN	NA
Thyroperoxidase (TPO) inhibition QSAR2 (Rat in vitro)	POS	NA	NA	POS_IN	NA
Thyroid Receptor α Binding (Human in vitro)					
mg/L			110.7643	208.6025	100.1151
µM			674.528	1270.34	609.6722
Positive for IC50 ≤ 10 µM					
Positive for IC50 ≤ 100 µM					
Domain		OUT	OUT	OUT	OUT
Thyroid Receptor β Binding (Human in vitro)					
mg/L		2843.621	5313.734	13.48465	373.5077
µM		17316.98	32359.38	82.11832	2274.573
Positive for IC50 ≤ 10 µM					
Positive for IC50 ≤ 100 µM					
Domain		IN	IN	OUT	OUT

Key POS = Positive; NEG = Negative; IN = within the applicability domain of the model; OUT = outside of the applicability domain of the model

The Danish QSAR Database reports that eugenol lacks the potential to interact with the thyroid receptor.

The Danish QSAR Database reports that eugenol gave positive results in respect of thyroid peroxidase (TPO) inhibition in two respective Leadscope global, binary composite QSAR models: QSAR1 and QSAR2 (and was within the applicability domain of these models). These models were developed to predict the potential of substances to inhibit thyroperoxidase using the US EPA ToxCast data sets (training set for QSAR1 = 877 chemicals; training set for QSAR2 = 1519 chemicals) and the commercial software Leadscope® Predictive Data Miner. The highest ranking structural features associated with activity were versions of phenols, anisole and aniline whereas the most frequent structural features associated with inactivity included ethers, esters, aryl halides and a tertiary amine [6]. The structure of eugenol includes both a phenol and an anisole group, and the substance therefore has the features associated with TPO inhibition activity. Moreover, positive results were obtained in the experimental training data set (from EpiSuite experimental databases or DK DTU QSAR models training sets).

Overall, OECD Conceptual Framework Level 1 (non-experimental) data for eugenol indicated that the substance has the potential to inhibit thyroperoxidase.

2.10.2.1.2 US EPA CompTox Chemicals Dashboard

The EDSP1 tab in the CompTox Chemicals Dashboard v4 includes 13 thyroid bioassays for eugenol. These assays are summarized in the table below and have been included in the ED Excel spreadsheet (Study ID matrix nos.: 1-13). Negative results were obtained in 12 of them. A positive result was obtained for eugenol in the thyroid peroxidase assay NCCT_TPO_AUR_dn; Study ID Matrix No. 5). The AC_{50} value was 5.57 µM (Hill Model). The limit of cytotoxicity for this assay was reported to be 1000 µM.

Table 2.10.1.2.2: Summary of 13 EDSP21 endocrine activity screening assays included in the ToxCast Thyroid Bioactivity Model

Assay endpoint	Assay type	Organism	Result	Study ID Matrix
ATG_THRa1_TRANS_up	mRNA induction	human	Inactive	1
ATG_THRa1_TRANS_dn	mRNA induction	human	Inactive	2
LTEA_HepaRG_THRSP_dn	mRNA induction	human	inactive	3
LTEA_HepaRG_THRSP_up	mRNA induction	human	inactive	4
NCCT_TPO_AUR_dn	**Thyroid peroxidase activity**	**rat**	**Active**	**5**
NCCT_HEK293T_CellTiterGLO	ATP content	human	Inactive	6
NCCT_QuantiLum_inhib_2_dn	Enzyme (monooxygenase) activity	*E. coli.*	Inactive	7
Tox21_TSHR_Agonist_ratio	cAMP measurement	human	Inactive	8
Tox21_TSHR_Antagonist ratio	cAMP measurement	human	Inactive	9
Tox21_TSHR_wt_ratio	cAMP measurement	human	Inactive	10
Tox21_TR_LUC_GH3_Agonist	Luciferase induction	Rat	Inactive	11
Tox21_TR_LUC_GH3_Antagonist	Luciferase induction	Rat	Inactive	12
Tox21_TR_LUC_GH3_Antagonist_viability	ATP content	Rat	Inactive	13

2.10.2.1.3 Have T-mediated parameters been sufficiently investigated?

The available dataset of *in vivo* mammalian toxicology studies for eugenol consists of short-term studies (14, 28 and 34 days) conducted in rodents, repeated dose study in dogs (21 day), sub-chronic studies (90 days and 19 weeks) conducted in rodents, carcinogenicity studies conducted in rodents and prenatal developmental toxicology studies conducted in rats and rabbits. However, much of the available data pre-dates revisions that were made to the OECD Test Guidelines to include EATS-mediated parameters. Moreover, no OECD TG 416 or 443 studies has been provided with the active substance eugenol.

Table 14 of the ECHA/EFSA GD on ED provides a list of T-mediated parameters that should be investigated in the OECD CF Level 4 and 5 *in vivo* OECD TG compliant mammalian toxicology studies. Using the currently available

[6] Rosenberg SA, Watt ED, Judson RS, Simmons SO, Paul Friedman K, Dybdahl M, Nikolov NG, Wedebye EB. QSAR models for thyroperoxidase inhibition and screening of U.S. and EU chemical inventories. *Computational Toxicology*. 2017, Vol 4,pp 11-21. https://doi.org/10.1016/j.comtox.2017.07.006.

set of toxicological data for eugenol, the Table 2.10.2.1.3/1 summarises the available information on T-mediated parameters.

Table 2.10.2.1.3/1: Summary of T-mediated parameters investigated in mammalian toxicology studies.

T-mediated parameters	OECD Test guideline	**Sufficiently investigated?** **Overall conclusion: No (not sufficiently investigated)** Based on the lack of TG 416 and/or 443 studies, and the absence of relevant *in vivo*-mechanistic and T-mediated parameters data such as thyroid-hormones measurements (T3/T4/TSH), colloid area histopathology, follicular cell height and thyroid weights.
T3/T4 levels	407 (optional), 408, 414, 421, 422, 443	Measurement of a single hormone on its own, without complementary parameters such as TSH, thyroid weight, histopathology of thyroid and pituitary, should not be used to draw conclusion regarding changes in the hypothalamus-pituitary-thyroid axis, but raises a concern for effects on the thyroid hormone system. T3 and/or T4 levels were not measured in sub-chronic toxicity and developmental toxicity studies provided. There are no OECD TG, 421, 422 and 443 studies available.
Thyroid-stimulating hormone level (TSH)	407 (optional), 408, 414, 421, 422, 443	Measurement of a single hormone on its own, without complementary parameters such as T3/T4, thyroid weight, histopathology of thyroid and pituitary, should not be used to draw conclusion regarding changes in the hypothalamus-pituitary-thyroid axis, but raises a concern for effects on the thyroid hormone system. TSH levels were not measured in sub-chronic toxicity and developmental toxicity studies provided. There are no OECD TG, 421, 422 and 443 studies available.
Colloid area (thyroid histopathology)	407, 422 (optional)	Studies conducted according to the recent versions of these guidelines are not available for eugenol. No effects on colloid area have been reported in the studies in which thyroid histopathology has been performed.
Follicular cell height (thyroid histopathology)	407, 422, 416	Studies conducted according to the recent versions of these guidelines are not available for eugenol. No effects on follicular cell height have been reported in the studies in which thyroid histopathology has been performed.
HDL/LDL ratio	408	This parameter is considered to be T-mediated only when a change is observed in other T-mediated parameters. There are two 90 day repeated dose toxicity studies available for eugenol conducted in rats and in mice respectively. These studies were performed as dose-range finding studies for 2 year carcinogenicity studies and do not conform to the OECD TG 408. The ratio of high density lipoprotein to low density lipoprotein is not investigated in these studies.
Liver weight	407, 408, 422, 451-3, 416, 443	This parameter is considered to be T-mediated only when a change is observed in other T-mediated parameters. Available sub-chronic 28 days, 34 days and 19 weeks toxicity studies have investigated the effects of eugenol on liver weights. However, no liver weights were recorded in the 90-day and 2-year carcinogenicity studies with eugenol. There are no studies conducted according to OECD TG 407, 416 or 443.
Thyroid histopathology	407, 408, 414, 421 (optional), 422 (optional), 451-3, 416 (optional), 443.	Thyroid histopathology has been examined in 90 days and 2 year carcinogenicity studies in rats and mice. Thyroid histopathology was not reported in two respective prenatal developmental toxicity studies conducted in rats and rabbits. There are no OECD TG 407 or 443 studies available for eugenol.

Thyroid weight	407 (optional), 408, 414, 421 (optional), 422 (optional), 451-3, 416, 443.	Thyroid weights were not reported in any of the available studies. There are no studies conducted according to OECD TG 416 or 443.

Circulating levels of T3, T4 and TSH thyroid hormones were not reported in sub-chronic toxicity and developmental toxicity studies provided.

Thyroid histopathology has been examined in 2 years carcinogenicity studies in rats and mice (ID: 84 and 85). These studies evaluated the effects of chronic exposure to eugenol on the incidence of thyroid C-Cell adenomas and carcinomas, follicular cell adenomas and carcinomas. On the other hand, thyroid histological investigations were not reported in two respective prenatal developmental toxicity studies conducted in rats and rabbits (ID:91 and 92), or in sub-chronic toxicity studies (ID: 81, 82 and 83). No OECD TG 416 and OECD TG 443 studies are available with the active substance eugenol.

Regarding the two parameters that are considered to be T-mediated (*as per* OECD150 and EFSA ED guideline) when other changes in T-mediated parameters are observed: HDL/LDL measurements are not reported in the available studies.

Moreover, there is no information on the effects of eugenol on thyroid weight, colloid area or follicular cell height in the available studies of the dossier.

Therefore, based on the lack of data on some relevant T-*in vivo* mechanistic and T-mediated parameters (e. g.: T3/T4/TSH measurements, thyroid weight colloid area histopathology and follicular cell height), it is concluded that T-mediated parameters have not been sufficiently investigated.

Table 2.10.2.1.3/2: T-mediated parameters not measured

OECD TG 407 - T-mediated parameters not investigated	
-Colloid area (thyroid histopathology) -Follicular cell height (thyroid histopathology) -Thyroid histopathology	
OECD TG 408 - T-mediated parameters not investigated	
- Thyroid weight - Liver weight - T3 and/or T4 level - Thyroid stimulating hormone level (TSH)	- Low-density lipoproteins (LDL) - High-density lipoproteins (HDL) -Thyroid histopathology
OECD TG 453 - T-mediated parameters not investigated	
-Liver weight -Thyroid weight	
OECD TG 414 - T-mediated parameters not investigated	
- Thyroid weight - Thyroid histopathology	- T3 and/or T4 level - Thyroid stimulating hormone level (TSH)

2.10.2.1.4 Lines of evidence for adverse effects and endocrine activity related to T-modality.

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
In vitro mechanistic	7	oxidoreductase_monooxygenase	E. Coli	0.5 hours	Uptake from the medium (in vitro)		No effect	No effect	There was a positive result in TPO activity assay. This is in agreement with QSAR1 and QSAR2 models (Danish QSAR Database)	Activity in TPO was described in one ToxCast model. There were no T3, T4 and TSH levels measurements in the overall dataset.	T
	3	thyroid hormone responsive	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	4	thyroid hormone responsive	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	1	Thyroid receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	2	Thyroid receptor	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	8	Thyroid receptor	Human	0.5 hours	Uptake from the medium (in vitro)		No effect	No effect			
	9	Thyroid receptor	Human	0.5 hours	Uptake from the medium (in vitro)		No effect	No effect			
	10	Thyroid receptor	Human	0.5 hours	Uptake from the medium (in vitro)		No effect	No effect			
	6	ATP content_cell cycle_cell death	Human	24 hours	Uptake from the		No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
					medium (in vitro)						
	11	Thyroid receptor	rat	28 hours	Uptake from the medium (in vitro)		No effect	No effect			
	12	Thyroid receptor	rat	28 hours	Uptake from the medium (in vitro)		No effect	No effect			
	13	Thyroid receptor	rat	28 hours	Uptake from the medium (in vitro)		No effect	No effect			
	5	Thyroperoxidase activity (TPO) (in vitro)	rat	0.5 hours	Uptake from the medium (in vitro)	5.57 µM	Change	Eugenol is active for thyroid peroxidase inhibition (TPO) assay. AC50 (hill model)= 5.57			
In vivo mechanistic	78	T3 and T4 level	Dog	21 day	Oral		Not measured	Not measured	Parameters not measured.		
	81	T3 and T4 level	Rat	13 week	Oral		Not measured	Not measured			
	82	T3 and T4 level	Mouse	13 week	Oral		Not measured	Not measured			
	87	T3 and T4 level	rat	10 day	Oral		Not measured	Not measured			
	88	T3 and T4 level	rabbit	13 day	Oral		Not measured	Not measured			
	89	T3 and T4 level	mouse	11 day	Oral		Not measured	Not measured			
	90	T3 and T4 level	hamster	10 day	Oral		Not measured	Not measured			
	91	T3 and T4 level	Rat	15 day	Oral		Not measured	Not measured			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	91	T3 and T4 level	Rat	15 day	Oral		Not measured	Not measured			
	92	T3 and T4 level	Rabbit	24 day	Oral		Not measured	Not measured			
	92	T3 and T4 level	Rabbit	24 day	Oral		Not measured	Not measured			
	81	Thyroid-stimulating hormone level (TSH)	Rat	13 week	Oral		Not measured	Not measured			
	82	Thyroid-stimulating hormone level (TSH)	Mouse	13 week	Oral		Not measured	Not measured			
	87	Thyroid-stimulating hormone level (TSH)	rat	10 day	Oral		Not measured	Not measured			
	88	Thyroid-stimulating hormone level (TSH)	rabbit	13 day	Oral		Not measured	Not measured			
	89	Thyroid-stimulating hormone level (TSH)	mouse	11 day	Oral		Not measured	Not measured			
	90	Thyroid-stimulating hormone level (TSH)	hamster	10 day	Oral		Not measured	Not measured			
	91	Thyroid-stimulating hormone level (TSH)	Rat	15 day	Oral		Not measured	Not measured			
	91	Thyroid-stimulating hormone level (TSH)	Rat	15 day	Oral		Not measured	Not measured			
	92	Thyroid-stimulating hormone level (TSH)	Rabbit	24 day	Oral		Not measured	Not measured			
	92	Thyroid-stimulating hormone level (TSH)	Rabbit	24 day	Oral		Not measured	Not measured			
T-mediated	79	Colloid area (thyroid histopathology)	Rat	28 day	Oral		Not measured	Not measured	Parameters not measured.	Colloid area, follicular cell height and HDL/LDL ratio were not measured. Data obtained from thyroid histopathology and liver weight did not suggest	
	80	Colloid area (thyroid histopathology)	Rat	34 day	Oral		Not measured	Not measured			
	79	Follicular cell height (thyroid histopathology)	Rat	28 day	Oral		Not measured	Not measured			
	80	Follicular cell height (thyroid histopathology)	Rat	34 day	Oral		Not measured	Not measured			
	81	HDL/LDL ratio (considered T-mediated only in combination with other thyroid endpoints)	Rat	13 week	Oral		Not measured	Not measured			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	82	HDL/LDL ratio (considered T-mediated only in combination with other thyroid endpoints)	Mouse	13 week	Oral		Not measured	Not measured		evidence of adversity.	
	78	Thyroid histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	Slight increase of follicular cell adenomas were observed in male mice. This finding was not significant and the incidence was low. On the other hand, the incidence of C-Cell adenoma in female rats was not dose-related. Overall, although some effects have been observed related to thyroid it is regarded that they have no enough evidence of adversity.		
	79	Thyroid histopathology	Rat	28 day	Oral		Not measured	Not measured			
	80	Thyroid histopathology	Rat	34 day	Oral		Not measured	Not measured			
	81	Thyroid histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Thyroid histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Thyroid histopathology	Rat	2 year	Oral	6000 ppm	Decrease	The incidence of C-Cell adenomas of the thyroid gland were decreased compared to controls in the high dose male group.			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Thyroid histopathology	Rat	2 year	Oral	>12500 ppm	No effect	The incidence of C-Cell adenomas of the thyroid gland was increased in female rats treated at the low dose of 6000 ppm. No increases were observed at the high dose of 12500 ppm. No dose-response relationship was observed.			
	85	Thyroid histopathology	Mouse	2 year	Oral	6000 ppm	Increase	Follicular cell adenomas incidence of the thyroid gland was increased in the high dose male group (6000 ppm). A statistically significant trend was observed, however, the incidence in top dose male group (6%) was not statistically significant compared with controls and was <10%.			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	85	Thyroid histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	87	Thyroid histopathology	rat	10 day	Oral			No effect			
	88	Thyroid histopathology	rabbit	13 day	Oral		Not measured	Not measured			
	89	Thyroid histopathology	mouse	11 day	Oral		Not measured	Not measured			
	90	Thyroid histopathology	hamster	10 day	Oral		Not measured	Not measured			
	91	Thyroid histopathology	Rat	15 day	Oral		Not measured	Not measured			
	91	Thyroid histopathology	Rat	15 day	Oral		Not measured	Not measured			
	92	Thyroid histopathology	Rabbit	24 day	Oral		Not measured	Not measured			
	92	Thyroid histopathology	Rabbit	24 day	Oral		Not measured	Not measured			
	79	Thyroid weight	Rat	28 day	Oral		Not measured	Not measured			
	80	Thyroid weight	Rat	34 day	Oral		Not measured	Not measured			
	81	Thyroid weight	Rat	13 week	Oral		Not measured	Not measured			
	82	Thyroid weight	Mouse	13 week	Oral		Not measured	Not measured			
	84	Thyroid weight	Rat	2 year	Oral		Not measured	Not measured			
	85	Thyroid weight	Mouse	2 year	Oral		Not measured	Not measured			
	87	Thyroid weight	rat	10 day	Oral		Not measured	Not measured			
	88	Thyroid weight	rabbit	13 day	Oral		Not measured	Not measured			
	89	Thyroid weight	mouse	11 day	Oral		Not measured	Not measured			
	90	Thyroid weight	hamster	10 day	Oral		Not measured	Not measured			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	91	Thyroid weight	Rat	15 day	Oral		Not measured	Not measured			
	91	Thyroid weight	Rat	15 day	Oral		Not measured	Not measured			
	92	Thyroid weight	Rabbit	24 day	Oral		Not measured	Not measured			
	92	Thyroid weight	Rabbit	24 day	Oral		Not measured	Not measured			
	92	Thyroid weight	Rabbit	24 day	Oral		Not measured	Not measured			
	79	Liver weight	Rat	28 day	Oral		No effect	No effect	No adverse effects on liver weight were found.		
	80	Liver weight	Rat	34 day	Oral		Change	The evaluation document reported that livers were weighted. Slight enlargement of the liver was observed in treated rats which was found microscopically to be due to slight liver cell enlargement. The doses in which effects were observed had not been specified in the study.			
	81	Liver weight	Rat	13 week	Oral		Not measured	Not measured			
	82	Liver weight	Mouse	13 week	Oral		Not measured	Not measured			
	83	Liver weight	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	84	Liver weight	Rat	2 year	Oral		Not measured	Not measured			
	85	Liver weight	Mouse	2 year	Oral		Not measured	Not measured			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
Sensitive to, but not diagnostic of, EATS	78	Adrenals histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	There was a slight enlargement of adrenals gland in rats after 34-day eugenol treatment. No other changes were reported in adrenals gland in other short-term or long-term toxicity studies with eugenol. This finding was considered equivocal.	No adverse effects were detected in sensitive EATS-related parameters.	N
	80	Adrenals histopathology	Rat	34 day	Oral		Change	The evaluation document reported that the slight enlargement of the adrenal glands was observed in treated rats with marked yellow discolouration. The doses in which effects were observed had not been specified in the study.			
	81	Adrenals histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Adrenals histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Adrenals histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Adrenals histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	81	Adrenals weight	Rat	13 week	Oral		Not measured	Not measured			
	82	Adrenals weight	Mouse	13 week	Oral		Not measured	Not measured			
	84	Adrenals weight	Rat	2 year	Oral		Not measured	Not measured			
	85	Adrenals weight	Mouse	2 year	Oral		Not measured	Not measured			
	81	Brain histopathology examination	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed		

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	82	Brain histopathology examination	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Brain histopathology examination	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Brain histopathology examination	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	81	Brain weight	Rat	13 week	Oral		Not measured	Not measured			
	82	Brain weight	Mouse	13 week	Oral		Not measured	Not measured			
	84	Brain weight	Rat	2 year	Oral		Not measured	Not measured			
	85	Brain weight	Mouse	2 year	Oral		Not measured	Not measured			
	87	Fetal development	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect	No treatment-related effects were observed on foetal development.		
	88	Fetal development	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Fetal development	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Fetal development	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	91	Fetal development	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Fetal development	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	91	Gestation length	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Gestation length	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	91	Litter size	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Litter size	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	87	Litter viability	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Litter viability	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Litter viability	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Litter viability	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	91	Litter viability	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Litter viability	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	87	Litter/pup weight	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	88	Litter/pup weight	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Litter/pup weight	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Litter/pup weight	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	91	Litter/pup weight	Rat	15 day	Oral	600 mg/kg bw/day	Decrease	The mean foetal weights in litters of rats treated at 600 mg/kg bw/day were slightly but significantly reduced (↓6%), but was not associated with any significant delay in skeletal development.			
	92	Litter/pup weight	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	87	Number of implantations, corpora lutea	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Number of implantations, corpora lutea	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Number of implantations, corpora lutea	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	90	Number of implantations, corpora lutea	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	91	Number of implantations, corpora lutea	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Number of implantations, corpora lutea	Rabbit	24 day	Oral	500 (350) mg/kg bw/day	No effect	No effect			
	87	Number of live births	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Number of live births	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Number of live births	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Number of live births	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	87	Numbers of embryonic or foetal deaths and viable foetuses	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Numbers of embryonic or foetal deaths and viable foetuses	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Numbers of embryonic or foetal deaths and viable foetuses	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Numbers of embryonic or foetal deaths and viable foetuses	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	92	Numbers of embryonic or foetal deaths and viable foetuses	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	91	Post implantation loss	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Post implantation loss	Rabbit	24 day	Oral	500 (350) mg/kg bw/day	No effect	No effect			
	91	Pre implantation loss	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Pre implantation loss	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	87	Sex ratio	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Sex ratio	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Sex ratio	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Sex ratio	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	91	Sex ratio	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Sex ratio	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	78	Pituitary histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	No treatment-related effects were observed.		
	81	Pituitary histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	82	Pituitary histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Pituitary histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Pituitary histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Pituitary histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Pituitary histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Pituitary weight	Rat	13 week	Oral		Not measured	Not measured			
	82	Pituitary weight	Mouse	13 week	Oral		Not measured	Not measured			
	84	Pituitary weight	Rat	2 year	Oral		Not measured	Not measured			
	85	Pituitary weight	Mouse	2 year	Oral		Not measured	Not measured			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	87	Presence of anomalies (external, visceral, skeletal)	rat	10 day	Oral	13 mg/kg bw/day	Increase	Increased incidences regarding ossification of ribs (mostly wavy; non-clearly dose-related) were described in all clove-oil treated groups. Increased incidences of incomplete closure of skull were recorded in 13 (36%), 60 (82%) and 280 (100%) mg/kg bw/day dose groups. No statistical analysis performed.	Developmental toxicity studies were performed in rats, rabbits, mice and hamsters with clove oil. Content of eugenol in clove oil was not presented. Moreover, statistical analysis were not performed in the skeletal abnormalities analysis, and HCD was not provided. Overall, the results were difficult to interpret.		
	88	Presence of anomalies (external, visceral, skeletal)	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Presence of anomalies (external, visceral, skeletal)	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	90	Presence of anomalies (external, visceral, skeletal)	hamster	10 day	Oral	177 mg/kg bw/day	Increase	Increased non clear dose-related ossification incidences in the sternebrae were recorded in the clove oil-treated groups (20%, 27%, 40% and 31% for 1.8, 8.2, 38.2 and 177 mg/kg bw/day dose groups, respectively). Moreover, increased incomplete ossification incidences of the extremities were described in the low and top dose groups (24 and 80%, respectively), whereas hyoid reduced or missing incidences were noted only in the top dose group (48%). No statistical analysis performed.			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	91	Presence of anomalies (external, visceral, skeletal)	Rat	15 day	Oral	600 mg/kg bw/day	Decrease	A statistically significant reduction in the percentage of foetuses with more than 6 ossified metatarsals occurred in litters of rats treated at 600 mg/kg bw/day. This finding was seen in isolation of any other effects on skeletal development.	Reduction in the percentage of foetuses with more than 6 ossified metatarsals was noted in foetuses from high dose treated dams in the rat study.		
	92	Presence of anomalies (external, visceral, skeletal)	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
Target organ toxicity	81	Bone marrow histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.	The main effects were detected in liver of female mouse in form of hepatocellular adenomas/ carcinomas, however, these incidences were within the HCD. Single observation of focal inflammation were noted in kidney and lung, however,	
	82	Bone marrow histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Bone marrow histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Bone marrow histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	80	Heart histopathology	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect	No treatment-related effects were observed.		

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	81	Heart histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect		these incidences were not reproduced in other toxicity studies.	
	82	Heart histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	83	Heart histopathology	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	84	Heart histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Heart histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	80	Heart weight	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	83	Heart weight	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	78	Kidney histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	Increased incidences of focal kidney inflammation were detected in 2-year mice study. These findings were not recorded in other repeated-dose studies		
	80	Kidney histopathology	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	81	Kidney histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Kidney histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	83	Kidney histopathology	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Kidney histopathology	Rat	2 year	Oral	>6000 ppm	No effect	A non-clear dose-related increase of chronic inflammation incidences of kidney was noted in male rats dose-groups.			
	84	Kidney histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect in females dose groups			
	85	Kidney histopathology	Mouse	2 year	Oral	6000 ppm	Increase	Increased incidence of focal inflammation of the kidney was observed in male mice treated at the top dose of 6000 ppm.			
	85	Kidney histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	80	Kidney weight	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	83	Kidney weight	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	78	Liver histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	In mice long-term toxicity study, increased incidences of hepatocellular adenomas/ carcinomas were recorded in top dose female group. No		
	81	Liver histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Liver histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	83	Liver histopathology	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect	other alterations were reported in livers in other short-term or long-term toxicity studies with eugenol.		
	84	Liver histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Liver histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	85	Liver histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	Hepatocellular adenomas and hepatocellular carcinomas of the liver were increased in low dose male mice; both combined incidences in males strengthened the evidence for an increased incidence in low dose mice. The rates in the high dose group were not different from those observed in controls. A dose-related trend was not observed.			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	85	Liver histopathology	Mouse	2 year	Oral	6000 ppm	Increase	The incidences of hepatocellular adenomas/carcinomas (combined) were significantly increased in female mice at top dose level, and showed a statistically significant trend.			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	86	Liver histopathology	Mouse	4 Single dose on day 1,8,15 and 22 of life	Oral	> ca 60 mg/kg bw/day	No effect	No effect			
	78	Lung histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	The increase of focal granulomatous inflammation in female mice, was considered a single event due to no further alterations		
	81	Lung histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Lung histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Lung histopathology	Rat	2 year	Oral	>6000 ppm	No effect	The incidence of alveolar/bronchiolar adenomas or carcinomas was significantly increased in male rats treated at the low dose 3000 pm. No significant increase was observed in the high dose (6000 ppm) group. No dose-response relationship was observed.	were found in lung in other studies		
	84	Lung histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Lung histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	85	Lung histopathology	Mouse	2 year	Oral	6000 ppm	Increase	Increased incidence of granulomatous inflammation of the lung were seen in females treated at the top dose of 6000 ppm.			
	86	Lung histopathology	Mouse	12 (+6 untreated) months	Oral	>750 mg/kg bw/day	No effect	No effect			
	81	Lymph nodes histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Lymph nodes histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Lymph nodes histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Lymph nodes histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Lymph nodes histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Lymph nodes histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Oesophagus histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Oesophagus histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Oesophagus histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Oesophagus histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Oesophagus histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Oesophagus histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	78	Pancreas histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	No treatment-related effects were observed.		
	81	Pancreas histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Pancreas histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Pancreas histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Pancreas histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Pancreas histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Pancreas histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Peripheral nerve histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Peripheral nerve histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Peripheral nerve histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Peripheral nerve histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Peripheral nerve histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Peripheral nerve histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Salivary glands histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Salivary glands histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Salivary glands histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Salivary glands histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Salivary glands histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Salivary glands histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Skin histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Skin histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Skin histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Skin histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Skin histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Skin histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Spinal cord histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Spinal cord histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Spinal cord histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Spinal cord histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Spinal cord histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Spinal cord histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	80	Spleen histopathology	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect	The spleen haemosiderosis described in 2-year study in rats were not supported by clinical biochemistry nor haematology analysis. Moreover, no further alterations were found in spleen in other studies.		
	81	Spleen histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Spleen histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	83	Spleen histopathology	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Spleen histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Spleen histopathology	Rat	2 year	Oral	12500 ppm	Increase	Increased incidence of splenic haemosiderosis was observed in female rats treated at the top dose of 12500 ppm			
	85	Spleen histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Spleen histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	80	Spleen weight	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	83	Spleen weight	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	78	Stomach histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	Hyperkeratosis and hyperplasia in forestomach was		

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	80	Stomach histopathology	Rat	34 day	Oral		Change	The evaluation document reported that in macroscopic examinations, mucosa of the forestomach contained coalescent areas covered with a thick, flaky, white material punctuated with minute ulcers. Microscopic examinations of the forestomach showed moderately severe hyperplasia and hyperkeratosis of the stratified squamous epithelium associated with focal ulceration. The doses in which effects were observed had not been specified in the study.	observed in rats in the 34-day repeated dose study conducted with eugenol. In the rat, mouse and hamster the forestomach occupies about two-thirds of the proximal area of the stomach and is lined by cornified, stratified squamous epithelium, and acts as a storage organ releasing relatively undigested food into glandular stomach in response to energy demand. This organ is also present in ruminants and camelids as dilation and modification of the esophagus. Thus, humans lack forestomach and consequently, the observed changes in rats do not present toxicological relevance for humans. In all likelihood, the effects observed in rats were triggered by eugenol gavage administration. Eugenol is classified as skin sensitizer		

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	81	Stomach histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	(H317), and causes severe/moderate/mild skin effects in human, rabbit and pig, and likely the direct eugenol application on forestomach mucosa causes epithelium damage.		
	82	Stomach histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Stomach histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Stomach histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Stomach histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	85	Stomach histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Thymus histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Thymus histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Thymus histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Thymus histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	85	Thymus histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Thymus histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	86	Thymus histopathology	Mouse	12 (+6 untreated) months	Oral	750 mg/kg bw/day	Increase	2/30 (7%) treated mice developed thymic lymphomas; the effects were not considered to be treatment related			
	81	Trachea histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Trachea histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Trachea histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Trachea histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Trachea histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Trachea histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
Systemic toxicity	76	Body weight	Rat	14 day	Oral	100000 ppm	Decrease	A decrease in mean bodyweight was observed in males at high dose 100000 ppm.	Signs of systemic toxicity occurred mainly at high doses, which included mortality, effects on bodyweight, food consumption, and clinical signs; these signs were related to general toxicity of higher doses. However, a case by case approach may be done, as toxic adverse effects were not observed in all studies.	Overall evidence of systemic toxicity.	
	77	Body weight	Mouse	14 day	Oral	25000 ppm	Decrease	A dose-related decrease in mean bodyweight >10% was observed in mice at doses ≥25000 ppm			
	79	Body weight	Rat	28 day	Oral	2000 mg/kg bw/day	Decrease	Bodyweight gain was depressed by 10-15% in treated rats			
	80	Body weight	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	81	Body weight	Rat	13 week	Oral	12500 ppm	Decrease	Final body weights were 10% less for male rats and 6% less in females rats in top dose group, as compared to controls; it was not reported whether the findings were significant. A decrease (12%) in bodyweight gain was detected in top dose male group.			
	82	Body weight	Mouse	13 week	Oral	6000 ppm	Decrease	A decrease in bodyweight gain was detected in the top dose male group (10%)			
	83	Body weight	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	84	Body weight	Rat	2 year	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Body weight	Rat	2 year	Oral	12500 ppm	Decrease	Bodyweights in female rats were reduced in the high dose group (12500 ppm) throughout the study			
	86	Body weight	Mouse	12 (+6 untreated) months	Oral	750 mg/kg bw/day	Decrease	Bodyweight gain was decreased at 4 and 8 months measurements (13% and 15%) compared with controls.			
	87	Body weight	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Body weight	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Body weight	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Body weight	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	91	Body weight	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Body weight	Rabbit	24 day	Oral	>500/350 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	79	Clinical chemistry and haematology	Rat	28 day	Oral		Not measured	Not measured			
	80	Clinical chemistry and haematology	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	83	Clinical chemistry and haematology	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	84	Clinical chemistry and haematology	Rat	2 year	Oral		Not measured	Not measured			
	84	Clinical chemistry and haematology	Rat	2 year	Oral		Not measured	Not measured			
	87	Clinical chemistry and haematology	rat	10 day	Oral		Not measured	Not measured			
	88	Clinical chemistry and haematology	rabbit	13 day	Oral		Not measured	Not measured			
	89	Clinical chemistry and haematology	mouse	11 day	Oral		Not measured	Not measured			
	90	Clinical chemistry and haematology	hamster	10 day	Oral		Not measured	Not measured			
	91	Clinical chemistry and haematology	Rat	15 day	Oral		Not measured	Not measured			
	92	Clinical chemistry and haematology	Rabbit	24 day	Oral		Not measured	Not measured			
	76	Clinical signs	Rat	14 day	Oral	>100000 ppm	No effect	No effect			
	77	Clinical signs	Mouse	14 day	Oral	>100000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	78	Clinical signs	Dog	21 day	Oral	250 mg/kg bw/day	Change	Dogs received a single dose. There were no consistent findings across the study but there were indications of increased pulse rats and reduced body temperature. Vomiting was seen at the top two dose levels (250 and 500 mg/kg bw/day).			
	78	clinical signs	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect			
	79	Clinical signs	Rat	28 day	Oral		No effect	No effect			
	80	Clinical signs	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	81	Clinical signs	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Clinical signs	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	83	Clinical signs	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Clinical signs	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Clinical signs	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	87	Clinical signs	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Clinical signs	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Clinical signs	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Clinical signs	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	91	Clinical signs	Rat	15 day	Oral	250 mg/kg bw/day	Change	Piloerection; ataxia, prostration, lethargy, noisy respiration, increased lachrymation were observed in rats treated at 600 mg/kg bw/day within 1 hr of dosing, resolved within 24 hrs. Piloerection and hunched posture were observed in rats treated at 250 mg/kg bw/day			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	92	Clinical signs	Rabbit	24 day	Oral	250 mg/kg bw/day	Change	One abortion, post dosing ataxia, breathing difficulties, pallor of the extremities, eyes and ears, and prostration were observed in rabbits treated at 500 (dosing reduced to 350 between GD 10 and 13) mg/kg bw/day. On the other hand, one abortion, ataxia and breathing difficulties were noted at 250 mg/kg bw/day. No significant treatment-related effects in adults treated at 100 mg/kg bw/day.			
	79	Food consumption	Rat	28 day	Oral		Not measured	Not measured			
	80	Food consumption	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	81	Food consumption	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Food consumption	Mouse	13 week	Oral		Not measured	Not measured			
	83	Food consumption	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	84	Food consumption	Rat	2 year	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Food consumption	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	87	Food consumption	rat	10 day	Oral		Not measured	Not measured			
	88	Food consumption	rabbit	13 day	Oral		Not measured	Not measured			
	89	Food consumption	mouse	11 day	Oral		Not measured	Not measured			
	90	Food consumption	hamster	10 day	Oral		Not measured	Not measured			
	91	Food consumption	Rat	15 day	Oral	600 mg/kg bw/day	Decrease	A reduction in food consumption occurred during the initial few days of dosing post-coitum in rats treated at 600 mg/kg bw/day. No effects were reported in rats treated at 250 or less mg/kg bw/day.			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	92	Food consumption	Rabbit	24 day	Oral	250 mg/kg bw/day	Decrease	A reduced trend in food consumption was observed in rabbits treated at 500 (dosing reduced to 350 between GD 10 and 13) and 250 mg/kg bw/day from the start of dosing to GD 18 (although results only were statistically significant through gestation day 6-12 and 9-12 for high and mid dose groups, respectively). No significant treatment-related effects in adults treated at 100 mg/kg bw/day.			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	76	Mortality	Rat	14 day	Oral	100000 ppm	Increase	At the highest dose tested of 100000 ppm, 20% of male and 100% of female rats died			
	77	Mortality	Mouse	14 day	Oral	50000 ppm	Increase	All mice treated at 100000 ppm and 60% of male mice at 50000 ppm died			
	78	Mortality	Dog	21 day	Oral	500 mg/kg bw/day	Increase	Dogs that received single dose. Ataxia in 3/4 dogs and deaths in 2/4 dogs were observed at the highest dose			
	78	Mortality	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect			
	79	Mortality	Rat	28 day	Oral		No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	80	Mortality	Rat	34 day	Oral	2000 mg/kg bw/day	Increase	A few deaths occurred at doses of 2000 mg/kg bw/day and the number increased with increasing doses. 8 animals survived to 34 days while 15 animals received the dose of 4000 mg/kg bw/day.			
	81	Mortality	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Mortality	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Mortality	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Mortality	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Mortality	Mice	2 year	Oral	3000 ppm	Decrease	A reduction in survival was detected in low and high dose male groups (12%)			
	86	Mortality	Mouse	12 (+6 untreated) months	Oral	>750 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	87	Mortality	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Mortality	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Mortality	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Mortality	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	91	Mortality	Rat	15 day	Oral	600 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Line(s) of evidence	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	92	Mortality	Rabbit	24 day	Oral	250 mg/kg bw/day	Change	5 rabbits treated at 500 mg/kg bw/day died. The top dose was subsequently reduced to 350 mg/kg bw/day (between GD 10 and 13). One further female treated at 350 mg/kg bw/ day was killed in extremis following abortion of her offspring on GD 25. 1 rabbit treated at 250 mg/kg bw/day died after showing clinical signs. Two further females of mid dose group were killed in extremis as consequence of dosing error and after abortion on GD 23, respectively.			

2.10.2.1.5 Assessment of the integrated lines of evidence and weight of evidence for T-mediated adversity and endocrine activity.

WoE for T-mediated adversity

Regarding T-mediated parameters:

-Thyroid histological investigations were conducted in rats and in mice in respective 2-year carcinogenicity studies (ID: 84 and 85).

In the 2-year carcinogenicity study in F344/N rats (ID: 84), male rats received eugenol via the diet at doses of 0, 3000 or 6000 ppm (equivalent to adjusted values of 128 and 260 mg/kg bw/day; or default values of 150 and 300 mg/kg bw/day, respectively), whereas female rats received eugenol via the diet at doses of 0, 6000 or 12500 ppm (equivalent to adjusted values of 302 and 648 mg/kg bw/day; or default values of 300 and 625 mg/kg bw/day, respectively). Statistically significant increase incidence of thyroid C-Cell adenoma was observed in the 6000 ppm female dose group compared with controls (22% vs 8% in controls). In the 12500 ppm dose female group, the incidence displayed lower values than noted in the 6000 ppm dose group and controls (4%), so a dose-related trend was not observed. On the other hand, no statistically significant differences were detected in the incidences of thyroid C-Cell carcinomas in any female dose groups.

In contrast, a statistically significant decrease in the incidence of thyroid C-Cell adenoma (10%, 10% and 0% for controls, low and high dose groups) and adenoma/carcinoma combined (18%, 16% and 4% for controls, low and high dose groups) was detected on thyroids of high dose male group compared with controls. On the other hand, no statistically significant differences were found in the incidences of thyroid C-Cell carcinomas between treated groups and controls.

In the 2-year carcinogenicity study in $B6C3F_1$ mice (ID: 85) animals received eugenol via the diet at doses of 0, 3000 and 6000 ppm (equivalent to adjusted values of 632/784 and 1250/1546 for males/females; or default values of 450 and 900 mg/kg bw/day). A slight increase incidence of follicular cell adenomas of the thyroid gland was observed in the high dose male mice. This incidence did not show statistically significant differences compared with controls, although a statistically significant increased trend was noted (0%, 0% and 6% for control, low and high dose groups, respectively).

- None of the available studies specifically assessed the T-mediated parameters: thyroid hormones (T3/T4/TSH), thyroid gland weight, colloid area histopathology and follicular cell height. However, the follicular cells of the thyroid glands were evaluated in rats and in mice as part of respective 2-year carcinogenicity studies, and no anomalies were described.

Regarding sensitive to, but not diagnostic of EATS parameters:

-In the developmental toxicity study in rats (ID: 91), these anomalies were found:

. The mean foetal weights in litters of rats treated at top dose level were slightly but significantly reduced, however, it was not associated with any significant delay in foetal development.

. Reduction in the percentage of foetuses with more than 6 ossified metatarsals occurred in litters of rats treated at high dose level. This finding was seen in isolation of any other effects on skeletal development.

-In the developmental toxicity study in rabbits (ID: 92), this anomaly was found:

. Increase in post implantation loss value was recorded in the top dose level. This finding was non-statistically significant and presented a wide standard deviation. This data was considered equivocal due to it was limited primarily to two females with significant numbers of late embryonic deaths. Moreover, the findings were not consistent with that seen for other females of this dose group.

Target organ toxicity

-In mice long-term toxicity study (ID: 85), increased incidence of hepatocellular adenoma was observed in the low dose male group (26%) compared with controls (8%). However, the occurrence did not show a dose-response pattern due to the incidence noted in the top dose group (20%) was lower than observed in the low dose group. On the other hand, the incidence of hepatocellular carcinomas reported in the low dose male group (40%) did not show a dose-response pattern, due to the incidence of hepatocellular carcinomas was even lower than controls (18% vs 20% in

control group). The increase incidences of hepatocellular adenomas and carcinomas (combined) in the high dose female group was considered an equivocal evidence of carcinogenicity due to both incidences did not display statistical significant results when were analysed individually, and the house location of animal´s cages might affect the incidence of hepatocellular adenomas and carcinomas.

- There was a slight enlargement of liver cells in rats after 34-day eugenol treatment (ID: 80).

- In long-term toxicity studies, increased incidence of alveolar/bronchiolar adenomas or carcinomas was recorded in male rats at the low dose level (ID: 84) No dose-response relationship was observed. On the other hand, increased incidence of granulomatous inflammation and adenomatous hyperplasia of the lung was seen in female mice treated at the top dose (ID: 85).

. Increased incidence of focal inflammation of the kidney was observed in male mice treated at the top dose in the 2-year carcinogenicity study (ID: 85). On the other hand, a non-clear dose-related increase of chronic inflammation incidences of kidney was noted in male rats dose-groups (ID: 84).

- Increased incidence of splenic haemosiderosis was observed in female rats treated at the top dose (ID: 84) in the 2-year carcinogenicity study. This finding was not supported by clinical biochemistry or haematology analysis. Moreover, no further alterations were found in spleen in other studies.

-Increased thymic lymphomas incidence was observed in long-term toxicity study in mice (ID: 86). The thymic lymphomas showed low incidence and were not reproduced in the further 2-year chronic toxicity studies.

-There was a slight enlargement of adrenal gland in rats after 34-day eugenol treatment (ID: 80).

- Hyperkeratosis and hyperplasia in forestomach was observed in rats in the 34-day repeated dose study (ID: 80) conducted with eugenol.

Therefore, based on a weight of evidence approach and taking into account histological assessment of the thyroid glands conduced in mice and in rats in respective 2-year carcinogenicity studies, no T-mediated adversity was detected. However, due to the lack of relevant T-mediated parameters data in the available studies, and the absence of TG 416 and/or TG 443 studies, it is concluded that T-mediated adversity has not been sufficiently investigated.

WoE for T-mediated activity

In vitro mechanistic test guidelines for the T modality are currently not available as well as specific *in vivo* mechanistic tests on mammals. The majority of the mammalian toxicity studies are not compliant with current guidelines and thus they do not include parameters useful to assess–T mediated activity. *In vitro* mechanistic data for eugenol are however available via the US EPA CompTox Chemicals Dashboard version 4.

The EDSP21 tab in the CompTox Chemicals Dashboard v4 includes 13 thyroid receptor bioassays for eugenol. Negative/inactive results were obtained in 12 of them, while a positive/active result was obtained for eugenol in the thyroid assay NCCT_TPO_AUR_dn; Study (ID: 5). The AC_{50} value was 5.57 µM (Hill Model). The limit of cytotoxicity for this assay was reported to be 1000 µM.

The available *in vitro* mechanistic bioassay data (OECD Conceptual Framework Level 2) indicate that eugenol has the potential to inhibit thyroperoxidase. These findings concur with QSAR models (e.g. Leadscope QSAR1 and QSAR2) for thyroperoxidase inhibition which indicates that eugenol has 2/3 of the high-ranking structural features (anisole and phenol groups) associated with inhibitory potential [6].

Overall, according to ECHA/EFSA ED guideline point 3.4.2, T-mediated activity has not been sufficiently investigated due to the absence of relevant thyroid parameters measures in mammals studies and the lack of TG 416 and/or 443 studies.

2.10.2.1.6 Selection of relevant scenario for the ED assessment of T-modality

According to the ECHA/EFSA ED GD, following the assessment of available ED evidence, relevant scenarios (see table below) should be identified to inform on the conclusion of the ED assessment for humans or to further steps of investigation that are required for the respective T-modality.

Based on the absence of relevant *in vivo*-mechanistic and T-mediated parameters and the lack of TG 416 and/or TG 443 studies, it has been concluded that T-mediated adversity and T activity have not been sufficiently investigated.

The relevant scenario for the T-modality is identified as 2a (iii).

Table 2.10.2.1.6: Identification of relevant scenario for T-modality

Adversity based on T-mediated parameters	Positive mechanistic OECD CF level 2/3 Test	Scenario	Next step of the assessment	Scenario selected
No (cd)	Yes/No	1a	Conclude: ED criteria not met because there is not "T-mediated" adversity	
Yes (sufficiently investigated)	Yes/No	1b	Perform MoA analysis	
No (not sufficiently investigated)	Yes	2a (i)	Perform MoA analysis (additional information may be needed for the analysis)	
No (not sufficiently investigated)	No (sufficiently investigated)	2a (ii)	Conclude: ED criteria not met because no T-mediated endocrine activity observed	
No (not sufficiently investigated)	No (not sufficiently investigated)	2a (iii)	Generate missing level 2 and 3 information. Alternatively, generate missing "T-mediated" parameters. Depending on the outcome move to corresponding scenario	x
Yes (not sufficiently investigated)	Yes/No	2b	Perform MoA analysis	

Co-RMS comment : The co-RMS considers that according to the ECHA/EFSA GD on ED, the relevant scenario for the T-modality is 2a(i). Based on the available data, the following are noted:

- Thyroid histopathology is sufficiently investigated in chronic toxicity studies in mice and rats and there is no evidence of adversity. It is noted that c-cell adenomas are not considered T-mediated effects.
- There are no T3/T4/TSH measurements in the available studies.
- There is a positive TPO assay (confirmed by *in silico* modelling) suggesting T-mediated endocrine activity.

In view of the above, potential T-mediated effects on the sensitive population (developing foetuses and newborn) have not been sufficiently investigated.

The co-RMS considers that additional information on the sensitive population (foetus and newborn) including plasma T4/T3 and TSH concentrations is considered necessary before concluding on T modality.

RMS response : RMS disagrees with the co-RMS proposal regarding the relevant scenario 2a(i) for T-modality. T-mediated parameters were not sufficiently investigated in the absence of TG 416 and/or 443 studies, and the absence of relevant *in vivo*-mechanistic and T-mediated parameters. Moreover, according to ECHA/EFSA ED guideline point 3.4.2, T-mediated activity has not been sufficiently investigated due to the absence of relevant thyroid parameters measurements in mammalian studies and the lack of TG 416 and/or 443 studies. In this regard, despite the positive TPO assay obtained in the US EPA ToxCast EDSP21 Chemical dashboard (in agreement with *in-silico* predictions), the RMS does not consider fulfilled the T-mediated activity requirement with this result, and is of the opinion that further data need to be generated to considered T-mediated activity as sufficiently investigated ([e.g. *in vitro* thyroperoxidase inhibition (rat and/or human) assay].

2.10.2.1.7 MoA analysis for T-modality

According to the ECHA/EFSA guidance in cases of Scenario 2a(iii), a MoA analysis for T-modality is not required.

2.10.2.1.8 Conclusion of the assessment of T-modality

According to the ECHA/EFSA GD on ED, the relevant scenario proposed for the T-modality is 2a (iii). It has been concluded that T-mediated adversity and T-mediated activity have not been sufficiently investigated based on the absece of relevant T-mediated parameters and the lack of TG 416 and/or TG 443 studies. On the other hand, based on the available data and using a weight of evidence approach, the evaluation of thyroid histopathology in two-year carcinogenicity studies, and supporting evidence from other repeated dose toxicity studies does not suggest an adversity mediated T-parameters. In addition, one positive/active result was obtained in the Thyroid Bioactivity Model US EPA for thyroid peroxidase inhibition (TPO), in agreement with QSAR *in-silico* predictions. Therefore, further data need to be generated before a conclusion on whether or not the ED criteria are met for the T-modality can be drawn [e.g. *in vitro* thyroperoxidase inhibition (rat and/or human) assay].

2.10.2.2 ED assessment for EAS-modality

2.10.2.2.1 Analysis of non-experimental data

In accordance with the OECD Conceptual Framework and the ECHA/EFSA GD on ED, Level 1, EAS-related non-test information was gathered for eugenol. Qualitative structural activity relationship (QSAR) data was obtained for eugenol from the Danish QSAR database, and results are summarised below.

Table 2.10.2.2.1/1: Results of Danish QSAR database for eugenol regarding EAS-modality.

	Exp.	Battery	CASE Ultra	Leadscope	SciQSAR
Estrogen Receptor α Binding, Full training set (Human *in vitro*)		NEG_IN	NEG_IN	NEG_IN	NEG_IN
Estrogen Receptor α Binding, Balanced Training Set (Human *in vitro*)		NEG_OUT	INC_OUT	NEG_IN	INC_OUT
Estrogen Receptor α Activation (Human *in vitro*)		NEG_IN	NEG_IN	NEG_IN	INC_OUT
Estrogen Receptor Activation, CERAPP data (*in vitro*)		NA	NA	POS_OUT	NA
Androgen Receptor Inhibition (Human *in vitro*)		NEG_IN	NEG_IN	NEG_IN	NEG_IN
Androgen Receptor Binding, CoMPARA data (*in vitro*)	NEG	NA	NA	INC OUT	NA
Androgen Receptor Inhibition, CoMPARA data (*in vitro*)	NEG	NA	NA	INC OUT	NA
Androgen Receptor Activation, CoMPARA data (*in vitro*)	NEG	NA	NA	NEG_IN	NA
Key: POS = Positive; NEG = Negative; IN = within the applicability domain of the model; OUT = outside of the applicability domain of the model					

The Danish QSAR Database reports that eugenol lacks the potential to interact with estrogen and androgen receptors. No alerts relevant to EAS-mediated endocrine properties were therefore identified for eugenol.

OECD ToolBox
The results of the OECD QSAR Toolbox v.4.2 for eugenol in respect of EAS-related endpoints are shown below.

Table 2.10.2.2.1/2. Results of the OECD QSAR Toolbox v.4.2 for eugenol

[1] Estrogen Receptor Binding, alerts in:	
parent only	Weak binder, OH group
metabolites from in vivo Rat metabolism simulator only	Strong binder, OH group; Moderate binder, OH group; Weak binder, OH group
metabolites from Rat liver S9 metabolism simulator only	Moderate binder, OH group; Weak binder, OH group
[2] rtER Expert System - USEPA, alerts in	
parent only	Alkoxyphenols
metabolites from in vivo Rat metabolism simulator only	No alert found
metabolites from Rat liver S9 metabolism simulator only	No alert found
OECD QSAR Toolbox v.4.2 profilers	
Profiler predictions are supporting information to be used together with the relevant QSAR predictions	

[1] Estrogen receptor binding: Weak binder, OH group

Estrogen receptor (ER) binding is a molecular initiating event similar to protein binding that leads to a series of adverse outcomes, which are typically considered reproductive and development hazards. It is an endpoint where

several comprehensive databases exist, which has lead to the development of several approaches for using (Q)SARs to predict ER-binding and possible endocrine disruption.

Since the ER-binding is a receptor mediated event, particular organic functional groups, size and shape are critical to binding potency. Chemicals with a single 5-or 6-member carbon ring structure with an unhindered hydroxyl-group (-OH) (a hydroxyl group in the para- or meta-position on the ring and without ortho substituents to the hydroxyl group) (5) are ER binders. Binding potency is related to the size and shape of non-hydroxylated-ring aspect of the molecule, which can be grossly measured by molecular weight.

The incorporated Toolbox ER binding profiling scheme is based on structural and parametric rules extracted from literature sources and supported by experimental data. The ER-binding profiler classifies chemicals as non-binders or binders depending on molecular weight (MW) and structural characteristics of the chemicals:

1. Very strong binders: Chemicals with MW between 200 and 500 Da and two rings with a hydroxyl group connected to each of them.

2. Strong binders: Chemicals with at least one 5-or 6-members carbon ring with an unhindered hydroxyl or amino group and MW between 200 and 500 Da.

3. Moderate binders: Chemicals with at least one 5-or 6-members carbon ring with an unhindered hydroxyl or amino group and MW between 170 and 200 Da.

4. Weak binders: Chemicals with at least one 5-or 6-members carbon ring with an unhindered hydroxyl or amino group and MW less than 170 Da.
If the target chemical does not meet some of the structural and parametric requirements listed above it is classified as Non binder:
- Non binder with impaired hydroxyl or amino group.
- Non binder, MW more than 500 Da.
- Non binders without hydroxyl or amino group.
- Non-binder, non-cyclic.

The OECD Toolbox v.4.2 predicts that eugenol has the potential to be an ER binder due to its cyclic molecular structure and the presence of a single non-impaired hydroxyl functional group.

[2] rtER Expert System USEPA: Alkoxyphenols

The rtER Expert System ver.1 – USEPA profiler consists of molecular definitions that mimic the structural criteria of chemical classes that are potential estrogen receptor-binders covered by US EPA Estrogen Receptor Expert System (ERES) The ERES profiler is an effects-based automated system used to predict estrogen receptor binding affinity. In the Toolbox, the rtER Expert System ver.1 – USEPA profiler is used for the purpose of categorization based on the structural definitions of the original ERES chemical classes. The rtER Expert System ver.1 – USEPA profiler is intended for categorization purpose and not for predicting relative binding affinity (RBA). rtER Expert System ver.1
USEPA profiler predicts that eugenol meets the criteria of chemical classes that are potential ER binders, on the basis that is an alkoxyphenol substance.

The rtER Expert System ver.1 – USEPA profiler consists of molecular definitions mimic the structural criteria of chemical classes potential estrogen receptor-binders covered by US EPA Estrogen Receptor Expert System (ERES) The ERES profiler is an effects-based automated system used to predict estrogen receptor binding affinity. The Estrogen Receptor Expert System (ERES) Profiler is an effects-based automated system used to predict estrogen receptor binding affinity

ToxCast: CERAPP Potency Level (ER-Related activity) and COMPARA (AR-related activity)

The ToxCast Model Dashboard includes predictions of the estrogen receptor activity of eugenol, based on the Collaborative Estrogen Receptor Activity Prediction Project (CERAPP[5]). The CERAPP is a large-scale modelling project which has investigated the efficacy of using predictive computational models trained on high-throughput screening data (e.g. from the EDSP21 initiative) to evaluate the ER-related activity of thousands of chemicals, and identify priorities for further testing.

On the other hand, the ToxCast Models Dashboard also includes predictions of the androgen receptor activity of eugenol based on the COMPARA. COMPARA is a large scale collaboration between 35 international groups using QSAR models to predict androgen receptor activity using a common training set of 1746 compounds provided by the US EPA. The result is consensus model of AR agonist activity that is run against the DSSTox chemical library that aims to identify priorities for further testing.
The CERAPP and COMPARA predictions for estrogen and androgen activity are summarised in the following table:

Table 2.10.2.2.1/3. Results of the CERAPP and COMPARA predictions for eugenol

Model	Receptor	Agonist	Antagonist	Binding
ToxCast Pathway Model (AUC)	Androgen	0	0	-
ToxCast Pathway Model (AUC)	Estrogen	0	0	-
COMPARA (Consensus)	Androgen	Inactive	Inactive	Inactive
CERAPP Potency Level (From Literature)	Estrogen	-	Inactive (Inactive)	-
CERAPP Potency Level (Consensus)	Estrogen	Active (Weak)	Active (Weak)	Active (Weak)

As noted, eugenol displayed inactive results for estrogen and androgen receptor activities.

2.10.2.2.2 US EPA CompTox Chemicals Dashboard

Estrogen receptor bioassays

The EDSP1 tab in the CompTox Chemicals Dashboard v4 includes 19 ER bioassays for eugenol. The results were positive/active for only 2 assays (ATG_ERa_TRANS_up and ATG_ERE_CIS_up, with an AC_{50} value of 43.26 and 24.58, respectively) and negative for 17 assays. The ATG_ERE_CIS_up assay presented the flags:"borderline active" and "less than 50% efficacy". However, active result from ATG_ERa_TRANS_up was not flagged and more reliable. The results are showed in table 2.10.2.2.2/1.

Table 2.10.2.2.2/1: Summary of US EPA ToxCast EDSP21- estrogenic bioactivity assays for eugenol

Assay endpoint	Assay type	Organism	Result	Study ID Matrix
ACEA_ER_80hr	real-time cell-growth kinetics	human	Inactive	14
ACEA_ER_AUC_viability	real-time cell-growth kinetics	human	Inactive	15
ATG_ERa_TRANS_up	**mRNA induction**	**human**	**Active**	**16**
ATG_ERE_CIS_up	**mRNA induction**	**human**	**Active**	**17**
NVS_NR_hER	radioligand binding	human	Inactive	18
OT_ER_ERaERa_0480	protein fragment complementation assay	human	Inactive	19
OT_ER_ERaERa_1440	protein fragment complementation assay	human	Inactive	20
OT_ER_ERaERb_0480	protein fragment complementation assay	human	Inactive	21
OT_ER_ERaERb_1440	protein fragment complementation assay	human	Inactive	22
OT_ER_ERbERb_0480	protein fragment complementation assay	human	Inactive	23
OT_ER_ERbERb_1440	protein fragment complementation assay	human	Inactive	24
OT_ERa_EREGFP_0120	fluorescent protein induction	human	Inactive	25
OT_ERa_EREGFP_0480	fluorescent protein induction	human	Inactive	26
TOX21_ERa_BLA_Agonist_ratio	beta lactamase induction	human	Inactive	27
TOX21_ERa_BLA_Antagonist_ratio	beta lactamase induction	human	Inactive	28
TOX21_ERa_BLA_Antagonist_viability	ATP content	human	Inactive	29
TOX21_ERa_LUC_VM7_Agonist	luciferase induction	human	Inactive	30
TOX21_ERa_LUC_VM7_Antagonist_specificity	luciferase induction	human	Inactive	31
TOX21_ERa_LUC_VM7_Antagonist_specificity_viability	ATP content	human	Inactive	32

Androgen receptor bioassays

Eugenol was tested in 15 assays included in the ToxCast AR Bioactivity Model. The results were inactive for 14 assays and active for 1 assay. NVS_NR_cAR was the assay with positive results. The AC_{50} was determined to be 19.08 µM (Hill Model). The limit of cytotoxicity was reported to be 1000 µM. However, a flag of "less than 50% efficacy was displayed.

Table 2.10.2.2.2/2: Summary of US EPA ToxCast EDSP21- androgenic bioactivity assays for eugenol

Assay endpoint	Assay type	Organism	Result	Study ID Matrix
ATG_AR_TRANS_up	mRNA induction	human	Inactive	33
NVS_NR_cAR	**radioligand binding**	**chimpanzee**	**Active**	**34**
OT_AR_ARELUC_AG_1440	luciferase induction	chinese hamster	Inactive	35
OT_AR_ARSRC1_0480	protein fragment complementation assay	human	Inactive	36
OT_AR_ARSRC1_0960	protein fragment complementation assay	human	Inactive	37
TOX21_AR_BLA_Agonist_ratio	beta lactamase induction	human	Inactive	38
TOX21_AR_BLA_Antagonist_ratio	beta lactamase induction	human	Inactive	39
TOX21_AR_BLA_Antagonist_viability	ATP content	human	Inactive	40
TOX21_AR_LUC_MDAKB2_Agonist	luciferase induction	human	Inactive	41
TOX21_AR_LUC_MDAKB2_Antagonist	luciferase induction	human	Inactive	42
TOX21_AR_LUC_MDAKB2_Antagonist_viability	ATP content	human	Inactive	43
TOX21_AR_LUC_MDAKB2_Antagonist_Specificity	luciferase induction	human	Inactive	44
TOX21_AR_LUC_MDAKB2_Antagonist_Specificity _viability	ATP content	human	Inactive	45
UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Agonist	Protein-protein binding	human	Inactive	46
UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Antagonist	Protein-protein binding	human	Inactive	47

Steroidogenesis bioassays

The EDSP21 tab in the CompTox Chemicals Dashboard includes 26 steroidogenesis bioassays for eugenol.

Of these assays, 24 consist of a series of *in vitro* mechanistic bioassays in H295R human adrenocortical carcinoma cells which assess the potential for eugenol to modulate steroid hormone biosynthesis (estrogen, androgen, progestogens and glucocorticoids).

The results were inactive for 23 assays and active for the 3 following assays:

- CEETOX_H295R_CORTIC_up. The AC_{50} was determined to be 43.56 µM. The limit of cytotoxicity for the assay was reported to be 1000 µM. (Study ID Matrix No. 57)
- CEETOX_H295R_ESTRADIOL_up. The AC_{50} was determined to be 32.56 µM. The bioassay has a "flag" indicating that eugenol has borderline activity in this assay. The limit of cytotoxicity for the assay was reported to be 1000 µM. (Study ID Matrix No. 63)
- CEETOX_H295R_ESTRONE_up. The AC_{50} was determined to be 36.26 µM. The bioassay has two "flags" indicating that: eugenol has borderline activity in this assay, and that "only highest concentration above baseline, active". Thus, only the highest concentration in the assay was above the baseline. The limit of cytotoxicity for the assay was reported to be 1000 µM. (Study ID Matrix No. 65)

These three CEETOX assays are performed using H295R human adrenocortical cells and measure the up-regulation of the hormones: corticosterone, estradiol and estrone, respectively, associated with the steroidogenesis pathway for the metabolism of cholesterol for steroid hormones in the adrenal glands.

These bioassays are listed in the table below and have been included in the ED Excel spreadsheet (Study ID matrix nos.: 48-73)

Table 2.10.2.2.2/3: Summary of US EPA ToxCast EDSP21- Steroidogenesis bioactivity assays for eugenol.

Assay endpoint	Assay type	Organism	Result	Study ID Matrix
CEETOX_H295R_11DCORT_dn	Hormone induction	human	Inactive	48
CEETOX_H295R_11DCORT_up	Hormone induction	human	Inactive	49
CEETOX_H295R_OHPREG_dn	Hormone induction	human	Inactive	50
CEETOX_H295R_OHPREG_up	Hormone induction	human	Inactive	51

Assay endpoint	Assay type	Organism	Result	Study ID Matrix
CEETOX_H295R_OHPROG_dn	Hormone induction	human	Inactive	52
CEETOX_H295R_OHPROG_up	Hormone induction	human	Inactive	53
CEETOX_H295R_ANDR_dn	Hormone induction	human	Inactive	54
CEETOX_H295R_ANDR_up	Hormone induction	human	Inactive	55
CEETOX_H295R_CORTIC_dn	Hormone induction	human	Inactive	56
CEETOX_H295R_CORTIC_up	**Hormone induction**	**human**	**Active**	**57**
CEETOX_H295R_CORTISOL_dn	Hormone induction	human	Inactive	58
CEETOX_H295R_CORTISOL_up	Hormone induction	human	Inactive	59
CEETOX_H295R_DOC_dn	Hormone induction	human	Inactive	60
CEETOX_H295R_DOC_up	Hormone induction	human	Inactive	61
CEETOX_H295R_ESTRADIOL_dn	Hormone induction	human	Inactive	62
CEETOX_H295R_ESTRADIOL_up	**Hormone induction**	**human**	**Active**	**63**
CEETOX_H295R_ESTRONE_dn	Hormone induction	human	Inactive	64
CEETOX_H295R_ESTRONE_up	**Hormone induction**	**human**	**Active**	**65**
CEETOX_H295R_PROG_dn	Hormone induction	human	Inactive	66
CEETOX_H295R_PROG_up	Hormone induction	human	Inactive	67
CEETOX_H295R_TESTO_dn	Hormone induction	human	Inactive	68
CEETOX_H295R_TESTO_up	Hormone induction	human	Inactive	69
TOX21_Aromatase_Inhibition	Luciferase induction	human	Inactive	70
CEETOX_H295R_MTT_cell_viability_up	Hormone induction	human	Inactive	71
CEETOX_H295R_MTT_cell_viability_dn	Hormone induction	human	Inactive	72
TOX21_Aromatase_inhibition_viability	ATP content	human	Inactive	73

2.10.2.2.3 Have EAS-mediated parameters been sufficiently investigated?

The available dataset of *in vivo* mammalian toxicology studies for eugenol consists of short-term studies (14, 28 and 34 days) conducted in rodents, repeated dose study in dogs (21 day), sub-chronic studies (90 days and 19 weeks) conducted in rodents, carcinogenicity studies conducted in rodents and prenatal developmental toxicology studies conducted in rats and rabbits. Neither two-generation reproductive toxicity study (OECD TG 416), nor the extended one-generation reproductive toxicity study (OECD TG 443) had been performed. Moreover, much of the available data pre-dates revisions that were made to the OECD Test Guidelines to include EAS-mediated parameters.

Table 14 of the ECHA/EFSA GD on ED provides a list of relevant EAS-mediated parameters that may be investigated in the OECD CF Level 4 and 5 *in vivo* OECD TG compliant mammalian toxicology studies. Using the currently available set of toxicological data for eugenol, the table 2.10.2.2.3/1 summarises the available information on EAS-mediated parameters.

Therefore, based on the assessment provided in the table below, it is considered that EAS-mediated adversity has not been sufficiently investigated due to no data have been specifically reported on most of the EAS-mediated parameters: sex hormones, accessory sex organ histopathology, age at first oestrus, age at balanopreputial separation, age at vaginal opening, anogenital distance (AGD), cervix histopathology, coagulating gland histopathology, oestrus cyclicity, genital abnormalities, nipple development, oviduct histopathology, sperm parameters, , vagina histopathology and smear; and weights of the following organs: coagulating gland, Cowper's gland, epididymis, LABC, prostate, testis, seminal vesicles, ovary and glans penis, These lack of most of EAS-mediated parameters is due to the absence of OECD TG 416 or 443 reproductive toxicity studies.

Table 2.10.2.2.3/1: Summary of EAS-mediated parameters investigated in mammalian toxicology studies

EAS-mediated parameters	**OECD Test guideline**	**Sufficiently investigated?** **Overall conclusion: No (not sufficiently investigated)** Based on the lack of OECD 416 and 443 studies.
Estradiol level	408 (optional)	No data
Follicle stimulating hormone (FSH) level	408 (optional)	No data

EAS-mediated parameters	OECD Test guideline	Sufficiently investigated? **Overall conclusion: No (not sufficiently investigated)** Based on the lack of OECD 416 and 443 studies.
Luteinising hormone (LH) level	408 (optional)	No data
Testosterone level	408 (optional)	No data
Accessory sex organs histopathology	408, 421, 451-3	No data
Age at first oestrus	OPPTS 890.1450	No data
Age at balanopreputial separation	426, 416, 443	No data
Age at vaginal opening	426, 416, 443	No data
Anogenital distance (AGD)	414, 421, 426, 416, 443	No data
Cervix histopathology	407, 408, 415, 422, 451-3, 416, 443	No data
Coagulating gland histopathology	407, 408, 415, 422, 451-3, 416, 443	No data
Coagulating gland weight	407, 421, 422, 416, 443	No data
Cowper's gland weight	421 (optimal), 422 (optional)	No data
Epididymis histopathology	407, 408, 415 (optional) 421, 422, 451-3, 416, 443	Epididymis histopathology was performed in the 90-day toxicity studies (OECD TG 408) in rodents and 2-year carcinogenicity studies in rodents (OECD TG 453).

EAS-mediated parameters	OECD Test guideline	Sufficiently investigated? **Overall conclusion: No (not sufficiently investigated)** Based on the lack of OECD 416 and 443 studies.
Epididymis weight	407, 408, 421, 422, 451-3, 416, 443	No data
Oestrus cyclicity	407 (optional), 408, 421, 422, 416, 443	Data of oestrus cyclicity was reported in a non-guideline and supportive study conducted in rats (ID: 75).
Glans penis weight	421 (optimal), 422 (optional)	No data
Genital abnormalities	414, 415, 421, 422, 416, 443	No data
LABC weight	421 (optimal), 422 (optional), OPPTS 890.1500	No data
Mammary gland histopathology (male)	407 (optional), 408, 422, 443, 451-3 (optional)	Mammary gland histopathology (male) was performed in the 90-day toxicity studies in rodents (TG 408) and 2-year carcinogenicity studies in rodents (OECD TG 453)
Mammary gland histopathology (female)	407, 408, 451-3, 443	Mammary gland histopathology (female) was performed in the 90-day toxicity studies in rodents (OECD TG 408) and 2-year carcinogenicity studies in rodents (OECD TG 453)
Nipple development	421, 422, 443	No data
Ovary histopathology	407, 408, 415 (optional) 421, 422, 426, 451-3, 416, 443	Ovary histopathology was performed in the 90-day toxicity studies in rodents (OECD TG 408) and 2-year carcinogenicity studies in rodents (OECD TG 453).
Ovary weight	407 (optional), 408, 421 (optional), 422, 451-3, 416, 443	No data
Oviduct histopathology	408, 415 (optional), 443	No data

EAS-mediated parameters	OECD Test guideline	Sufficiently investigated? **Overall conclusion: No (not sufficiently investigated)** Based on the lack of OECD 416 and 443 studies.
Prostate histopathology (with seminal vesicles and coagulating glands)	407, 408, 415 (optional) 421, 422, 426, 451-3, 416, 443	Prostate histopathology was performed in the 90-day toxicity studies in rodents (OECD TG 408) and 2-year carcinogenicity studies in rodents (OECD TG 453).
Prostate weight	407, 408, 421, 422, 416, 443	No data
Seminal vesicles histopathology	407, 408, 415 (optional), 422, 451-3, 416, 443	Seminal vesicles histopathology was performed in the 90-day toxicity studies in rodents (OECD TG 408) and 2-year carcinogenicity studies in rodents (OECD TG 453).
Seminal vesicles weight	407, 408, 421, 422, 416, 443	No data
Sperm morphology	408 (optional), 416, 443	No data
Sperm motility	408 (optional), 416, 443	No data
Sperm numbers	408 (optional), 416, 443	No data
Testis histopathology	407, 408, , 415 (optional) 421, 422, 451-3, 416, 443	Testis histopathology was performed in the subchronic 34, 90-day and 19 weeks toxicity studies in rodents, and in 2-year carcinogenicity studies in rodents.
Testis weight	407, 408, 421, 422, 451-3, 416, 443	Testes were weighted in the 34 days and 19-week subchronic toxicity study in rats, but no data were presented.
Uterus histopathology (with cervix)	407, 408, 415 (optional), 421 (optional), 422, 451-3, 416, 443	Uterus histopathology was performed in the 90-day toxicity studies (OECD TG 408) in rodents and 2-year carcinogenicity studies (OECD TG 453) in rodents.
Uterus weight (with cervix)	407 (optional), 408, 414 (gravid uterus), 415 (optional), 421 (optional), 422, 451-3, 416, 443	Uterus weight was measured in the reproductive developmental toxicity studies conducted with eugenol (OECD TG 414) in rats and rabbits.

EAS-mediated parameters	OECD Test guideline	Sufficiently investigated? **Overall conclusion: No (not sufficiently investigated)** Based on the lack of OECD 416 and 443 studies.
Vagina histopathology	407, 408, 415 (optional), 422, 451-3, 416, 443	No data
Vaginal smear	407 (optional), 408, 421, 422, 416, 443	No data

Table 2.10.2.2.3/2: EAS-mediated parameters not measured

ECD TG 407 - EAS-mediated parameters not investigated
- Cervix histopathology - Coagulating gland histopathology - Coagulating gland weight - Epididymis histopathology - Epididymis weight - Mammary gland histopathology (male and female) - Ovary histopathology - Prostate histopathology - Prostate weight - Seminal vesicles histopathology - Seminal vesicles weight. -Testis histopathology. - Testis weight - Uterus histopathology (with cervix) - Vaginal histopathology
OECD TG 408 - EAS-mediated parameters not investigated
- Accessory sex organs histopathology. - Cervix histopathology. - Coagulating gland histopathology - Epididymis weight - Oestrus cyclicity - Ovary weight - Oviduct histopathology. - Prostate weight - Seminal vesicles weight. - Uterus weight (with cervix) - Vaginal histopathology - Vaginal smear
OECD TG 453 - EAS-mediated parameters not investigated
- Accessory sex organs. - Cervix histopathology. - Coagulating gland histopathology. - Epididymis weight. - Ovary weight. - Vaginal histopathology.
OECD TG 414 - EAS-mediated parameters not investigated

- Anogenital distance measurement	- Genital abnormalities

Regarding to the EAS-mediated endocrine activity:

E-modality: It is considered sufficiently investigated based on the estrogenic activity output data from the US EPA ToxCast Bioactivity Model. However, further data need to be generated to clarify the potential anti-estrogenic activity observed in the *vitro* estrogen/androgen receptor binding assay conducted by Howes *et al.* (ID:74). A study in line with OECD TG 455 is required.

A-modality: It is not considered sufficiently investigated based on the lack of the "Stably transfected human androgen receptor transcriptional activation assay" (OECD TG 458).

S-modality: It is not considered sufficiently investigated based on the lack of the H295R Steroidogenesis assay (OECD TG 456) and/or a study in line with OPPTS 890.1200 (Aromatase assay).

Therefore, both EAS-mediated adversity and EAS-mediated endocrine activity have not been sufficiently investigated.

2.10.2.2.4 Lines of evidence for adverse effects and endocrine activity related to EAS-modality.

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
In vitro mechanistic	14	Estrogen receptor	Human	80 hours	Uptake from the medium (in vitro)		No effect	No effect	Eugenol showed positive results in two estrogen receptor bioactivation assays. Moreover, estrogen receptor binding assays revealed that eugenol is an ER-antagonist, in which eugenol was able to weakly compete with 17β-estradiol for binding to both the ERα and ERβ. Additionally, eugenol showed dose-dependent anti-estrogenic activity in yeast screen assay.	The weight of evidence of the *in vitro* EAS-modalities showed that eugenol could be a potential ER-antagonist, without ruling out a possible interaction with components of the steroidogenesis pathway. Further information is needed in order to clarify these endpoints.	E
	15	Estrogen receptor	Human	80 hours	Uptake from the medium (in vitro)		No effect	No effect			
	16	Estrogen receptor	Human	24 hours	Uptake from the medium (in vitro)	43.26 µM	Change	Eugenol is active for estrogen (ESR1) mRNA induction assay. AC50 (hill model)= 43.26 µM.			
	17	Estrogen receptor	Human	24 hours	Uptake from the medium (in vitro)	24.58 µM	Change	Eugenol is active for estrogen (ESR1) mRNA induction assay. AC50 (hill model)= 24.58 µM. The assay presents two flags: Borderline active and less than 50% efficacy			
	18	Estrogen receptor	Human	18 hours	Uptake from the medium (in vitro)		No effect	No effect			
	19	Estrogen receptor	Human	8 hours	Uptake from the medium (in vitro)		No effect	No effect			
	20	Estrogen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	21	Estrogen receptor	Human	8 hours	Uptake from the medium (in vitro)		No effect	No effect			
	22	Estrogen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	23	Estrogen receptor	Human	8 hours	Uptake from the medium (in vitro)		No effect	No effect			
	24	Estrogen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	25	Estrogen receptor	Human	2 hours	Uptake from the medium (in vitro)		No effect	No effect			
	26	Estrogen receptor	Human	8 hours	Uptake from the medium (in vitro)		No effect	No effect			
	27	Estrogen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	28	Estrogen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	29	Estrogen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	30	Estrogen receptor	Human	22 hours	Uptake from the medium (in vitro)		No effect	No effect			
	31	Estrogen receptor	Human	22 hours	Uptake from the medium (in vitro)		No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	32	Estrogen receptor	Human	22 hours	Uptake from the medium (in vitro)		No effect	No effect			
	74	Estrogen receptor	Yeast		Uptake from the medium (in vitro)		Induction	A dose-dependent anti-estrogenic activity (without cytotoxicity) was observed; inhibits stimulation of ß-galactosidase activity induced by 17ß-estradiol			
	74	Estrogen receptor	Yeast		Uptake from the medium (in vitro)		No effect	no estrogenic activity in the estrogen-responsive yeast screen			
	74	Estrogen receptor	recombinant human ERα and ERß		Uptake from the medium (in vitro)		Induction	Eugenol was able to compete with [2,4,6,7-3H]17 -estradiol for binding to ERα and ERß, but required concentrations in the order of 10E4 to 10E5 times higher than 17 β-estradiol.			
	33	Androgen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect	Eugenol displayed positive result in one androgen receptor		A

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	34	Androgen receptor	chimpanzee	72 hours	Uptake from the medium (in vitro)	19.08 µM	Change	Eugenol is active for androgen binding assay (AR). AC50 (hill model)= 19.08 µM. The assay presents the flag: Less than 50% efficacy.	binding assay. However, this assay was flagged as having less than 50% efficacy. Thus, it makes the results of this experiment no conclusive regarding a potential disruption effect of eugenol with AR.		
	35	Androgen receptor	Chinese Hamster	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	36	Androgen receptor	Human	8 hours	Uptake from the medium (in vitro)		No effect	No effect			
	37	Androgen receptor	Human	16 hours	Uptake from the medium (in vitro)		No effect	No effect			
	38	Androgen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	39	Androgen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	40	Androgen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	41	Androgen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	42	Androgen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	43	Androgen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	44	Androgen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	45	Androgen receptor	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	46	Androgen receptor	Human	3 hours	Uptake from the medium (in vitro)		No effect	No effect			
	47	Androgen receptor	Human	3 hours	Uptake from the medium (in vitro)		No effect	No effect			
	74	Androgen receptor	Yeast		Uptake from the medium (in vitro)		No effect	No evidence for stimulation of ß-galactosidase activity in androgen screen and no anti-androgenic activity			
	56	Corticosterone	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect	The available *in vitro* mechanistic bioassay data shows that eugenol may have some potential to interact with components of the steroidogenesis pathway *in vitro*, potentially affecting		S
	57	Corticosterone	Human	48 hours	Uptake from the medium (in vitro)	43.56 µM	Change	Eugenol is active for corticosterone (NR3C1) induction assay. AC50 (hill model)= 43.56 µM			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	60	11-Deoxycorticosterone (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect	the production of estradiol, estrone and glucocorticoids.		
	61	11-Deoxycorticosterone (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	48	11-Deoxycortisol (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	49	11-Deoxycortisol (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	50	17-alpha-hydroxypregnelone (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	51	17-alpha-hydroxypregnelone (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	52	17-alpha-hydroxyprogesterone (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	53	17-alpha-hydroxyprogesterone (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	54	Androstenedione (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	55	Androstenedione (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	6	ATP content_cell cycle_cell death	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	71	Cellular proliferation	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	72	Cellular proliferation	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	73	Cellular proliferation	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	58	Cortisol (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	59	Cortisol (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	70	CYP19	Human	24 hours	Uptake from the medium (in vitro)		No effect	No effect			
	62	Estradiol level (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	63	Estradiol level (in vitro)	Human	48 hours	Uptake from the medium (in vitro)	32.56 µM	Change	Eugenol is active for estradiol (ESR1) induction assay. AC50 (hill model)= 32.56 µM. The assay presents the flag: Borderline active			
	64	Estrone (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	65	Estrone (in vitro)	Human	48 hours	Uptake from the medium (in vitro)	36.26 µM	Change	Eugenol is active for estrone (ESR1) induction assay. AC50 (hill model)= 36.26 µM. The assay presents the flag: Borderline active and only highest concentration above baseline, active			
	66	Progesterone (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	67	Progesterone (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	68	Testosterone level (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
	69	Testosterone level (in vitro)	Human	48 hours	Uptake from the medium (in vitro)		No effect	No effect			
In vivo mechanistic	75	Estradiol level	Rat	15 day	intramuscular injection	0.4 ml/day	Increase	Eugenol treatment increased serum estradiol levels (87%) compared to controls	Eugenol increased estradiol and progesterone levels, whereas decreased testosterone levels in	No *in vivo* mechanistic assays level 3 were presented. A non-guideline single dose	EAS

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	75	Progesterone	Rat	15 day	intramuscular injection	0.4 ml/day	Increase	Eugenol treatment increased serum progesterone levels (48%) compared to controls	a single dose assay in female rats throughout 15 days schedule administration.	study in rats showed changes in sex hormones. No reliability warranted.	
	75	Testosterone level	Rat	15 day	intramuscular injection	0.4 ml/day	Decrease	Eugenol treatment decreased serum testosterone levels (78%) compared to controls			
EAS-mediated	81	Accessory sex organs histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed	Increased incidences of endometrial stromal polyps/sarcoma, and cystic hyperplasia were detected in the uterus of rats. Moreover, an increased incidences of follicular cysts was recorded in the ovaries of mice. A potential EAS-adverse effect might be further investigated. No further alterations were found in reproductive organs and associated parameters.	EAS
	82	Accessory sex organs histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	87	Ano-Genital distance	rat	10 day	Oral		Not measured	Not measured	Not measured		
	88	Ano-Genital distance	rabbit	13 day	Oral		Not measured	Not measured			
	89	Ano-Genital distance	mouse	11 day	Oral		Not measured	Not measured			
	90	Ano-Genital distance	hamster	10 day	Oral		Not measured	Not measured			
	91	Ano-Genital distance	Rat	15 day	Oral		Not measured	Not measured			
	92	Ano-Genital distance	Rabbit	24 day	Oral		Not measured	Not measured			
	79	Coagulating gland histopathology	Rat	28 day	Oral		Not measured	Not measured	No treatment-related effects were observed		
	79	Coagulating gland histopathology	Rat	28 day	Oral		Not measured	Not measured			
	80	Coagulating gland histopathology	Rat	34 day	Oral		Not measured	Not measured			
	80	Coagulating gland histopathology	Rat	34 day	Oral		Not measured	Not measured			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	81	Coagulating gland histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Coagulating gland histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Coagulating gland histopathology	Rat	2 year	Oral		Not measured	Not measured			
	85	Coagulating gland histopathology	Mouse	2 year	Oral		Not measured	Not measured			
	79	Cowper's gland weight	Rat	28 day	Oral		Not measured	Not measured	Not measured		
	80	Cowper's gland weight	Rat	34 day	Oral		Not measured	Not measured			
	79	Epididymis histopathology	Rat	28 day	Oral		Not measured	Not measured	No treatment-related effects were observed		
	80	Epididymis histopathology	Rat	34 day	Oral		Not measured	Not measured			
	81	Epididymis histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Epididymis histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Epididymis histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Epididymis histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	79	Epididymis weight	Rat	28 day	Oral		Not measured	Not measured			
	80	Epididymis weight	Rat	34 day	Oral		Not measured	Not measured			
	81	Epididymis weight	Rat	13 week	Oral		Not measured	Not measured			
	82	Epididymis weight	Mouse	13 week	Oral		Not measured	Not measured			
	84	Epididymis weight	Rat	2 year	Oral		Not measured	Not measured			
	85	Epididymis weight	Mouse	2 year	Oral		Not measured	Not measured			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	75	Estrus cyclicity	Rat	15 day	Intramuscular injection	0.4 ml/day	Increase	Eugenol enhanced the duration of proestrus, metestrus, diestrus phases, and total duration of cycles (29%, 22%, 16% and 17%, respectively) compared with controls	Eugenol increased duration estrus cyclicity in a single dose assay in female rats throughout 15 days schedule administration		
	81	Estrus cyclicity	Rat	13 week	Oral		Not measured	Not measured			
	82	Estrus cyclicity	Mouse	13 week	Oral		Not measured	Not measured			
	87	Genital abnormalities	rat	10 day	Oral		Not measured	Not measured	Not measured		
	88	Genital abnormalities	rabbit	13 day	Oral		Not measured	Not measured			
	89	Genital abnormalities	mouse	11 day	Oral		Not measured	Not measured			
	90	Genital abnormalities	hamster	10 day	Oral		Not measured	Not measured			
	91	Genital abnormalities	Rat	15 day	Oral		Not measured	Not measured			
	92	Genital abnormalities	Rabbit	24 day	Oral		Not measured	Not measured			
	81	Mammary gland histopathology (female)	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were		

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Mammary gland histopathology (female)	Rat	2 year	Oral	6000 ppm	Decrease	Fibroadenomas of the mammary gland in female rat were decreased in low and high dose groups compared to controls.	observed in mammary gland.		
	85	Mammary gland histopathology (female)	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	86	Mammary gland histopathology (female)	Mouse	12 (+6 untreated) months	Oral	750 mg/kg bw/day	Increase	1/30 (3%) treated mice developed mammary adenoacanthoma; the effects were not considered to be treatment related			
	81	Mammary gland histopathology (male)	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Mammary gland histopathology (male)	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Mammary gland histopathology (male)	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Mammary gland histopathology (male)	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Ovary histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	Increase of ovary follicular cysts was noted in the top dose group in 2-year mice study. However, no HCD was provided and this finding was not supported by statistical analysis.		
	82	Ovary histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Ovary histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Ovary histopathology	Mouse	2 year	Oral	6000 ppm	Increase	Increase of ovary follicular cysts in the top dose female group compared with			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
								controls (33% vs 22% in controls).			
	75	Ovary weight	Rat	15 day	Intramuscular injection	0.4 ml/day	Increase	Eugenol treatment increased ovary somatic index (32%) compared to controls			
	78	Ovary weight	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect			
	81	Ovary weight	Rat	13 week	Oral		Not measured	Not measured			
	82	Ovary weight	Mouse	13 week	Oral		Not measured	Not measured			
	84	Ovary weight	Rat	2 year	Oral		Not measured	Not measured			
	85	Ovary weight	Mouse	2 year	Oral		Not measured	Not measured			
	79	Prostate histopathology (with seminal vesicles and coagulating glands)	Rat	28 day	Oral		Not measured	Not measured	No treatment-related effects were observed in prostate.		
	80	Prostate histopathology (with seminal vesicles and coagulating glands)	Rat	34 day	Oral		Not measured	Not measured			
	81	Prostate histopathology (with seminal vesicles and coagulating glands)	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Prostate histopathology (with seminal vesicles and coagulating glands)	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Prostate histopathology (with seminal vesicles and coagulating glands)	Rat	2 year	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	85	Prostate histopathology (with seminal vesicles and coagulating glands)	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	79	Prostate weight	Rat	28 day	Oral		Not measured	Not measured			
	80	Prostate weight	Rat	34 day	Oral		Not measured	Not measured			
	81	Prostate weight	Rat	13 week	Oral		Not measured	Not measured			
	79	Seminal vesicles histopathology	Rat	28 day	Oral		Not measured	Not measured	No treatment-related effects were observed in seminal vesicles.		
	80	Seminal vesicles histopathology	Rat	34 day	Oral		Not measured	Not measured			
	81	Seminal vesicles histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Seminal vesicles histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Seminal vesicles histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Seminal vesicles histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	79	Seminal vesicles weight	Rat	28 day	Oral		Not measured	Not measured			
	80	Seminal vesicles weight	Rat	34 day	Oral		Not measured	Not measured			
	81	Seminal vesicles weight	Rat	13 week	Oral		Not measured	Not measured			
	82	Seminal vesicles weight	Mouse	13 week	Oral		Not measured	Not measured			
	79	Testis histopathology	Rat	28 day	Oral		Not measured	Not measured	No treatment-related effects were observed in testes.		
	80	Testis histopathology	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	83	Testis histopathology	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Testis histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Testis histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	79	Testis weight	Rat	28 day	Oral		Not measured	Not measured			
	80	Testis weight	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	81	Testis weight	Rat	13 week	Oral		Not measured	Not measured			
	82	Testis weight	Mouse	13 week	Oral		Not measured	Not measured			
	83	Testis weight	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	84	Testis weight	Rat	2 year	Oral		Not measured	Not measured			
	85	Testis weight	Mouse	2 year	Oral		Not measured	Not measured			
	78	Uterus histopathology (with cervix)	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	Increased incidences of endometrial stromal polyps/sarcoma, and cystic hyperplasia were detected in the		
	81	Uterus histopathology (with cervix)	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Uterus histopathology (with cervix)	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Uterus histopathology (with cervix)	Rat	2 year	Oral	12500 ppm	Increase	There was a positive trend (P<0.05) and a (P=0.051) increased incidence of endometrial stromal polyps/sarcoma of the uterus of female rats observed in the high dose group (uterine cystic hyperplasia). The 34% incidence in the high dose group was above the historical average for the laboratory.	uterus after eugenol long-term exposure.		
	84	Uterus histopathology (with cervix)	Rat	2 year	Oral	12500 ppm	Increase	There was an increase of cystic hyperplasia in the uterus of high dose female group (22% vs 3% in controls), and a slight increase in the low dose group (4%) compared with controls.			
	85	Uterus histopathology (with cervix)	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	81	Uterus weight (with cervix)	Rat	13 week	Oral		Not measured	Not measured			
	82	Uterus weight (with cervix)	Mouse	13 week	Oral		Not measured	Not measured			
	85	Uterus weight (with cervix)	Mouse	2 year	Oral		Not measured	Not measured			
	87	Uterus weight (with cervix)	rat	10 day	Oral		Not measured	Not measured			
	88	Uterus weight (with cervix)	rabbit	13 day	Oral		Not measured	Not measured			
	89	Uterus weight (with cervix)	mouse	11 day	Oral		Not measured	Not measured			
	90	Uterus weight (with cervix)	hamster	10 day	Oral		Not measured	Not measured			
	91	Uterus weight (with cervix)	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Uterus weight (with cervix)	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	81	Vagina histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment related effect was observed.		
	82	Vagina histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Vagina histopathology	Rat	2 year	Oral		Not measured	Not measured			
	78	Vaginal smears	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	75	[Not in list]	Rat	15 day	Intramuscular injection	0.4 ml/day	Change	Eugenol treatment increased total protein levels in ovary (68%) and uterus (66%), and decreased in vagina (18%) compared to controls.			
	75	[Not in list]	Rat	15 day	Intramuscular injection	0.4 ml/day	Change	Eugenol treatment decreased total carbohydrate levels in ovary (21%), and increased in uterus (19%), and vagina (39%) compared to controls.			
	75	[Not in list]	Rat	15 day	Intramuscular injection	0.4 ml/day	Change	Eugenol treatment increased total lipid levels in ovary (92%) and uterus (59%), and decreased in vagina (14%) compared to controls.			
Sensitive to, but not diagnostic of, EATS	78	Adrenals histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	There was a slight enlargement of adrenals gland in rats	No adverse effects were detected in	N

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	80	Adrenals histopathology	Rat	34 day	Oral		Change	The evaluation document reported that the slight enlargement of the adrenal glands was observed in treated rats with marked yellow discolouration. The doses in which effects were observed had not been specified in the study.	after 34-day eugenol treatment. No other changes were reported in adrenals gland in other short-term or long-term toxicity studies with eugenol. This finding was considered equivocal.	sensitive EATS-related parameters.	
	81	Adrenals histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Adrenals histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Adrenals histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Adrenals histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	81	Adrenals weight	Rat	13 week	Oral		Not measured	Not measured			
	82	Adrenals weight	Mouse	13 week	Oral		Not measured	Not measured			
	84	Adrenals weight	Rat	2 year	Oral			Not measured			
	85	Adrenals weight	Mouse	2 year	Oral			Not measured			
	81	Brain histopathology examination	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed		
	82	Brain histopathology examination	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Brain histopathology examination	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Brain histopathology examination	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	81	Brain weight	Rat	13 week	Oral		Not measured	Not measured			
	82	Brain weight	Mouse	13 week	Oral		Not measured	Not measured			
	84	Brain weight	Rat	2 year	Oral		Not measured	Not measured			
	85	Brain weight	Mouse	2 year	Oral		Not measured	Not measured			
	87	Fetal development	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect	No treatment-related effects were observed on foetal development.		
	88	Fetal development	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Fetal development	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Fetal development	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	91	Fetal development	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Fetal development	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	91	Gestation length	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	92	Gestation length	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	91	Litter size	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Litter size	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	87	Litter viability	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Litter viability	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Litter viability	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Litter viability	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	91	Litter viability	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Litter viability	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	87	Litter/pup weight	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Litter/pup weight	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Litter/pup weight	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	90	Litter/pup weight	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	91	Litter/pup weight	Rat	15 day	Oral	600 mg/kg bw/day	Decrease	The mean foetal weights in litters of rats treated at 600 mg/kg bw/day were slightly but significantly reduced (↓6%), but was not associated with any significant delay in skeletal development.			
	92	Litter/pup weight	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	87	Number of implantations, corpora lutea	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Number of implantations, corpora lutea	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Number of implantations, corpora lutea	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Number of implantations, corpora lutea	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	91	Number of implantations, corpora lutea	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	92	Number of implantations, corpora lutea	Rabbit	24 day	Oral	500 (350) mg/kg bw/day	No effect	No effect			
	87	Number of live births	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Number of live births	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Number of live births	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Number of live births	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	87	Numbers of embryonic or foetal deaths and viable foetuses	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Numbers of embryonic or foetal deaths and viable foetuses	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Numbers of embryonic or foetal deaths and viable foetuses	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Numbers of embryonic or foetal deaths and viable foetuses	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	92	Numbers of embryonic or foetal deaths and viable foetuses	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	91	Post implantation loss	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	92	Post implantation loss	Rabbit	24 day	Oral	500 (350) mg/kg bw/day	No effect	No effect			
	91	Pre implantation loss	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Pre implantation loss	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	87	Sex ratio	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Sex ratio	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Sex ratio	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Sex ratio	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	91	Sex ratio	Rat	15 day	Oral	>600 mg/kg bw/day	No effect	No effect			
	92	Sex ratio	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
	78	Pituitary histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	No treatment-related effects were observed.		
	81	Pituitary histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Pituitary histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Pituitary histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Pituitary histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Pituitary histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Pituitary histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Pituitary weight	Rat	13 week	Oral		Not measured	Not measured			
	82	Pituitary weight	Mouse	13 week	Oral		Not measured	Not measured			
	84	Pituitary weight	Rat	2 year	Oral		Not measured	Not measured			
	85	Pituitary weight	Mouse	2 year	Oral		Not measured	Not measured			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	87	Presence of anomalies (external, visceral, skeletal)	rat	10 day	Oral	13 mg/kg bw/day	Increase	Increased incidences regarding ossification of ribs (mostly wavy; non-clearly dose-related) were described in all clove-oil treated groups. Increased incidences of incomplete closure of skull were recorded in 13 (36%), 60 (82%) and 280 (100%) mg/kg bw/day dose groups. No statistical analysis performed.	Developmental toxicity studies were performed in rats, rabbits, mice and hamsters with clove oil. Content of eugenol in clove oil was not presented. Moreover, statistical analysis was not performed in the skeletal abnormalities analysis, and HCD was not provided. Overall, the results were difficult to interpret.		
	88	Presence of anomalies (external, visceral, skeletal)	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Presence of anomalies (external, visceral, skeletal)	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	90	Presence of anomalies (external, visceral, skeletal)	hamster	10 day	Oral	177 mg/kg bw/day	Increase	Increased non-clear dose-related ossification incidences in the sternebrae were recorded in the clove oil-treated groups (20%, 27%, 40% and 31% for 1.8, 8.2, 38.2 and 177 mg/kg bw/day dose groups, respectively). Moreover, increased incomplete ossification incidences of the extremities were described in the low and top dose groups (24 and 80%, respectively), whereas hyoid reduced or missing incidences were noted only in the top dose group (48%). No statistical analysis performed.			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	91	Presence of anomalies (external, visceral, skeletal)	Rat	15 day	Oral	600 mg/kg bw/day	Decrease	A statistically significant reduction in the percentage of foetuses with more than 6 ossified metatarsals occurred in litters of rats treated at 600 mg/kg bw/day. This finding was seen in isolation of any other effects on skeletal development.	Reduction in the percentage of foetuses with more than 6 ossified metatarsals was noted in foetuses of high dose treated dam in the rat study.		
	92	Presence of anomalies (external, visceral, skeletal)	Rabbit	24 day	Oral	>500 (350) mg/kg bw/day	No effect	No effect			
Target organ toxicity	81	Bone marrow histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.	The main effects were detected in liver of female mouse in form of hepatocellular adenomas/carcinomas, however, these incidences were within the HCD. Single observation of focal inflammation were noted in kidney and lung, however, these incidences were not reproduced in other toxicity studies.	
	82	Bone marrow histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Bone marrow histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Bone marrow histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	80	Heart histopathology	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect	No treatment-related effects were observed.		

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	81	Heart histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Heart histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	83	Heart histopathology	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	84	Heart histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Heart histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	80	Heart weight	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	83	Heart weight	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	78	Kidney histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	Increased incidences of focal kidney inflammation were detected in 2-year mice study. These findings were not recorded in other repeated-dose studies		
	80	Kidney histopathology	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	81	Kidney histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Kidney histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	83	Kidney histopathology	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	84	Kidney histopathology	Rat	2 year	Oral	>6000 ppm	No effect	A non-clear dose-related increase of chronic inflammation			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
								incidences of kidney was noted in male rats dose-groups			
	84	Kidney histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect in females dose groups			
	85	Kidney histopathology	Mouse	2 year	Oral	6000 ppm	Increase	Increased incidence of focal inflammation of the kidney was observed in male mice treated at the top dose of 6000 ppm.			
	85	Kidney histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	80	Kidney weight	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	83	Kidney weight	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	78	Liver histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	In mice long-term toxicity study, increased incidences of hepatocellular adenomas/ carcinomas were recorded in top dose female group No other alterations were reported in livers in other short-term or		
	81	Liver histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Liver histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	83	Liver histopathology	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Liver histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect	long-term toxicity studies with eugenol.		
	84	Liver histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Liver histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	Hepatocellular adenomas and hepatocellular carcinomas of the liver were increased in low dose male mice; both combined incidences in males strengthened the evidence for an increased incidence in low dose mice. The rates in the high dose group were not different from those observed in controls. A dose-related trend was not observed.			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	85	Liver histopathology	Mouse	2 year	Oral	6000 ppm	Increase	The incidences of hepatocellular adenomas/ carcinomas (combined) were significantly increased in female mice at top dose level, and showed a statistically significant trend.			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	86	Liver histopathology	Mouse	4 Single dose on day 1,8,15 and 22 of life	Oral	> ca 60 mg/kg bw/day	No effect	No effect			
	78	Lung histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	The increase of focal granulomatous inflammation in female mice, was considered a single event due to no further alterations		
	81	Lung histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Lung histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Lung histopathology	Rat	2 year	Oral	>6000 ppm	No effect	The incidence of alveolar/bronchiolar adenomas or carcinomas was significantly increased in male rats treated at the low dose 3000 pm. No significant increase was observed in the high dose (6000 ppm) group. No dose-response relationship was observed.	were found in lung in other studies		
	84	Lung histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Lung histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	85	Lung histopathology	Mouse	2 year	Oral	6000 ppm	Increase	Increased incidence of granulomatous inflammation of the lung were seen in females treated at the top dose of 6000 ppm.			
	86	Lung histopathology	Mouse	12 (+6 untreated) months	Oral	>750 mg/kg bw/day	No effect	No effect			
	81	Lymph nodes histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Lymph nodes histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Lymph nodes histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Lymph nodes histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Lymph nodes histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Lymph nodes histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Oesophagus histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Oesophagus histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Oesophagus histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Oesophagus histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Oesophagus histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Oesophagus histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	78	Pancreas histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	No treatment-related effects were observed.		
	81	Pancreas histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Pancreas histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Pancreas histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Pancreas histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Pancreas histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Pancreas histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Peripheral nerve histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Peripheral nerve histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Peripheral nerve histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Peripheral nerve histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Peripheral nerve histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Peripheral nerve histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Salivary glands histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Salivary glands histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Salivary glands histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Salivary glands histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Salivary glands histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Salivary glands histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Skin histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Skin histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Skin histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Skin histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Skin histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Skin histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Spinal cord histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Spinal cord histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Spinal cord histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Spinal cord histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Spinal cord histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Spinal cord histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	80	Spleen histopathology	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect	The spleen haemosiderosis described in 2-year study in rats were not supported by clinical biochemistry nor haematology analysis. Moreover, no further alterations were found in spleen in other studies.		
	81	Spleen histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Spleen histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	83	Spleen histopathology	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Spleen histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Spleen histopathology	Rat	2 year	Oral	12500 ppm	Increase	Increased incidence of splenic haemosiderosis was observed in female rats treated at the top dose of 12500 ppm			
	85	Spleen histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Spleen histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	80	Spleen weight	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	83	Spleen weight	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	78	Stomach histopathology	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect	Hyperkeratosis and hyperplasia in forestomach was		

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	80	Stomach histopathology	Rat	34 day	Oral		Change	The evaluation document reported that in macroscopic examinations, mucosa of the forestomach contained coalescent areas covered with a thick, flaky, white material punctuated with minute ulcers. Microscopic examinations of the forestomach showed moderately severe hyperplasia and hyperkeratosis of the stratified squamous epithelium associated with focal ulceration. The doses in which effects were observed had not been specified in the study.	observed in rats in the 34-day repeated dose study conducted with eugenol. In the rat, mouse and hamster the forestomach occupies about two-thirds of the proximal area of the stomach and is lined by cornified, stratified squamous epithelium, and acts as a storage organ releasing relatively undigested food into glandular stomach in response to energy demand. This organ is also present in ruminants and camelids as dilation and modification of the esophagus. Thus, humans lack forestomach and consequently, the observed changes in rats do not present toxicological relevance for humans. In all likelihood, the effects observed in rats were triggered by eugenol gavage administration. Eugenol is classified		

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	81	Stomach histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	as skin sensitizer (H317), and causes severe/moderate/mild skin effects in human, rabbit and pig, and likely the direct eugenol application on forestomach mucosa causes epithelium damage.		
	82	Stomach histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Stomach histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Stomach histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Stomach histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Stomach histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	81	Thymus histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Thymus histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Thymus histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Thymus histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Thymus histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Thymus histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	86	Thymus histopathology	Mouse	12 (+6 untreated) months	Oral	750 mg/kg bw/day	Increase	2/30 (7%) treated mice developed thymic lymphomas; the effects were not considered to be treatment related			
	81	Trachea histopathology	Rat	13 week	Oral	>12500 ppm	No effect	No effect	No treatment-related effects were observed.		
	82	Trachea histopathology	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Trachea histopathology	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Trachea histopathology	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Trachea histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			
	85	Trachea histopathology	Mouse	2 year	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
Systemic toxicity	76	Body weight	Rat	14 day	Oral	100000 ppm	Decrease	A decrease in mean bodyweight was observed in males at high dose 100000 ppm.	Signs of systemic toxicity occurred mainly at high doses, which included mortality, effects on bodyweight, food consumption, and clinical signs; these signs were related to general toxicity of higher doses. However, a case by case approach may be done, as toxic adverse effects were not observed in all studies.	Overall evidence of systemic toxicity.	
	77	Body weight	Mouse	14 day	Oral	25000 ppm	Decrease	A dose-related decrease in mean bodyweight >10% was observed in mice at doses ≥25000 ppm			
	79	Body weight	Rat	28 day	Oral	2000 mg/kg bw/day	Decrease	Bodyweight gain was depressed by 10-15% in treated rats			
	80	Body weight	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	81	Body weight	Rat	13 week	Oral	12500 ppm	Decrease	Final body weights were 10% less for male rats and 6% less in females rats in top dose group, as compared to controls; it was not reported whether the findings were significant. A decrease (12%) in bodyweight gain was detected in top dose male group.			
	82	Body weight	Mouse	13 week	Oral	6000 ppm	Decrease	A decrease in bodyweight gain was detected in the top dose male group (10%)			
	83	Body weight	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	84	Body weight	Rat	2 year	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Body weight	Rat	2 year	Oral	12500 ppm	Decrease	Bodyweights in female rats were reduced in the high dose group (12500 ppm) throughout the study			
	86	Body weight	Mouse	12 (+6 untreated) months	Oral	750 mg/kg bw/day	Decrease	Bodyweight gain was decreased at 4 and 8 months measurements (13% and 15%) compared with controls.			
	87	Body weight	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Body weight	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Body weight	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Body weight	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	91	Body weight	Rat	15 day	Oral	600 mg/kg bw/day	No effect	No effect			
	92	Body weight	Rabbit	24 day	Oral	>500/350 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	79	Clinical chemistry and haematology	Rat	28 day	Oral			Not measured			
	80	Clinical chemistry and haematology	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	83	Clinical chemistry and haematology	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	84	Clinical chemistry and haematology	Rat	2 year	Oral			Not measured			
	84	Clinical chemistry and haematology	Rat	2 year	Oral			Not measured			
	87	Clinical chemistry and haematology	rat	10 day	Oral			Not measured			
	88	Clinical chemistry and haematology	rabbit	13 day	Oral			Not measured			
	89	Clinical chemistry and haematology	mouse	11 day	Oral			Not measured			
	90	Clinical chemistry and haematology	hamster	10 day	Oral			Not measured			
	91	Clinical chemistry and haematology	Rat	15 day	Oral			Not measured			
	92	Clinical chemistry and haematology	Rabbit	24 day	Oral			Not measured			
	76	Clinical signs	Rat	14 day	Oral	>100000 ppm	No effect	No effect			
	77	Clinical signs	Mouse	14 day	Oral	>100000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	78	Clinical signs	Dog	21 day	Oral	250 mg/kg bw/day	Change	Dogs received a single dose. There were no consistent findings across the study but there were indications of increased pulse rats and reduced body temperature. Vomiting was seen at the top two dose levels (250 and 500 mg/kg bw/day).			
	78	clinical signs	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect			
	79	Clinical signs	Rat	28 day	Oral		No effect	No effect			
	80	Clinical signs	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	81	Clinical signs	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Clinical signs	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	83	Clinical signs	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Clinical signs	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Clinical signs	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	87	Clinical signs	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Clinical signs	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Clinical signs	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Clinical signs	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	91	Clinical signs	Rat	15 day	Oral	250 mg/kg bw/day	Change	Piloerection, ataxia, prostration, lethargy, noisy respiration, increased lachrymation were observed in rats treated at 600 mg/kg bw/day within 1 hr of dosing, resolved within 24 hrs. Piloerection and hunched posture were observed in rats treated at 250 mg/kg bw/day			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	92	Clinical signs	Rabbit	24 day	Oral	250 mg/kg bw/day	Change	One abortion, post dosing ataxia, breathing difficulties, pallor of the extremities, eyes and ears, and prostration were observed in rabbits treated at 500 (dosing reduced to 350 between GD 10 and 13) mg/kg bw/day. On the other hand, one abortion, ataxia and breathing difficulties were noted at 250 mg/kg bw/day. No significant treatment-related effects in adults treated at 100 mg/kg bw/day.			
	79	Food consumption	Rat	28 day	Oral			Not measured			
	80	Food consumption	Rat	34 day	Oral	>4000 mg/kg bw/day	No effect	No effect			
	81	Food consumption	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Food consumption	Mouse	13 week	Oral			Not measured			
	83	Food consumption	Rat	19 week	Oral	>900 mg/kg bw/day	No effect	No effect			
	84	Food consumption	Rat	2 year	Oral	>6000 ppm	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	84	Food consumption	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	87	Food consumption	rat	10 day	Oral			Not measured			
	88	Food consumption	rabbit	13 day	Oral			Not measured			
	89	Food consumption	mouse	11 day	Oral			Not measured			
	90	Food consumption	hamster	10 day	Oral			Not measured			
	91	Food consumption	Rat	15 day	Oral	600 mg/kg bw/day	Decrease	A reduction in food consumption occurred during the initial few days of dosing post-coitum in rats treated at 600 mg/kg bw/day. No effects were reported in rats treated at 250 or less mg/kg bw/day.			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	92	Food consumption	Rabbit	24 day	Oral	250 mg/kg bw/day	Decrease	A reduced trend in food consumption was observed in rabbits treated at 500 (dosing reduced to 350 between GD 10 and 13) and 250 mg/kg bw/day from the start of dosing to GD 18 (although results only were statistically significant through gestation day 6-12 and 9-12 for high and mid dose groups, respectively). No significant treatment-related effects in adults treated at 100 mg/kg bw/day.			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	76	Mortality	Rat	14 day	Oral	100000 ppm	Increase	At the highest dose tested of 100000 ppm, 20% of male and 100% of female rats died			
	77	Mortality	Mouse	14 day	Oral	50000 ppm	Increase	All mice treated at 100000 ppm and 60% of male mice at 50000 ppm died			
	78	Mortality	Dog	21 day	Oral	500 mg/kg bw/day	Increase	Dogs that received single dose. Ataxia in 3/4 dogs and deaths in 2/4 dogs were observed at the highest dose			
	78	Mortality	Dog	21 day	Oral	>100 mg/kg bw/day	No effect	No effect			
	79	Mortality	Rat	28 day	Oral		No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	80	Mortality	Rat	34 day	Oral	2000 mg/kg bw/day	Increase	A few deaths occurred at doses of 2000 mg/kg bw/day and the number increased with increasing doses. 8 animals survived to 34 days while 15 animals received the dose of 4000 mg/kg bw/day.			
	81	Mortality	Rat	13 week	Oral	>12500 ppm	No effect	No effect			
	82	Mortality	Mouse	13 week	Oral	>6000 ppm	No effect	No effect			
	84	Mortality	Rat	2 year	Oral	>6000 ppm	No effect	No effect			
	84	Mortality	Rat	2 year	Oral	>12500 ppm	No effect	No effect			
	85	Mortality	Mice	2 year	Oral	3000 ppm	Decrease	A reduction in survival was detected in low and high dose male groups (12%)			
	86	Mortality	Mouse	12 (+6 untreated) months	Oral	>750 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	87	Mortality	rat	10 day	Oral	>280 mg/kg bw/day	No effect	No effect			
	88	Mortality	rabbit	13 day	Oral	>172 mg/kg bw/day	No effect	No effect			
	89	Mortality	mouse	11 day	Oral	>215 mg/kg bw/day	No effect	No effect			
	90	Mortality	hamster	10 day	Oral	>177 mg/kg bw/day	No effect	No effect			
	91	Mortality	Rat	15 day	Oral	600 mg/kg bw/day	No effect	No effect			

Grouping	Study ID Matrix	Effect target	Species	Exposure	Route of exposure	Effect dose	Effect direction	Observed effect	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
	92	Mortality	Rabbit	24 day	Oral	250 mg/kg bw/day	Change	5 rabbits treated at 500 mg/kg bw/day died. The top dose was subsequently reduced to 350 mg/kg bw/day (between GD 10 and 13). One further female treated at 350 mg/kg bw/day was killed in extremis following abortion of her offspring on GD 25. 1 rabbit treated at 250 mg/kg bw/day died after showing clinical signs. Two further females of mid dose group were killed in extremis as consequence of dosing error and after abortion on GD 23, respectively.			

2.10.2.2.5 Assessment of the integrated lines of evidence and weight of evidence for EAS-mediated adversity and endocrine activity.

WoE for EAS-mediated adversity

This section provides the lines of evidence for the *in vivo* mammalian toxicology studies (Level 4 and 5) using test substance eugenol in respect of the EAS-modality. The following sections provide an analysis of the integrated lines of evidence and report the weight of evidence in respect of EAS-mediated adversity.

Regarding EAS-mediated parameters

-The main *in vivo* effects of concern were recorded in the 2-year carcinogenicity study conducted in rats with eugenol (ID: 84). In this study an increased incidence of uterine endometrial stromal polyp or sarcoma and cystic hyperplasia were described in the uterus at top dose level.

Statistically significant increase trend in the incidence of uterine endometrial stromal polyp or sarcoma was observed in the high female dose group (15%, 12% and 34% for controls, low and high dose groups, respectively). In the 12500 ppm dose female group, the incidence was non-statistically significant compared to controls (p=0.051), but as noted, the p value was just in the border of the statistical significance. However, this value was higher than mean incidence of HCD reported by Southern Research Institute (15%) If we analyse the incidences individually, the incidence of uterine endometrial stromal polyps was 15, 12 and 32% for control, low and high dose female groups, respectively. On the other hand, the incidence of uterine endometrial stromal sarcoma was 0, 0 and 2% for control, low and high dose female groups, respectively.

On the other hand, increase incidence of cystic hyperplasia was detected in the uterus of high dose female rat group (3%, 4% and 22% for control, low and high dose groups, respectively). In the 2-year carcinogenicity study conducted in mice (ID: 85), an increase of ovary follicular cysts was noted in the top dose female group (22%, 22% and 33% for control, low and high dose groups, respectively). However, historical control data were not provided for these lesions, and no statistical analysis was performed in non-neoplastic changes in the studies, so these results were not supported by statistical significance.

No further alterations were found in EAS-mediated parameters in the two-year carcinogenicity studies.

- Increased incidence of mammary adenoacanthoma was noted in the chronic toxicity study in mice (ID: 86). These effects were not considered to be treatment related. On the other hand, fibroadenomas of the mammary gland were decreased in female rats at low and high dose levels (ID: 84).

Regarding sensitive to, but not diagnostic of EATS parameters:

-In the developmental toxicity study in rats (ID: 91), these anomalies were found:

. The mean foetal weights in litters of rats treated at top dose level were slightly but significantly reduced, however, it was not associated with any significant delay in foetal development.

. Reduction in the percentage of foetuses with more than 6 ossified metatarsals occurred in litters of rats treated at high dose level. This finding was seen in isolation of any other effects on skeletal development.

-In the developmental toxicity study in rabbits (ID: 92), this anomaly was found:

. Increase in post implantation loss value was recorded in the top dose level. This finding was non-statistically significant and presented a wide standard deviation. This data was considered equivocal due to it was limited primarily to two females with significant numbers of late embryonic deaths. Moreover, the findings were not consistent with that seen for other females of this dose group.

Target organ toxicity

- In mice long-term toxicity study (ID: 85), increased incidence of hepatocellular adenoma was observed in the low dose male group (26%) compared with controls (8%). However, the occurrence did not show a dose-response pattern due to the incidence noted in the top dose group (20%) was lower than observed in the low dose group. On the other hand, the incidence of hepatocellular carcinomas reported in the low dose male group (40%) did not show a dose-response pattern, due to the incidence of hepatocellular carcinomas was even lower than controls (18% vs 20% in control group). The increase incidences of hepatocellular adenomas and carcinomas (combined) in the high dose female group was considered an equivocal evidence of carcinogenicity due to both incidences did not display

statistical significant results when were analysed individually, and the house location of animal´s cages might affect the incidence of hepatocellular adenomas and carcinomas.

- There was a slight enlargement of liver cells in rats after 34-day eugenol treatment (ID: 80).

- In long-term toxicity studies, increased incidence of alveolar/bronchiolar adenomas or carcinomas was recorded in male rats at the low dose level (ID: 84) No dose-response relationship was observed. On the other hand, increased incidence of granulomatous inflammation and adenomatous hyperplasia of the lung was seen in female mice treated at the top dose (ID: 85).

. Increased incidence of focal inflammation of the kidney was observed in male mice treated at the top dose in the 2-year carcinogenicity study (ID: 85). On the other hand, a non-clear dose-related increase of chronic inflammation incidences of kidney was noted in male rats dose-groups (ID: 84).

- Increased incidence of splenic haemosiderosis was observed in female rats treated at the top dose (ID: 84) in the 2-year carcinogenicity study. This finding was not supported by clinical biochemistry or haematology analysis. Moreover, no further alterations were found in spleen in other studies.

-Increased thymic lymphomas incidence was observed in long-term toxicity study in mice (ID: 86). The thymic lymphomas showed low incidence and were not reproduced in the further 2-year chronic toxicity studies.

-There was a slight enlargement of adrenal gland in rats after 34-day eugenol treatment (ID: 80).

- Hyperkeratosis and hyperplasia in forestomach was observed in rats in the 34-day repeated dose study (ID: 80) conducted with eugenol.

Overall, based on the available data, the weight of evidence does not support an unequivocal EAS-mediated adversity. The increased incidences of endometrial polyps or sarcoma, and the cystic hyperplasia in the uterus of 2-year treated rats might be carefully evaluated due to they could be considered as signs of adversity. Besides, a slight increase of ovary follicular cysts was described in the top dose of 2-year treated mice. The fact that effects were not supported by statistical significance, the absence of data from OECD TG 416 or 443 studies, and no other histopathological alterations were found in the uterus or ovaries in sub-chronic toxicity studies conducted with eugenol, make its interpretation difficult. Uterine endometrial stromal polyps are a common spontaneous lesion in female rats, and are expected to occur in aged rats7, however these incidences in eugenol-long-term treated rats exceed the historical control range from conducted laboratory. Additionally, there is no evidence that uterine stromal polyps in rodents are hormone sensitive, so the relevance in mammals is still not clear7. On the other hand, increased incidences of ovary follicular cyst in mice were also observed.

Therefore, it has not been sufficiently demonstrated that the effects observed in the 2-year carcinogenicity studies conducted in rodents are not relevant for humans, and that these effects are not related with an endocrine mode of action.

WoE for EAS-mediated activity

The available dataset of *in vitro* mechanistic assays showed positive/active results in each of the EAS-mediated parameter. Toxcast ER bioactivity model showed two active/positive assays (ID: 16 and 17). The positive result in ATG_ERE_CIS_up (ID: 17) is of low relevance due to the flags displayed in the study (borderline active and less than 50% efficacy), but the active result from CERAPP (consensus) and the positive in ATG_ERa_TRANS_up (ID: 16) should be considered more carefully.

On the other hand, Toxcast AR bioactivity model displayed one active/positive result (ID: 34). However, the fact was flagged as having less than 50% efficacy makes the results of this experiment on their own insufficient for concluding that eugenol has any potential to interact with the AR.

To end, Toxcast Steroidogenesis bioactivity model showed that eugenol may have some potential to interact with components of the steroidogenesis pathway *in vitro*, potentially affecting the production of estradiol, estrone and glucocorticoids (ID: 57, 63 and 65). However it must be considered that the estradiol and estrone induction assays are both “flagged”, so reliability of results should be taken cautiously.

An *in vitro* estrogen/androgen receptor binding assay (Howes *et al.*, ID:74) showed that eugenol could play an ER-antagonist role. This study demonstrated that eugenol compete with ^{3}H-estradiol for binding to both the ERα and

[7] Davis B., *et al*. Endometrial stromal polyps in rodents: Biology, etiology, and relevance to disease in women. *Toxicologic Pathology*,; 2012; 40: pp 419-424.

ERβ, but required concentrations in the order of 10^4 to 10^5. Howes *et al.* used a first version of currently ER STTA assay (OECD TG 455), based on recombinant yeast approach.

Therefore, further information is needed to clarify the *in vitro* mechanistic results that suggest that eugenol could be a weak ER-binding and ER-antagonist, and if it exists a biologically plausible link between the *in vitro* outcome and the *in vivo* endometrial polyps/sarcoma and cysts hyperplasia found in the uterus of rats.

Overall, further data need to be generated regarding EAS-related activity:

E modality: A study in line with OECD TG 455.

A modality: A study in line with OECD TG 458.

S modality: A study in line with OECD TG 456 (H295R Steroidogenesis Assay) and/or a study in line with OPPTS 890.1200 (Aromatase assay).

In case of OECD TG 458, 456 and OPPTS 890.1200 are negative, a study in line with OECD TG 441 (Hershberger Assay) is required.

2.10.2.2.6 Selection of relevant scenario for the ED assessment of EAS-modality

No OECD TG 443 or OECD TG 416 studies have been conducted with eugenol, and there were no measurements of most EAS-mediated parameters in the studies provided. Based on the available information, equivocal signs of EAS-mediated adversity have been identified. On the other hand, positive results were obtained in the *in vitro* mechanistic assays from EAS-Bioactivity Models (US EPA). Even so, further data according to the EFSA/ECHA ED guidance need to be generated. Overall, it can be concluded that EAS-mediated adversity and EAS-mediated activity has not been sufficiently investigated, it corresponds to the scenario 2a (iii).

The relevant scenario for the EAS-modality is identified as 2a (iii).

Table 2.10.2.2.6: Identification of relevant scenario for EAS-modality

Adversity based on EAS-mediated parameters	Positive mechanistic OECD CF level 2/3 Test	Scenario	Next step of the assessment	Scenario selected
No (sufficiently investigated)	Yes/No	1a	Conclude: ED criteria not met because there is not "EAS-mediated" adversity	
Yes (sufficiently investigated)	Yes/No	1b	Perform MoA analysis	
No (not sufficiently investigated)	Yes	2a (i)	Perform MoA analysis (additional information may be needed for the analysis)	
No (not sufficiently investigated)	No (sufficiently investigated)	2a (ii)	Conclude: ED criteria not met because no EAS-mediated endocrine activity observed	
No (not sufficiently investigated)	No (not sufficiently investigated)	2a (iii)	Generate missing level 2 and 3 information. Alternatively, generate missing "EAS-mediated" parameters. Depending on the outcome move to corresponding scenario	x
Yes (not sufficiently investigated)	Yes/No	2b	Perform MoA analysis	

Co-RMS comment : The co-RMS considers that according to the ECHA/EFSA GD on ED, the relevant scenario for the EAS-modality is 1a.

The isoeugenol study may represent a worst-case for the assessment of eugenol, but further justification would be needed to completely dismiss the study. It is noted that several EAS-mediated parameters have been investigated in this study, including sperm parameters, AGD, oestrus cyclicity, and sexual maturation, and no effect was observed at doses below the MTD.

It is also noted that the uterine endometrial stromal polyps observed in the 2-year carcinogenicity study in rats are spontaneous lesions with different aetiology and biology to human endometrial polyps and are not hormone dependent.

In view of the above, the co-RMS considers that according to the ECHA/EFSA GD on ED, the relevant scenario for the EAS-modalities is 1a.

However, in case isoeugenol is not considered a relevant surrogate for eugenol for ED assesssment, then the following are noted concerning endocrine activity:

- *E-modality:* negative ER model. Modality is sufficiently investigated; no further data is needed and no concern is identified.
- *A-modality*: agree that a study in line with OECD TG 458 should be conducted and if negative an OECD TG 441 will be needed.
- *S-modality:* agree that a study in line with OECD TG 456 and a study in line with OPPTS 890.1200 should be performed.

RMS response : RMS disagrees with the co-RMS proposal regarding the relevant scenario 1a for EAS-modalities. EAS-mediated parameters were not sufficiently-investigated based on the absence of TG 416 and/or 443 studies.

Regarding E-modality, the RMS is of the opinion that the active result from CERAPP (consensus), the positive *in vitro* ATG_ERa_TRANS_up from ER Toxcast bioassays, and the findings from Howes *et al.* (B.6.8.3.5) should be taken into account for the overall ED assessment, due to present concern regarding potential estrogenic activity for eugenol. Therefore, in order to confirm or rule out these results, the RMS considers that further data need to be generated before a conclusion on whether or not the ED criteria are met for the E-modality can be drawn (e.g. a study in line with OECD TG 455).

RMS agrees with the co-RMS conclusions for A and S-modalities.

2.10.2.2.7 MoA analysis for EAS-modalities

According to the ED EFSA/ECHA guidance (2018), in cases of Scenario 2a(iii), a MoA analysis for EAS-modalities is not required.

2.10.2.2.8 Conclusion on the assessment of EAS-modalities

Based on the available data, it was concluded that EAS-mediated adversity and EAS-mediated activity have not been sufficiently investigated. Therefore, a scenario 2a (iii) has been established. Effects on EAS-mediated parameters were detected and positive results were obtained in the *in vitro* Toxcast Bioactivity assays for EAS modalities. Overall, additional studies in line with the ED EFSA/ECHA guidance are required:

E modality: A study in line with OECD TG 455.

A modality: A study in line with OECD TG 458.

S modality: A study in line with OECD TG 456 (H295R Steroidogenesis Assay) and/or a study in line with OPPTS 890.1200 (Aromatase assay).

In case of OECD TG 458, 456 and OPPTS 890.1200 are negative, a study in line with OECD TG 441 (Hershberger Assay) is required.

2.10.2.2.9 Overall conclusion on the ED assessment for humans

T-modality: It was concluded that T-mediated adversity and T-activity have not been sufficiently investigated due to relevant T-mediated parameters were not measured and the absence of TG 416 and/or 443 studies. On the other hand, the evaluation of the available data, and using a weight of evidence approach, does not suggest a T-mediated adversity. In addition, one positive/active result was obtained in the Thyroid Bioactivity Model US EPA for thyroid peroxidase inhibition (TPO), in agreement with QSAR *in-silico* predictions. Therefore, further data need to be generated before a conclusion on whether or not the ED criteria are met for the T-modality can be drawn [e.g. *in vitro* thyroperoxidase inhibition (rat and/or human) assay]. If the mentioned assay is negative, the scenario 2a(ii) applies and ED criteria are not met for T modality. However, if a positive result is obtained, the scenario 2a(i) applies

and further data will be needed to support the MoA analysis: a study in line with OECD TG 443 (with cohort 1a/1b including the mating of cohort 1b to produce the F2 generation, OECD 2018) or with OECD TG 416.

EAS-modality:

Based on the available data, EAS-mediated adversity and EAS-activity have not been sufficiently investigated. Relevant EAS-mediated parameters were not measured and studies in line with TG 416 and/or 443 were not conducted with the active substance eugenol. The data evaluation showed effects in EAS-mediated parameters (uterine endometrial polyp/stroma and cyst hyperplasia, and ovaries follicular cysts), however, the weight of evidence does not suggest a clear manifestation of adversity. Overall, further data need to be generated before a conclusion on whether or not the ED criteria are met for the EAS-modalities can be drawn:

E modality: A study in line with OECD TG 455.

If the above assay is positive, a study in line with OECD TG 440 (Uterotrophic *in vivo* assay) might be required in order to confirm or rule out the estrogenic activity of eugenol from the *in vitro* assay.

A modality: A study in line with OECD TG 458.

S modality: A study in line with OECD TG 456 (H295R Steroidogenesis Assay) and/or a study in line with OPPTS 890.1200 (Aromatase assay).

In case of OECD TG 458, 456 and OPPTS 890.1200 are negative, a study in line with OECD TG 441 (Hershberger Assay) is required.

If the mentioned studies are negative, the scenario 2a(ii) applies and ED criteria are not met for EAS modalities. However, if these studies are positive for at least one modality, the scenario 2a(i) applies and further data will be needed to support the MoA analysis: a study in line with OECD TG 443 (with cohort 1a/1b including the mating of cohort 1b to produce the F2 generation, OECD 2018) or with OECD TG 416 (according to the latest version of 2001).

2.10.3 ED assessment for non-target organims

For the assessment of the endocrine disrupting properties of eugenol, the applicant has provided a document ("Eugenol: Assessment of endocrine properties", by Staphyt Ltd. and Eden Research, 2021, KCA 5.8.3/01a), which has been taken into account by the RMS.

For non-target organisms, there are no available studies with eugenol investigating endpoints for EATS-mediated activity or endpoints that are sensitive to, but not diagnostic of, EATS modalities

A invertebrate study, a *Daphnia magna* reproduction test is available (Vol. 3 CA B.9.2.5.1/01). These invertebrate assays are considered as OECD Conceptual Framework Level 4 studies (OECD GD 150, 2018), however, invertebrates studies are not considered in this assessment since invertebrate organisms are out of the scope of the ED assessment as per the EFSA/ECHA Guidance due to the scarce knowledge on the endocrinology for non-target invertebrates and the limited amount of information provided in these studies for the identification of potential ED-related effects.

Furthermore, no literature papers were considered of possible relevance to the assessment of endocrine disrupting properties of eugenol (Vol. 3 CA B.9.11.1).

It should be noted that in Section 3.1 of the EFSA/ECHA (2018) guidance, there may be cases in which due to the knowledge on the physico-chemical and (eco)toxicological properties of the substance an ED assessment does not appear scientifically necessary. In such cases, it swould be justified.

The applicant has included the following weight of evidence (WoE) ED assessment for non-target organisms to justifiy that additional testing on terrestrial or aquatic vertebrate non-target organisms is not scientifically justified and no further data are therefore presented :

Eugenol is a naturally occurring terpene oil found in a wide variety of plant species from 0.02 to 180000 mg/kg, for blueberry and clove respectively (please, see eugenol Addendum – Confirmatory Data Table B.7.1.1, and Vol. 3 CA B.9.1.3). Following field application of the representative formulated product, Mevalone, initial environmental exposure of eugenol will decline rapidly in relation to the applied dose due to volatilisation and degradation. It is observed that the DT50 in soil for eugenol is less than one day and the DT50 in air obtained from the Atkinson model is 1.975 hours (Vol. 3 CA B.8; EFSA Journal 2012;10(11):2914 for details). Consequently, the duration of exposure under typical conditions will be very limited, particularly in relation to background levels of eugenol in the environment. This is also confirmed by the results of the residue trials conducted with Mevalone on grapevines

and apples (Vol. 3 CA B.7.3). A total of 11 trials in grapes were conducted in Northern EU countries (Austria, Germany and Northern France) and in Southern EU countries (Spain, Portugal and Italy) in 2006 and 2020. All 2020 trials were conducted according to the critical GAP for the renewal and are therefore relevant to support the use of Mevalone in the EU. In the 2020 season trials in grapes, residues of eugenol were not detected in the untreated control samples and not detected or detected up to 0.02 mg/kg in the treated samples. All residues of eugenol in grapes had declined to not detectable by 1 day after the last application. Furthermore, a total of 6 trials in apples were conducted in Northern EU countries (Austria, Germany and Northern France) and in Southern EU countries (Spain, Southern France and Italy) in 2020 with an LOQ of 0.01 mg/kg. All trials were conducted according to the critical GAP for the renewal and are therefore relevant to support the use of Mevalone in the EU. No residues of eugenol were detected at or above the LOQ of 0.01 mg/kg in any of the treated samples even on the day of the last application of Mevalone according to the critical GAP.

Furthermore, eugenol is of low acute toxicity to birds (acute oral avian LD50 >10000 mg Mevalone/kg bw (corresponding to >320 mg eugenol/kg bw based on the nominal eugenol content of 3.2% w/w); Vol. 3 CP B.9.1.1, Table 9.1.1-1. In an 8-day dietary toxicity study (please see eugenol DAR, Volume 3, Annex B.9, 2011, B.9.1.2) with Mevalone there were no deaths or reductions in feed consumption or body weight at the maximum dose tested. The dietary LD50 value was equivalent to 5866 mg product/kg bw/day (corresponding to > 187.7 mg eugenol/kg bw based on the nominal eugenol content of 3.2% w/w). In the interests of minimising vertebrate testing, it is not justified to conduct a new reproductive avian toxicity study for an active substance that is ubiquitous in the environment, degrades rapidly following application as a plant protection product and is of known low acute oral avian toxicity. Essentially, minimal long-term exposure to birds is expected, including during the reproductive period. Coupled with the lack of EATS-mediated activity observed in a range of in vitro assays and no endocrine related effects observed in available in vivo mammalian studies, no endocrine effects on birds are expected. Similarly, additional vertebrate testing of aquatic organisms, including amphibians (e.g. Xenopus) or fish, is not justified based on minimal chronic exposure (eugenol is rapidly degradable, volatile and shows low potential for bioconcentration) and lack of expected toxicity based on the available vertebrate (mammalian) data package. Low toxicity to fish is confirmed based on the acute fish toxicity (96-hour LC50 >10 mg eugenol/L for rainbow trout; Vol. 3 CA B.9.2.1/01). The available aquatic data have been assessed in the CLH report for eugenol and it is proposed that eugenol is not classified for short-term (acute) aquatic hazard or long-term aquatic hazard. As discussed in Section 2 above, the ED criteria are not met based on the available mammalian data. Given the high level of conservation of the endocrine system across taxonomic groups of vertebrates (EFSA/ECHA (2018) guidance, Section 3.1 and Section 4.2), natural occurrence of eugenol, as well as the favourable physico-chemical and (eco)toxicological characteristics of eugenol, it is highly unlikely that the rapidly degradable substance would result in any effects on EATS-mediated activity in other terrestrial or aquatic non-target organisms.

Information on natural background levels of eugenol was provided as confirmatory data (Eugenol Addendum – Confirmatory Data, August 2016). The RMS (UK) concluded that *«... the confirmatory data requirement are not met, as it cannot be established that background exposure is greater or similar to predicted exposures...».* EFSA agreed with RMS conclusion (EFSA Supporting publication 2017:EN-1165). No new data has been submitted to enable a comparison between the natural background exposure and the exposure due to the use of the plant protection product. Therefore, a negligible exposure has not been demonstrated.

Additionally, the data of residues of eugenol in plants other than grape or pome (0.02 – 180000 ,g/kg, in plant tissue) were included as confirmatory data information on natural background levels of eugenol (Eugenol Addendum – Confirmatory Data, August 2016). It was concluded that there is insufficient information to establish a background exposure concentration of eugenol on the basis of this data since there are significant uncertainties regarding the reliability of these data. Furthermore, under some circumstances background exposure could exceed the predicted exposure based on the proposed use (please, see Vol. 3 CA B.9.1.1.3 for further justification).

Moreover, considering the Table 9 of EFSA/Echa GD on ED (EFSA Journal 2018 :16(6)-5311) no studies are available included in the « OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals » with the active substance eugenol.

Furthermore, the available toxicology (mammalian) data for eugenol showed that EATS-mediated adversity and EATS-activity have not been sufficiently investigated, therefore, further data need to be generated before a conclusion on whether or not the ED criteria are met for the EATS-modalities can be drawn (see point 2.10.2.2.9).

2.10.3.1 ED assessment for T-modality

2.10.3.1.1 Lines of evidence for adverse effects and endocrine activity related to T-modality

There are no available guideline studies with eugenol investigating endpoints for adverse effects and endocrine activity related to T-modality in non-target organisms.

2.10.3.1.1.1 Assessment of the integrated lines of evidence and weight of evidence

The assessment of the available mammalian studies concluded that based on the battery of *in vitro* assays (13 assays on thyroid activity associated with the EDSP21 tab in the CompTox Chemicals Dashboard), eugenol is active (assay NCCT_TPO_AUR_dn) for thyroid peroxidase inhibition (TPO). This somehow is in agreement with the alerts structural data raised by the models QSAR1 and QSAR 2 which predicted that eugenol has potential inhibit Thyroperoxidase (TPO) activity. Furthermore, T-mediated adversity and T-activity have not been sufficiently investigated due to relevant T-mediated parameters were not measured and the absence of TG 416 and/or 443 studies. On the other hand, the evaluation of the available data, and using a weight of evidence approach, does not suggest a T-mediated adversity.
No evidence for T-mediated activity or adversity was found for non-target organisms other than mammals, however, considering that amphibians' studies are not available in the dossier; T-mediated activity cannot considered as sufficiently investigated. Therefore, further information should be submitted for addressing the potential T-mediated adversity in non-target organisms.

According to EFSA/ECHA GD on ED (2018).

- *To have the **T-mediated adversity** with regard to other non-target organisms sufficiently investigated the results from all the 'T-mediated' parameters foreseen to be investigated in the Larval Amphibian Growth and Development Assay (LAGDA; OECD TG 241) would be needed. However, if the T-mediated parameters foreseen to be investigated in an Amphibian Metamorphosis Assay (AMA, OECD TG 231) are negative, this would be sufficient to support that T-mediated adversity is unlikely because no T-related endocrine activity has been observed".*

Amphibians' studies are not available in the dossier; consequently, RMS considers that further information should be needed for addressing the potential T-mediated adversity in non-target organisms. In particular, and following the EFSA/ECHA GD on ED (2018), an Amphibian Metamorphosis Assay (AMA, OECD TG 231) should be submitted by applicant to address T-modality endocrine activity of eugenol.

Considering the information in the mammalian toxicity section, and in line with the XETA Annex of the EFSA/ECHA ED Guidance, the XETA (Xenopus Eleutheroembryo Thyroid Assay, OECD 248, 2019 is not appropiate to investigate T-activity since a poteintial thyroid peroxidase inhibition (TPO) was found at OECD Framework Level 1 (non-experimental) and Level 2 (*in vitro* ToxCast mechanistic, assay NCCT_TPO_AUR_dn).

2.10.3.1.2 Initial analyisis fo the evidence and identification of relevant scenario

For non-target organisms, there are no available studies with eugenol investigating endpoints for adverse effects and endocrine activity related to T-modality. According to the ECHA/EFSA ED guidance (EFSA Journal 2018;16(6):5311), eugenol falls into the 2a (iii) scenario where T modalities have not been sufficiently investigated in non-target organisms. "To consider the T-modality sufficiently investigated, a Leverl 3 Study : 'Amphibian metamorphosis assay' (AMA; OECD TG 231 (OECD, 2009c)) should be conducted" (ECHA/EFSA ED guidance (EFSA Journal 2018;16(6):5311)).

The relevant scenario for the T-modality is identifiedd as 2a (iii)

Table 2.10.3.1.2-1. Identification of relevant scenario for T-modality

Adversity based on T-mediated parameters	Positive mechanistic OECD CF level 2/3 Test	Scenario	Next step of the assessment	Scenario selected
No (cd)	Yes/No	1a	Conclude: ED criteria not met because there is not "T-mediated" adversity	
Yes (sufficiently investigated)	Yes/No	1b	Perform MoA analysis	
No (not sufficiently investigated)	Yes	2a (i)	Perform MoA analysis (additional information may be needed for the analysis)	
No (not sufficiently investigated)	No (sufficiently investigated)	2a (ii)	Conclude: ED criteria not met because no T-mediated endocrine activity observed	

Adversity based on T-mediated parameters	Positive mechanistic OECD CF level 2/3 Test	Scenario	Next step of the assessment	Scenario selected
No (not sufficiently investigated)	**No (not sufficiently investigated)**	**2a (iii)**	**Generate missing level 2 and 3 information. Alternatively, generate missing "T-mediated" parameters. Depending on the outcome move to corresponding scenario**	**X**
Yes (not sufficiently investigated)	Yes/No	2b	Perform MoA analysis	

2.10.3.1.3 MoA analysis for T-modality

According to the ED EFSA/ECHA guidance (2018), in cases of Scenario 2a (iii), a MoA analysis for EAS-modalities is not required.

2.10.3.1.4 Conclusion on the ED assessment for T-modality

The available toxicology (mammalian) data for eugenol showed that T-mediated adversity and T-activity have not been sufficiently investigated, therefore, further data need to be generated before a conclusion on whether or not the ED criteria are met for the T-modality can be drawn (see point 2.10.2.2.9).

T-mediated adversity and T-activity are not considered sufficiently investigated on non target organisms other than mammals, because Amphibians' studies are not available. A level 3 study Amphibian Metamorphosis Assay (AMA, OECD TG 231) should be submitted before a conclusion on whether or not the ED criteria are met for the T-modality can be drawn. Once provided, the need of further data will be triggered by the following scenarios:

1. If the above study is negative, the scenario 2a(ii) applies and ED criteria are not met for T modality.
2. If positive, the scenario 2a(i) applies and further data will be needed to support the MoA analysis, i.e. a Level 4 study following OECD TG 241 (LAGDA, Larval Amphibian Growth and Development Assay).

2.10.3.2 ED assessment for EAS-modality

2.10.3.2.1 Lines of evidence for adverse effects and endocrine activity related to EAS-modalities

There are no available guideline studies with eugenol investigating endpoints for adverse effects and endocrine activity related to EAS-modalities in non-target organisms.

2.10.3.2.1.1 Assessment of the integrated lines of evidence and weight of evidence

The assessment of available in vivo toxicity data to study EAS-mediated adversity and EAS-activity on mammals (toxicology) have not been sufficiently investigated. Relevant EAS-mediated parameters were not measured and studies in line with TG 416 and/or 443 were not conducted with the active substance eugenol. The data evaluation showed effects in EAS-mediated parameters (uterine endometrial polyp/stroma and cyst hyperplasia, and ovaries follicular cysts), however, the weight of evidence does not suggest a clear manifestation of adversity. Overall, further data need to be generated before a conclusion on whether or not the ED criteria are met for the EAS-modalities on mammals can be drawn:

The available dataset of *in vitro* mechanistic assays showed positive/active results in each of the EAS-mediated parameter:

- Toxcast ER bioactivity model showed two active/positive assays (ID: 16 and 17). The positive result in ATG_ERE_CIS_up (ID: 17) is of low relevance due to the flags displayed in the study (borderline active and less than 50% efficacy), but the active result from CERAPP (consensus) and the positive in ATG_ERa_TRANS_up (ID: 16) should be considered more carefully.
- Toxcast AR bioactivity model displayed one active/positive result (ID: 34). However, the fact was flagged as having less than 50% efficacy makes the results of this experiment on their own insufficient for concluding that eugenol has any potential to interact with the AR.
- Toxcast Steroidogenesis bioactivity model showed that eugenol may have some potential to interact with components of the steroidogenesis pathway in vitro, potentially affecting the production of estradiol, estrone

and glucocorticoids (ID: 57, 63 and 65). However it must be considered that the estradiol and estrone induction assays are both "flagged", so reliability of results should be taken cautiously.

No evidence for EAS-mediated activity or adversity was found for non-target organisms other than mammals, however, considering that studies with eugenol investigating endpoints for adverse effects and endocrine activity related to EAS-modalities in non-target organisms are not available in the dossier; EAS-mediated adversity cannot considered as sufficiently investigated. Therefore, further information should be submitted for addressing the potential EAS-mediated adversity in non-target organisms.
According to EFSA/ECHA GD on ED (2018).

- "*To consider the **E, A, S modalities** for non-target organisms other than mammals sufficiently investigated, preferably the 'Fish short term reproduction assay' (FSTRA; OECD TG 229) should have been conducted; however the 21-day fish assay OECD TG 230 (OECD, 2009b) is acceptable as well...*"

In order to make sufficient data available to reach a conclusion on EAS modalities in non-target organisms RMS considers that a "Fish short-term reproduction assay (FSTRA, OECD 229)" included gonad histhopatology should be submitted to address the E, A, S-modalities endocrine activity of eugenol.

2.10.3.2.2 Initial analysis of the evidence and identification of the relevant scenario

For non-target organisms, there are no available studies with eugenol investigating endpoints for adverse effects and endocrine activity related to EAS-modalities. According to the ECHA/EFSA ED guidance (EFSA Journal 2018;16(6):5311), eugenol falls into the 2a (iii) scenario EAS-modalities, where EAS modalities have not been sufficiently investigated, a Leverl 3 study : Fish short term reproduction assay assay' (FSTRA; OECD TG 231 (OECD, 2009c)), included gonad histopathology, should be submitted before a conclusion on whether or not the ED criteria are met for the EAS-modalities can be drawn.

The relevant scenario for the EAS-modality is identified as 2a (iii).

Table 2.10.3.2.2-2: Identification of relevant scenario for EAS-modality

Adversity based on EAS-mediated parameters	Positive mechanistic OECD CF level 2/3 Test	Scenario	Next step of the assessment	Scenario selected
No (sufficiently investigated)	Yes/No	1a	Conclude: ED criteria not met because there is not "EAS-mediated" adversity	
Yes (sufficiently investigated)	Yes/No	1b	Perform MoA analysis	
No (not sufficiently investigated)	Yes	2a (i)	Perform MoA analysis (additional information may be needed for the analysis)	
No (not sufficiently investigated)	No (sufficiently investigated)	2a (ii)	Conclude: ED criteria not met because no EAS-mediated endocrine activity observed	
No (not sufficiently investigated)	**No (not sufficiently investigated)**	**2a (iii)**	**Generate missing level 2 and 3 information. Alternatively, generate missing "EAS-mediated" parameters. Depending on the outcome move to corresponding scenario**	**X**
Yes (not sufficiently investigated)	Yes/No	2b	Perform MoA analysis	

2.10.3.2.3 MoA analysis for EAS-modalities

According to the ED EFSA/ECHA guidance (2018), in cases of Scenario 2a (iii), a MoA analysis for EAS-modalities is not required.

2.10.3.2.4 Conclusion on the ED assessment for EAS-modalities

The available toxicology (mammalian) data for eugenol showed that EAS-mediated adversity and EAS-activity have not been sufficiently investigated, therefore, further data need to be generated before a conclusion on whether or not the ED criteria are met for the EAS-modalities can be drawn (see point 2.10.2.2.9)..

EAS-mediated adversity and EAS-activity are not considered sufficiently investigated on non-target organisms other than mammals, because no studies with eugenol investigating endpoints for adverse effects and endocrine activity related to EAS-modalities are available. A level 3 study Fish Short Term Reproduction Assay (FSTRA, OECD TG 229), including histopathology assessment, should be submitted before a conclusion on whether or not the ED criteria are met for the EAS-modalities can be drawn. Once provided, the need of further data will be triggered by the following scenarios:

1. If the above study is negative, the scenario 2a(ii) applies and ED criteria are not met for EAS modalities.
2. If positive, the scenario 2a(i) applies and further data will be needed to support the MoA analysis, i.e. a level 5 study following OECD TG 240 (MEOGRT, Medaka Extended One Generation Reproduction Test) would be necessary.

2.10.3.2.5 Overall conclusions on the ED assessment for non-target organisms

The ED-assessment for **wild mammals** is based on the same dataset as used for the human health assessment, but with additional consideration for the population relevant of any adverse effects observed for the EATS-modalities. Given that in the human health assessment, it was concluded that EATS-mediated adversity and EATS-activity have not been sufficiently investigated and further data should be generated before a conclusion on whether or not the ED criteria are met for the EAS-modalities on wild mammals can be drawn (see point 2.10.2.2.9).
It was concluded that EATS-mediated adversity and EATS-activities for non-target organisms other than mammals have not been sufficiently investigated. Consequently, the scenario 2a (iii) applies.

Therefore, level 3 tests should be conducted as follows:

- A test according to OECD TG 229 (Fish Short Term Reproduction Assay, FSTRA), including histophatology assessment.
- A test according to OECD TG 231 (Amphibian Metamorphosis Assay ; AMA).

The Co-RMS has proposed to perform an ELS test (OECD TG 210) to study chronic risk to fish and EAS-adversity of the active substance eugenol. This test is a Level 4 studio that gives information on general toxicity and on parameters that might be “sensitive to, but not diagnostic of EATS” such as hatchability and development.

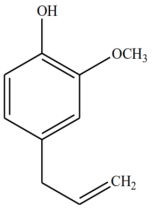
If the mentioned studies are negative, the scenario 2a(ii) applies and ED criteria are not met for EATS modalities. However, if these studies are positive, the scenario 2a(i) applies and further data will be needed to support the MoA analysis: a study in line with OECD TG 240 (MEOGRT, Medaka Extended One Generation Reproduction Test) and/or with OECD TG 241 (LAGDA, Larval Amphibian Growth and Development Assay).

2.11 PROPOSED HARMONISED CLASSIFICATION AND LABELLING ACCORDING TO THE CLP CRITERIA *[SECTIONS 1-6 OF THE CLH REPORT]*

2.11.1 Identity of the substance [section 1 of the CLH report]

2.11.1.1 Name and other identifiers of the substance

Table 82: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	Eugenol ; 2-Methoxy-4-(prop-2-en-1-yl)phenol
Other names (usual name, trade name, abbreviation)	Eugenol
ISO common name (if available and appropriate)	No ISO common name
EC number (if available and appropriate)	202-589-1
EC name (if available and appropriate)	eugenol
CAS number (if available)	97-53-0
Other identity code (if available)	967
Molecular formula	$C_{10}H_{12}O_2$
Structural formula	
SMILES notation (if available)	COc1cc(CC=C)ccc1O
Molecular weight or molecular weight range	164.20 g/mol
Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)	The active substance is not a mixture of isomers. Therefore, consideration of isomeric composition is not relevant.
Description of the manufacturing process and identity of the source (for UVCB substances only)	Not a UVCB substance.
Degree of purity (%) (if relevant for the entry in Annex VI)	990 g/kg minimum

2.11.1.2 Composition of the substance

Table 83: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	**Concentration range (% w/w minimum and maximum in multi-constituent substances)**	**Current CLH in Annex VI Table 3.1 (CLP)**	**Current self-classification and labelling (CLP)**
Eugenol CAS number: 97-53-0	99%	No Annex VI entry	

Table 84: Impurities (non-confidential information) if relevant for the classification of the substance

Impurity (Name and numerical identifier)	**Concentration range (% w/w minimum and maximum)**	**Current CLH in Annex VI Table 3.1 (CLP)**	**Current self-classification and labelling (CLP)**	**The impurity contributes to the classification and labelling**
Methyleugenol	-	-	-	No

Table 85: Additives (non-confidential information) if relevant for the classification of the substance

Additive (Name and numerical identifier)	**Function**	**Concentration range (% w/w minimum and maximum)**	**Current CLH in Annex VI Table 3.1 (CLP)**	**Current self-classification and labelling (CLP)**	**The additive contributes to the classification and labelling**
Eugenol does not contain additives					

Table 86: Test substances (non-confidential information)

Identification of test substance	**Purity**	**Impurities and additives (identity, %, classification if available)**	**Other information**	**The study(ies) in which the test substance is used**

2.11.2 Proposed harmonized classification and labelling

2.11.2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 87: Proposed harmonised classification and labelling according to the CLP criteria

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry											
Proposed harmonised classification in CLH report*		eugenol; 2-methoxy-4-(prop-2-en-1-yl)phenol	202-589-1	97-53-0	Skin Sens. 1A	H317	GHS07 Wng	H317			
Dossier submitters proposal		eugenol; 2-methoxy-4-(prop-2-en-1-yl)phenol	202-589-1	97-53-0	**Add** Acute Tox. 4 Skin Irrit. 2 Eye Irrit. 2 STOT SE 3 Aquatic Chronic 2	**Add** H302 H315 H319 H336 H411	**Add** GHS07 GHS09 Wng	**Add** H302 H315 H319 H336 H411		**Add** Oral: ATE = 1930 mg/kg/bw	
Resulting Annex VI entry if agreed by RAC and COM		eugenol: 2-methoxy-4-(prop-2-en-1-yl)phenol	202-589-1	97-53-0	Acute Tox. 4 Skin Irrit. 2 Eye Irrit. 2 Skin Sens. 1A STOT SE 3 Aquatic Chronic 2	H302 H315 H319 H317 H336 H411	GHS07 GHS09 Wng	H302 H315 H319 H317 H336 H411		Oral: ATE = 1930 mg/kg/bw	

* Referred to a CLH report prepared by DK and currently under ECHA revision, with a proposal only for the Skin sensitisation hazard class.

2.11.2.2 Additional hazard statements / labelling

Not applicable

Table 88: Reason for not proposing harmonised classification and status under CLH public consultation

Hazard class	Reason for no classification	Within the scope of CLH public consultation
Explosives	Data conclusive but not sufficient for classification	Yes
Flammable gases (including chemically unstable gases)	Hazard class not applicable	No
Oxidising gases	Hazard class not applicable	No
Gases under pressure	Hazard class not applicable	No
Flammable liquids	Data conclusive but not sufficient for classification.	Yes
Flammable solids	Hazard class not applicable	No
Self-reactive substances	Data conclusive but not sufficient for classification.	Yes
Pyrophoric liquids	Data conclusive but not sufficient for classification	Yes
Pyrophoric solids	Hazard class not applicable	No
Self-heating substances	Data conclusive but not sufficient for classification	Yes
Substances which in contact with water emit flammable gases	Data conclusive but not sufficient for classification	Yes
Oxidising liquids	Data conclusive but not sufficient for classification	Yes
Oxidising solids	Hazard class not applicable	No
Organic peroxides	Hazard class not applicable	No
Corrosive to metals	Data conclusive but not sufficient for classification	Yes
Acute toxicity via oral route	**Harmonised classification proposed: Acute Tox. 4 (H302)**	Yes
Acute toxicity via dermal route	Hazard class not applicable (corrosive)	Yes
Acute toxicity via inhalation route	Data conclusive but not sufficient for classification	Yes
Skin corrosion/irritation	**Harmonised classification proposed: Skin Irrit. 2 (H315)**	Yes
Serious eye damage/eye irritation	**Harmonised classification proposed: Eye Irrit. 2 (H319)**	Yes
Respiratory sensitisation	Data lacking	No
Skin sensitisation	**Harmonised classification proposal not required (Denmark CLH proposal for this hazad class is in progress: Skin Sens. 1A (H317))**	No
Germ cell mutagenicity	Data conclusive but not sufficient for classification	Yes
Carcinogenicity	Data conclusive but not sufficient for classification	Yes
Reproductive toxicity	Data lacking for sexual function and fertility effects but data conclusive but not sufficient for classification for developmental toxicity.	Yes
Specific target organ toxicity-single exposure	**Harmonised classification proposed: STOT SE 3 (H336)**	Yes

Hazard class	Reason for no classification	Within the scope of CLH public consultation
Specific target organ toxicity-repeated exposure	Data conclusive but not sufficient for classification	Yes
Aspiration hazard	Data lacking	No
Hazardous to the aquatic environment	Aquatic Chronic 2	Yes
Hazardous to the ozone layer	Data conclusive but not sufficient for classification	Yes

2.11.3 History of the previous classification and labelling

Eugenol is an active substance for plant protection products (PPP) approved under Regulation (EC) 1107/2009 by Commission Implementing Regulation (EU) no. 546/2013.

Eugenol has not classification under the Dangerous Substances Directive 67/548/EEC (DSD) or Regulation (EC) no. 1272/2008 (CLP). Currently there is a submitted CLH Report of Denmark based only in skin sensitisation with a proposal of classification as Skin Sens. 1A.

2.11.4 Identified uses

2.11.5 Data sources

2.12 RELEVANCE OF METABOLITES IN GROUNDWATER

There are no metabolites of relevance.

2.12.1 STEP 1: Exclusion of degradation products of no concern

2.12.2 STEP 2: Quantification of potential groundwater contamination

2.12.3 STEP 3: Hazard assessment – identification of relevant metabolites

2.12.3.1 STEP 3, Stage 1: screening for biological activity

2.12.3.2 STEP 3, Stage 2: screening for genotoxicity

2.12.3.3 STEP 3, Stage 3: screening for toxicity

2.12.4 STEP 4: Exposure assessment – threshold of concern approach

2.12.5 STEP 5: Refined risk assessment

2.12.6 Overall conclusion

2.13 CONSIDERATION OF ISOMERIC COMPOSITION IN THE RISK ASSESSMENT

Eugenol does not contain isomers

2.13.1 Identity and physical chemical properties

2.13.2 Methods of analysis

2.13.3 Mammalian toxicity

2.13.4 Operator, Worker, Bystander and Resident exposure

2.13.5 Residues and Consumer risk assessment

2.13.6 Environmental fate

2.13.7 Ecotoxicology

2.14 RESIDUE DEFINITIONS

2.14.1 Definition of residues for exposure/risk assessment

Food of plant origin: Sum of free and conjugated eugenol expressed as eugenol (fruit crops)

Food of animal origin: Not required

Soil: Eugenol

Groundwater: Eugenol

Surface water: Eugenol

Sediment: Eugenol

Air: Eugenol

Body fluids and tissues: active substance and its sulphate and glucuronide conjugates, measured in urine samples.

2.14.2 Definition of residues for monitoring

Food of plant origin: Not required
Not required, since it is proposed to include eugenol into Annex IV to Regulation (EC) No. 396/2005 (eugenol seems to fit the Criterion 4 to SANCO/11188/2013: The consumer exposure to residues of eugenol linked to use as plant protection product is considered as negligible compared to other uses in the food chain and/or natural background).

Food of animal origin: Not required

Soil: Eugenol

Groundwater: Eugenol

Surface water: Eugenol

Sediment: Eugenol

Air: Eugenol

Body fluids and tissues: active substance and its sulphate and glucuronide conjugates, measured in urine samples.

Level 3

EUGENOL

3 PROPOSED DECISION WITH RESPECT TO THE APPLICATION

3.1 BACKGROUND TO THE PROPOSED DECISION

3.1.1 Proposal on acceptability against the decision making criteria – Article 4 and annex II of regulation (EC) No 1107/2009

3.1.1.1 Article 4

		Yes	No	
i)	It is considered that Article 4 of Regulation (EC) No 1107/2009 is complied with. Specifically the RMS considers that authorisation in at least one Member State is expected to be possible for at least one plant protection product containing the active substance for at least one of the representative uses.			***Assessment not finalized*** **Identity** Eugenol The CS formulation, MEVALONE (3AEY), containing 33 g/L eugenol, 66 g/L geraniol and 66 g/L thymol as active substances is the representative formulation for the renewal of approval. The CS formulation MEVALONE (3AEY) is a Capsule Suspension formulation with slow release performance. Eugenol as one of its three active substances has a minimum concentration of 990 g/kg (99% w/w). The representative use that was assessed was as fungicide in viticulture and Pome fruits **Toxicology:** Applicant should submit further information on data required for endocrine disruption assessment (EATS-modalities). **Residues/consumer risk assessment** Eugenol and methyleugenol are naturally occurring in a wide variety of fruits, vegetables, herbs and spices Residue trials have demonstrated no detectable residues in grapes at harvest. In apples, residues of eugenol were <LOQ and methyleugenol was not detected. Aceptable chronic consumer risk was identified. It should be indicated that the chronic risk is subestimated in this provisional approach, since available data do not include the conjugates of eugenol (Included in the RD for RA). However, it can be concluded that since there is a broad security margin for the calculated chronic risk assessment (only 0.7% of ADI), a chronic risk situation for the consumer is not foreseen to be reached. **Ecotoxicology**

				• **Birds:** No acute risk. No data or a strong justification are available to address the reproductive risk on birds. Therefore, further information should be submitted. • **Fish:** No acute risk. No data is available to address the long-term risk on fish. Therefore, further information should be submitted. No risk is expected in mammals, bees, non-target arthropods other than bees, non-target soil meso- and macrofauna, Soil nitrogen transformation or non-target terrestrial plants. .

3.1.1.2 Submission of further information

		Yes	No	
i)	It is considered that a complete dossier has been submitted		X	See data gaps in the list of studies to be generated, still ongoing or available but not peer reviewed in point 3.1.4.
ii)	It is considered that in the absence of a full dossier the active substance may be approved even though certain information is still to be submitted because: (a) the data requirements have been amended or refined after the submission of the dossier; or (b) the information is considered to be confirmatory in nature, as required to increase confidence in the decision.			***Assessment not finalized*** Please refer to Point 3.1.4. List of studies to be generated, still ongoing or available but not peer reviewed. ***Identity:*** ***Further information:*** *Applicant should submit further information on:* • The information on the potential impact of water treatment processes on the active substance and its metabolites in water for drinking water uses is required for the consumer risk assessment performed in the residue section of the DRAR. ***Toxicology:*** *Applicant should submit further information on :* • data required for endocrine disruption assessment (EATS-modalities). ***Fate and behaviour in the environment*** *Applicant should submit further information about:* • The route of degradation in soil under irradiated conditions is not considered fully addressed. The characterization of photoproducts in soil should be submitted. • The identity of the hydrolysis products 'Unknown 1' and 'Unknown 2' should be resolved. More information is needed regarding to the percentages of AR reached by 'other unknowns.

				• The identity of the unknown peaks from the direct photochemical degradation study should be resolved. • A data gap has been identifed to address the route and rate of degradation under natural water/sediment system. • An analysis of the short-range transport in air should be included. • A data gap has been proposed for information to address the effect of water treatment processes on the nature of residues of the active substance when surface water is abstracted for drinking water to address Article 4 (approval criteria for active substances) 3(b) of Regulation (EC) No 1107/2009. ***Ecotoxicology section:*** *Applicant should submit further information about:* • Further information should be submitted to address the chronic risk on birds. • A new chronic toxicity study on fish.
3.1.1.3 Restrictions on approval				
		Yes	No	
	It is considered that in line with Article 6 of Regulation (EC) No 1107/2009 approval should be subject to conditions and restrictions.			
3.1.1.4 Criteria for the approval of an active substance				
Dossier				
		Yes	No	
	It is considered the dossier contains the information needed to establish, where relevant, Acceptable Daily Intake (ADI), Acceptable Operator Exposure Level (AOEL) and Acute Reference Dose (ARfD).	X		
	It is considered that the dossier contains the information necessary to carry out a risk assessment and for enforcement purposes (relevant for substances for which one or more representative uses includes use on feed or food crops or leads indirectly to residues in food or feed). In particular it is considered that the dossier: (a) permits any residue of concern to be defined; (b) reliably predicts the residues in food and feed, including succeeding crops			*[Insert brief overall summary of consideration of residues & consumer assessment here]* *[Explain if this applies to all or some of the representative uses/use scenarios/products]*

	(c) reliably predicts, where relevant, the corresponding residue level reflecting the effects of processing and/or mixing; (d) permits a maximum residue level to be defined and to be determined by appropriate methods in general use for the commodity and, where appropriate, for products of animal origin where the commodity or parts of it is fed to animals; (e) permits, where relevant, concentration or dilution factors due to processing and/or mixing to be defined.			
	It is considered that the dossier submitted is sufficient to permit, where relevant, an estimate of the fate and distribution of the active substance in the environment, and its impact on non-target species.			*[Explain if this applies to all or some of the representative uses/use scenarios/products]*
Efficacy				
		Yes	No	
	It is considered that it has been established for one or more representative uses that the plant protection product, consequent on application consistent with good plant protection practice and having regard to realistic conditions of use is sufficiently effective.	X		Terpene compounds such as eugenol, geraniol and thymol generally possess antifungal activity, having effects on spore germination, hyphal penetration, mycelial growth and hyphal growth. For the uses grapes/BOTRCI and UNCINE, the representative formulation, MEVALONE, is currently commercially available and supported by efficacy data evaluated under Uniform Principles for national registrations. For pome fruits/postharvest storage diseases (PHYTSP, ALTESP and BOTRCI), currently, this use is not registered, however, it is considered that the GAP is realistic from an efficacy point of view considering the studies provided by the applicant (studies submitted for new registration in Central zone in July 2021). Eugenol is a contact action fungicide. It prevents the development of fungal mycelium from spores or destroys existing mycelium by a direct action on the cell membranes. Due to the mode of action, no problems with resistance or cross-resistance are expected. Eugenol, is a plant extract included in the terpene alcohols chemical group, classified by FRAC into plant oils, FRAC codes F7: cell membrane disruption /46, with resistance not known. MEVALONE formulation has been applied in various EU member states for many years without reports of adverse effects on treated crops. Available efficacy used to obtain registration of the representative formulation in various countries shows the absence of phytotoxicity when the product is used

				according to the GAP. Consequently, no negative impact is expected on treated crops when used according to recommendations." There is no evidence of any undesirable or unintended side-effects.
Relevance of metabolites				
		Yes	No	
	It is considered that the documentation submitted is sufficient to permit the establishment of the toxicological, ecotoxicological or environmental relevance of metabolites.			<u>Fate and behaviour in the environment</u> Further information is needed.
Composition				
		Yes	No	
	It is considered that the specification defines the minimum degree of purity, the identity and maximum content of impurities and, where relevant, of isomers/diastereo-isomers and additives, and the content of impurities of toxicological, ecotoxicological or environmental concern within acceptable limits.	X		Eugenol has a minimum purity of 990 g/kg (99% w/w). Methyl eugenol is regarded as Eugenol relevant impurity at a maximum level of 1 g/kg (0.1% w/w).
	It is considered that the specification is in compliance with the relevant Food and Agriculture Organisation specification, where such specification exists.			No FAO specification is available at the time of submission.
	It is considered for reasons of protection of human or animal health or the environment, stricter specifications than that provided for by the FAO specification should be adopted			Not applicable. No FAO specification is available.
Methods of analysis				
		Yes	No	
	It is considered that the methods of analysis of the active substance, safener or synergist as manufactured and of determination of impurities of toxicological, ecotoxicological or environmental concern or which are present in quantities greater than 1 g/kg in the active substance, safener or synergist as manufactured, have been validated and shown to be sufficiently specific, correctly calibrated, accurate and precise.	X		Available validated methods are suitable for analysis of methyl eugenol in the active substance. Refer to Volume 4, confidential for further details on composition.
	It is considered that the methods of residue analysis for the active substance and relevant metabolites in plant, animal and environmental matrices and drinking water, as appropriate, shall have been validated and shown to be sufficiently sensitive with respect to the levels of concern.	X		Suitable validated methods are available for residue analysis for the active substance and relevant impurity. However, for pre-registration methods in plant matrices the methods do not perform acid hydrolysis. According to the proposed residue definition for risk assessment if the residue definion is eventually set as RD-RA nº1 (sum of free and conjugated eugenol expressed as eugenol), the residue definition would not be covered.

	It is confirmed that the evaluation has been carried out in accordance with the uniform principles for evaluation and authorisation of plant protection products referred to in Article 29(6) of Regulation 1107/2009.	X		
Impact on human health				
Impact on human health - ADI, AOEL, ARfD				
		Yes	No	
	It is confirmed that (where relevant) an ADI, AOEL and ARfD can be established with an appropriate safety margin of at least 100 taking into account the type and severity of effects and the vulnerability of specific groups of the population.	X		The RMS is of the opinion that, with the available studies, the following values should be set: ADI = 0.17 mg/kg bw/day AOEL = 1.0 mg/kg bw/day ARfD: Not required as no acute effects were observed.
Impact on human health – proposed genotoxicity classification				
		Yes	No	
	It is considered that, on the basis of assessment of higher tier genotoxicity testing carried out in accordance with the data requirements and other available data and information, including a review of the scientific literature, reviewed by the Authority, **the substance SHOULD BE classified or proposed for classification**, in accordance with the provisions of Regulation (EC) No 1272/2008, **as mutagen category 1A or 1B.**		X	Three new studies of genotoxicity have been submitted for the renewal process of eugenol. Twenty six studies that were previously addressed have been presented and a reassessment has been performed by the RMS for this renewal. Eugenol was negative *in vitro* for gene mutation (Ames test and MLA). Positive results were obtained *in vitro* for structural chromosome aberration, sister chromatid exchange and DNA adducts formation. On the other hand, results from *in vivo* assays were mostly negative (micronucleus, chromosomal aberration, gene mutation and DNA damage tests). The only positive results were obtained in two micronucleus tests performed by intraperitoneal administration or after oral eugenol administration at a very high dose (14794.4 mg/kg orally). Overall, based on all the available information and using a weight of evidence approach, it may be concluded that eugenol should not be classified as mutagenic.
Impact on human health – proposed carcinogenicity classification				
		Yes	No	
i)	It is considered that, on the basis of assessment of the carcinogenicity testing carried out in accordance with the data requirements for the active substances, safener or synergist and other available data and information, including a review of the scientific literature, reviewed by the Authority, **the substance SHOULD BE classified or proposed for**		X	No new studies of long-term/carcinogenicity have been submitted for the renewal process of eugenol. After re-evaluating the data from the available carcinogenicity studies, it can be concluded that eugenol does not meet the criteria for carcinogenicity classification.

	classification, in accordance with the provisions of Regulation (EC) No 1272/2008, **as carcinogen category 1A or 1B.**			
ii)	Linked to above classification proposal. It is considered that exposure of humans to the active substance, safener or synergist in a plant protection product, under realistic proposed conditions of use, is negligible, that is, the product is used in closed systems or in other conditions excluding contact with humans and where residues of the active substance, safener or synergist concerned on food and feed do not exceed the default value set in accordance with Article 18(1)(b) of Regulation (EC) No 396/2005.			
Impact on human health – proposed reproductive toxicity classification				
		Yes	No	
i)	It is considered that, on the basis of assessment of the reproductive toxicity testing carried out in accordance with the data requirements for the active substances, safeners or synergists and other available data and information, including a review of the scientific literature, reviewed by the Authority, **the substance SHOULD BE classified or proposed for classification**, in accordance with the provisions of Regulation (EC) No 1272/2008, **as toxic for reproduction category 1A or 1B.**		X	No reproductive generation studies have been provided for the renewal process of eugenol. A multigenerational reproduction study performed in rats with isoeugenol, although the extrapolation to eugenol is doubtful. No effects on sexual function and fertility were found after isoeugenol treatment. Regarding developmental toxicity, no new studies have been submitted for the renewal process, but no adverse effects on development were observed in any of the studies already available. Based on the available information till the date, it could be considered that eugenol does not meet the criteria for reproductive toxicity classification. Pending on discussion about read-across to isoeugenol data.
ii)	Linked to above classification proposal. It is considered that exposure of humans to the active substance, safener or synergist in a plant protection product, under realistic proposed conditions of use, is negligible, that is, the product is used in closed systems or in other conditions excluding contact with humans and where residues of the active substance, safener or synergist concerned on food and feed do not exceed the default value set in accordance with Article 18(1)(b) of Regulation (EC) No 396/2005.			
Impact on human health – proposed endocrine disrupting properties classification				
		Yes	No	
i)	It is considered that **the substance SHOULD BE identified as having endocrine disrupting properties** in accordance with the provisions of point 3.6.5 in Annex II of Regulation (EC) No 1107/2009			Upon a preliminary assessment of the endocrine disrupting properties of eugenol:

				-T-modality: It was concluded that T-mediated adversity and T-mediated activity have been not sufficiently investigated (scenario 2aiii) due to relevant T-mediated parameters were not measured and the absence of TG 416 and/or 443 studies with eugenol. The evaluation of the available data from thyroid histopathology in two-year carcinogenicity studies in rats and mice, and supporting evidence from other repeated dose toxicity studies do not suggest an adversity mediated T-parameters. One positive/active result was obtained in the Thyroid Bioactivity Model US EPA for thyroid peroxidase inhibition (TPO), in agreement with QSAR *in-silico* predictions. Therefore, further data need to be generated before a conclusion on whether or not the ED criteria are met for the T-modality can be drawn [e.g. *in vitro* thyroperoxidase inhibition (rat and/or human) assay]. If the mentioned assay is negative, the scenario 2a(ii) applies and ED criteria are not met for T modality. However, if a positive result is obtained, the scenario 2a(i) applies and further data will be needed to support the MoA analysis: a study in line with OECD TG 443 (with cohort 1a/1b including the mating of cohort 1b to produce the F2 generation, OECD 2018) or with OECD TG 416. -EAS modalities: It was concluded that EAS-mediated adversity and EAS-mediated activity have been not sufficiently investigated (scenario 2aiii) due to relevant EAS-mediated parameters were not measured and the absence of TG 416 and/or 443 studies with eugenol. *In vitro* EAS-modalities assays suggested that eugenol could be a potential ER-antagonist. On the other hand, equivocal signs of EAS-mediated adversity were found (uterine endometrial polyp/stroma and cyst hyperplasia, and ovaries follicular cysts) in the long-term/chronic toxicity studies in rats and mice. Further data need to be generated before a conclusion on whether or not the ED criteria are met for the EAS-modalities can be drawn. E modality: A study in line with OECD TG 455. If the above assay is positive, a study in line with OECD TG 440 (Uterotrophic *in vivo* assay) might be required in order to confirm or rule out the estrogenic activity of eugenol from the *in vitro* assay. A modality: A study in line with OECD TG 458. S modality: A study in line with OECD TG 456 (H295R Steroidogenesis Assay) and/or a study in line with OPPTS 890.1200 (Aromatase assay).

				In case of OECD TG 458, 456 and OPPTS 890.1200 are negative, a study in line with OECD TG 441 (Hershberger Assay) is required. If the mentioned studies are negative, the scenario 2a(ii) applies and ED criteria are not met for EAS modalities. However, if these studies are positive for at least one modality, the scenario 2a(i) applies and further data will be needed to support the MoA analysis: a study in line with OECD TG 443 (with cohort 1a/1b including the mating of cohort 1b to produce the F2 generation, OECD 2018) or with OECD TG 416 (according to the latest version of 2001).
ii)	Linked to above identification proposal. It is considered that exposure of humans to the active substance, safener or synergist in a plant protection product, under realistic proposed conditions of use, is negligible, that is, the product is used in closed systems or in other conditions excluding contact with humans and where residues of the active substance, safener or synergist concerned on food and feed do not exceed the default value set in accordance with Article 18(1)(b) of Regulation (EC) No 396/2005.			*[if yes provide a brief explanation of conditions of use and cross refer to the section containing full details to support the contention of negligible exposure]*
Fate and behaviour in the environment				
Persistent organic pollutant (POP)				
		Yes	No	
	It is considered that the active substance **FULFILS** the criteria of a persistent organic pollutant (POP) as laid out in Regulation 1107/2009 Annex II Section 3.7.1.		X	***1.- Persistence criterion*** ***Soil:*** *The route and rate of degradation of eugenol were studied in four soils in the laboratory under aerobic conditions. Eugenol declined rapidly in each soil type. The DT_{50} and DT_{90} values were <1 day and <3 days respectively.* *No studies on anaerobic degradation were performed. Based on the fast aerobic degradation and the application timing of Mevalone, it is unlikely that eugenol residues would be found in anaerobic conditions.* *The photolysis study showed that degradation of eugenol was rapid in both irradiated and dark control conditions, with a DT50 of < 1 hour.* *Based on the rapid degradation rates of eugenol in soil, it is not expected to be persistent in a viable soil environment.* *Overall, eugenol does not fulfill the persistence criterion in soil set out in points 3.7.1.1 (POP criteria), 3.7.2.1 (PBT criteria), 3.7.3.1 (vPvB criteria) of annex II of the regulation 1107/2009.*

				Aquatic system: *Eugenol was shown to be hydrolytically stable at pH 4 and 7, and a DT_{50} of 120 days at pH 9.* *The photolysis DT_{50} in aqueous pH 7 buffer is 9.2 days, at environmental temperatures.* *In the aerobic mineralisation study DT_{50}'s of 1.8-2 days were calculated. Mineralisation to carbon dioxide was the major degradation product with maximum levels reaching 59.4-69.1%.* *Eugenol can be classified as readily biodegradable under the test conditions. Studies on degradation water/sediment systems have not been provided.* *Eugenol is not expected to fulfills the persistence criterion in aquatic systems set out in point 3.7.2.1 (PBT criteria)* ***2.- Bioaccumulation criterion*** *The log P_{ow} values for eugenol are 2.47, 2.49 and 2.44 at pH values 4, 7 and 9, respectively. These values are less than 3 and therefore do not trigger further consideration of bioconcentration in fish.* ***3.- Toxicity criterion*** *Based on the chronic toxicity on algae and aquatic invertebrates, it can be concluded that eugenol does not fulfill the criterion of toxicity to aquatic organisms set out in the Annex II of the regulation 1107/2009.* ***4.- Atmospheric Long range transport*** *The Atkinson calculation outputs a DT50 of eugenol in air of 0.165 hours and therefore it is considered that eugenol is not of potential concern for LRT.*
Persistent, bioaccumulative and toxic substance (PBT)				
		Yes	No	
	It is considered that the active substance **FULFILS** the criteria of a persistent, bioaccumulative and toxic (PBT) substance as laid out in Regulation 1107/2009 Annex II Section 3.7.2.		X	*See previous paragraph*
Very persistent and very bioaccumulative substance (vPvB).				
		Yes	No	

	It is considered that the active substance **FULFILS** the criteria of a a very persistent and very bioaccumulative substance (vPvB) as laid out in Regulation 1107/2009 Annex II Section 3.7.3.		X	*See previous paragraph*
Ecotoxicology				
		Yes	No	
i	It is considered that the risk assessment demonstrates risks to be acceptable in accordance with the criteria laid down in the uniform principles for evaluation and authorisation of plant protection products referred to in Article 29(6) under realistic proposed conditions of use of a plant protection product containing the active substance, safener or synergist. The RMS is content that the assessment takes into account the severity of effects, the uncertainty of the data, and the number of organism groups which the active substance, safener or synergist is expected to affect adversely by the intended use.			• **Birds:** No acute risk. No data or a strong justification are available to address the reproductive risk on birds. Therefore, further information should be submitted. • **Fish:** No acute risk. No data is available to address the long-term risk on fish. Therefore, further information should be submitted. No risk is expected in mammals, , bees, non-target arthropods other than bees, non-target soil meso- and macrofauna, Soil nitrogen transformation or non-target terrestrial plants.
ii	It is considered that, the substance **SHOULD BE identified as having endocrine disrupting properties** that may cause adverse effects on non-target organisms in accordance with the provisions of point 3.8.2 in Annex II of Regulation (EC) No 1107/2009.		X	No ED adversity for eugenol has been observed on NTO. However, the EATS-mediated adversity was not considered sufficiently investigated. Therefore, it is necessary to generate further information (Level 3 studies): • A study according to OECD TG 231 (AMA) • A study according to OECD 229 (FSTRA).
iii	Linked to the consideration of the endocrine properties immediately above. It is considered that the exposure of non-target organisms to the active substance in a plant protection product under realistic proposed conditions of use is negligible.		X	New information should be submitted. Please, see above.
iv	It is considered that it is established following an appropriate risk assessment on the basis of Community or internationally agreed test guidelines, that the use under the proposed conditions of use of plant protection products containing this active substance, safener or synergist: — will result in a negligible exposure of honeybees, or — has no unacceptable acute or chronic effects on colony survival and development, taking into account effects on honeybee larvae and honeybee behaviour.		X	The risks to bees is considered acceptable following the proposed representative uses in vineyards and pome fruit without the need for mitigation,.

Residue definition				
		Yes	No	
	It is considered that, where relevant, a residue definition can be established for the purposes of risk assessment and for enforcement purposes.	X		It is proposed that a residue definition for monitoring plant matrices is not required, since it is proposed to include eugenol into Annex IV to Regulation (EC) No. 396/2005. For RA: Sum of free and conjugated eugenol expressed as eugenol (fruit crops) Soil ; Water ; Sediment ; Air : Eugenol
Fate and behaviour concerning groundwater				
		Yes	No	
	It is considered that it has been established for one or more representative uses, that consequently after application of the plant protection product consistent with realistic conditions on use, the predicted concentration of the active substance or of metabolites, degradation or reaction products in groundwater complies with the respective criteria of the uniform principles for evaluation and authorisation of plant protection products referred to in Article 29(6) of Regulation 1107/2009.			The representative product Mevalone is a CS formulation containing 33 g/L eugenol, 66 g/L geraniol and 66 g/L thymol. Mevalone is the common representative product of the three active substances eugenol, geraniol and thymol intended to be renewed at the same time. The active substances are for use as a fungicide, for application on grapes and pome fruits. Relevant predefined scenarios for the respective crops were chosen. Application timing was chosen under consideration of the appropriate growth stages for the different FOCUS crops and scenarios. PEC_{gw} values were below the 0.1 µg/L limit for eugenol using all models. The risk to groundwater was determined to be acceptable for all uses of Mevalone containing eugenol.

3.1.2 Proposal – Candidate for substitution

Candidate for substitution				
		Yes	No	
	It is considered that the active substance shall be approved as a candidate for substitution			

3.1.3 Proposal – Low risk active substance

Low-risk active substances				
		Yes	No	
	It is considered that the active substance **shall be considered of low risk.** If the active substance is not a micro-organism, in particular it is considered that: (a) the substance **should NOT be classified or proposed for classification** in accordance to Regulation (EC) No 1272/2008 as any of the following: — carcinogenic category 1A, 1B or 2, — mutagenic category 1A, 1B or 2, — toxic to reproduction category 1A, 1B or 2, — skin sensitiser category 1, — serious damage to eye category 1, — respiratory sensitiser category 1, — acute toxicity category 1, 2 or 3, — specific Target Organ Toxicant, category 1 or 2, — toxic to aquatic life of acute and chronic category 1 on the basis of appropriate standard tests, — explosive, — skin corrosive, category 1A, 1B or 1C; (b) it has **not been identified as priority substance under Directive 2000/60/EC**; (c) it is **not deemed to be an endocrine disruptor** in accordance to Annex II of Regulation (EC) No 1107/2009; (d) it **has no neurotoxic or immunotoxic effects**; (e) it **is not persistent** (half-life in soil is more than 60 days) or its **bio-concentration factor is lower than 100**. (f) it is a **semiochemical** and verifies points (a) to (d).		X	***Toxicology:*** The RMS proposes the following classification of eugenol according to Criteria of Regulation (EC) No. 1272/2008: - Skin Sens. 1 (H317)

	Paragraph (e) doesn't apply to naturally occurring active substances. If the active substance is a micro-organism, in particular it is considered that at strain level the micro-organism has not demonstrated multiple resistance to anti-microbials used in human or veterinary medicine. If the active substance is a baculovirus, in particular it has not demonstrated adverse effects on non-target insects.			

3.1.4 List of studies to be generated, still ongoing or available but not peer reviewed

Data gap	Relevance in relation to representative use(s)	Study status		
		No confirmation that study available or on-going.	Study on-going and anticipated date of completion	Study available but not peer-reviewed
3.1.4.1 Identity of the active substance or formulation				
None				
3.1.4.2 Physical and chemical properties of the active substance and physical, chemical and technical properties of the formulation				
None				
3.1.4.3 Data on uses and efficacy				
None				
3.1.4.4 Data on handling, storage, transport, packaging and labelling				
None				
3.1.4.5 Methods of analysis				
Methods in crop matrices for pre-registration : The methods do not perform acid hydrolysis. According to the proposed residue definition for risk assessment if the residue definion is eventually set as RD-RA nº1, the residue definition would not be covered.	All intended uses	X		
3.1.4.6 Toxicology and metabolism				
Data required for endocrine disruption assessment (EAST-modalities)	All representative uses	X		

Data gap	Relevance in relation to representative use(s)	Study status		
		No confirmation that study available or on-going.	Study on-going and anticipated date of completion	Study available but not peer-reviewed
3.1.4.7 Residue data				
3.1.4.8 Environmental fate and behaviour				
The applicant is called to submit further information about the characterization of photoproducts in soil.	All intended uses	X		
The applicant is called to identity of the hydrolysis products 'Unknown 1' and 'Unknown 2'. More information is needed regarding to the percentages of AR reached by 'other unknowns from the hydrolysis study.	All intended uses	X		
The applicant is called to identity the unknown peaks from the direct photochemical degradation study.	All intended uses	X		
A data gap has been identifed to address the route and rate of degradation under natural water/sediment system.	All intended uses	X		
Applicant is called to submit an analysis of the short-range transport in air for eugenol.	All intended uses	X		
A data gap has been proposed for information to address the effect of water treatment processes on	All intended uses	X		

Data gap	Relevance in relation to representative use(s)	Study status		
		No confirmation that study available or on-going.	Study on-going and anticipated date of completion	Study available but not peer-reviewed
the nature of residues of the active substance when surface water is abstracted for drinking water to address Article 4 (approval criteria for active substances) 3(b) of Regulation (EC) No 1107/2009.				
3.1.4.9 Ecotoxicology				
The applicant is called to submit further information to assess the chronic risk on birds.	All intended uses	X		
The applicant is called to submit a new chronic toxicity study on fish	All intended uses	X		
The applicant is called to submit further information/data for addressing the potential ED activity of eugenol on non-target organisms. In particular, level 3 tests should be conducted as follows: • A test according to OECD TG 229 (Test No. 229: Fish Short Term Reproduction Assay). • A test according to OECD TG 231 (Test No. 231: Amphibian Metamorphosis Assay) In case of positive result/s based on the level 3 test, additional testing (OECD TG 241 and/or OECD	All intended uses	X		

Data gap	Relevance in relation to representative use(s)	Study status		
		No confirmation that study available or on-going.	Study on-going and anticipated date of completion	Study available but not peer-reviewed
TG 240) might be needed in order to further investigate the adversity.				

3.1.5 Issues that could not be finalised

An issue is listed as an issue that could not be finalised where there is not enough information available to perform an assessment, even at the lowest tier level, for the representative uses in line with the Uniform Principles, as laid out in Commission Regulation (EU) No 546/2011, and where the issue is of such importance that it could, when finalised, become a concern (which would also be listed as a critical area of concern if it is of relevance to all representative uses).

	Area of the risk assessment that could not be finalised on the basis of the available data	**Relevance in relation to representative use(s)**
1	Endocrine disruption properties (human toxicology)	All representative uses
2	Impact of water treatment process on eugenol when water is abstracted for drinking water	All representative uses
3	The characterization of photoproducts in soil.	All representative uses
4	The characterization of photoproducts in aquatic systems.	All representative uses
5	A data gap has been identifed to address the route and rate of degradation under natural water/sediment system.	All representative uses
6	The analysis of the short-range transport in air for eugenol.	All representative uses
7	Chronic risk to birds	All representative uses
8	Chronic risk to fish	All representative uses
9	EAST mediated adversity of eugenol with regard to non-target organisms	All representative uses

3.1.6 Critical areas of concern

An issue is listed as a critical area of concern:
(a) where the substance does not satisfy the criteria set out in points 3.6.3, 3.6.4, 3.6.5 or 3.8.2 of Annex II of Regulation (EC) No 1107/2009 and the applicant has not provided detailed evidence that the active substance is necessary to control a serious danger to plant health which cannot be contained by other available means including non-chemical methods, taking into account risk mitigation measures to ensure that exposure of humans and the environment is minimised, or
(b) where there is enough information available to perform an assessment for the representative uses in line with the Uniform Principles, as laid out in Commission Regulation (EU) 546/2011, and where this assessment does not permit to conclude that for at least one of the representative uses it may be expected that a plant protection product containing the active substance will not have any harmful effect on human or animal health or on groundwater or any unacceptable influence on the environment.

An issue is also listed as a critical area of concern where the assessment at a higher tier level could not be finalised due to a lack of information, and where the assessment performed at the lower tier level does not permit to conclude that for at least one of the representative uses it may be expected that a plant protection product containing the active substance will not have any harmful effect on human or animal health or on groundwater or any unacceptable influence on the environment.

Critical area of concern identified	Relevance in relation to representative use(s)
7.Chronic risk for birds	All representative uses
8. Chronic risk to fish	All representative uses

3.1.7 Overview table of the concerns identified for each representative use considered

(If a particular condition proposed to be taken into account to manage an identified risk, as listed in 3.3.1, has been evaluated as being effective, then 'risk identified' is not indicated in this table.)
All columns are grey as the material tested in the toxicological studies has not been demonstrated to be representative of the technical specification.

Representative use		Vineyards	Apples
Operator risk	Risk identified		
	Assessment not finalised	X[1]	X[1]
Worker risk	Risk identified		
	Assessment not finalised	X[1]	X[1]
Bystander risk	Risk identified		
	Assessment not finalised	X[1]	X[1]
Consumer risk	Risk identified		
	Assessment not finalised		
Risk to wild non target terrestrial vertebrates	Risk identified		
	Assessment not finalised	X[7,8]	X[7,8]
Risk to wild non target terrestrial organisms other than vertebrates	Risk identified	X[9]	
	Assessment not finalised		
Risk to aquatic organisms	Risk identified		
	Assessment not finalised	X[8]	X[8]
Groundwater exposure active substance	Legal parametric value breached		
	Assessment not finalised		
Groundwater exposure metabolites	Legal parametric value breached		
	Parametric value of 10µg/L[(a)] breached		
	Assessment not finalised		
Comments/Remarks			

The superscript numbers in this table relate to the numbered points indicated within chapter 3.1.5 and 3.1.6. Where there is no superscript number, see level 2 for more explanation.
(a): Value for non relevant metabolites prescribed in SANCO/221/2000-rev 10-final, European Commission, 2003

3.1.8 Area(s) where expert consultation is considered necessary

It is recommended to organise a consultation of experts on the following parts of the assessment report:

Area(s) where expert consultation is considered necessary	**Justification**
	[specify the reasons why expert consultation is considered necessary]

3.1.9 Critical issues on which the Co RMS did not agree with the assessment by the RMS

Points on which the co-rapporteur Member State did not agree with the assessment by the rapporteur member state. Only the points relevant for the decision making process should be listed.

Issue on which Co-RMS disagrees with RMS	**Opinion of Co-RMS**	**Opinion of RMS**
Toxicological values (ADI, AOEL, ARfD and AAOEL)	Additional uncertainty factors and setting of acute reference values.	Additional uncertainty factors applied only at the end of the process in case of lacking data and not setting of acute reference values.
Relevant scenario for EAST modalities.	Relevant scenario for T-modality is 2a(i) and for EAS modality is 1a.	Relevant scenarios are 2a(iii) for T-modality and for EAS modality.

3.2 PROPOSED DECISION

It is proposed that:

[redacted]

[redacted]

3.3 RATIONAL FOR THE CONDITIONS AND RESTRICTIONS TO BE ASSOCIATED WITH THE APPROVAL OR AUTHORISATION(S), AS APPROPRIATE

3.3.1 Particular conditions proposed to be taken into account to manage the risks identified

Proposed condition/risk mitigation measure	Relevance in relation to representative use(s)
[redacted]	

3.4 APPENDICES

GUIDANCE DOCUMENTS USED IN THIS ASSESSMENT

General

- COMMISSION IMPLEMENTING REGULATION (EU) No. 844/2012 of 18 September 2012; setting out the provisions necessary for the implementation of the renewal procedure for active substances, as provided for in Regulation (EC) No. 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market.
- COMMISSION REGULATION (EU) No. 283/2013 of 1 March 2013; setting out the data requirements for active substances, in accordance with Regulation (EC) No. 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products in the market.
- COMMISSION REGULATION (EU) No. 544/2011 of 10 June 2011, implementing Regulation (EC) No. 1107/2009 of the European Parliament and of the Council as regards of data requirements for active substances.
- REGULATION (EC) No. 1907/2006 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 18 December 2006. Concerning the Registration, Evaluation, Authorisation and Restrictions of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No. 793/93 and Commission Regulation (EC) No. 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC.
- REGULATION (EC) No. 1272/2008 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 December 2008; on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No. 1907/2006.

Section identity, physical chemical and analytical methods

Section physico chemical properties

- Manual on development and use of FAO and WHO specifications for pesticides: PLANT PRODUCTION AND PROTECTION PAPER 228; FAO/WHO Joint Meeting on Pesticide Specifications (JMPS); First edition-third revision; 2016.
- CIPAC MT 31: Free acidity or alkalinity, (Handbook F, p. 96), (2007)
- CIPAC MT 39.3: Low temperature stability of liquid formulations, (Handbook J, p. 126), (2007)
- CIPAC MT 46: Accelerated storage procedure, (Handbook J, p. 148), (2007)
- CIPAC MT 46.3: Accelerated storage procedure, (Handbook F, p. 128), (2000)
- CIPAC MT 47: Persistent foaming, (Handbook F, p. 152), (2007)
- CIPAC MT 59.3: Sieve analysis, wet sieving, (Handbook F, p. 179), (2007)
- CIPAC MT 75: Determination of pH values, (Handbook F, p. 205) ,(1994)
- CIPAC MT 75.3: Determination of pH values, (Handbook J, p. 131) ,(2000)
- CIPAC MT 157: Water solubility, (Handbook F, p. 379), (2007)
- CIPAC MT 160: Spontaneity of dispersion, of suspension concentrates, (Handbook F, p. 391), (2007)
- CIPAC MT 161: Suspensibility of aqueous suspension concentrates, (Handbook F, p. 394), (2007)
- CIPAC MT 181: Solubility in Organic Solvents, (Handbook H, p. 314), (2007)
- COUNCIL REGULATION (EC) No. 440/2008 of 30 May 2008
- Laying down test methods pursuant to Regulation (EC) No. 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

- OECD Test Guideline 69: Guidance Document on the Validation of (Q)SAR Models, (2007)
- OECD Test Guideline 101: UV-VIS Absorption Spectra (Spectrometric Method), (1981)
- OECD Test Guideline 102: Melting Point/Melting Range, (1995)
- OECD Test Guideline 103: Boiling Point, (1995)
- OECD Test Guideline 104: Vapour Pressure, (2006)
- OECD Test Guideline 105: Water Solubility, (1995)
- OECD Test Guideline 107: Partition Coefficient (n-octanol/water): Shake Flask Method, (1995)
- OECD Test Guideline 109: Density of Liquids and Solids, (2012)
- OECD Test Guideline 112: Dissociation Constants in Water (Titration Method), (1981)
- OECD Test Guideline 114: Viscosity of liquids, (2012).
- OECD Test Guideline 115: Surface Tension of Aqueous Solutions, (1995).
- OPPTS 830.6314 Oxidation/Reduction: Chemical Incompatibility, (1996).
- UN Test N.4: Test method for self-heating substances. (UNECE, 2009).
- Estimation of volatile emission potential method of pesticides by thermogravimetry, Department of Pesticide Regulation, United State of California, Attachment B, Method date: 2-9-05

Section analytical methods

- SANCO 3030/99 rev. 5 of 22 March 2019: Technical Active Substance and Plant protection products: Guidance for generating and reporting methods of analysis in support of pre- and post-registration data requirements for Annex (Section 4) of Regulation (EU) No 283/2013 and Annex (Section 5) of Regulation (EU) No. 284/2013.
- SANCO 3029/ 99 rev. 4 of 11/07/00. Residues: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414.
- SANCO /825/00 rev. 8.1 of 16/11/2010. Guidance document on pesticide residue analytical methods.
- SANTE 2017/10632 Rev. 3 of 22 November 2017: Technical Guideline on the Evaluation of Extraction Efficiency of Residue Analytical Methods.
- OECD 501 of 8 January 2007. Metabolism in crops.
- EU guideline for residue data 7028/VI/95 rev.3, APPENDIX A of 22/07/1997. Metabolism and distribution in plants.

Section Data on application and efficacy

Section Toxicology

- ECHA Guidance on the application of the CLP criteria. Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures. Version 5.0 July 2017.
- Guidance Document on Considerations for Waiving or Bridging of Mammalian Acute Toxicity Tests, Series on Testing & Assessment No. 237; ENV/JM/MONO(2016)32
- Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009, EFSA/ECHA (2018), Adopted on 5 June 2018.
- Guidance on dermal absorption. 2017. Buist H, Craig P, Dewhurst I, HougaardBennekou S, Kneuer C, Machera K, Pieper C, Court Marques D, Guillot G, Ruffo F and Chiusolo A. EFSA Journal 2017; 15(6):4873. doi: 10.2903/j.efsa.2017.4873
- Note for agreement by Member States's Competent Authorities in the SCoPAFF: Phytopharmaceutical legislation section [SANTE/2018/10591 rev.1]

- Guidance of EFSA Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/20091. EFSA Journal 2011; 9(2): 2092.
- Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data. EFSA Journal 2012; 10 (3): 2579.
- Guidance document on the assessment of the equivalence of technical materials of substances regulated under Regulation (EC) No 1107/2009. SANCO/10597/2003 –rev. 10.1. 13 July 2012.
- Outcome of the pesticides peer review meeting on general recurring issues in mammalian toxicology. EFSA Supporting publication 2016: EN-1074
- Scientific opinion: clarification of some aspects related to genotoxicity assessment. November 2017. EFSA Journal. doi: 10.2903/j.efsa.2017.5113
- Retrospective analysis of the immunotoxic effects of plant protection products as reported in the Draft Assessment Reports for their peer review at EU level (Dewhurst, I., Koshy, L, Samuel, S. and Shillaker, D., 2015, EFSA supporting publication 2015:EN-782).
- Guidance for immunotoxicity risk assessment for chemicals. IPCS harmonization project document; no. 10. World Health Organization and International Programme on Chemical Safety. (2012).
- Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption
- Scientific opinion: Statement on the applicability of the Margin of Exposure approach for the safety assessment of impurities1 which are both genotoxic and carcinogenic in substances added to food/feed. EFSA Journal 2012;10(3):2578.
- Scientific opinion. Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data. EFSA Journal 2012;10(3):2579.
- OECD Test Guideline 403: Acute Inhalation Toxicity (2009).
- OECD Test Guideline 404: Acute Dermal Irritation/Corrosion (2015).
- OECD Test Guideline 405: In Vivo Eye Irritation/Serious Eye Damage (2017).
- OECD Test Guideline 406: Skin Sensitisation Guinea Pig Maximisation Test and Buehler Test (2021).
- OECD Test Guideline 407: Repeated Dose 28-Day Oral Toxicity Study in Rodents (2008)
- OECD Test Guideline 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents (2018)
- OECD Test Guideline 414: Prenatal developmental toxicity study (2018).
- OECD Test Guideline 416: Two-Generation Reproduction Toxicity Study (2001).
- OECD Test Guideline 417: Toxicokinetics (2010)
- OECD Test Guideline 420: Acute Oral Toxicity – Fixed Dose Procedure (2002)
- OECD Test Guideline 429: Skin Sensitisation: Local Lymph Node Assay (LLNA) (2010)
- OECD Test Guideline 432: In Vitro 3T3 NRU Phototoxicity Test (2019)
- OECD Test Guideline 453: Combined chronic toxicity\carcinogenicity studies (2018).
- OECD Test Guideline 471: Bacterial Reverse Mutation Test (2020).
- OECD Test Guideline 473: In Vitro Mammalian Chromosomal Aberration Test (2016).
- OECD Test Guideline 474: Mammalian Erythrocyte Micronucleus Test (2016).
- OECD Test Guideline 475: Mammalian Bone Marrow Chromosomal Aberration Test (2016).
- OECD Test Guideline 479: Genetic Toxicology: In Vitro Sister Chromatid Exchange Assay in Mammalian Cells (1986, deleted 2014)
- OECD Test Guideline 486: Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells In Vivo (1997)

- OECD Test Guideline 490: In Vitro Mammalian Cell Gene Mutation Tests Using the Thymidine Kinase Gene (2016)

Section Residue and consumer risk assessment

Section fate and behavior in environment

- EFSA (European Food Safety Authority), 2007. Scientific Opinion of the Panel on Plant Protection Products and their Residues on a request from EFSA related to the default Q10 value used to describe the temperature effect on transformation rates of pesticides in soil. The EFSA Journal (2007) 622, 1-32.
- EFSA (European Food Safety Authority), 2011. Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009 (OJ L 309, 24.11.2009, p. 1-50). EFSA Journal 2011;9(2):2092. 49 pp. doi:10.2903/j.efsa.2011.2092.
- EFSA (European Food Safety Authority) 2014b. EFSA Guidance Document for evaluating laboratory and field dissipation studies to obtain DegT50 values of active substances of plant protection products and transformation products of these active substances in soil. EFSA Journal 2014;12(5):3662.
- EFSA (European Food Safety Authority), 2015. Technical report on the outcome of the pesticides peer review meeting on general recurring issues in ecotoxicology. EFSA supporting publication 2015:EN-924. 62 pp.
- FOCUS 1997. Soil persistence models and EU registration. The final report of the work of the Soil Modelling Work group of FOCUS. 77 pp.
- FOCUS 2000. FOCUS groundwater scenarios in the EU review of active substances. Report of the FOCUS Groundwater Scenarios Workgroup, EC Document Reference SANCO/321/2000 rev.2, 202 pp.
- FOCUS 2001. FOCUS Surface Water Scenarios in the EU Evaluation Process under 91/414/EEC. Report of the FOCUS Working Group on Surface Water Scenarios, EC Document Reference SANCO/4802/2001-rev.2. 245 pp., as updated by the Generic Guidance for FOCUS surface water scenarios, version 1.1 dated March 2012
- FOCUS 2006. Guidance Document on Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU Registration. Report of the FOCUS Work Group on Degradation Kinetics, EC Document Reference Sanco/10058/2005 version 2.0, 434 pp. June 2006.
- FOCUS 2008. “Pesticides in Air: Considerations for Exposure Assessment”. Report of the FOCUS Working Group on Pesticides in Air, EC Document Reference SANCO/10553/2006 Rev 2 June 2008. 327 pp.
- FOCUS 2011. Guidance Document on Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU Registration. Version 1.0 (23/11/2011).
- FOCUS (2014a) : Generic guidance for Estimating Persistence and Degradation Kinetics from Environmental Fate Studies on Pesticides in EU Registration. version 1.1 (18/12/2014). 440 pp.
- FOCUS 2014b. Assessing Potential for Movement of Active Substances and their Metabolites to Ground Water in the EU. The Final Report of the Ground Water Work Group of FOCUS, SANCO/13144/2010 version 3, 613 pp., 10 October 2014.
- FOCUS 2014c. Generic guidance for Tier 1 FOCUS ground water assessments. Technical Report Version 2.2, FOrum for the Co-ordination of pesticide fate models and their Use
- FOCUS 2015. Generic guidance for FOCUS surface water scenarios. Technical Report Version 1.4, FOrum for the Co-ordination of pesticide fate models and their USe. May 2015.
- SANCO 221/2000 rev 10 : European Commission, 2003. Guidance Document on Assessment of the Relevance of Metabolites in Groundwater of Substances Regulated under Council Directive 91/414/EEC. SANCO/221/2000-rev. 10 - final, 25 February 2003.

- OECD 307. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Aerobic and Anaerobic Transformation in Soil, 2002.
- OECD "Phototransformation of Chemicals on Soil Surfaces" (Draft, January 2002).
- OECD 106. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Adsorption - Desorption Using a Batch Equilibrium Method, 2000.
- OECD 111 (2002); OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Hydrolysis as a Function of pH, 2004.
- OECD 301 D. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Ready Biodegradability, 1992.
- OECD Guideline 309. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Aerobic Mineralisation in Surface Water – Simulation Biodegradation Test, 2004.
- OECD 308. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Aerobic and Anaerobic Transformation in Aquatic Sediment Systems, 2002.

Section ecotoxicology

- EFSA (European Food Safety Authority), 2009. Guidance on the Risk Assessment for Birds and Mammals: EFSA Journal 2009; 7(12):1438
- EFSA (European Food Safety Authority), 2013. Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters: EFSA Journal 2013;11(7):3290
- Guidance document on regulatory testing and risk assessment procedures for plant protection products with non-target arthropods ESCORT II (2000)
- EFSA (European Food Safety Authority), 2013 Guidance Document on the risk assessment of plant protection products on bees (Apis mellifera, Bombus spp. and solitary bees). EFSA Journal 2013;11(7):3295, 268 pp., doi:10.2903/j.efsa.2013.3295
- Guidance Document on Terrestrial Ecotoxicology Under Council Directive 91/414/EEC: SANCO/10329/2002
- EFSA (European Food Safety Authority), 2011. Guidance Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009: EFSA Journal 2011;9(2):2092
- ECHA and EFSA (European Chemicals Agency and European Food Safety Authority), 2018. Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 (EFSA/ECHA, 2018). EFSA Journal, Vol 16, Issue 6, June 2018, e05311 https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2018.5311
- EFSA GD for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 (EFSA Journal 2018; 16(6): 5311, 135 pp.
- Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009.
- Blümer et al., 2000. Laboratory residual contact test with the predatory mite Typhlodromus pyri Scheuten (Acari: Phytoseiidae) for regulatory testing of plant protection products. M.P. CANDOLFI et al. (2000): Guidelines to evaluate side-effects of plant protection products to non-target arthropods. IOBC, BART and EPPO Joint Initiative.
- ECHA Guidance to Regulation (EC) no 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, version 5 July 2017.
- EFSA (European Food Safety Authority), 2009. Guidance Document on Risk Assessment for Birds and Mammals on request from EFSA. EFSA Journal 2009;7(12):1438, 358 pp. doi:10.2903/j.efsa.2009.1438.

- EFSA (European Food Safety Authority), 2013. Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. EFSA Journal 2013;11(7):3290, 268 pp. doi:10.2903/j.efsa.2013.3290
- EFSA (European Food Safety Authority), 2013. EFSA Guidance Document on the risk assessment of plant protection products on bees (Apis mellifera, Bombus spp. and solitary bees). EFSA Journal 2013;11(7):3295, 268 pp., doi:10.2903/j.efsa.2013.3295.
- European Commission, 2002a. Guidance Document on Terrestrial Ecotoxicology Under Council Directive 91/414/EEC. SANCO/10329/2002 rev.2 final, 17 October 2002
- Mead-Briggs et al., 2000. A laboratory test for evaluating the effects of plant protection products on the parasitic wasp, Aphidius rhopalosiphi (DeStephani-Perez) (Hymenoptera: Braconidae). M.P. CANDOLFI et al. (2000): Guidelines to evaluate side-effects of plant protection products to non-target arthropods. IOBC, BART and EPPO Joint Initiative.
- OECD 201 (1984, update 2002). OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Freshwater Alga and Cyanobacteria, Growth Inhibition Test, 2011.
- OECD 202 (1984). OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Daphnia sp., Acute Immobilisation Test, 2004.
- OECD 203 (1992). OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Fish, Acute Toxicity Test, 1992.
- OECD 204 (1984). OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Fish, Prolonged Toxicity Test: 14-day Study, 1984.
- OECD 205. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Avian Dietary Toxicity Test, 1984.
- OECD 206. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Avian Reproduction Test, 1984.
- OECD 207 (1984) and ISO 11268-1 (1993). OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Earthworm, Acute Toxicity Tests, 1984.
- OECD 209. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Activated Sludge, Respiration Inhibition Test (Carbon and Ammonium Oxidation), 2010.
- OECD 210. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Fish, Early-life Stage Toxicity Test, 2013.
- OECD 211. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Daphnia magna Reproduction Test, 2012.
- OECD 211. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Daphnia magna Reproduction Test.
- OECD 213. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Honeybees, Acute Oral Toxicity Test, 1998.
- OECD 214. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Honeybees, Acute Contact Toxicity Test, 1998.
- OECD 215. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Fish, Juvenile Growth Test, 2000.
- OECD 216. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Soil Microorganisms: Nitrogen Transformation Test, 2000.
- OECD 217. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Soil Microorganisms: Carbon Transformation Test, 2000.
- OECD 218. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Sediment-Water Chironomid Toxicity Test Using Spiked Sediment, 2004.
- OECD 222. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Earthworm Reproduction Test (Eisenia fetida/ Eisenia andrei, 2016.

- OECD 223. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Avian Acute Oral Toxicity Test, 2016.
- OECD 226. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Predatory mite (Hypoaspis (Geolaelaps) aculeifer) reproduction test in soil, 2016.
- OECD 232. OECD GUIDELINE FOR THE TESTING OF CHEMICALS. Collembolan Reproduction Test in Soil, 2016.
- SETAC (Society of Environmental Toxicology and Chemistry), 2001. Guidance Document on Regulatory Testing and Risk Assessment procedures for Plant Protection Products with Non-Target Arthropods. ESCORT 2.

3.5 REFERENCE LIST

Section identity, physical chemical and analytical methods

- Draft Assessment Report (DAR) on the active substance eugenol prepared by the rapporteur Member State United Kingdom in the framework of Directive 91/414/EEC. July 2011.
- Final addendum to Draft Assessment Report (DAR) on eugenol. RMS: United Kingdom. June 2012
- Addendum-confirmatory data to Draft Assessment Report (DAR) on eugenol under regulation 1107/2009. RMS: United Kingdom. August 2016.
- Conclusion on the peer review of the pesticide risk assessment of the active substance eugenol. EFSA Journal 2012; 20(11):2914.
- Peer review report on eugenol: October 2012.
- Final Review Report for the active substance eugenol. SANCO/10577/2013 rev.3 May 2013.
- Outcome of the consultation with Member States, the applicant and EFSA on the pesticide risk assessment for eugenol in light of confirmatory data. January 2017. doi:10.2903/sp.efsa.2017.EN-1165.

Section data on application and efficacy

- Draft Assessment Report (DAR) on the active substance eugenol prepared by the rapporteur Member State United Kingdom in the framework of Directive 91/414/EEC. July 2011.
- Final addendum to Draft Assessment Report (DAR) on eugenol. RMS: United Kingdom. June 2012
- Addendum-confirmatory data to Draft Assessment Report (DAR) on eugenol under regulation 1107/2009. RMS: United Kingdom. August 2016.
- Conclusion on the peer review of the pesticide risk assessment of the active substance eugenol. EFSA Journal 2012; 20(11):2914.
- Peer review report on eugenol: October 2012.
- Final Review Report for the active substance eugenol. SANCO/10577/2013 rev.3 May 2013.
- Outcome of the consultation with Member States, the applicant and EFSA on the pesticide risk assessment for eugenol in light of confirmatory data. January 2017. doi:10.2903/sp.efsa.2017.EN-1165.

Section toxicology

- Draft Assessment Report (DAR) on the active substance eugenol prepared by the rapporteur Member State United Kingdom in the framework of Directive 91/414/EEC. July 2011.
- Final addendum to Draft Assessment Report (DAR) on eugenol. RMS: United Kingdom. June 2012
- Addendum-confirmatory data to Draft Assessment Report (DAR) on eugenol under regulation 1107/2009. RMS: United Kingdom. August 2016.
- Conclusion on the peer review of the pesticide risk assessment of the active substance eugenol. EFSA Journal 2012; 20(11):2914.
- Peer review report on eugenol: October 2012.
- Final Review Report for the active substance eugenol. SANCO/10577/2013 rev.3 May 2013.
- Outcome of the consultation with Member States, the applicant and EFSA on the pesticide risk assessment for eugenol in light of confirmatory data. January 2017. doi:10.2903/sp.efsa.2017.EN-1165.
- NTP technical report on the toxicology and carcinogenesis studies of isoeugenol (Cas No. 97-54-1) in F344/N rats and B6C3F1 mice. NTP TR 551. NIH Publication No. 10-5892. September 2010.

- Opinion of the Scientific Committee on Food on Methyleugenol (4-Allyl-1,2-dimethoxybenzene). SCF/CS/FLAV/FLAVOUR/4 ADD1 FINAL. September 2001.
- Flavouring Group Evaluation 60 (FGE.60): Consideration of eugenol and related hydroxyallylbenzene derivatives evaluated by JECFA (65th meeting) structurally related to ring-substituted phenolic substances evaluated by EFSA in FGE.22 (2006). Opinion of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC). (Question EFSA-2008-32L). The EFSA Journal (2009) ON-965, 1-53.

Section residue and consumer risk assessment

Section fate and behavior in environment

Section ecotoxicology