EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2015. Scientific Opinion on Flavouring Group Evaluation 25, Revision 3 (FGE.25Rev3): Aliphatic hydrocarbons from chemical group 31

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SCIENTIFIC OPINION

Scientific opinion on Flavouring Group Evaluation 25, Revision 3 (FGE.25Rev3): Aliphatic hydrocarbons from chemical group 31[1]

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids of the European Food Safety Authority was requested to evaluate 14 flavouring substances in the Flavouring Group Evaluation 25, Revision 3, using the Procedure in Commission Regulation (EC) No 1565/2000. None of the substances was considered to have a genotoxic potential. This revision is made due to the inclusion of new toxicity data on the supporting substances β-caryophyllene [FL-no: 01.007] and myrcene [FL-no: 01.008] considered in FGE.78Rev2 to cover the assessment of 4(10)-thujene [FL-no: 01.059], 2,6-dimethylocta-2,4,6-triene [FL-no: 01.035], cis-3,7-dimethyl-1,3,6-octatriene [FL-no: 01.064] and 1-octene [FL-no: 01.070]. The substances were evaluated through a stepwise approach (the Procedure) that integrates information on structure-activity relationships, intake from current uses, toxicological threshold of concern, and available data on metabolism and toxicity. The Panel concluded that all 14 substances [FL-no: 01.001, 01.027, 01.028, 01.033, 01.034, 01.035, 01.038, 01.039, 01.046, 01.054, 01.057, 01.059, 01.064, 01.070] do not give rise to safety concerns at their levels of dietary intake, estimated on the basis of the MSDI approach. Besides the safety assessment of these flavouring substances, the specifications for the materials of commerce have also been considered. Adequate specifications including complete purity and identity criteria for the materials of commerce have been provided for all 14 candidate substances.

KEY WORDS

flavourings, aliphatic, hydrocarbons, FGE.25.

[1] On request from the European Commission, Question No EFSA-Q-2013-00193, EFSA-Q-2013-00849, EFSA-Q-2013-00850 and EFSA-Q-2013-00851 adopted on 18 March 2015.

[2] Panel members: Claudia Bolognesi, Laurence Castle, Jean-Pierre Cravedi, Karl-Heinz Engel, Paul Fowler, Konrad Grob, Rainer Gürtler, Trine Husøy, Wim Mennes, Maria Rosaria Milana, André Penninks, Maria de Fatima Tavares Poças, Vittorio Silano, Andrew Smith, Christina Tlustos, Fidel Toldra, Detlef Wölfle and Holger Zorn. Correspondence: fip@efsa.europa.eu

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SUMMARY

Following a request from the European Commission, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (the Panel) was asked to deliver a scientific opinion on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Panel was requested to evaluate 14 flavouring substances in the Flavouring Group Evaluation 25, Revision 3 (FGE.25Rev3), using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000. These 14 flavouring substances belong to chemical group 31, Annex I of the Commission Regulation (EC) No 1565/2000.

The present revision of FGE.25, Revision 3 includes the assessment of a 90-day study on ß-caryophyllene [FL-no: 01.007] supporting the candidate substance 4(10)-thujene [FL-no: 01.059] and a 90-day study on myrcene [FL-no: 01.008] supporting the candidate substances 2,6-dimethylocta-2,4,6-triene [FL-no: 01.035], cis-3,7-dimethyl-1,3,6-octatriene [FL-no: 01.064] and 1-octene [FL-no: 01.070].

Since the publication of FGE.25Rev2, Industry has informed EFSA that 23 substances [former FL-no: 01.021, 01.022, 01.023, 01.030, 01.031, 01.032, 01.036, 01.037, 01.042, 01.043, 01.044, 01.047, 01.050, 01.051, 01.052, 01.053, 01.055, 01.056, 01.058, 01.060, 01.066, 01.067 and 01.078] are no longer supported for use as flavouring substances in Europe by Industry. The FGE.25Rev3 therefore deals with 14 flavouring substances in total.

The 14 candidate substances are aliphatic hydrocarbons from chemical group 31, which have been divided into four subgroups: I) acyclic alkanes, II) acyclic alkenes, III) cyclohexene hydrocarbons, IV) is now empty as all the substances are no longer supported by Industry, V) bicyclic, non-aromatic hydrocarbon ,VI) is now empty as all the substances are no longer supported by Industry.

Seven of the 14 flavouring substances possess chiral centres and three can exist as geometrical isomers.

All of the 14 candidate substances are classified into structural class I, according to the decision tree approach presented by Cramer et al. (1978).

Twelve of the 14 candidate substances have been reported to occur naturally in a wide range of food items.

In its evaluation, the Panel as a default used the “Maximised Survey-derived Daily Intake” (MSDI) approach to estimate the per capita intakes of the flavouring substances in Europe. However, when the Panel examined the information provided by the European Flavour Industry on the use levels in various foods, it appeared obvious that the MSDI approach in a number of cases would grossly underestimate the intake by regular consumers of products flavoured at the use level reported by the Industry, especially in those cases where the annual production values were reported to be small. In consequence, the Panel had reservations about the data on use and use levels provided and the intake estimates obtained by the MSDI approach.

In the absence of more precise information that would enable the Panel to make a more realistic estimate of the intakes of the flavouring substances, the Panel decided also to perform an estimate of the daily intakes per person using a “modified Theoretical Added Maximum Daily Intake” (mTAMDI) approach based on the normal use levels reported by Industry. In those cases where the mTAMDI approach indicated that the intake of a flavouring substance might exceed its corresponding threshold of concern, the Panel decided not to carry out a formal safety assessment using the Procedure. In these cases the Panel requires more precise data on use and use levels.

According to the default MSDI approach, 12 of the 14 flavouring substances in this group have intakes in Europe from 0.0085 to 14 µg/*capita* per day, which are below the threshold of concern value for structural class I (1800 µg/person per day) substances. For limonene [FL-no: 01.001] and l-limonene

[FL-no: 01.046] the intakes are 4000 and 2100 µg/*capita* per day, which are above the threshold of concern value for structural class I (1800 µg/person per day).

On the basis of the reported annual production volumes in Europe (MSDI approach), the total combined intakes of the candidate and supporting substances can be calculated for the substances in subgroup I, II, III and V (those subgroups still containing substances) evaluated through the Procedure.

Subgroup I (acyclic alkanes): the combined intake of the five candidate substances, all from structural class I and evaluated via the A-side of the Procedure (Appendix A), is 3.0 µg/*capita* per day, which does not exceed the threshold of 1800 µg/person per day.

Subgroup II (acyclic alkenes): the combined intake of the three candidate substances, all from structural class I and evaluated via the B-side of the Procedure (Appendix A), is 23 µg/*capita* per day, which does not exceed the threshold of 1800 µg/person per day.

Subgroup III (cyclohexene hydrocarbons): the combined intake of the three candidate substances, all from structural class I and evaluated via the A-side of the Procedure (Appendix A), is 6100 µg/*capita* per day, which does exceed the threshold of 1800 µg/person per day. The total combined intake of the three candidate and four supporting substances (also from structural class I) is approximately 42 000 µg/*capita* per day. This intake exceeds the threshold of 1800 µg/person per day for a structural class I substance. However, together, limonene [FL-no: 01.001], *l*-limonene [FL-no: 01.046] and *d*-limonene (supporting substance [FL-no: 01.045]) account for approximately 40 000 µg/*capita* per day. The total combined intake of 42 000 µg/*capita* per day for the candidate and the supporting substances corresponds to 700 µg/kg bw per day for a person with a body weight of 60 kg. Thus, based on the NOAEL for *d*-limonene of 215 mg/kg bw per day, a margin of safety of 307 can be calculated, and accordingly these substances are not expected to be of safety concern at the estimated level of intake.

Subgroup V (bicyclic, non-aromatic hydrocarbons): as no sufficient data are available to conclude that the candidate substance [FL-no: 01.059] will be metabolised to innocuous products, it is evaluated via the B-side of the Procedure (Appendix A). The total combined intake of the one candidate and eight supporting substances (also from structural class I) is approximately 3800 µg/*capita* per day. This intake exceeds the threshold of 1800 µg/person per day for a structural class I substance. However, three supporting substances, pin-2(10)-ene [FL-no: 01.003], pin-2(3)-ene [FL-no: 01.004] and β-caryophyllene [FL-no: 01.007] together account for approximately 3400 µg/*capita* per day. The total combined intake of 3800 µg/*capita* per day for the candidate and the supporting substances corresponds to 63 µg/kg bw per day for a person with a body weight of 60 kg. Thus, based on the NOAEL for β-caryophyllene of 222 mg/kg bw per day, a margin of safety of 3500 can be calculated, and accordingly this substance is not expected to be of safety concern at the estimated level of intake.

The available information on metabolism of the 14 candidate substances evaluated through the Procedure or the supporting substances for this FGE was limited. For the following 10 candidate substances it can be concluded that they will be metabolised into innocuous metabolites: [FL-no: 01.033, 01.034, 01.038, 01.054 and 01.057] from subgroup I and [FL-no: 0.001, 01.027, 01.028, 01.039 and 01.046] from subgroup III. For two candidate substances there are data, which show that they may be metabolised to toxic metabolites [FL-no: 01.064 and 01.070]. For the remaining two candidate substances [FL-no: 01.035 and 01.059], the information is too limited and it cannot be assumed that they are metabolised to innocuous metabolites.

It was noted that where toxicity data were available they were consistent with the conclusions in the present flavouring group evaluation using the Procedure.

It is concluded that the 10 candidate substances which are expected to be metabolised to innocuous substances, [FL-no: 01.033, 01.034, 01.038, 01.054 and 01.057] from subgroup I and [FL-no: 01.001,

01.027, 01.028, 01.039 and 01.046] from subgroup III, would not give rise to safety concerns at their estimated intakes arising from their use as flavouring substances based on the MSDI approach.

For 4(10)-thujene [FL-no: 01.059], from subgroup V, which is not expected to be metabolised to innocuous substances, a margin of safety could be calculated based upon a NOAEL (222 mg/kg/bw) for the supporting substance ß-caryophyllene [FL-no: 01.007]. Compared to the MSDI of 4(10)-thujene of 14 µg/*capita* per day corresponding to 0.2 µg/kg bw per day, the NOAEL provides a margin of safety of 9.5×10^5.

For the three remaining substances, 2,6-dimethylocta-2,4,6-triene [FL-no: 01.035], cis-3,7-dimethyl-1,3,6-octatriene [FL-no: 01.064] and 1-octene [FL-no: 01.070] a margin of safety could be calculated based upon a NOAEL (44 mg/kg bw) for the supporting substance myrcene [FL-no: 01.008]. Compared to the MSDI of 2,6-dimethylocta-2,4,6-triene [FL-no: 01.035], cis-3,7-dimethyl-1,3,6-octatriene [FL-no: 01.064] and 1-octene [FL-no: 01.070] of 9.1, 14 and 0.0085 µg/*capita* per day equal to 0.15, 0.23 and 0.00014 µg/kg bw per day, the NOAEL provides a margin of safety of 2.9×10^5, 1.9×10^5 and 3.1×10^8.

The mTAMDI values for 11 candidate substances are above the threshold for structural class I of 1800 µg/person per day. For these substances more reliable exposure data are requested in order for them to be considered using the Procedure. For limonene [FL-no: 01.001] the mTAMDI is 1800 µg/person per day and for each of l-limonene and 1-octene [FL-no: 01.046 and 01.070] the mTAMDI is 1600 µg/person per day.

In order to determine whether this conclusion could be applied to the materials of commerce, it is necessary to consider the available specifications. The specifications including complete purity criteria and identity for the materials of commerce have been provided for all 14 flavouring substances.

Thus, for all 14 candidate substances [FL-no: 01.001, 01.027, 01.028, 01.033, 01.034, 01.035, 01.038, 01.039, 01.046, 01.054, 01.057, 01.059, 01.064 and 01.070] the Panel concluded that they would present no safety concern at their estimated levels of intake based on the MSDI approach.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The use of flavourings in food is regulated under Regulation (EC) No 1334/2008 of the European Parliament and Council of 16 December 2008[4] on flavourings and certain food ingredients with flavouring properties for use in and on foods. On the basis of Article 9(a) of this Regulation, an evaluation and approval are required for flavouring substances.

The Union list of flavourings and source materials was established by Commission Implementing Regulation (EC) No 872/2012[5]. The list contains flavouring substances for which the scientific evaluation should be completed in accordance with Commission Regulation (EC) No 1565/2000[6].

FGE.78Rev1

On 19 May 2011, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) adopted an opinion on Flavouring Group Evaluation 78, Revision 1 (FGE.78Rev1): Consideration of aliphatic and alicyclic and aromatic hydrocarbons evaluated by JECFA (63rd meeting) structurally related to aliphatic and aromatic hydrocarbons evaluated by EFSA in FGE.25Rev2[7].

The substances [FL-no: 01.008, 01.018, 01.040 and 01.061] were among the 14 substances for which the Panel had "reservations (no European production volumes available, preventing them from being evaluated using the Procedure, and/or missing information on stereoisomerism/composition of mixture)" and also among those for which "additional toxicity data was requested".

FGE.25Rev2

On 19 May 2011, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) adopted an opinion on Flavouring Group Evaluation 25, Revision 2 (FGE.25Rev2): Aliphatic and aromatic hydrocarbons from chemical group 31[8].

The substances with [FL-no: 01.035, 01.064, 01.070 and 01.035] were among the 27 candidate substances for which "additional toxicity data" were required by EFSA. For [FL-no: 01.035] also "additional information on composition" was requested.

FGE.18Rev2

On 30 September 2010, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) adopted an opinion on Flavouring Group Evaluation 18, Revision 2 (FGE.18Rev2): Aliphatic, alicyclic and aromatic saturated and unsaturated tertiary alcohols, aromatic tertiary alcohols and their esters from chemical groups 6 and 8.

For the flavouring substance [FL-no: 02.146], the Panel considered that "additional data" are needed including "information on specifications/stereoisomerism/composition of mixture".

[4] Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. OJ L 354, 31.12.2008, p.34-50.

[5] Commission implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC.OJ L 267, 2.10.2012, p. 1-161.

[6] Commission Regulation No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. OJ L 180, 19.7.2000, p. 8-16.

[7] EFSA Journal 2011;9(6):2178

[8] EFSA Journal 2011;9(6):2177

On 21 November 2012, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) adopted a statement on the re-evaluation of 3,7-dimethylocta-1,5,7-trien-3-ol [FL-no: 02.146] based on additional data on a supporting substance[9].

The Panel concluded that "linalool [FL-no: 02.013] is not sufficiently structurally related to 3,7-dimethylocta-1,5,7-trien-3-ol [FL-no: 02.146] for a re-evaluation of [FL-no: 02.146]. Accordingly, "a 90-day study on 3,7- dimethylocta-1,5,7-trien-3-ol [FL-no: 02.146] or on a sufficiently structurally related substance has to be provided in order to establish on appropriate NOAEL".

New data and relationship with other substances

On 5 and 11 July 2013, the applicant submitted additional data on the following acyclic terpene hydrocarbons [FL-no: 01.008, 01.018, 01.040, 01.061, 01.035, 01.064, 01.070 and 02.146, represented by myrcene [FL-no: 01.008].

As regards the related substances also evaluated in these opinions, namely [FL-no: 01.003, 01.004, 01.007, 01.009, 01.017, 01.024, 01.026, 01.029 and 01.059], data was submitted and are currently being evaluated (EFSA-Q-2013-00185 to – 00193).

As regards substance with [FL-no: 01.014], data should be submitted by 31 December 2013[10].

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The European Commission requests the European Food Safety Authority (EFSA) to finalise its safety assessment on this group of flavouring substances in accordance with Commission Regulation (EC) No 1565/2000.

SUPPORTING DOCUMENTS

Submission by the European Flavour Association

INTERPRETATION OF THE TERMS OF REFERENCE

The above background and terms of reference include also a previous mandate received from the European Commission on 6 February 2013[11]. The present scientific opinion FGE.25Rev3 covers the safety assessment of the following flavouring substances: 4(10)-thujene with [FL-no: 01.059], 2,6-Dimethylocta-2,4,6-triene with [FL-no: 01.035], cis-3,7-Dimethyl-1,3,6-octatriene with [FL-no: 01.064] and 1-Octene with [FL-no: 01.070].

[9] EFSA Journal 2012;10(12):2995
[10] This substance is in the process of being deleted from the Union List (DG SANTE, 2015)
[11] SANCO.E3/SH/km D (2013) Ares(2013)15188

ASSESSMENT

1. History of the Evaluation of the Substances in the Present FGE

The first version of the Flavouring Group Evaluation 25 (FGE.25) dealt with 32 aliphatic and aromatic hydrocarbons which have been divided into eight subgroups: I) acyclic alkanes, II) acyclic alkenes, III) cyclohexene hydrocarbons, IVa) benzene hydrocarbons, IVb) napthalene hydrocarbons, IVc) diphenylmethane, V) bi-and tricyclic, non-aromatic hydrocarbons and VI) macrocyclic, non-aromatic hydrocarbons. For one candidate substance, 2-methylbuta-1,3-diene [former FL-no: 01.049] (synonym: isoprene) evaluated in FGE.25 there was evidence of a genotoxic potential *in vivo* and of carcinogenic effects in experimental animals. Therefore, this substance could not be evaluated through the Procedure and could not be considered safe when used as a chemically defined flavouring substance. Subsequently, the substance has been deleted from the Register (Mennicke et al., 1983).

The first Revision of FGE.25 (FGE.25Rev1) included the assessment of three additional candidate substances [FL-no: 01.059, 01.070 and 01.078]. For two of these substances additional genotoxicity data on structurally related substances have been provided. A survey in open literature did not result in further data.

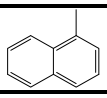
The second Revision of FGE.25 (FGE.25Rev2) included the assessment of three additional candidate substances [FL-no: 01.001, 01.021 and 01.046]. No toxicity or metabolism data were provided for these three substances. A survey in open literature did not result in further data for these three substances. In the FGE.25 and FGE.25Rev1, the Panel considered that additional toxicity data were needed for 26 of the substances evaluated through the Procedure as no adequate toxicity study from which a NOAEL could be established was available, neither on the candidate substances nor on supporting substances. Additional toxicity and genotoxicity data had become available for the supporting substance myrcene [FL-no: 01.008] as had additional genotoxicity data for one of the 26 substances [FL-no: 01.047] and on cedrene washed (unspecified cedrene). Since the publication of FGE.25Rev1 new tonnage data for [FL-no: 01.035, 01.047 and 01.064] had become available (Flavour Industry, 2010), and included in this revision. Industry has also submitted additional information on stereoisomeric composition [FL-no: 01.027, 01.032, 01.034, 01.035, 01.050, 01.055, 01.056 and 01.060], composition of mixture [FL-no: 01.078] and missing ID-test [FL-no: 01.078].

Since the publication of FGE.25Rev2, 23 substances [FL-no: 01.021, 01.022, 01.023, 01.030, 01.031, 01.032, 01.036, 01.037, 01.042, 01.043, 01.044, 01.047, 01.050, 01.051, 01.052, 01.053, 01.055, 01.056, 01.058, 01.060, 01.066, 01.067 and 01.078] are no longer supported for use as flavouring substances in Europe by Industry and will therefore not be considered any further (DG SANCO, 2012; DG SANCO, 2013). Information from the previous version of FGE.25 on these substances is collected in Appendix D. However, information on these substances will be kept in the main text if relevant for the remaining candidate substances. The 23 substances are listed here below.

FL-no	EU Register name
01.021	delta-Cadinene
01.022	α-Cedrene
01.023	1(5),11-Guaiadiene
01.030	β-Cubebene
01.031	1,2-Dihydro-1,1,6-trimethylnaphthalene
01.032	2,3-Dihydrofarnesene
01.036	Diphenylmethane
01.037	Dodec-1-ene
01.042	Germacra-1(10),4(14),5-triene
01.043	3,7,10-Humulatriene
01.044	Isolongifolene
01.047	Longifolene
01.050	3-Methylhexane
01.051	2-Methylnaphthalene

FL-no	EU Register name
01.052	α-Muurolene
01.053	Naphthalene
01.055	β-Phellandrene
01.056	α-Santalene
01.058	1,2,3,4-Tetrahydro-1,1,6-trimethylnaphthalene
01.060	1,1,7-Trimethyltricyclo[2.2.1.0.(2.6)]heptane
01.066	2-Cedrene
01.067	8(14)-Cedrene
01.078	2,4-Nonadiene

As a consequence the following supporting substance has been deleted from this revision; moreover 1-methylnaphthalene is also in the process of being deleted from the Union List (DG SANTE, 2015)

FL-no JECFA no	EU Register name	Structural formula
01.014 1335	1-Methylnaphthalene	

The table below gives information on publication dates and links to the published versions.

FGE	Opinion Adopted by EFSA	Link	No. of Candidate Substances
FGE.25	1 April 2008	http://www.efsa.europa.eu/en/efsajournal/doc/918.pdf	32
FGE.25Rev1	23 September 2009	http://www.efsa.europa.eu/en/scdocs/scdoc/1334.htm	34
FGE.25Rev2	18 May 2011	http://www.efsa.europa.eu/en/efsajournal/pub/2177.htm	37
FGE.25Rev3	18 March 2015		14

The present Revision of FGE.25, FGE.25Rev3, deals with additional toxicity data from a 90-day study provided for β-caryophyllene [FL-no: 01.007] considered in FGE.78Rev2 (EFSA, in press). This information is used for the assessment of 4(10)-thujene [FL-no: 01.059]. Furthermore, new short term study and genotoxicity data have been provided for [FL-no: 01.007] (EFFA, 2012). Additional toxicity data from a 90-day study provided for the supporting substance myrcene [FL-no: 01.008] are also evaluated. This information is used for the assessment of 2,6-dimethylocta-2,4,6-triene [FL-no: 01.035], cis-3,7-dimethyl-1,3,6-octatriene [FL-no: 01.064] and 1-octene [FL-no: 01.070]. Furthermore, new information on European production figures has been provided for [FL-no: 01.035, 01.064 and 01.070] (IOFI, 2013) and use levels have been provided for [FL-no: 01.001, 01.046 and 01.070] (EFFA, 2015).

2. Presentation of the Substances in Flavouring Group Evaluation 25, Revision 3

2.1. Description

The present Flavouring Group Evaluation 25, Revision 3 (FGE.25Rev3) using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000 (EC, 2000) (The Procedure – shown in schematic form in Appendix A of this FGE), deals with 14 aliphatic hydrocarbons (candidate substances) from chemical group 31, Annex I of Commission Regulation (EC) No 1565/2000 (EC, 2000). The candidate substances in the group have been divided into the following subgroups:

I) Acyclic alkanes,

II) Acyclic alkenes,

III) Cyclohexene hydrocarbons,

IV) The group was divided into IVa) benzene hydrocarbons, IVb) naphthalene hydrocarbons, IVc) diphenylmethane; the substances previously allocated to these groups are no longer supported for use as flavouring substances in Europe,

V) Bicyclic, non-aromatic hydrocarbons and

VI) Macrocyclic, non-aromatic hydrocarbons. The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry.

One flavouring substance, 2-methylbuta-1,3-diene [former FL-no: 01.049] (synonym: isoprene) evaluated in FGE.25 has been deleted from the Register of flavouring substances as it showed genotoxic potential *in vivo* and carcinogenic effects in experimental animals. Therefore this substance will not be further discussed as a candidate substance in the current Revision 3 of FGE.25 (FGE.25Rev3).

The 14 candidate substances under consideration, with their chemical Register names, FLAVIS -(FL-), Chemical Abstract Service- (CAS-), Council of Europe- (CoE-) and Flavor and Extract Manufacturers Association- (FEMA-) numbers, structure and specifications, are listed in Table 1.

A summary of the safety evaluation of the candidate substances under consideration in the present evaluation are listed in Table 7.

The 14 candidate substances are closely related structurally to 18 flavouring substances (supporting substances) evaluated at the 63rd JECFA meeting (JECFA, 2005b) in the groups of “Aliphatic and alicyclic hydrocarbons”. The supporting substances are listed in Table 8.

SUMMARY OF SPECIFICATION DATA

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 25, Revision 3

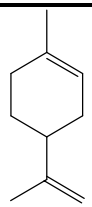
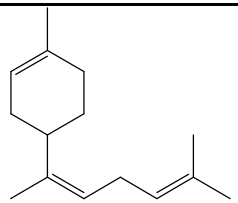
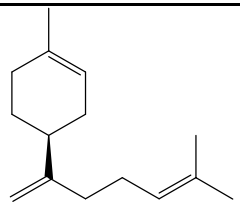
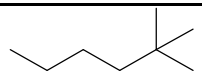
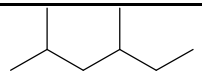
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C [c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]	Specification comments
01.001	Limonene		2633 491 138-86-3	Liquid $C_{10}H_{16}$ 136.23	Insoluble Soluble	178 MS 95 %	1.4760-1.4820 0.843-0.851	With respect to specific gravity it is noted that limonene and l-limonene are submitted by different applicants.
01.027	Bisabola-1,8,12-triene		17627-44-0	Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble Freely soluble	99 (5 hPa) MS 95 %	1.483-1.489 0.912-0.918	Racemate, mixture of (E)- and (Z)-isomers (EFFA, 2010). 50 - 70 % (E)-isomer (EFFA, 2013). CASrn in Register refers to the racemate.
01.028	beta-Bisabolene		495-61-4	Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble Freely soluble	130 (13 hPa) MS 95 %	1.489-1.495 0.879-0.885	CASrn in Register refers to the (4S)-isomer.
01.033	2,2-Dimethylhexane		590-73-8	Liquid C_8H_{18} 114.23	Practically insoluble or insoluble Freely soluble	107 MS 95 %	1.390-1.396 0.693-0.699	
01.034	2,4-Dimethylhexane		589-43-5	Liquid C_8H_{18} 114.23	Practically insoluble or insoluble Freely soluble	109 MS 95 %	1.390-1.396 0.697-0.703	Racemate (EFFA, 2010).

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 25, Revision 3

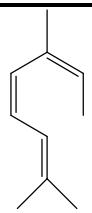
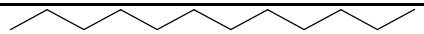
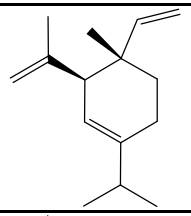
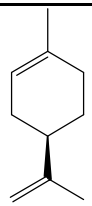
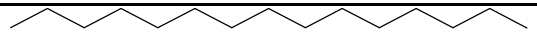
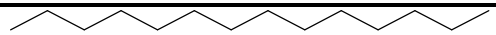
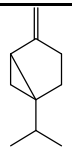
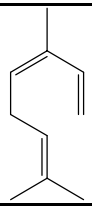
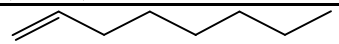
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C [c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]	Specification comments
01.035	2,6-Dimethylocta-2,4,6-triene		673-84-7	Liquid $C_{10}H_{16}$ 136.24	Practically insoluble or insoluble Freely soluble	75 (13 hPa) MS 95 %	1.539-1.545 0.809-0.815	Mixture of (E)- and (Z)-isomers (EFFA, 2010). 4E,6E (25 - 50 %); 4E,6Z (25 - 30 %); 4Z,6E (25 - 30 %); 4Z,6Z (10 - 20 %) (EFFA, 2013). CASrn in Register does not specify stereoisomeric composition.
01.038	Dodecane		112-40-3	Liquid $C_{12}H_{26}$ 170.34	Practically insoluble or insoluble Freely soluble	216 MS 95 %	1.417-1.423 0.746-0.752	
01.039	delta-Elemene		10996 20307-84-0	Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble Freely soluble	107 (13 hPa) MS 95 %	1.480-1.486 0.856-0.862	CASrn in Register refers to the (3R, 4R)-isomer.
01.046	l-Limonene		2633 491 5989-54-8	Liquid $C_{10}H_{16}$ 136.23	Insoluble Soluble	177 MS 95 %	1.469 - 1.473 0.837 - 0.841	With respect to specific gravity it is noted that limonene and l-limonene are submitted by different applicants. CASrn in Register refers to the (4S)-isomer.
01.054	Pentadecane		629-62-9	Liquid $C_{15}H_{32}$ 212.42	Practically insoluble or insoluble Freely soluble	270 10 MS 95 %	1.428-1.434 0.765-0.771	

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 25, Revision 3

FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [(a)] Solubility in ethanol [(b)]	Boiling point, °C [(c)] Melting point, °C ID test Assay minimum	Refrac. Index [(d)] Spec.gravity [(e)]	Specification comments
01.057	Tetradecane		 629-59-4	Liquid $C_{14}H_{30}$ 198.39	Practically insoluble or insoluble Freely soluble	252 5 MS 95 %	1.422-1.428 0.759-0.765	
01.059	4(10)-Thujene		 11018 3387-41-5	Liquid $C_{10}H_{16}$ 136.24	Practically insoluble or insoluble Freely soluble	165 MS 96 %	1.463-1.469 0.840-0.846	Assay value of 96%: mixture of 70 % 4(10)-thujene, 6 % alpha-pinene, 19 % beta-pinene, 1 % myrcene, <4 % not identified (EFFA).
01.064	cis-3,7-Dimethyl-1,3,6-octatriene		 3338-55-4	Liquid $C_{10}H_{16}$ 136.24	Practically insoluble or insoluble Freely soluble	177 MS 95 %	1.483-1.489 0.796-0.802	Synonym: cis-beta-ocimene.
01.070	1-Octene		4293 111-66-0	Liquid C_8H_{16} 112.22	Insoluble Soluble	121 IR NMR MS 97 %	1.410-1.416 0.718-0.722	

(a): Solubility in water, if not otherwise stated.
(b): Solubility in 95 % ethanol, if not otherwise stated.
(c): At 1013.25 hPa, if not otherwise stated.
(d): At 20°C, if not otherwise stated.
(e): At 25°C, if not otherwise stated.

2.2. Stereoisomers

It is recognised that geometrical and optical isomers of substances may have different properties. Their flavour may be different, they may have different chemical properties resulting in possible variability in their absorption, distribution, metabolism, elimination and toxicity. Thus information must be provided on the configuration of the flavouring substance, i.e. whether it is one of the geometrical/optical isomers, or a defined mixture of stereoisomers. The available specifications of purity will be considered in order to determine whether the safety evaluation carried out for candidate substances for which stereoisomers may exist can be applied to the material of commerce. Flavouring substances with different configurations should have individual chemical names and codes (CAS number, FLAVIS number etc.).

Seven of the 14 candidate substances possess chiral centres. For five of these chiral substances the chemical names and CAS numbers specify the stereoisomers. For two of the substances [FL-no: 01.027 and 01.034] information on the stereoisomeric composition has been provided (EFFA, 2010) (see Table 1).

Due to the presence and the position of double bonds three of the 14 candidate substances can exist as geometrical isomers. For two of these flavouring substances [FL-no: 01.027 and 01.035] Industry has provided the information on the ratios of these isomers (see Table 1). For [FL-no: 01.064] CAS number and name specify the configuration of the double bond.

2.3. Natural Occurrence in Food

Twelve of the 14 candidate substances have been reported to occur in various types of alcoholic beverages, chicken, egg (boiled), fish (raw), guinea hen, lamb fat, cheese, milk, butter, various herbs, various fruits and vegetables, tea, mace and liquorice.

Table 2: Candidate Substances Reported to Occur in Food (TNO, 2000; TNO, 2010)

FL-no:	Name:	Quantitative data reported
01.001	Limonene	Up to 1.6 mg/kg in carrot, up to 0.3 in black currants, up to 1.4 mg/kg in tea and very high content in citrus oil
01.028	β-Bisabolene	33.3 mg/kg in parsley, up to 1.18 mg/kg in carrot, 0.003 mg/kg in artichoke, 0.0003 mg/kg in guava fruit, trace amounts in mace and in nutmeg
01.033	2,2-Dimethylhexane	Up to 0.9 mg/kg in tea
01.034	2,4-Dimethylhexane	Up to 2 mg/kg in tea
01.035	2,6-Dimethylocta-2,4,6-triene	0.45 mg/kg in mango, 0.03 mg/kg in orange juice, up to 0.08 mg/kg in blackcurrants, trace amounts in sage
01.038	Dodecane	Up to 3.5 mg/kg in butter, up to 0.1 mg/kg in passiflora, 0.1 mg/kg in beans, 0.1 mg/kg in cocoa, 0.1 mg/kg in tea, up to 0.1 mg/kg in lamb, 0.08 mg/kg in dill, up to 0.05 mg/kg in strawberry, 0.02 mg/kg in chicken, up to 0.01 mg/kg in loquat, up to 0.01 mg/kg in papaya, 0.01 mg/kg in pea, up to 0.004 mg/kg in egg, 0.009 mg/kg in Guinea hen, 0.0006 mg/kg in raw fish, trace amounts in liquorice
01.039	delta-Elemene	Trace amounts in mandarin juice

Table 2: Candidate Substances Reported to Occur in Food (TNO, 2000; TNO, 2010)

FL-no:	Name:	Quantitative data reported
01.054	Pentadecane	Up to 2.3 mg/kg in butter, up to 0.4 mg/kg in mango, up to 0.346 mg/kg in cheese, 0.1 mg/kg in chicken, 0.1 mg/kg in tea, up to 0.05 mg/kg in strawberry, up to 0.03 mg/kg in raw fish, 0.029 mg/kg in Guinea hen, up to 0.02 mg/kg in egg (boiled), 0.014 mg/kg in milk powder, up to 0.01 mg/kg in papaya, 0.02 mg/kg in tamarind, 0.00001 mg/kg in aubergine, trace amounts in liquorice
01.057	Tetradecane	Up to 1.9 mg/kg in butter, 0.3 mg/kg in liquorice, up to 0.3 mg/kg in mango, up to 0.3 mg/kg in dill, 0.021 mg/kg in guinea hen, up to 0.15 mg/kg in cheese, 0.1 mg/kg in cocoa, 0.1 mg/kg in tea, 0.088 mg/kg in passiflora, up to 0.05 mg/kg in strawberry, up to 0.01 mg/kg in papaya, up to 0.003 mg/kg in egg, 0.001 mg/kg in milk 0.0005 mg/kg in chicken, 0.0008 mg/kg in raw fish, trace amounts in thymus, aubergine, coconut and lamb
01.059	4(10)-Thujene	Up to 1000 mg/kg in caraway seed (oil), up to 1.9 mg/kg in blackcurrant, up to 5.2 mg/kg in carrot, up to 49000 mg/kg in cardamom (oil), up to 4000 mg/kg in coriander seed (oil), up to 4800 mg/kg in cumin seed (oil), 239000 mg/kg in pepper (oil) (different species), 334000 mg/kg in ginger (oil), up to 87600 mg/kg in laurel (oil), up to 510000 mg/kg in nutmeg (oil)
01.064	*cis*-3,7-Dimethyl-1,3,6-octatriene	Up to 13.6 mg/kg in guava fruit, up to 7.5 mg/kg in mango, 5.3 mg/kg in celery, 2.7 mg/kg in parsley, 2 mg/kg in dill, up to 0.6 mg/kg in tea, up to 0.5 mg/kg in papaya, up to 0.2 mg/kg in blackcurrant, 0.18 mg/kg in grapefruit, 0.05 mg/kg in cocoa, up to 0.01 mg/kg in passiflora, trace amounts in nectarine
01.070	1-Octene	Up to 0.009 mg/kg in butter (1.7 mg/kg in heated butter), up to 0.001 mg/kg in boiled egg, 0.002 mg/kg in guinea hen

According to TNO two of the substances have not been reported to occur naturally in any food items (Table 3):

Table 3: Candidate Substances Not Reported to Occur in Food (TNO, 2000; TNO, 2011)

FL-no:	Name:
01.027	Bisabola-1,8,12-triene
01.046	*l*-limonene

3. Specifications

Purity criteria for the 14 substances have been provided by the Flavour Industry (EFFA, 2005a; EFFA, 2006a; EFFA, 2006b; Flavour Industry, 2006; Flavour Industry, 2009) (see Table 1).

Judged against the requirements in Annex II of Commission Regulation (EC) No 1565/2000 (EC, 2000), this information is adequate for all 14 candidate substances (see Section 2.2 and Table 1).

4. Intake Data

Annual production volumes of the flavouring substances as surveyed by the Industry can be used to calculate the "Maximised Survey-derived Daily Intake" (MSDI) by assuming that the production figure only represents 60 % of the use in food due to underreporting and that 10 % of the total EU population are consumers (SCF, 1999).

However, the Panel noted that due to year-to-year variability in production volumes, to uncertainties in the underreporting correction factor and to uncertainties in the percentage of consumers, the reliability of intake estimates on the basis of the MSDI approach is difficult to assess.

The Panel also noted that in contrast to the generally low *per capita* intake figures estimated on the basis of this MSDI approach, in some cases the regular consumption of products flavoured at use levels reported by the Flavour Industry in the submissions would result in much higher intakes. In such cases, the human exposure thresholds below which exposures are not considered to present a safety concern might be exceeded.

Considering that the MSDI model may underestimate the intake of flavouring substances by certain groups of consumers, the SCF recommended also taking into account the results of other intake assessments (SCF, 1999).

One of the alternatives is the "Theoretical Added Maximum Daily Intake" (TAMDI) approach, which is calculated on the basis of standard portions and upper use levels (SCF, 1995) for flavourable beverages and foods in general, with exceptional levels for particular foods. This method is regarded as a conservative estimate of the actual intake by most consumers because it is based on the assumption that the consumer regularly eats and drinks several food products containing the same flavouring substance at the upper use level.

One option to modify the TAMDI approach is to base the calculation on normal rather than upper use levels of the flavouring substances. This modified approach is less conservative (e.g., it may underestimate the intake of consumers being loyal to products flavoured at the maximum use levels reported) (EC, 2000). However, it is considered as a suitable tool to screen and prioritise the flavouring substances according to the need for refined intake data (EFSA, 2004a).

4.1. Estimated Daily *per Capita* Intake (MSDI Approach)

The intake estimation is based on the Maximised Survey-derived Daily Intake (MSDI) approach, which involves the acquisition of data on the amounts used in food as flavourings (SCF, 1999). These data are derived from surveys on annual production volumes in Europe. These surveys were conducted in 1995 by the International Organization of the Flavour Industry (IOFI), in which flavour manufacturers reported the total amount of each flavouring substance incorporated into food sold in the EU during the previous year (IOFI, 1995). The intake approach does not consider the possible natural occurrence in food.

Average *per capita* intake (MSDI) is estimated on the assumption that the amount added to food is consumed by 10 % of the population[12] (Eurostat, 1998). This is derived for candidate substances from estimates of annual volume of production provided by Industry and incorporates a correction factor of 0.6 to allow for incomplete reporting (60 %) in the Industry surveys (SCF, 1999).

In the present FGE.25Rev3 the total annual production volume of the 14 candidate substances from use as flavouring substances in Europe was reported to be approximately 51 000 kg[13] (EFFA, 2005a;

[12] EU figure 375 million. This figure relates to EU population at the time for which production data are available, and is consistent (comparable) with evaluations conducted prior to the enlargement of the EU. No production data are available for the enlarged EU.

[13] The substances which are no longer supported have such low production volumes that their removal from this FGE hardly affects the total annual production volume.

EFFA, 2005b; EFFA, 2006a; EFFA, 2006b; EFFA, 2008; Flavour Industry, 2006). For the 18 supporting substances the total annual volume of production in Europe is approximately 330 000 kg. *d*-Limonene [FL-no: 01.045] accounts for 280 000 kg and 47 000 kg is accounted for by seven other supporting substances: [FL-no: 01.003, 01.004, 01.005, 01.006, 01.007, 01.008 and 01.020] (JECFA, 2005b).

On the basis of the annual volumes of production reported for the 14 candidate substances, the MSDI values for each of these flavourings have been estimated (Table 7).

Nearly 100 % of the total annual volume of production for the candidate substances is accounted for by two substances [FL-no: 01.001 and 01.046]. The estimated daily *per capita* intake from use as flavouring substance is 4000 and 2100 µg, respectively. The daily *per capita* intakes for each of the remaining substances are less than 28 µg (Table 7).

4.2. Intake Estimated on the Basis of the Modified TAMDI (mTAMDI)

The method for calculation of modified Theoretical Added Maximum Daily Intake (mTAMDI) values is based on the approach used by SCF up to 1995 (SCF, 1995).

The assumption is that a person may consume a certain amount of flavourable foods and beverages per day.

For all candidate substances information on food categories and normal and maximum use levels[14,15] were submitted by the Flavour Industry (EFFA, 2005a; EFFA, 2006a; EFFA, 2007, EFFA, 2015). For the present calculation of mTAMDI, the reported normal use levels were used. In the case where different use levels were reported for different food categories the highest reported normal use level was used.

Table 4: Use of Candidate Substances

Food category	Description	Flavourings used
01.0	Dairy products, excluding products of category 2	14
02.0	Fats and oils, and fat emulsions (type water-in-oil)	14
03.0	Edible ices, including sherbet and sorbet	14
04.1	Processed fruits	14
04.2	Processed vegetables (incl. mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds	None
05.0	Confectionery	14
06.0	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery	14
07.0	Bakery wares	14
08.0	Meat and meat products, including poultry and game	14
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms	14
10.0	Eggs and egg products	FL-no: 01.001, 01.046, 01.070
11.0	Sweeteners, including honey	FL-no: 01.001, 01.046, 01.070
12.0	Salts, spices, soups, sauces, salads, protein products etc.	14
13.0	Foodstuffs intended for particular nutritional uses	14
14.1	Non-alcoholic ("soft") beverages, excl. dairy products	14
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts	14
15.0	Ready-to-eat savouries	14
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) - foods that could not be placed in categories 1 – 15	14

[14] "Normal use" is defined as the average of reported usages and "maximum use" is defined as the 95th percentile of reported usages (EFFA, 2002).

[15] The normal and maximum use levels in different food categories (EC, 2000) have been extrapolated from figures derived from 12 model flavouring substances (EFFA, 2004).

According to the Flavour Industry the normal use levels for the 14 candidate substances, for which use levels have been provided, are in the range of 1 - 20 mg/kg food, and the maximum use levels are in the range of 1 - 100 mg/kg (EFFA, 2002; EFFA, 2005a; EFFA, 2006a; EFFA, 2007, EFFA, 2015) Table B.1.2, Appendix B.

The mTAMDI values are for 11candidate substances from structural class I (see Table 6) above the threshold for structural class I of 1800 µg/person per day. For limonene [FL-no: 01.001] the mTAMDI is 1800 µg/person per day and for each of l-limonene and 1-octene [FL-no: 01.046 and 01.070] the mTAMDI is 1600 µg/person per day.

For detailed information on use levels and intake estimations based on the mTAMDI approach, see Section 7 and Appendix B.

5. Absorption, Distribution, Metabolism and Elimination

Generally, the available data indicate that the aliphatic and alicyclic hydrocarbons may participate in similar metabolic pathways. Being lipophilic and of relatively low molecular weight, these hydrocarbons may be assumed to be absorbed in the gastrointestinal tract. Subsequently, they can be oxidised to polar oxygenated metabolites, e.g. by CYP-450 enzymes. The phase I metabolites can then be conjugated and excreted mainly in the urine. The candidate and supporting substances are expected to be metabolised either by side chain oxidation or epoxidation of the exocyclic or endocyclic double bonds. Alkyl oxidation initially yields hydroxylated metabolites that may be excreted in conjugated form or undergo further oxidation, yielding more polar metabolites, which can also be excreted. If a double bond is present, intermediate epoxide metabolites may be formed, which are transformed either by hydrolysis to yield diols or by conjugation with glutathione to yield mercapturic acid derivatives. The saturated alkanes in this group may be anticipated to be metabolised via omega and omega-1, -2, -3 or -4 oxidation. Whereas omega oxidation would ultimately lead to the formation of carboxylic acids, the other oxidations would give rise to secondary alcohols and ketones. The carboxylic acids may be expected to participate in the endogenous fatty acid metabolism.

However, for most of the four subgroups (see Appendix C) the information available was scarce and the similarity between candidate and supporting substances was limited. In addition, proper mass balance data were not available. The few mass balance data available indicated only slow elimination. For several subgroups no data were available at all. In Table 5 the final conclusion for each of the candidate substances have been presented together with a brief explanatory statement. For subgroup III there are only data for one supporting substance, *d*-limonene [FL-no: 01.045], which is oxidised in both side chains to yield alcohols and carboxylic acids, which may be conjugated and eliminated in the urine.

A more detailed description of the metabolism is given in Appendix C.

Table 5: Can Innocuous Metabolites be Expected to be Formed Based on Available Data?

FL-no:	Substance name	Innocuous metabolites?
Subgroup I: ACYCLIC ALKANES		
01.033	2,2-Dimethylhexane	Yes
01.034	2,4-Dimethylhexane	Yes
01.038	Dodecane	Yes
01.054	Pentadecane	Yes
01.057	Tetradecane	Yes
Subgroup II: ACYCLIC ALKENES		
01.035	2,6-Dimethylocta-2,4,6-triene	No
01.064	cis-3,7-Dimethyl-1,3,6-octatriene	No (presence of terminal double bond which may give rise to reactive metabolites without counteracting metabolic options)
01.070	1-Octene	No (presence of terminal double bond which may give rise to reactive metabolites without counteracting metabolic options)
Subgroup III: CYCLOHEXENE HYDROCARBONS		
01.027	Bisabola-1,8,12-triene	Yes
01.028	β-Bisabolene	Yes
01.039	delta-Elemene	Yes
01.001	Limonene	Yes
01.046	l-Limonene	Yes
Subgroup IV: AROMATIC HYDROCARBONS		
The substances previously allocated to this subgroup are no longer supported for use as flavouring substances in Europe by Industry		
Subgroup V: BICYCLIC, NON-AROMATIC HYDROCARBON		
01.059	4(10)-Thujene	No
Subgroup VI: MACROCYCLIC, NON-AROMATIC HYDROCARBONS		
The substances previously allocated to this subgroup are no longer supported for use as flavouring substances in Europe by Industry		

6. Application of the Procedure for the Safety Evaluation of Flavouring Substances

The application of the Procedure is based on intakes estimated on the basis of the MSDI approach. Where the mTAMDI approach indicates that the intake of a flavouring substance might exceed its corresponding threshold of concern, a formal safety assessment is not carried out using the Procedure. In these cases the Panel requires more precise data on use and use levels. For comparison of the intake estimations based on the MSDI approach and the mTAMDI approach, see Section 7.

For the safety evaluation of the 14 candidate substances from chemical group 31, the Procedure as outlined in Appendix A was applied, based on the MSDI approach. The stepwise evaluations of these 14 substances are summarised in Table 7.

Step 1

All 14 candidate substances evaluated using the Procedure are classified into structural class I [FL-no: 01.001, 01.027, 01.028, 01.033, 01.034, 01.035, 01.038, 01.039, 01.046, 01.054, 01.057, 01.059, 01.064 and 01.070], according to the decision tree approach presented by Cramer *et al*. (Cramer et al., 1978).

Step 2

On the basis of the metabolism information available, candidate substances of subgroup I [FL-no: 01.033, 01.034, 01.038, 01.054 and 01.057] and candidate substances of subgroup III [FL-no: 01.001, 01.027, 01.028, 01.039 and 01.046] may be predicted to be metabolised to innocuous products at the estimated levels of intake based on the MSDI approach. Accordingly the evaluation of these 10 substances, all belonging to structural Cramer class I, will proceed along the A-side of the Procedure scheme.

Two candidate substances from subgroup II [FL-no: 01.064 and 01.070] contain terminal double bonds in the absence of other functional groups that may provide alternative routes of detoxication. Therefore, for these two substances it cannot be concluded that they will be metabolised to innocuous products, and accordingly they proceed along the B-side of the Procedure scheme.

For the remaining two candidate substances [FL-no: 01.035 and 01.059] there are not sufficient data available on biotransformation to conclude that they will be metabolised to innocuous products, and therefore their evaluation will proceed along the B-side of the Procedure scheme.

Step A3

The five candidate substances from subgroup I [FL-no: 01.033, 01.034, 01.038, 01.054 and 01.057] and three candidate substances from subgroup III [FL-no: 01.027, 01.028 and 01.039], proceeding via the A-side, have been assigned to structural class I and have estimated European daily *per capita* intakes ranging from 0.012 to 2.7 µg (Table 6). These intakes are below the threshold of concern of 1800 µg/person per day for structural class I. Accordingly, it is concluded that these eight candidate substances do not pose a safety concern as flavouring substances when used at estimated levels of intake, based on the MSDI approach. Two candidate substances from subgroup III [FL-no: 01.001 and 01.046] have an estimated European daily *per capita* intakes of 4000 and 2100 µg, respectively, which are above the threshold of concern of 1800 µg/person per day for structural class I. These two candidate substances will therefore proceed to step A4 of the Procedure scheme.

Step A4

The candidate substances [FL-no: 01.001 and 01.046] or their metabolites are not endogenous.

Step A5

The two candidate substances [FL-no: 01.001 and 01.046] are supported by the substance [FL-no: 01.045] for which an adequate carcinogenicity study is available. From this study a no observed adverse effect level (NOAEL) of 215 mg/kg bw per day can be derived. The estimated daily *per capita* intake is 4000 µg for [FL-no: 01.001] and 2100 µg for [FL-no: 01.046], corresponding to 0.07 mg/kg bw per day and 0.035 mg/kg bw per day at a body weight of 60 kg, respectively. Thus, a margin of safety of 3070 can be calculated for [FL-no: 01.001] and a margin of safety of 6140 can be calculated for [FL-no: 01.046]. These two substances are accordingly not expected to be of safety concern at the estimated levels of intake.

Step B3

The four candidate substances [FL-no: 01.035, 0.059, 01.064 and 01.070] proceeding via the B-side and which have been assigned to Cramer structural class I have estimated European daily *per capita* intakes between 0.0085 and 14 µg (Table 6). These intakes are below the threshold of concern of 1800 µg/person per day for structural class I. Accordingly, these four substances proceed to step B4 of the Procedure.

Step B4

For one of these substances, 4(10)-thujene [FL-no: 01.059] a margin of safety could be calculated based upon a NOAEL (222 mg/kg bw per day) for the supporting substance ß-caryophyllene [FL-no: 01.007]. Compared to the MSDI of 4(10)-thujene of 14 µg/*capita* per day equal to 0.2 µg/kg bw per day, the NOAEL provides a margin of safety of 9.5×10^5.

For the three remaining substances, 2,6-dimethylocta-2,4,6-triene [FL-no: 01.035], cis-3,7-dimethyl-1,3,6-octatriene [FL-no: 01.064] and 1-octene [FL-no: 01.070] margins of safety could be calculated based upon a NOAEL (44 mg/kg bw per day) for the supporting substance myrcene [FL-no: 01.008]. Compared to the MSDI of 2,6-dimethylocta-2,4,6-triene [FL-no: 01.035], cis-3,7-dimethyl-1,3,6-octatriene [FL-no: 01.064] and 1-octene [FL-no: 01.070] of 9.1, 14 and 0.0085 µg/*capita* per day equal to 0.15, 0.23 and 0.00014 µg/kg bw per day, the NOAEL provides margins of safety of 2.9×10^5, 1.9×10^5 and 3.1×10^8.

7. Comparison of the Intake Estimations Based on the MSDI Approach and the mTAMDI Approach

For 11 of the 14 candidate substances, the mTAMDIs are above the threshold for structural class I of 1800 µg/person per day. For limonene [FL-no: 01.001] the mTAMDI is 1800 µg/person per day and for each of l-limonene and 1-octene [FL-no: 01.046 and 01.070] the mTAMDI is 1600 µg/person per day.For comparison of the intake estimates based on the MSDI approach and the mTAMDI approach, see Table 6.

For 11 of the 14 candidate substances, further information is required. This would include more reliable intake data and then, if required, additional toxicological data.

Table 6: Estimated intakes based on the MSDI approach and the mTAMDI approach

FL-no	EU Register name	MSDI (µg/*capita*/day)	mTAMDI (µg/person/day)	Structural class	Threshold of concern (µg/person/day)
01.027	Bisabola-1,8,12-triene	0.024	3900	Class I	1800
01.028	beta-Bisabolene	2.7	3900	Class I	1800
01.033	2,2-Dimethylhexane	1.2	3900	Class I	1800
01.034	2,4-Dimethylhexane	1.2	3900	Class I	1800
01.038	Dodecane	0.012	3900	Class I	1800
01.039	delta-Elemene	0.012	3900	Class I	1800
01.054	Pentadecane	0.61	3900	Class I	1800
01.057	Tetradecane	0.012	3900	Class I	1800
01.035	2,6-Dimethylocta-2,4,6-triene	9.1	3900	Class I	1800
01.059	4(10)-Thujene	14	3100	Class I	1800
01.064	cis-3,7-Dimethyl-1,3,6-octatriene	14	3900	Class I	1800
01.070	1-Octene	0.0085	1600	Class I	1800
01.001	Limonene	4000	1800	Class I	1800
01.046	l-Limonene	2100	1600	Class I	1800

8. Considerations of Combined Intakes from Use as Flavouring Substances

Because of structural similarities of candidate and supporting substances, it can be anticipated that many of the flavourings are metabolised through the same metabolic pathways and that the metabolites may affect the same target organs. Further, in case of combined exposure to structurally related flavourings, the pathways could be overloaded. Therefore, combined intake should be considered. As flavourings not included in this FGE may also be metabolised through the same pathways, the combined intake estimates presented here are only preliminary. Currently, the combined intake estimates are only based on MSDI exposure estimates, although it is recognised that this may lead to underestimation of exposure. After completion of all FGEs, this issue should be readdressed.

The total estimated combined daily *per capita* intake of structurally related flavourings is estimated by summing the MSDI for individual substances.

The combined intakes have been calculated on the basis of the annual production volumes from use as flavouring substances in Europe (EFFA, 2005a; EFFA, 2005b; EFFA, 2006a; EFFA, 2006b; Flavour Industry, 2006; JECFA, 2005b).

Subgroup I (acyclic alkanes): The combined intake of the five candidate substances, all from structural class I and evaluated via the A-side of the Procedure (Appendix A), is 3.0 µg/*capita* per day, which does not exceed the threshold of 1800 µg/person per day. There are no supporting substances in subgroup I.

Subgroup II (acyclic alkenes): The combined intake of the three candidate substances, all from structural class I and evaluated via the B-side of the Procedure (Appendix A), is 23 µg/*capita* per day, which does not exceed the threshold of 1800 µg/person per day. Subgroup III (cyclohexene hydrocarbons): The combined intake of the five candidate substances, all from structural class I and evaluated via the A-side of the Procedure (Appendix A), is 6100 µg/*capita* per day, which exceeds the threshold of 1800 µg/person per day. The total combined intake of the five candidate and six supporting substances (also from structural class I) is approximately 42000 µg/*capita* per day. This intake exceeds the threshold of 1800 µg/person per day for a structural class I substance. However, together, limonene [FL-no: 01.001], *l*-limonene [FL-no: 01.046] and *d*-limonene (supporting substance [FL-no: 01.045]) account for approximately 40000 µg/*capita* per day. The total combined intake of 42000 µg/*capita* per day for the candidate and the supporting substances corresponds to 700 µg/kg bw per day for a person with a body weight of 60 kg. Thus, based on the NOAEL for *d*-limonene of 215 mg/kg bw per day, derived from a chronic 2-year toxicity study, a margin of safety of 307 can be calculated, and accordingly these substances are not expected to be of safety concern at the estimated level of intake.

Subgroup V (bicyclic, non-aromatic hydrocarbons): The total combined intake of the one candidate and eight supporting substances, all from structural class I and evaluated via the B-side of the Procedure (Appendix A) is approximately 3800 µg/*capita* per day. This intake exceeds the threshold of 1800 µg/person per day for a structural class I substance. However, together, three supporting substances, pin-2(10)-ene [FL-no: 01.003], pin-2(3)-ene [FL-no: 01.004] and β-caryophyllene [FL-no: 01.007] account for approximately 3400 µg/*capita* per day. The total combined intake of 3800 µg/*capita* per day for the candidate and the supporting substances corresponds to 63 µg/kg bw per day for a person with a body weight of 60 kg. Thus, based on the NOAEL for β-caryophyllene of 222 mg/kg bw per day, a margin of safety of 3500 can be calculated, and accordingly these substances are not expected to be of safety concern at the estimated level of intake.

9. Toxicity

9.1. Acute Toxicity

Data are available for two candidate substances, 15 supporting substances and one structurally related substance (1-methyl cyclohexa-1,3-diene [former FL-no: 01.077]). Oral LD_{50} values in rat and mouse ranged from 1590 to 13 000 mg/kg body weight (bw).

The acute toxicity data are summarised in Table 9.

9.2. Subacute, Subchronic, Chronic and Carcinogenicity Studies

Data are available for two supporting substances from subgroup II (myrcene [FL no: 01.008] and undeca-1,3,5-triene [FL no: 01.061], for one supporting substance from subgroup III (*d*-limonene [FL no: 01.045]), for three supporting substances for subgroup V (pinene [FL no: 01.003], β-caryophyllene [FL-no: 01.007] and camphene [FL no: 01.009]) (see also Table 10). In the text below the relevant studies are discussed.

Subgroup II

Myrcene [01.008]

Mice, 90 day study (NTP, 2010)

Male and female $B6C3F_1$ mice (10/sex/group) were administered 0, 250, 500, 1000, 2000 or 4000 mg/kg bw of myrcene by gavage 5 days a week for 14 weeks. Body weights and clinical observations were recorded weekly. Blood was collected from mice surviving to the end of the study for haematological analyses and micronuclei evaluation. Sperm morphology and vaginal cytology evaluations were conducted at the end of the study on animals in the control and three lowest dose groups. At necropsy, organ weights were measured and complete histopathological examination was performed on animals from the dose groups 0, 1000, 2000 and 4000 mg/kg bw, and all animals that died early.

All animals in the 4000 mg/kg bw group died within the first three days while 9 of 10 males and 8 of 10 females in the 2000 mg/kg bw group died prior to week 5. In animals that died prior to study termination, clinical signs included lethargy, abnormal breathing and/or thin appearance. The final mean body weights and body weight gain of the 1000 mg/kg bw males and 500 mg/kg bw females were significantly less than those of vehicle controls. Because of the low survival in the two top doses, further results are not reported for those doses.

A significant, approximately 15-20 % decrease in hematocrit, haemoglobin and erythrocyte count values was observed in the 1000 mg/kg bw dose group in both females and males at week 14. A dose-dependent significant increase in the relative liver weight (approximately 7 %, 6 % and 17 %) were observed for all doses in male mice, and a significant increase in absolute liver weight were observed for the low dose males only. In female mice a dose-dependent significant increase in relative kidney weight was observed at all doses (approximately 14 %, 12 % and 22 %), with a significant increase in absolute kidney weight in the 1000 mg/kg bw dose group only. Also for female mice dose-dependent increases in absolute and relative liver weight (approximately 8 %, 17 % and 26 %) were observed, but the increase reached statistical significance only at 500 and 1000 mg/kg bw. There were no significant changes seen in the weights of the reproductive organs, in the sperm parameters, or in oestrous cyclicity at any dose level. No significant histopathological changes in other organs examined, including kidney, were observed in mice receiving up to 1000 mg/kg bw myrcene for 14 weeks.

As a significant dose-dependent increase in the relative kidney weight was observed for female mice at all treatment doses, no NOAEL for this study could be allocated.

Rats, 90-day study (NTP, 2010)

Male and female F344N Fisher rats (10/sex/group) were administered 0, 250, 500, 1000, 2000 or 4000 mg/kg bw of myrcene by gavage 5 days a week for 14 weeks. Body weights and clinical observations were recorded weekly. Blood was collected from rats surviving to the end of the study for clinical chemistry and haematological analyses. Sperm morphology and vaginal cytology evaluations were conducted at the end of the study on animals in the control and three lowest dose groups. At necropsy, organ weights were measured and complete histopathological examination was performed on animals from the dose groups 0, 2000 and 4000 mg/kg bw, and all animals that died early. Tissues were examined in the lower dose groups to a no-effect level, including renal pathology in all dose groups. Additionally sections of kidney from both sexes were stained using the Mallory-Heidenhain technique for investigation of hyaline droplet formation (indicative of development of α2μ-globulin).

All animals in the group receiving 4000 mg/kg bw myrcene died within the first week of the study, except for one male that died at day 11. One male receiving 500 mg/kg bw, one male and one female receiving 1000 mg/kg bw and two males and four females receiving 2000 mg/kg bw died before the end of the study. Final mean body weight and mean body weight gains of males and females administered 500 mg/kg bw or more were significantly less than those of vehicle control. At termination at week 14, dose-related decreases in plasma creatinine concentration in both males and females were observed, statistically significant at 500 mg/kg bw and above in males and at 250 mg/kg bw and above in females. These decreases were suggested by the authors to be associated with the decreased body weight gains observed in treated rats. No other consistent changes in clinical chemistry parameters were found.

Absolute kidney and liver weights were significantly increased in both male and female rats receiving myrcene. Also a dose-dependent increase in the relative liver and kidney weights were observed for males (25-150 % in kidney, 13-46 % in liver) and females (27-100 % in kidney, 13-67 % in liver). Microscopically, the incidence of renal tubular necrosis was significantly increased in all dosed groups of males and females, with increasing severity from minimal to moderate related to dose. Both control and treated rats showed development of chronic progressive nephropathy (CPN), although the incidence was higher in treated rats. Treatment-related increases in the incidences and severity of hyaline droplet accumulation were found in the 250, 500 and 1000 mg/kg bw males, accompanied by granular casts in the outer medulla of the kidney. Hyaline droplet formation was not observed in the 2000 mg/kg bw males, although the animals showed a high incidence of renal tubular necrosis, nephrosis and CPN. No evidence of hyaline droplet accumulation was found in female rats, however treated females showed both nephrosis and CPN. A significant increase in nephrosis was observed in the 1000 and 2000 mg/kg bw dose groups of both males and females, with a dose-related increase in severity from minimal to moderate. Nephrosis is an uncommon lesion defined as renal tubule epithelial degeneration and regeneration.

A dose-related effect of myrcene in the nose was observed in both sexes as degeneration of the olfactory epithelium and necrosis of the respiratory epithelium (significant only at 2000 mg/kg bw) accompanied by chronic inflammatory change (significant at 1000 and 2000 mg/kg bw).

The incidence of splenic atrophy were significantly increased in both sexes receiving 2000 mg/kg bw, accompanied by thymic necrosis in one male and three females. In the mesenteric lymph node, the incidence of atrophy were increased in males receiving 2000 mg/kg bw and females receiving 1000 or 2000 mg/kg bw. The lymphoid changes in these organs were considered by the authors to be secondary to morbidity rather than a direct toxic effect of myrcene.

It has been argued that increased hyaline droplet accumulation in male rats is characteristic of α2μ-globulin nephropathy (Hard et al., 1993), which is a male rat specific effect with little relevance for humans. The evidence provided for the mechanistic background of the hyaline droplet formation was considered too limited to completely disregard the nephrotoxic effects in male rats as irrelevant for humans. Involvement of α2μ-globulin accumulation (e.g. by immunohistochemical techniques) was not demonstrated. In addition, also renal toxicity was observed in the female animals. Therefore, based on the presence of renal tubular necrosis in all test groups, a NOAEL could not be assigned.

Mice, 2-year carcinogenicity study (NTP, 2010)

Groups of $B6C3F_1$ mice (50/sex/group) were administered 0, 250, 500 or 1000 mg myrcene/kg bw per day in corn oil by gavage once per day, five days a week for 104 (females) weeks or 105 weeks (males). The animals were observed twice per day and weighed once per week for 12 weeks and once per month thereafter. Complete necropsies and microscopic examination were performed on all animals. Histological examinations were performed on all animals and tissues from all major organs were examined.

Mean body weights of males receiving 1000 mg/kg bw and females receiving 500 or 1000 mg/kg bw were less than those of the vehicle control. Survival of the high-dose group was significantly reduced for both males and females. Due to the high mortality in the 1000 mg/kg bw dose group, the results from this group are not described further.

In the liver there was an increase in hepatocellular carcinoma in both males and females, with a significant increase in incidence in males at the 500 mg/kg bw dose level (incidence 1 in controls, 4 in the 250 mg/kg bw group and 9 in the 500 mg/kg bw group) and females at 250 mg/kg bw, but not at 500 mg/kg bw. Males and to a lesser extent females showed an increase in hepatocellular adenoma in the liver at both 250 and 500 mg/kg bw. Liver hypertrophy was observed to increase with dose both in incidence and severity, reaching statistical significance only at 500 mg/kg bw in both males and females. Eosinophilic foci and cytoplasmic vacuolization were noted in both male and female treatment groups.

Treatment-related changes in other organs included increases in hyperplasia, inflammation, necrosis and ulcer of the forestomach, which were most likely attributable to gavage administration of an irritant substance. Bone marrow atrophy and mandibular and mesenteric lymph node atrophy was observed in both males and females at the 500 mg/kg bw dose. In addition, male mice showed atrophy of the spleen at the 500 mg/kg bw dose.

Taking into account the high sensitivity of this mice strain to tumour development in the liver, these effects were not regarded relevant to humans. A NOAEL of 250 mg/kg bw for myrcene was allocated, based on the increase in bone marrow atrophy and lymph node atrophy observed in both males and females at 500 mg/kg bw dose.

Rats, two-year carcinogenicity study (NTP, 2010)

A chronic two-year bioassay on myrcene using the standard NTP protocol with F344/N rats was conducted. Doses were determined from the results of the prior 13-week subchronic study. Groups of F344/N rats (50/sex/group) were administered 0, 250, 500 or 1000 mg myrcene/kg bw per day in corn oil by gavage once per day, five days a week for 104 weeks. The animals were observed twice per day and weighed once per week for 12 weeks and once per month thereafter. Complete necropsies and microscopic examination were performed on all animals. Histological examinations were performed on all animals and tissues from all major organs were examined.

Survival rates of females were comparable across all control and treatment groups. Survival of males in the low- and mid-dose groups was similar to that in the controls. However, no males in the high-dose group survived past 83 weeks of the study, due to renal toxicity. Body weight gain was significantly reduced in high-dose males and females, while the mean body weight of the 500 mg/kg females were less than those of the vehicle controls during much of the study but were similar by the end of the study. Due to the high mortality of males in the 1000 mg/kg bw group, the data from this dose group are not presented further.

The incidences of renal tubular adenoma and of renal tubule adenoma or carcinoma (combined) showed a significant and dose-dependent increase in the 250 and 500 mg/kg bw males. Two renal tubule adenomas occurred in the 1000 mg/kg bw females, the incidence being higher than the

historical control mean for the laboratory. In males high increase in both the incidence and severity of renal tubular nephrosis were observed, already at the lowest dose, with 84 and 92 % incidence at 250 and 500 mg/kg bw, respectively. A significant but not dose related increase in papilla mineralisation, epithelial hyperplasia and inflammation was seen in the dose groups 250 and 500 mg/kg bw. In female rats a dose-dependent increase in nephropathy (CPN) were observed for the doses 250, 500 and 1000 mg/kg bw and a dose-dependent increase in the incidence and severity of renal tubule nephrosis at the doses 500 and 1000 mg/kg bw. In addition a non-dose related significant increase in renal tubular hyperplasia was reported at the doses (250, 500, 1000 mg/kg bw). Nephrosis, observed both in male and female rats, was reported by the authors to be an uncommon lesion defined as renal tubule epithelial degeneration and regeneration. This indicates that myrcene might cause nephrotoxicity by a mechanism other than, or in addition to, α2μ-globulin nephropathy. The observation of renal neoplasms in female rats also suggests a mechanism of carcinogenesis that may be related to nephrosis and is distinct from the α2μ-globulin mechanism.

Other histopathological changes seen in the study included chronic active inflammation in the nose and forestomach, suggesting that the substance is an irritant. In females, the incidence of thyroid gland C-cell adenomas was significantly increased in the 250 mg/kg bw group, but the incidence did not increase with increasing dose. In the lung, the incidence of alveolar/bronchiolar adenoma in the 250 mg/kg bw group exceeded the control means, but was not observed at higher doses.

Due to the observation of renal tubular adenomas and carcinomas in all dose groups in male rats, accompanied by an increased incidence and severity of nephrosis in both sexes, a NOAEL for myrcene from this study could not be allocated.

Overall conclusion for the NTP study on myrcene

No overall NOAEL from the NTP study on myrcene could be allocated due to the observation renal toxicity in male and female rats at all dose groups. The Panel has considered deriving a BMDL from the NTP study of myrcene. However, a BMDL from this study could not be derived because of a lack of dose-response since nearly 100 % incidence of nephropathy was observed in rats already at the lowest dose of myrcene.

Since the publication of FGE.25Rev2 (EFSA CEF Panel, 2011) new data on myrcene have become available (Bauter, 2013b). The results are described in more detail in FGE.78Rev 2 (EFSA CEF Panel, in press).

In an OECD 408 compliant 90-day study the subchronic toxicity of myrcene (93.3 % pure) was evaluated in male and female rats, based on daily exposure to the test substance in the diet (Bauter, 2013b). Four groups of adult Sprague-Dawley rats (10/sex/group) were maintained on diets prepared to contain 0, 700, 2100 or 4200 mg/kg feed of myrcene. However, the myrcene content of the diet decreased considerably over 7 days, so that every week a new batch of the diet was prepared. Therefore, the Panel decided to take the concentration of myrcene in the diet on the last day of the week for quantification of the exposure, rather than the (logarithmic) mean over the whole week. Accordingly, an adjusted calculated mean daily intake of 8.0, 40 and 44 mg/kg bw per day, respectively for males, and 9.6, 48 and 53 mg/kg bw per day, respectively, for females for 90 days was calculated.

There were no mortalities, clinical, or ophthalmological changes attributable to myrcene administration. There were no statistically significant, dietary concentration-dependent changes in body weight, body weight gain, food consumption, or food efficiency in males and females attributed to the administration of myrcene during the study.

There were no clinical pathological findings, changes in macroscopic or microscopic histopathology, or organ weight changes in the groups administered myrcene. Some incidental changes in clinical chemistry and hematology parameters were within approximate historical control values, did not

correlate with macroscopic or histopathological findings, were without biologic impact, and were considered not toxicologically relevant. A few histopathological changes were considered incidental, spontaneous in nature as observed for the age and strain of rat used in this study, and had no established relationship to administration of the test substance.

Under the conditions of the study and based on the toxicological endpoints evaluated, the no-adverse-effect level (NOAEL) for administration of myrcene in the diet was determined to be the highest dose-group, calculated to provide an estimated daily intake of 44 mg/kg bw per day for males and 53 mg/kg bw per day for females, respectively.

The Panel took note of the fact that two 90-day studies are available on myrcene: the NTP study and the study by Bauter (2013b). While the NTP study showed kidney toxicity in all animals at 250 mg/kg body wt per day, the study by Bauter (2013b) observed no toxicity at all in the kidneys at 44 and 53 mg/kg bw per day in males and females resp. The Panel decided to accept the NOAEL of the study by Bauter (2013b) (44 mg/kg bw per day) because the NTP study is a gavage study, which leads to peak exposure levels compared to the Bauter study in which the compound was added in the food. Thus, the Bauter study reflects the dietary administration (consumption with food matrix, over a more extended period of time) in consumers much better than gavage (NTP) study. Additionally, in the Bauter study no kidney toxicity was observed at all and the NOAEL derived from the Bauter study is six-fold lower than the effect level in the NTP study.

Subgroup III

d-Limonene [FL-no: 01.045]

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated the supporting substance *d*-limonene as an additive in its forty-first meeting in 1993, and withdrew the previous ADI for *d*-limonene and allocated an ADI "not specified" (JECFA, 1993). This assessment was mainly based on an NTP study with *d*-limonene from 1990 (NTP, 1990). In the carcinogenicity study, F344 rats (n = 50/dose/sex) were treated with 0, 75 and 150 mg/kg *d*-limonene (males) and 0, 300 and 600 mg/kg (females) by gavage in corn oil for five days a week. B6C3F$_1$ mice (n = 50/dose/sex) were treated with 0, 250 and 500 mg/kg *d*-limonene (males) and 0, 500 and 1000 mg/kg (females) in corn oil by gavage for five days a week. In the high dose female rats, the survival was reduced. No effect on survival or any other toxic effect was observed in the females of the low dose group. The major toxicological effect in the male rats was found in the kidneys where a dose dependent increase in both renal mineralization and epithelial hyperplasia, and a dose-dependent increase in renal tubular cell adenomas and adenocarcinomas was observed. These effects were accompanied with dose related increase in α2μ-globulin in the kidney of male rats. No increase in α2μ-globulin, kidney nephropathy or renal adenomas or carcinomas was found in female rats. Therefore, the toxicological effects seen in the male rat kidneys are due to α2μ-globulin accumulation related nephropathy specifically seen in male rats, which is not relevant for humans (Hard et al., 1993). A reduction in the mean body weights at the high dose group was observed for male and female rats (4 – 7 %) and high dose female mice (5-15 %). For the male rats this reduction in mean body weight may have been linked to the toxicity in the kidneys. Based on the decreased body weights in female mice, a NOAEL of 500 mg/kg bw per day (5 days/week) could be derived, but considering the decrease in survival in the female rats exposed at 600 mg/kg bw per day (5 days/week) an overall NOAEL of 300 mg/kg bw per day (5 days/week) should be derived from these NTP studies. This would correspond to 215 mg/kg bw per day for daily exposure.

Subgroup V

Supporting substance

ß-Caryophyllene [FL-no: 01.007]

In a 14 day range finding dietary study (Bauter, 2011), groups (3/sex/dietary intake level) of male and female Hsd:SD® rats were fed a diet designed to provide 0 (dietary control), 6000, 18 000 and 48 000 mg/kg feed of β-caryophyllene daily. These estimated dietary levels correspond to the measured intakes of 0, 516, 1547 and 3569 mg/kg bw per day for males and 0, 528, 1582 and 4438 mg/kg bw per day for females, respectively. Clinical observations were recorded daily and body weights and food consumption observations were made on days 0, 7 and 14. There were no mortalities. Hyperactivity observed in 1/3 (33 %) of males and females in group 4 (48 000 mg/kg feed) in the latter part of the study may be possibly attributed to test substance administration. Dose-dependent decreases in male food consumption and food efficiency with significant corresponding decreases in group 4 male body weight and body weight gain were considered a result of test substance administration, but were not correlated with any other clinical signs. Females did not exhibit significant differences from female control. Findings at terminal sacrifice included all (100 %) males and females of group 4 with distention (cecum) and slight redness of the stomach, small intestines, and cecum. Based upon the limited toxicological endpoints evaluated, the study authors selected doses for the 90-day study.

90-Day Study on β-Caryophyllene [FL-no: 01.007]

In an OECD 408 compliant 90-day study, 4 groups of rats (10/sex/dietary intake level) of male and female CRL Sprague-Dawley CD®IGS rats were fed a diet designed to provide 0 (dietary control), 3500, 7000 and 21 000 mg/kg feed and 3500, 14 000 and 56 000 mg/kg feed of β-caryophyllene for males and females, respectively, daily (Bauter, 2013a). These dietary levels corresponded to measured daily intakes of 0, 222, 456 and 1367 mg/kg bw for males and 0, 263, 1033 and 4278 mg/kg bw for females, respectively (Bauter, 2013a). The purity of the β-caryophyllene preparation was between 99 % (start of study) and 96 % (end of study). Clinical observations of toxicity were performed on day 0 and weekly until sacrifice. Animals were weighed on day 0 at the start of the study and weekly thereafter. Food consumption and efficiency were measured and calculated weekly. Blood chemistry and haematology were performed on blood drawn via sublingual bleed during week 12 after overnight fast. Urine was collected during the 15 hours prior to the blood draw. Prior to initiation of the study and on day 91 the eyes of all rats were examined by focal illumination and indirect ophthalmoscopy. At termination of the study all survivors were sacrificed and subject to full necropsy.

There were no mortalities, clinical signs of toxicity or ophthalmological changes associated with the presence of β-caryophyllene in the diet. There were statistically significant and concentration-related reductions in body weight gain, food consumption and food efficiency in males and females at the 21 000 mg/kg feed and 56 000 mg/kg feed concentration groups, respectively (body weight of high dose groups: males 77.3 %, females 82.6 % as compared to controls).

Although for some parameters statistically significant differences were found when compared to concurrent controls, haematology, clinical chemistry, coagulation and urine analysis parameters for the middle and high concentrations for both males in general were within the range of historical controls. Most of these changes were small, and observed in the highest dose group. Thus, in the female test groups, a statistically significant increase for platelet count was reported at the highest dietary level; such an increase was not observed in the male test groups. A dose dependent increase in white blood cells in males reached statistical significance at the middle and high dose; several other blood cells showed significant changes at the highest dose as well in males; the effects in females were less pronounced. There were no histopathology findings correlating to these variations.

In females a dose-dependent decrease in serum glucose concentrations and an increase in triglyceride levels reached statistically significance only at the highest dose level. In conjunction with changes reported in the liver these changes were attributed to metabolic changes as a result of high concentrations of β-caryophyllene in the diet. Pathological findings include increases in absolute and relative liver weights; these were found statistically significant in the mid- and high-dose groups of both sexes. Histopathological liver changes at the mid and high intake levels for both sexes were characterized by centrilobular to midzonal distributed hepatocellular hypertrophy. Based on hepatocyte hypertrophy in both sexes, the increases in absolute and/or relative liver weights at the mid- and highest dietary levels, and the absence of any other significant abnormality upon histopathological examination, the study authors conclude that the hepatocyte hypertrophy is the result of hepatic enzyme induction; this has, however, not been confirmed by measurements of relevant enzymes.

Necropsy revealed enlarged kidneys in one male at the highest test concentration and significant increases in relative kidney weight of male high-dose as well as of female mid- and high-dose groups. Microscopic examination revealed mononuclear infiltration in only one of ten females of the highest dose group. No other microscopically visible alterations were reported in the kidneys of female rats. The increase in kidney weights in female remains unexplained. Microscopical examination of the kidney of males revealed an increased incidence of nephropathy characterised by regeneration of proximal cortical tubules with thickened membranes, mononuclear cell infiltration and tubular casts at all dose levels. The severity of the nephropathy exhibited a dose-dependent shift from low to high grades. Kidney cells of affected males also were reported to have necrotic nuclei and an increase in eosinophilic cytoplasm. Likewise, in the kidneys of all treated males eosinophilic cytoplasmic droplets were present, with a dose-dependent shift to higher grades In a supplementary study the kidney slides of exclusively male rats were stained also with Mallory Heidenhain stain (Zook and Garlick, 2013), which results in enhanced staining of the cytoplasmic droplets (De Rijk et al., 2003; Frazier et al., 2012); this confirmed the observations of the eosine staining. An increased hyaline droplet accumulation in male rats exclusively is characteristic of α_{2u}-globulin nephropathy (Hard et al., 1993), which is considered a male rat specific effect with little relevance for humans. Although no specific immunohistochemical staining of α_{2u} –globulin has been done to confirm the presence of this protein, the Panel considers the evidence sufficient to conclude that this kidney toxicity in male rats exclusively is not relevant for humans.

Microscopic examination of the mesenteric lymph nodes revealed the presence of erythrocytes in the sinuses at the mid- and high-intake levels for both sexes. Additionally, reduced spleen weights for males at the highest dietary level were considered related to general reductions in lymphoid system weights.

The Panel concluded that under the conditions of the present 90-day dietary toxicity study and based on the toxicological findings in haematology in males, the liver, the mesenteric lymph node pathology in both sexes and non-explained effects in female kidneys only the lowest dose provides a no-adverse-effect level (NOAEL) for β-caryophyllene, which is the lowest in male rats: 222 mg/kg bw per day.

Repeated dose toxicity data are summarised in Table 10.

9.3. Developmental / Reproductive Toxicity Studies

No developmental or reproductive studies are available for the candidate substances but for three supporting substances.

The developmental/reproductive toxicity data are summarised in Table 11.

9.4. Genotoxicity Studies

Data from *in vitro* tests are available for two candidate substances (subgroup I: [FL-no: 01.038 and FL-no: 01.057]) and 10 supporting flavouring substances (one from subgroup II, four from subgroup

III, and five from subgroup V (for pin-2(3)-ene [FL-no: 01.004] also data for separate stereoisomers were available (+ and -)- α-pinene), and one structurally related substance, (2-methylbuta-1,3-diene) not used as flavouring substance from subgroup II. Data for three supporting substances [FL-no: 01.008 (subgroup II), FL-no: 01.019 (subgroup III), FL-no: 01.004 (subgroup V), and data for the structurally related substance from subgroup II are considered valid.

Data from *in vivo* tests are available for two supporting substances (one from subgroup II and one from subgroup III) and for one substance structurally related to subgroup II (2-methylbuta-1,3-diene).

Candidate substances

Subgroup I

The two candidate substances [FL-no: 01.038 and 01.057] tested *in vitro* for bacterial gene mutations gave negative results in bacterial reverse gene mutation tests and for mammalian cell gene mutations.

Subgroup II

For the three candidate substances in subgroup II [FL-no: 01.035, 01.064 and 01.070] there are no genotoxicity data available, but it was noted that in contrast to the structurally related substance 2-methyl-1,3-butadiene, these substances do not contain conjugated terminal double bonds, except [FL-no: 01.064].

The available *in vivo* studies on the structurally related substance 2-methylbuta-1,3-diene (isoprene) reported a negative result in a valid chromosomal aberration assay in the bone marrow of mice after 12 days of inhalatory exposure to isoprene. However, isoprene induced sister chromatid exchanges (SCE) in the bone marrow and micronuclei in peripheral blood cells of mice after 12 days of inhalatory exposure in two valid studies carried out within NTP. Induction of micronuclei in peripheral blood cells of mice has also been reported after inhalatory exposure for 13 weeks. In contrast, inhalatory exposure of isoprene to male and female rats for four weeks did not result in an increase in the frequency of micronuclei in the lung fibroblasts. The validity of the latter two studies cannot be evaluated due to limited details available. Isoprene has been reported to bind covalently to haemoglobin *in vivo* (IARC, 1999).

The genotoxic and carcinogenic potential of isoprene has been evaluated by IARC (1999a). It was concluded that there is sufficient evidence of carcinogenicity in experimental mammalians and that isoprene is *'possibly carcinogenic to humans' (Group 2B)* (IARC, 1999). Isoprene has been classified in the EU as a *'Muta. Cat. 3; R68'* and *'Carc. Cat. 2; R45'* (EFSA, 2004b).

The available data on *in vivo* genotoxicity of isoprene indicate a genotoxic potential of the substance. In the light of the evidence of carcinogenic activity of isoprene in rats and mice (NTP, 1999) and the genotoxic effects of isoprene in mice and the fact that the structurally related substance 1,3-butadiene is classified as a genotoxic carcinogen, the Panel concluded that there is reason for concern with respect to genotoxicity and carcinogenicity of isoprene. This substance has been deleted from the Register.

For the supporting substances myrcene, several *in vitro* genotoxicity tests and three *in vivo* genotoxicity studies were available. All the *in vitro* genotoxicity tests on myrcene were negative. Two micronucleus tests on peripheral blood cells and one chromosomal aberration assay with myrcene gave negative results.

Conclusion on Genotoxicity for subgroup II

The supporting substance myrcene [FL-no: 01.008] (that is considered by the Panel a more adequate supporting substance for the substances in subgroup II) has like isoprene, two conjugated terminal

double bonds but has a longer chain length, with 10 carbon atoms, like [FL-no: 01.064]. The genotoxicity data available on myrcene do not give rise to concern with respect to genotoxicity. Therefore, the Panel has not concern for genotoxicity for the three substances in subgroup II.

Subgroup III

For the five candidate substances in subgroup III no genotoxicity studies were available. For the four supporting substances, *d*-limonene [FL-no: 01.045], gamma-terpinene [FL-no: 01.020], α-terpinene [FL-no: 01.019] and α-phellandrene [FL-no: 01.006], several *in vitro* studies on genotoxicity were available and they were all negative. Also two *in vivo* Comet assay with *d*-limonene and a study with *d*-limonene in BigBlue™ rats were found negative. Therefore, the Panel has no concern for genotoxicity for the substances in subgroup III.

Subgroup V

For the candidate substance in subgroup V there are no genotoxicity data available. For the supporting substances, only negative results were reported in the available studies except for delta-3-carene (see Table 12). Delta-3-carene was studied individually as a component in wood fumes and wood fume condensates. A bacterial reverse gene mutation study (insufficiently reported) showed that delta-3-carene induced gene mutations in TA100 and TA102 strains in the absence of metabolic activation at high concentrations only, while it was negative in the presence of metabolic activation (Kurttio et al., 1990).

Information on the supporting substance β-caryophyllene [FL-no: 01.007] has been provided by EFFA (EFFA, 2012). The new data submitted cover a bacterial reverse mutation assay and an *in vivo* mouse erythrocyte micronucleus test.

In vitro

No evidence of genotoxic potential was observed when *S. typhimurium* strains TA98 and TA100 and *E. coli* WP2uvrA were incubated with five test concentrations between 2300 and 9000 µg/plate in the presence or absence of rat liver (S9) bioactivation system using the plate incorporation method (Di Sotto et al., 2008). The positive and negative controls provided the appropriate response in the tester strains. However, the study design and reporting exhibits major deviations from OECD guideline 471 and is considered of insufficient quality.

In vivo

In an *in vivo* micronucleus induction assay, groups of mice (National Institute of Hygiene, Mexico) (5/sex/dose) were administered a single dose of 0, 20, 200 and 2000 mg/kg bw of β-caryophyllene by corn oil gavage. Blood was drawn and smears for analysis were prepared at 24, 48, 72 and 96 hours post dose. No significant increase in the induction of micronucleated polychromatic erythrocytes (MNPE) was observed for the treatments groups while all positive controls provided the appropriate response (Molina-Jasso et al., 2009). In a follow up study, groups of the same strain of mice (5/sex/dose) were administered daily doses of 0, 20, 200 and 2000 mg/kg bw for three consecutive days by corn oil gavage with blood sampled and smears for analysis prepared at 24, 48, 72 and 96 hours post administration. There was no significant increase in MNPE however there was a slight increase in MNPE at the highest dose tested from 48 - 96 hours post dose. The authors did not consider this an indication of genotoxic potential due to the high dose administered over three consecutive days (Molina-Jasso et al., 2009). The Panel noted that the limit dose (2000 mg/kg) was applied in both treatment regimens without signs of toxicity (altered PCE/NCE ratio). The study is compliant with OCED guideline 474, except the reporting of individual data and historical controls; therefore, this study is considered of limited validity.

Altogether, the Panel has no concern for genotoxicity for the substances in subgroup V.

Data on the genotoxicity of the flavouring substances in this group are limited and the genotoxicity could not be assessed adequately for these substances. However, the Panel concluded that the available data do not preclude evaluating the 14 candidate substances using the Procedure.

Data on genotoxicity are summarised in Table 12 and 13.

New Mutagenicity/Genotoxicity Studies on β-Caryophyllene [FL-no: 01.007]

Information on the representative substance β-caryophyllene [FL-no: 01.007] has been provided by EFFA (EFFA, 2012). The new data submitted cover a bacterial reverse mutation assay and an *in vivo* mouse erythrocyte micronucleus test.

In vitro

No evidence of genotoxic potential was observed when *S. typhimurium* strains TA98 and TA100 and *E. coli* WP2uvrA were incubated with 5 test concentrations between 2300 and 9000 µg/plate in the presence or absence of rat liver (S9) bioactivation system using the plate incorporation method (Di Sotto et al., 2008). The positive and negative controls provided the appropriate response in the tester strains. However, the study design and reporting exhibits major deviations from OECD guideline 471 (see Table12).

In vivo

In an *in vivo* micronucleus induction assay, groups of mice (5/sex/dose; mouse strain from National Institute of Hygiene (NIH), Mexico) were administered by gavage as a single dose of 0, 20, 200 and 2000 mg/kg bw of β-caryophyllene solved in corn oil. Blood was drawn and smears for analysis were prepared at 24, 48, 72 and 96 hours post dose. No significant increase in the induction of micronucleated polychromatic erythrocytes (MNPE) was observed for the treatment groups while all positive controls provided the appropriate response (Molina-Jasso et al., 2009). In a follow up study groups of NIH mice (5/sex/dose) were administered daily doses of 0, 20, 200 and 2000 mg/kg bw for three consecutive days by gavage with blood sampled and smears for analysis prepared at 24, 48, 72 and 96 hours post administration. The Panel noted that the limit dose (2000 mg/kg) was applied in both treatment regimens without signs of toxicity (altered reticulocyte/NCE ratio). A slight, non-significant increase in the MNPE frequency was observed in the highest dose group at 48, 72 and 96 hours, respectively. However, at none of these time points the effects were clearly dose-related since the low and medium doses resulted in lower MNPE frequencies than that observed in control animals. Overall, the Panel concluded that β-caryophyllene did not cause a significant increase in MNPE. However, the study exhibits deviations from OECD guideline 474 and therefore, is considered to be of limited validity (see Table 13).

Although the newly submitted data are of limited validity they do not preclude the substances to be evaluated using the Procedure.

For a summary of *in vitro / in vivo* genotoxicity data on β-caryophyllene, see Table 14.

CONCLUSIONS

The present revision of FGE.25, Revision 3 includes the assessment of a 90-day study on ß-caryophyllene [FL-no: 01.007] supporting the candidate substance 4(10)-thujene [FL-no: 01.059] and a 90-day study on myrcene [FL-no: 01.008] supporting the candidate substances 2,6-dimethylocta-2,4,6-triene [FL-no: 01.035], cis-3,7-dimethyl-1,3,6-octatriene [FL-no: 01.064] and 1-octene [FL-no: 01.070].

Since the publication of FGE.25Rev2, the Industry has informed EFSA that 23 substances [former FL-no: 01.021, 01.022, 01.023, 01.030, 01.031, 01.032, 01.036, 01.037, 01.042, 01.043, 01.044, 01.047, 01.050, 01.051, 01.052, 01.053, 01.055, 01.056, 01.058, 01.060, 01.066, 01.067 and 01.078] are no longer supported for use as flavouring substances in Europe by Industry. The FGE.25Rev3 therefore deals with 14 flavouring substances in total.

The 14 candidate substances are aliphatic hydrocarbons from chemical group 31, which belong to the subgroups I) acyclic alkanes, II) acyclic alkenes, III) cyclohexene hydrocarbons and V) bicyclic, non-aromatic hydrocarbons.

Seven of the 14 flavouring substances possess chiral centres and three can exist as geometrical isomers.

All of the 14 candidate substances are classified into structural class I, according to the decision tree approach presented by Cramer et al. (1978).

Twelve of the 14 candidate substances have been reported to occur naturally in a wide range of food items.

According to the default MSDI approach, 12 of the 14 flavouring substances in this group have intakes in Europe from 0.0085 to 14 µg/*capita* per day, which are below the threshold of concern value for structural class I (1800 µg/person per day) substances. For limonene [FL-no: 01.001] and l-limonene [FL-no: 01.046] the intakes are 4000 and 2100 µg/*capita* per day, which are above the threshold of concern value for structural class I (1800 µg/person per day).

On the basis of the reported annual production volumes in Europe (MSDI approach), the total combined intakes of the candidate and supporting substances can be calculated for the substances in subgroup I , II, III and V.

Subgroup I (acyclic alkanes): the combined intake of the five candidate substances, all from structural class I and evaluated via the A-side of the Procedure (Appendix A), is 3.0 µg/*capita* per day, which does not exceed the threshold of 1800 µg/person per day. There are no supporting substances in subgroup I.

Subgroup II (acyclic alkenes): the combined intake of the three candidate substances, all from structural class I and evaluated via the B-side of the Procedure (Appendix A), is 23 µg/*capita* per day, which does not exceed the threshold of 1800 µg/person per day.

Subgroup III (cyclohexene hydrocarbons): the combined intake of the five candidate substances, all from structural class I and evaluated via the A-side of the Procedure (Appendix A), is 6100 µg/*capita* per day, which does exceed the threshold of 1800 µg/person per day. The total combined intake of the five candidate and six supporting substances (also from structural class I) is approximately 42 000 µg/*capita* per day. This intake exceeds the threshold of 1800 µg/person per day for a structural class I substance. However, together, limonene [FL-no: 01.001], *l*-limonene [FL-no: 01.046] and *d*-limonene (supporting substance [FL-no: 01.045]) account for approximately 40 000 µg/*capita* per day. The total combined intake of 42 000 µg/*capita* per day for the candidate and the supporting substances corresponds to 700 µg/kg bw per day for a person with a body weight of 60 kg. Thus, based on the NOAEL for *d*-limonene of 215 mg/kg bw per day, derived from a chronic 2-year toxicity study, a

margin of safety of 307 can be calculated, and accordingly these substances are not expected to be of safety concern at the estimated level of intake.

Subgroup V (bicyclic, non-aromatic hydrocarbon): the total combined intake of the one candidate and eight supporting substances, all from structural class I and evaluated via the B-side of the Procedure (Appendix A) is approximately 3800 µg/*capita* per day. This intake exceeds the threshold of 1800 µg/person per day for a structural class I substance. However, together, three supporting substances, pin-2(10)-ene [FL-no: 01.003], pin-2(3)-ene [FL-no: 01.004] and β-caryophyllene [FL-no: 01.007] account for approximately 3400 µg/*capita* per day. The total combined intake of 3800 µg/*capita* per day for the candidate and the supporting substances corresponds to 63 µg/kg bw per day for a person with a body weight of 60 kg. Thus, based on the NOAEL for β-caryophyllene of 222 mg/kg bw per day, a margin of safety of 3500 can be calculated, and accordingly these substances are not expected to be of safety concern at the estimated level of intake.

The available information on metabolism of the 14 candidate substances evaluated through the Procedure or the supporting substances for this FGE was very limited. Overall, for the following 10 candidate substances it can be concluded that they will be metabolised into innocuous metabolites: [FL-no: 01.033, 01.034, 01.038, 01.054 and 01.057] from subgroup I and [FL-no: 01.001, 01.027, 01.028, 01.039 and 01.046] from subgroup III. For four candidate substances [FL-no: 01.064, 01.070, 01.035 and 01.059], it cannot be assumed that they are metabolised to innocuous metabolites.

It was noted that where toxicity data were available they were consistent with the conclusions in the present flavouring group evaluation using the Procedure.

It is concluded that the 10 candidate substances which are expected to be metabolised to innocuous substances, [FL-no: 01.033, 01.034, 01.038, 01.054 and 01.057] from subgroup I and [FL-no: 01.001, 01.027, 01.028, 01.039 and 01.046] from subgroup III, would not give rise to safety concerns at their estimated intakes arising from their use as flavouring substances based on the MSDI approach.

For 4(10)-thujene [FL-no: 01.059], one of the substances from subgroup V which are not expected to be metabolised to innocuous substances, a margin of safety could be calculated based upon a NOAEL (222 mg/kg/bw) for the supporting substance ß-caryophyllene [FL-no: 01.007]. Compared to the MSDI of 4(10)-thujene of 14 µg/*capita* per day equal to 0.2 µg/kg bw per day, the NOAEL provides a margin of safety of 9.5×10^5.

For the three remaining substances, 2,6-dimethylocta-2,4,6-triene [FL-no: 01.035], cis-3,7-dimethyl-1,3,6-octatriene [FL-no: 01.064] and 1-octene [FL-no: 01.070] margins of safety could be calculated based upon a NOAEL (44 mg/kg bw) for the supporting substance myrcene [FL-no: 01.008]. Compared to the MSDI of 2,6-dimethylocta-2,4,6-triene [FL-no: 01.035], cis-3,7-dimethyl-1,3,6-octatriene [FL-no: 01.064] and 1-octene [FL-no: 01.070] of 9.1, 14 and 0.0085 µg/*capita* per day equal to 0.15, 0.23 and 0.00014 µg/kg bw per day, the NOAEL provides margins of safety of 2.9×10^5, 1.9×10^5 and 3.1×10^8.

The mTAMDI values for 11 candidate substances are above the threshold for structural class I of 1800 µg/person per day. For these substances more reliable exposure data are requested in order for them to be considered using the Procedure. For limonene [FL-no: 01.001] the mTAMDI is 1800 µg/person per day and for each of l-limonene and 1-octene [FL-no: 01.046 and 01.070] the mTAMDI is 1600 µg/person per day.

Intake estimates according to the mTAMDI for the 11 candidate substances for which use levels exceed the thresholds of concern for the structural class, and more reliable exposure data are requested. On the basis of such additional data, the flavouring substances should be considered using the Procedure. Subsequently, additional data might become necessary.

In order to determine whether this conclusion could be applied to the materials of commerce, it is necessary to consider the available specifications.

Specifications including complete purity criteria and identity for the materials of commerce have been provided for all 14 flavouring substances.

Thus, for all 14 candidate substances [FL-no: 01.001, 01.027, 01.028, 01.033, 01.034, 01.035, 01.038, 01.039, 01.046, 01.054, 01.057, 01.059 and 01.070] the Panel concluded that they would present no safety concern at their estimated levels of intake based on the MSDI approach.

SUMMARY OF SAFETY EVALUATION

Table 7: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

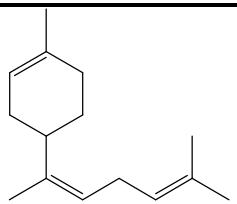
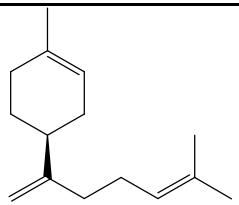
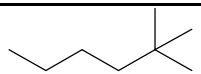
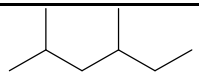
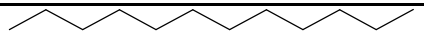
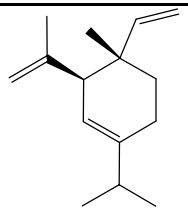
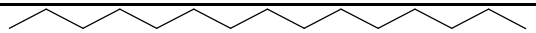
FL-no	EU Register name	Structural formula	MSDI [(a)] (µg/*capita* per day)	Class [(b)] Evaluation procedure path [(c)]	Outcome on the named compound (d or e)	Outcome on the material of commerce (f, g, or h)	Evaluation remarks
01.027	Bisabola-1,8,12-triene		0.024	Class I A3: Intake below threshold	d	g	
01.028	beta-Bisabolene		2.7	Class I A3: Intake below threshold	d	f	
01.033	2,2-Dimethylhexane		1.2	Class I A3: Intake below threshold	d	f	
01.034	2,4-Dimethylhexane		1.2	Class I A3: Intake below threshold	d	f	
01.038	Dodecane		0.012	Class I A3: Intake below threshold	d	f	
01.039	delta-Elemene		0.012	Class I A3: Intake below threshold	d	f	
01.054	Pentadecane		0.61	Class I A3: Intake below threshold	d	f	

Table 7: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

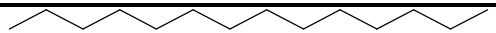
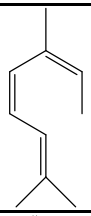
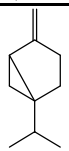
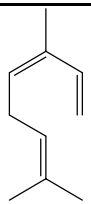
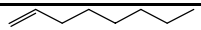
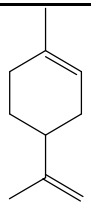
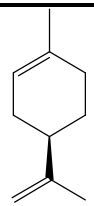
FL-no	EU Register name	Structural formula	MSDI [(a)] (µg/*capita* per day)	Class [(b)] Evaluation procedure path [(c)]	Outcome on the named compound [(d or e)]	Outcome on the material of commerce [(f, g, or h)]	Evaluation remarks
01.057	Tetradecane		0.012	Class I A3: Intake below threshold	d	f	
01.035	2,6-Dimethylocta-2,4,6-triene		9.1	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.059	4(10)-Thujene		14	Class I B3: Intake below threshold, B4: Adequate NOAEL exists	d		
01.064	cis-3,7-Dimethyl-1,3,6-octatriene		14	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.070	1-Octene		0.0085	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.001	Limonene		4000	Class I A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	d	f	

Table 7: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

FL-no	EU Register name	Structural formula	MSDI [(a)] (µg/*capita* per day)	Class [(b)] Evaluation procedure path [(c)]	Outcome on the named compound [(d or e)]	Outcome on the material of commerce [(f, g, or h)]	Evaluation remarks
01.046	l-Limonene		2100	Class I A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	d	f	

(a): EU MSDI: Amount added to food as flavour in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita per day.
(b): Thresholds of concern: Class I = 1800 µg/person per day, Class II = 540 µg/person per day, Class III = 90 µg/person per day.
(c): Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.
(d): No safety concern based on intake calculated by the MSDI approach of the named compound.
(e): Data must be available on the substance or closely related substances to perform a safety evaluation.
(f): No safety concern at the estimated level of intake of the material of commerce meeting the specification requirement (based on intake calculated by the MSDI approach).
(g): Tentatively regarded as presenting no safety concern (based on intake calculated by the MSDI approach) pending further information on the purity of the material of commerce and/or information on stereoisomerism.
(h): No conclusion can be drawn due to lack of information on the purity of the material of commerce.

SUPPORTING SUBSTANCES SUMMARY

Table 8: Supporting Substances Summary

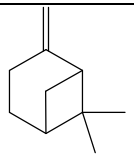
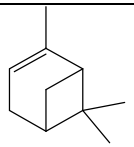
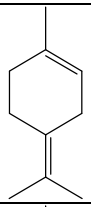
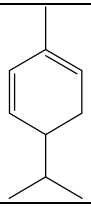
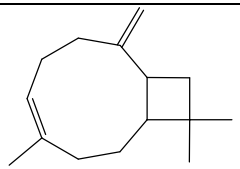
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	JECFA no Specification available	MSDI (EU) [a] (μg/*capita* per day)	SCF status [b] JECFA status [c] CoE status [d]	Comments
01.003	Pin-2(10)-ene		2903 2114 127-91-3	1330 JECFA specification (JECFA, 2005a).	1300	No safety concern (JECFA, 2005b) Category B (CoE, 1992)	JECFA name: beta-Pinene. EFSA conclusion: additional data required (EFSA, 2009b).
01.004	Pin-2(3)-ene		2902 2113 80-56-8	1329 JECFA specification (JECFA, 2005a)	1800	No safety concern (JECFA, 2005b) Category B (CoE, 1992)	JECFA name: Alpha-Pinene. EFSA conclusion: additional data required (EFSA, 2009b).
01.005	Terpinolene		3046 2115 586-62-9	1331 JECFA specification (JECFA, 2005a).	660	No safety concern (JECFA, 2005b) Category B (CoE, 1992)	
01.006	alpha-Phellandrene		2856 2117 99-83-2	1328 JECFA specification (JECFA, 2005a).	79	No safety concern (JECFA, 2005b) Category B (CoE, 1992)	
01.007	beta-Caryophyllene		2252 2118 87-44-5	1324 JECFA specification (JECFA, 2005a).	330	No safety concern (JECFA, 2005b) Category B (CoE, 1992)	EFSA conclusion: additional data required (EFSA, 2009b).

Table 8: Supporting Substances Summary

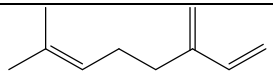
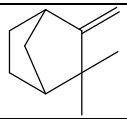
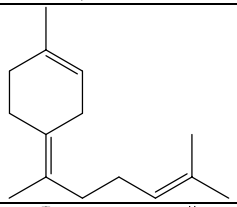
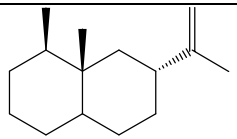
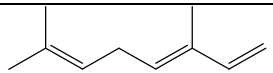
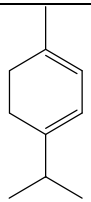
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	JECFA no Specification available	MSDI (EU) [a] (μg/*capita* per day)	SCF status [b] JECFA status [c] CoE status [d]	Comments
01.008	Myrcene		2762 2197 123-35-3	1327 JECFA specification (JECFA, 2005a)	290	No safety concern (JECFA, 2005b) Category B (CoE, 1992)	EFSA conclusion: B4-No, additional data required (EFSA, 2009b).
01.009	Camphene		2229 2227 79-92-5	1323 JECFA specification (JECFA, 2005a)	13	No safety concern (JECFA, 2005b) Category B (CoE, 1992)	EFSA conclusion: additional data required (EFSA, 2009b).
01.016	1,4(8),12-Bisabolatriene		3331 10979 495-62-5	1336 JECFA specification (JECFA, 2005a).	13	No safety concern (JECFA, 2005b)	JECFA name: Bisabolene.
01.017	Valencene		3443 11030 4630-07-3	1337 JECFA specification (JECFA, 2005a)	53	No safety concern (JECFA, 2005b)	EFSA conclusion: additional data required (EFSA, 2009b).
01.018	beta-Ocimene	 (E)- isomer shown	3539 11015 13877-91-3	1338 JECFA specification (JECFA, 2005a).	55	No safety concern (JECFA, 2005b)	JECFA name: 3,7-Dimethyl-1,3,6-octatriene.
01.019	alpha-Terpinene		3558 11023 99-86-5	1339 JECFA specification (JECFA, 2005a)	28	No safety concern (JECFA, 2005b)	JECFA name: p-Mentha-1,3-diene.

Table 8: Supporting Substances Summary

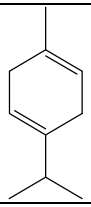
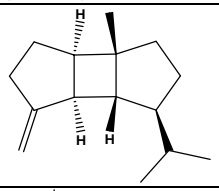
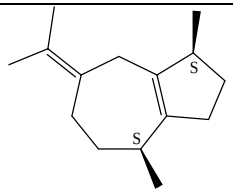
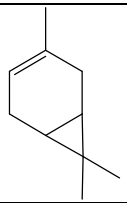
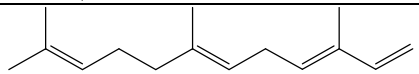
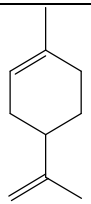
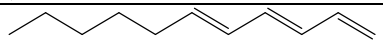
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	JECFA no Specification available	MSDI (EU) [a] (µg/*capita* per day)	SCF status [b] JECFA status [c] CoE status [d]	Comments
01.020	gamma-Terpinene		3559 11025 99-85-4	1340 JECFA specification (JECFA, 2005a)	1200	No safety concern (JECFA, 2005b)	JECFA name: p-Mentha-1,4-diene.
01.024	beta-Bourbonene		11931 5208-59-3	1345 JECFA specification (JECFA, 2005a).	0.012	No safety concern (JECFA, 2005b)	EFSA conclusion: additional data required (EFSA, 2009b). MSDI based on USA production figure.
01.026	1(5),7(11)-Guaiadiene		88-84-6	1347 JECFA specification (JECFA, 2005a).	0.012	No safety concern (JECFA, 2005b)	JECFA name: Guaiene. EFSA conclusion: additional data required (EFSA, 2009b).
01.029	delta-3-Carene		3821 10983 13466-78-9	1342 JECFA specification (JECFA, 2005a)	290	No safety concern (JECFA, 2005b)	EFSA conclusion: additional data required (EFSA, 2009b).
01.040	alpha-Farnesene		3839 10998 502-61-4	1343 JECFA specification (JECFA, 2005a).	0.61	No safety concern (JECFA, 2005b)	

Table 8: Supporting Substances Summary

FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	JECFA no Specification available	MSDI (EU) [(a)] (µg/*capita* per day)	SCF status [(b)] JECFA status [(c)] CoE status [(d)]	Comments
01.045	d-Limonene		2633 491 5989-27-5	1326 JECFA specification (JECFA, 2005a).	34000	No safety concern (JECFA, 2005b)	ADI not specified (JECFA, 2006).
01.061	Undeca-1,3,5-triene		3795 16356-11-9	1341 JECFA specification (JECFA, 2005a).	0.24	No safety concern (JECFA, 2005b)	JECFA name: 1,3,5-Undecatriene. EFSA conclusion: additional data required (EFSA, 2009b).

(a): EU MSDI: Amount added to food as flavouring substance in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita per day.
(b): Category 1: Considered safe in use, Category 2: Temporarily considered safe in use, Category 3: Insufficient data to provide assurance of safety in use, Category 4: Not acceptable due to evidence of toxicity.
(c): No safety concern at estimated levels of intake.
(d): Category A: Flavouring substance, which may be used in foodstuffs, Category B: Flavouring substance which can be used provisionally in foodstuffs.

TOXICITY DATA

Table 9: Acute Toxicity

Chemical Name [FL-no] *	Species	Sex	Route	LD_{50} (mg/kg bw)	Reference	Comments
Tetradecane [01.057]	Rat	NR	Oral	> 5000	(Enichem Augusta Ind., 1987)	Study not available
	Rat	NR	Oral	> 5000	(PETRESA, 19??b)	Study not available
(Undeca-1,3,5-triene [01.061])	Rat	M, F	Oral	> 8000	(Pellmont, 1973)	Data not possible to interprete
	Mouse	M, F	Oral	2000 - 4000	(Pellmont, 1973)	Data not possible to interprete
(Myrcene [01.008])	Rat	M	Oral	> 5000	(Moreno, 1972a)	Identity of compound not stated as other than code number
(β-Ocimene [01.018])	Rat	NR	Oral	5000	(Moreno, 1976a)	One dose tested
(*d*-Limonene [01.045])	Rat	M	Oral	> 5000	(Moreno, 1972d)	Identity of compound not stated as other than code number
	Rat	M, F	Oral	M: 4400 F: 5100	(Tsuji et al., 1975a)	Study is on dogs, rats not mentioned in English text. Study in Japanese
	Mouse	M, F	Oral	M: 5600 F: 6600	(Tsuji et al., 1975a)	Study is on dogs, mice not mentioned in English text. Study in Japanese
(Terpinolene [01.005])	Rat	NR	Oral	4.39 ml/kg (3784)	(Levenstein, 1975)	Observation period not given.
(α-Terpinene [01.019])	Rat	NR	Oral	1680	(Moreno, 1973b)	
(gamma-Terpinene [01.020])	Rat	M, F	Oral	3650	(Moreno, 1973a)	
(α-Phellandrene [01.006])	Rat	M, F	Oral	> 5700	(Moreno, 1972b)	Submitted study is on dermal toxicity.
	Rat	M, F	Oral	1.87 ml/kg (1590)	(Brownlee, 1940)	Substance mixed with acacia. 6 animals per dose group
β-Bisabolene [01.028]	Rat	NR	Oral	> 5000	(Moreno, 1974a)	One dose tested
	Mouse	M, F	Oral	> 13,360	(Hoffman-LaRoche, Inc., 1967)	No of dose levels not given.
(delta-3-Carene [01.029])	Rat	M	Oral	4800	(Moreno, 1972c)	Identity of compound not stated
(Pin-2(3)-ene [01.004])	Rat	M, F	Oral	3700	(Moreno, 1972e)	Identity of compound not stated
(Pin-2(10)-ene [01.003])	Rat	NR	Oral	> 5000	(Moreno, 1975)	One dose tested.
(Camphene [01.009])	Rat	NR	Oral	> 5000	(Moreno, 1974b)	One dose tested
(Valencene [01.017])	Rat	M	Oral	> 5000	(Moreno, 1980)	One dose tested
(β–Caryophyllene [01.007])	Rat	M, F	Oral	> 5000	(Hart and Wong, 1971)	One dose tested
(1(5),7(11)-Guaiadiene [01.026])	Rat	NR	Oral	> 5000	(Moreno, 1976b)	One dose tested
(1-methyl cyclohexa-1,3-diene [01.077])	Rat	M, F	Oral	> 2000	(Felice, 2005)	Acute toxic class method. 2000 mg/kg is only dose tested, no death was observed.

* Supporting substances are listed in brackets.

Table 10: Subacute / Subchronic / Chronic / Carcinogenicity Studies

Chemical Name [FL-no]	Species; Sex No./Group	Route	Dose levels	Duration	NOAEL (mg/kg bw per day)	Reference	Comments
(Undeca-1,3,5-triene [01.061])	Rat; M, F 10	Diet	Calculated to provide 10 mg Galbelica/kg bw per day corresponding to 2 mg undecatriene, 8 mg pinene and the rest dodecene.	14 days	Corresponding to 2 mg undecatriene[1]	(Shapiro, 1988)	Not valid. See footnote[1].
(*d*-Limonene [01.045])	Rat; M 5	Gavage	0, 75, 150 or 300 mg/kg bw per day 5 days a week	6 or 27 days (5 or 25 doses)	75	(Kanerva et al., 1987)	Study is on kidney toxicity specifically.
	Rat; M 5 – 10	Gavage	0, 2, 5, 10, 30 or 75 mg/kg bw per day, 5 days/week	90 days	5	(Webb et al., 1989)	Study is on kidney toxicity specifically.
	Dog; M, F 6	Gavage	0, 0.4, 1.2 or 3.6 ml/kg bw per day corresponding to 0, 340, 1000 or 3000 mg/kg bw per day	180 days	M: 340 F: 340	(Tsuji et al., 1975a)	Study is in Japanese, only summary and tables in English. Quality of study not possible to assess.
	Dog; M, F 10	Gavage (divided doses)	0, 0.12 or 1.2 ml/kg bw per day corresponding to 0, 100 or 1000 mg/kg bw per day.	180 days	100	(Webb et al., 1990)	
	Mouse; M, F 10	Gavage	0, 413, 825, 1650, 3300 or 6600 mg/kg bw per day for 12 days over 16-day period	16 days	1650	(NTP, 1990)	
	Mouse; M, F 20	Gavage	0, 125, 250, 500, 1000 or 2000 mg/kg bw per day 5 days per week	90 days	M: 500 F: 500	(NTP, 1990)	Clinical signs of rough hair coat and decrased activity were observed at the two highest doses.
	Mouse; M, F M:50 F:50	Gavage	M: 0, 250 or 500 mg/kg bw per day F: 0, 500, 1000 mg/kg bw per day 5 days per week	2 years	M: 250 F: 500	(NTP, 1990)	
	Rat; M, F 10	Gavage	0, 413, 825, 1650, 3300 or 6600 mg/kg bw per day for 12 days over 16-day period	16 days	1650	(NTP, 1990)	
	Rat; M, F 20	Gavage	0, 150, 300, 600, 1200 or 2400 mg/kg bw per day 5 days per week	90 days	M: None F: 1200	(NTP, 1990)	Dose-dependent nephropathy in all treated males.

Table 10: Subacute / Subchronic / Chronic / Carcinogenicity Studies

Chemical Name [FL-no]	Species; Sex No./Group	Route	Dose levels	Duration	NOAEL (mg/kg bw per day)	Reference	Comments
	Rat; M, F M:50 F:50	Gavage	M: 0, 75 or 150 mg/kg bw per day F: 0, 300 or 600 mg/kg bw per day 5 days per week	2 years	M: None F: 300	(NTP, 1990)	
(Pin-2(10)-ene [01.003])	Rat; M, F 10	Diet	Calculated to provide 10 mg Galbelica/kg bw per day corresponding to 8 mg pin-2(10)-ene	14 days	Corresponding to 8 mg pin-2(10)-ene[1]	(Shapiro, 1988)	Not valid. See footnote[1].
(Camphene [01.009])	Rat; M, F 10	Gavage	0, 62.5, 250, 1000 mg/ kg bw per day	28 days	M: None F: 250	(Hoechst, 1991)	Study report very limited in details. Study according to OECD Guideline 407.
(Myrcene [01.008])	Mouse; M,F M:10 F:10	Gavage	0, 250, 500, 1000, 2000 or 4000 mg/kg bw for 5 days a week	90 days	250	(NTP, 2010)	This study was performed at a either a single dose or multiple dose levels that produced no effects. Therefore, this dose level is not a true NOEL, but the highest dose tested that produced no adverse effects. The actual NOEL would be higher.
	Mouse; M, F M:50 F:50	Gavage	0, 250, 500 or 1000 mg/kg bw for 5 days a week	2 years	250	(NTP, 2010)	
	Rat; M, F M:10 F:10	Gavage	0.250, 500, 1000, 2000 or 4000 mg/kg bw per day	90 days		(NTP, 2010)	Study showed nephrotoxicity at the lowest dose level (250 mg/kg body wt)
	Rat; M, F M:50 F:50	Gavage	0 ,250, 500 or 1000 mg/kg bw for 5 days a week	2 years		(NTP, 2010)	Study showed nephrotoxicity at the lowest dose level and kidnet tumors at hihger dose levels
	Rat; M, F M:10 F:10	Diet	0, 8,0 , 40 , and 44 mg/kg bw per day (males) 0, 9,6 , 48 , and 53 mg/kg bw per day (females)	90 days	44 (males) 53 (females)	(Bauter, 2013b)	No toxicity at highest dose level

Table 10: Subacute / Subchronic / Chronic / Carcinogenicity Studies

Chemical Name [FL-no]	Species; Sex No./Group	Route	Dose levels	Duration	NOAEL (mg/kg bw per day)	Reference	Comments
(β-Caryophyllene [01.007])	Rat; M, F M:10 F:10	Diet	0, 220, 460 and 1.370 mg/kg bw per day (males) 0, 260, 1.030 and 4.280 mg/kg bw per day (females),	90 days	222 (males) 263(females)	(Bauter, 2013a)	Study according to OECD Guideline 408.
	Rat; M, F M:3 F:3	Diet	0, 520, 1.550 and 3.570mg/kg bw per day (males) 0, 530, 1.580 and 4.440 mg/kg bw per day (females)	14 days	-	(Bauter, 2011)	

[1] Study performed using a dose of 10 mg/kg body weight per day of Galbelica, which is a solution composed of 80 % β-pinene and 20 % 1,3,5-undecatriene and dodecene.

Table 11: Developmental and Reproductive Toxicity Studies

Chemical Name [FL-no]	Study type Durations	Species/Sex No / group	Route	Dose levels	NOAEL (mg/kg bw per day), Including information of possible maternal toxicity	Reference	Comments
(Myrcene [01.008])	Developmental Toxicity: Gestation Days 6 - 15	Rat; F 16 - 29	Gavage	250, 500 or 1200 mg/kg bw per day	Maternal: 500[1] Foetal: 500	(Delgado et al., 1993a)	Study considered valid.
	Peri- and Postnatal Developmental Toxicity: Gestation Day 15 to Postnatal Day 21	Rat; F 12 - 18	Gavage	0, 250, 500, 1000, 1500 mg/kg bw per day	Maternal: 500[2] Peri- and Post-natal: 250	(Delgado et al., 1993b)	Study considered valid.
	Reproductive and developmental toxicity: Prior to mating until postnatal day 21	Rat; M, F 60	Gavage	0, 100, 300, 500 mg/kg bw per day	Maternal/paternal: 500 mg/kg bw per day Foetal: 300 mg/kg bw per day	(Paumgartten et al., 1998)	Study considered valid.
(d-Limonene [01.045])	Developmental Toxicity: Gestation Days 9 - 15	Rat; F 20	Oral	0, 591 or 2869 mg/kg bw per day	Maternal: 591 Foetal: 591	(Tsuji et al., 1975b)	Study in Japanese, only summary and tables in English. Quality of study not possible to assess.
	Developmental Toxicity: Gestation Days 7 - 12	Mouse; F 15	Oral	0, 591 or 2363 mg/kg bw per day	Maternal: 591 Foetal: 591	(Kodama et al., 1977a)	Study in Japanese, only summary and tables in English. Quality of study not possible to assess.
	Developmental toxicity: gestation days 6 - 18	Rabbit; F 10, 18 in highest dose group	Oral	0, 250, 500 or 1000 mg/kg bw per day	Maternal: 250 Foetal: 1000	(Kodama et al., 1977b)	Study in Japanese, only summary and tables in English. Quality of study not possible to assess.
(Pin-2(3)-ene [01.004])	Developmental toxicity: Gestation days 9 - 14	Rat; F 12 - 17	Oral	Not relevant	Not relevant	(Hasegawa and Toda, 1978)	Study is not considered valid. Study is on a mixture of menthol, menthone, pinene, borneol cineol, camphene rheochrysin in olive oil. Pin-2(3)-ene content is 17 %. Study in Japanese.
	Developmental toxicity: Gestation Days 6 - 15	Rat; F 20	Gavage		Maternal: 250[3] Foetal: 1000	(Leuschner, 1992; LPT Research, 1992)	Study not submitted.

1 Test substance was myrcene.
2 Study performed using a dose of 10 mg/kg body weight per day of Galbelica, which is a solution composed of 80 % β-pinene and 20 % 1,3,5-undecatriene.
3 Test substance was 78 % pure.

Table 12: Genotoxicity (*in vitro*)

Chemical Name [FL-no] *	Test System	Test Object	Concentration	Result	Reference	Comments
Cedrene washed[6] [CAS no 11028-42-5]	Ames test	*S. typhimurium* TA97, TA98; TA100; TA1535; TA102	8-5000[4]	Negative[1]	(Gocke, 1999)	Validity cannot be evaluated as substance is not specified. Cedarwood oil terpenes and tertonoids.
	Ames test	*S. typhimurium* TA97, TA98; TA100; TA1535; TA102	1.6-1000[5]	Negative[1]	(Gocke, 1999)	Validity cannot be evaluated as substance is not specified. Cedarwood oil terpenes and tertonoids.
Dodecane [01.038]	Ames test	*S. typhimurium* TA98; TA100	NR	Negative[1]	(Tummey et al., 1992)	Only part of abstract available. Validity of the study cannot be evaluated due to insufficient report of experimental details and results.
	Mammalian cell gene mutation test (mouse lymphoma assay)	Mouse lymphocytes	NR	Negative[1]	(Tummey et al., 1992)	Only part of abstract available. Validity of the study cannot be evaluated due to insufficient report of experimental details and results.
	Mammalian cell gene mutation test	V79 Chinese hamster ovary cells	0.12 mM (20 µg/ml)	Negative[3]	(Lankas et al., 1978)	Published non-GLP study. Some important details of study design and results are not reported. Thus, the validity of the study cannot be evaluated. Study designed to evaluate the ability of various alkanes to enhance the mutagenicity induced by the chemical carcinogen methylazoxymethanol acetate. Dodecane showed no mutagenic activity per se, but increased the mutagenesis induced by pretreatment with the carcinogen.
Tetradecane [01.057]	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	50, 150, 500, 1500, 5000 µg/plate	Negative[1]	(PETRESA, 19??a)	(Study carried out by Huntingdon Research Centre, Report PEQ 5C/85914, sponsored by PETRESA; year not indicated) Unpublished GLP-study carried out in accordance with OECD guideline 471 as stated in the IUCLID datasheet submitted. IUCLID abstract available only. Validity of the study cannot be evaluated.

Table 12: Genotoxicity (*in vitro*)

Chemical Name [FL-no] *	Test System	Test Object	Concentration	Result	Reference	Comments
	Mammalian cell gene mutation test	V79 Chinese hamster ovary cells	0.12 mM (23 µg/ml)	Negative[3]	(Lankas et al., 1978)	Published non-GLP study. Some important details of study design and results are not reported. Thus, the validity of the study cannot be evaluated. Study designed to evaluate the ability of various alkanes to enhance the mutagenicity induced by the chemical carcinogen methylazoxymethanol acetate. Tetradecane showed no mutagenic activity per se, but increased the mutagenesis induced by pretreatment with the carcinogen.
	Ames test (preincubation method)	*S. typhimurium* TA98; TA100; UTH8414; UTH8413	0, 50, 100, 500, 1000, 2000 µg/plate	Negative[1]	(Connor et al., 1985)	Published non-GLP study with insufficient report of some details of method and results. Thus, the validity of the study cannot be evaluated. Cytotoxicity not reported.
(2-Methylbuta-1,3-diene) or isoprene	Ames test	*S. typhimurium* TA98; TA100; TA1530; TA1535; TA1538	25 % atmosphere concentration	Negative[1]	(De Meester et al., 1981)	Published non-GLP study not in accordance with OECD guideline 471. Part of a larger study evaluating the effects of various experimental conditions (different liver cell preparations and concentrations) on the mutagenic activity of butadiene, hexachlorobutadiene and isoprene. Some important details of study design and results are not reported. Thus, the validity of the study cannot be evaluated. Plates were exposed to a 25 % 2-methylbuta-1,3-diene atmosphere for 24 hours.
	Ames test (preincubation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537	0, 100, 333, 1000, 3333, 10000 µg/plate	Negative[1]	(Mortelmans et al., 1986) (NTP, 1999)	Published summary report including detailed results from studies on 270 compounds tested in various laboratories within the NTP to a large extent in accordance with OECD guideline 471.
	Ames test	*S. typhimurium* TA102; TA104	NR	Negative	(Kushi et al., 1985)	Published abstract only, of which part of the text including results is missing. No information on the use of a metabolic activation system. Validity of the study cannot be evaluated.
	Ames test (preincubation method)	*S. typhimurium* TA98; TA100; TA1535 *E. coli* WP2uvrA/pKM101	0, 500, 1000, 2000, 5000 µg/plate	Negative[1]	(Madhusree et al., 2002)	Published non-GLP study with limited report of experimental details and results. Thus, the validity of the study cannot be evaluated.

Table 12: Genotoxicity (*in vitro*)

Chemical Name [FL-no] *	Test System	Test Object	Concentration	Result	Reference	Comments
	Sister chromatid exchange test	Chinese hamster ovary cells	0, 50, 160, 500, 1600 µg/ml (-S9) 0, 160, 500, 1600, 5000 µg/ml (+S9).	Negative[1]	(NTP, 1999; Galloway et al., 1987)	Published summary report including detailed results from studies on 108 chemicals tested within the NTP to a large extent in accordance with OECD guideline 479.
	Chromosomal aberration assay	Chinese hamster ovary cells	0, 1600, 3000, 5000 µg/ml	Negative[1]	(NTP, 1999; Galloway et al., 1987)	Published summary report including detailed results from studies on 108 chemicals tested within the NTP to a large extent in accordance with OECD guideline 473.
(Myrcene [01.008])	Chromosomal aberration assay	Human lymphocytes	1000 µg/ml	Negative[1]	(Kauderer et al., 1991)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Mammalian cell gene mutation assay	Chinese hamster ovary V79 cells	1000 µg/ml	Negative[1]	(Kauderer et al., 1991)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Sister chromatid exchange test	Human lymphocytes	1000 µg/ml	Negative[1]	(Kauderer et al., 1991)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Sister chromatid exchange test	Chinese hamster ovary cells and hepatic tumour cell line	500 µg/ml	Negative[1]	(Röscheisen et al., 1991)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Ames test (plate incorporation method)	*S. typhimurium* TA97a ; TA98; TA100; TA1535	Up to 1500 µg/plate (16 concentrations)	Negative	(Gomes-Carneiro et al., 2005)	Valid studies which were carried out with a selection of 6 of the the concentrations mentioned. In the first run concentrations up to cytotoxicity were studied; in a second run only non-toxic concentrations were tested.
	Ames	*S. typhimurium* TA97; TA98; TA100; TA1535	10 – 10 000 µg/plate	Negative[1]	(NTP, 2010)	
	Reverse mutation	*E. coli* WP2*uvrA*/pKM101	50 – 10 000 µg/plate	Negative[1]	(NTP, 2010)	
	Ames	*S. typhimurium* TA97a; TA98; TA100; TA1535	10 - 5000	Negative[1]	(Gomes-Carneiro et al., 2005)	
	Ames	*S. typhimurium* TA97a; TA98; TA100; TA1535	1 - 1500	Negative[1]	(Gomes-Carneiro et al., 2005)	

Table 12: Genotoxicity (*in vitro*)

Chemical Name [FL-no] *	Test System	Test Object	Concentration	Result	Reference	Comments
(*d*-Limonene [01.045])	Ames test (preincubation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537	0.03, 0.3, 3, 30 µM/plate (4.1, 41, 410, 4100 µg/plate)	Negative[1]	(Florin et al., 1980)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	Up to 150,000 µg/plate	Negative[1]	(Heck et al., 1989)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Ames test	*S. typhimurium* TA102	Up to 5000 µg/plate	Negative[1]	(Müller et al., 1993)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537	Up to 3333 µg/plate	Negative[1]	(Haworth et al., 1983)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Ames test (preincubation method)	*S. typhimurium* TA98; TA100, UTH8413 and UTH8414	0, 10 to 500 µg/plate (5 concentrations)	Negative[1]	(Connor et al., 1985)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Forward mutation assay	L5178Y Mouse lymphoma	Up to 100 µg/ml	Negative[1]	(Heck et al., 1989)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Forward mutation assay	L5178Y Mouse Lymphoma	Up to 100 µg/ml	Negative[1]	(Myhr et al., 1990)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Chromosomal aberration assay	Chinese hamster ovary cells	500 µg/ml	Negative[1]	(Anderson et al., 1990)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Sister chromatid exchange test	Chinese hamster ovary cells	162 µg/ml	Negative[1]	(Anderson et al., 1990)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Sister chromatid exchange test	Chinese hamster ovary cells	1000 µM (136.2 µg/ml)	Negative	(Sasaki et al., 1989)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
(gamma-Terpinene [01.020])	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	50,000 µg/plate	Negative[1]	(Heck et al., 1989)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Unscheduled DNA synthesis	Rat hepatocytes	30 µg/ml	Negative	(Heck et al., 1989)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
(α-Terpinene [01.019])	Ames test (plate incorporation method)	*S. typhimurium* TA97a ; TA98; TA100; TA1535	Up to 1500 µg/plate (13 concentrations)	Negative	(Gomes-Carneiro et al., 2005)	Valid studies which were carried out with a selection of 6 of the the concentrations mentioned. In the first run concentrations up to cytotoxicity were studied; in a second run only non-toxic concentrations were tested.

Table 12: Genotoxicity (*in vitro*)

Chemical Name [FL-no] *	Test System	Test Object	Concentration	Result	Reference	Comments
(α-Phellandrene [01.006])	Sister chromatid exchange test	Chinese hamster ovary cells	1000 µM(136.2 µg/ml)	Negative	(Sasaki et al., 1989)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
(delta-3-Carene [01.029])	Ames test (plate incorporation method)	*S. typhimurium* TA98; TA100; TA102	up to 5 µl/plate (up to 4300 µg/plate; 5 concentrations)	Positive[3] Negative[2]	(Kurttio et al., 1990)	Published non-GLP study with insufficiently reported results. Limited validity. Positive without metabolic activation in TA100 and TA102 and at doses of 2.5 µl/plate and higher.
(Pin-2(3)-ene [01.004])	Ames test	*S. typhimurium* TA98; TA100	100 µl/plate (85,800 µg/ plate)	Negative[2]	(Rockwell and Raw, 1979)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537	0.03, 0.3, 3, 30 µM/plate (4.1, 41, 410, 4100 µg/ plate)	Negative[1]	(Florin et al., 1980)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	25000 µg/plate	Negative[1]	(Heck et al., 1989)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	25 µl/plate (21,450 µg/ plate)	Negative[1]	(Jagannath, 1984)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	5 µl/plate (4290 µg/plate)	Negative[1]	(DeGraff, 1983)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Ames test	*S. typhimurium* TA98; TA100; UTH8414; UTH8413	0, 10 to 500 µg/plate (5 concentrations)	Negative[1]	(Connor et al., 1985)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Unscheduled DNA synthesis	Rat hepatocytes	10000 µg/ml	Negative	(Heck et al., 1989)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
(+)-α-pinene (pin-2(3)-ene) (isomer of [01.004])	Ames test (plate incorporation method)	*S. typhimurium* TA97a ; TA98; TA100; TA1535	Up to 1000 µg/plate (18 concentrations)	Negative	(Gomes-Carneiro et al., 2005)	Valid studies.
(-)-α-pinene (pin-2(3)-ene) (isomer of [01.004])	Ames test (plate incorporation method)	*S. typhimurium* TA97a ; TA98; TA100; TA1535	Up to 4000 µg/plate (19 concentrations)	Negative	(Gomes-Carneiro et al., 2005)	Valid studies.

Table 12: Genotoxicity (*in vitro*)

Chemical Name [FL-no] *	Test System	Test Object	Concentration	Result	Reference	Comments
(Pin-2(10)-ene [01.003])	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	5000 µg/plate	Negative[1]	(Heck et al., 1989)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Ames test (preincubation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537	0.03, 0.3, 3, 30 µM/plate (4.1, 41, 410, 4100 µg/plate)	Negative[1]	(Florin et al., 1980)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Sister chromatid exchange	Chinese hamster ovary cells	1000 µM (136.2 µg/ml)	Negative	(Sasaki et al., 1989)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
(Camphene [01.009])	Ames test	*S. typhimurium* TA98; TA100	100 µl/plate (84,500 µg/ plate)	Negative[2]	(Rockwell and Raw, 1979)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Ames test	*S. typhimurium* TA98; TA100; UTH8414; UTH8413	0, 10 to 1000 µg/plate (5 concentrations)	Negative[1]	(Connor et al., 1985)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Sister chromatid exchange test	Chinese hamster ovary cells	1000 µM (136.2 µg/ml)	Negative	(Sasaki et al., 1989)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
(β–Caryophyllene [01.007])	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	150,000 µg/plate	Negative[1]	(Heck et al., 1989)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	150 µl/plate	Negative[1]	(Lorillard, 1984)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Ames test (plate incorporation method)	*S. typhimurium* TA98; TA100; TA102; TA1535; TA1537	10,000 µg/plate	Negative[1]	(Longfellow, 1998)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Sister chromatid exchange test	Chinese hamster ovary cells	1000 µM (204.4 µg/ml)	Negative	(Sasaki et al., 1989)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).

Table 12: Genotoxicity (*in vitro*)

Chemical Name [FL-no] *	Test System	Test Object	Concentration	Result	Reference	Comments
	Ames test (plate incorporation method)	*S. typhimurium* strains TA98; TA100 *E. coli* WP2uvrA	2300 to 9000 µg/plate	Negative[1]	(Di Sotto et al., 2008)	Study not in compliance with OECD 471: Assay does not include TA 1535 and TA 1537 (or TA97, 97a, resp) - 5 concentrations tested (plate-incorpration) but not given in detail (authors stated that in range finder up to 9 mg/plate without cytotoxicity) results not given in detail (no values, no raw data). Methods and results poorly reported: no historical contral data. The study is considered to be of insufficient quality.

* Supporting substances are listed in brackets.
NR: Not Reported.
[1] With and without S9 metabolic activation.
[2] With metabolic activation.
[3] Without metabolic activation.
[4] Plate incorporation.
[5] Pre-incubation.
[6] An Ames test with cedrene washed (unspecified cedrene) was also submitted, but an adequate identification of the substance studied was not possible. Therefore the study is not further discussed.

Table 13: Genotoxicity (*in vivo*)

Chemical Name [FL-no] *	Test System	Test Object	Route	Dose	Result	Reference	Comments
(2-Methylbuta-1,3-diene)	*In vivo* Chromosomal aberration assay	Mouse (B6C3F1) bone marrow (male mice)	Inhalation	0, 438, 1750, 7000 ppm for 6 hours per day for 12 exposures over a period of 16 days (Trial 1) 0, 70, 220, 700 ppm for 6 hours per day for 12 exposures over a period of 16 days (Trial 2)	Negative	(Tice et al., 1987; Tice, 1988; Shelby, 1990)	Unpublished study report and published summary report of a valid multiple endpoint cytogenicity study sponsered by NTP, roughly in accordance with OECD guideline 475 (special dosage regimen used).
	In vivo Sister chromatid exchange test	Mouse (B6C3F1) bone marrow (male mice)	Inhalation	0, 438, 1750, 7000 ppm for 6 hours per day for 12 exposures over a period of 16 days (Trial 1) 0, 70, 220, 700 ppm for 6 hours per day for 12 exposures over a period of 16 days (Trial 2)	Positive	(Tice et al., 1987; Tice, 1988; Shelby, 1990)	Unpublished study report and published summary report of valid cytogenicity study sponsered by NTP. The study is considered valid. Significant ($0.01<p<0.05$) increase in the frequency of SCE in the bone marrow cells at all concentrations. In addition, a significant delay in bone marrow cellular proliferation kinetics (lengthening of the generation time) was detected. The mitotic index was not significantly altered.
	In vivo Micronucleus test	Mouse (B6C3F1) peripheral blood cells (male mice)	Inhalation	0, 438, 1750, 7000 ppm for 6 hours per day for 12 exposures over a period of 16 days	Positive	(Tice et al., 1987; Tice, 1988)	Unpublished study report and published summary report of valid cytogenicity study sponsored by NTP, roughly in accordance with OECD guideline 474 (special dosage regimen used). The study is considered valid. Significant ($p<0.001$) increase in the frequency of micronucleated polychromatic and normochromatic erythrocytes, and percentage of PCE. A significant ($p<0.001$) and dose-dependent decrease in the percentage of circulating polychromatic erythrocytes (suppression of erythropoiesis) was noted.

Table 13: Genotoxicity (*in vivo*)

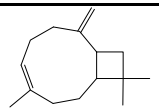
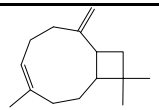
Chemical Name [FL-no] *	Test System	Test Object	Route	Dose	Result	Reference	Comments
	In vivo Micronucleus test	Mouse(B6C3F1) peripheral blood cells (male and female mice)	Inhalation	0, 70, 220, 700, 2200, 7000 ppm for 13-weeks	Positive	(Jauhar et al., 1988)	Study carried out within NTP. Only short summary and tabulated results available from NTP TR 486 (NTP, 1999). Percentage of polychromatic erythrocytes among erythrocytes in peripheral blood was increased probable due to a possible adaptation to cytotoxicity with chronic exposure. Due to the limited details available on this study, the validity cannot be evaluated.
	In vivo Micronucleus test	Rat lung fibroblasts (male and female rats)	Inhalation	0, 220, 700, 7000 ppm for 13-weeks	Negative	(Khan and Heddle, 1991)	Study carried out within NTP. Only tabulated results available from NTP TR 486 (NTP, 1999). Unusual study protocol. Validity of the study cannot be evaluated.
(Myrcene [01.008])	*In vivo* Chromosomal aberration assay	Rat (Wistar) bone marrow	Gavage	0, 100, 500, 1000 mg/kg bw (single exposure)	Negative	(Zamith et al., 1993)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	In vivo Micronucleus test	Mouse (B6C3F1) peripheral blood cells	Gavage	0, 250, 500, 1000, 2000 mg/kg bw (single exposure)	Negative	(NTP, 2003)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	Micronucleus assay	Mouse peripheral blood cells	Gavage	250, 500, 1000 mg/kg bw/ day	Negative	(NTP, 2010)	
(*d*-Limonene [01.045])	*In vivo* Comet assay	Mouse (ddY) / Rat (Wistar).	Oral	0, 2000 mg/kg	Negative	(Sekihashi et al., 2002)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	In vivo Mammalian spot test	Mouse embryos from C57BL/6JHan x T stocks	Intraperitoneal injection	215 mg/kg bw	Negative	(Fahrig, 1984)	Abstracted by JECFA at their 63rd meeting (JECFA, 2006).
	In vivo Comet assay	Rats (Sprague-Dawley) (males) (Kidneys)	Gavage	0, 1000, 2000 mg/kg bw (single exposure)	Negative	(Nesslany et al., 2007)	

Table 13: Genotoxicity (*in vivo*)

Chemical Name [FL-no] *	Test System	Test Object	Route	Dose	Result	Reference	Comments
	In vivo transgenic mutagenisity assay	Rats (Big blue) (males) (liver, kidney, bladder)	Diet	0, 525 mg/kg bw per day (10 days)	Negative	(Turner et al., 2001)	The author do not specify whether the tested compound is *d*- or *l*-limonene, and the purity of the compound is not stated. However, the stability of the limonene in the diet was measured.
(β-caryophyllene [01.007])	micronucleus induction assay	Mouse (National Institute of Hygiene (NIH), Mexico)	Gavage	0, 20, 200 and 2000 mg/kg bw	Negative	(Molina-Jasso et al., 2009)	The study is compliance with OCED guideline except: number of micronucleated immature erythrocytes are not given separately for each animal and historical controls not given. The study is reliable with restrictions and therefore, considered to be of limited validity.

* Supporting substances are listed in brackets.

Table 14: Genotoxicity Data of β-Caryophyllene Submitted by EFFA (EFFA, 2012)

FL-no JECFA -no	EU Register name JECFA name	Structural formula	End-point	Test system	Concentration	Results	Reference	Comments
In vitro								
01.007 1324	β-Caryophyllene		Reverse mutation	*S. typhimurium* TA98, TA100, *E. coli* WP2uvrA	2300–9000 µg/plate	Negative[1]	(Di Sotto et al., 2008)	Study not in compliance with OECD 471: - assay does not include TA1535 and TA1537 (or TA97, 97a, resp) -5 concentrations tested (plate-incorpration) but not given in detail (authors stated that in range finder up to 9 mg/plate without cytotoxicity) -results not given in detail (no values, no raw data). Methods and results poorly reported -no historical contral data insufficient quality.
In vivo								
01.007 1324	β-Caryophyllene		Micronucleated polychromatic erythrocytes	Mice	0, 20, 200 and 2000 mg/kg bw[2]	Negative	(Molina-Jasso et al., 2009)	In compliance with OECD 474 -except: number of micronucleated immature erythrocytes not given separately for each animal; historical controls not given. Reliable with restrictions=limited validity.

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Appendix A. Procedure for the Safety Evaluation

The approach for a safety evaluation of chemically defined flavouring substances as referred to in Commission Regulation (EC) No 1565/2000 (EC, 2000), named the "Procedure", is shown in schematic form in Figure A.1. The Procedure is based on the Opinion of the Scientific Committee on Food expressed on 2 December 1999 (SCF, 1999), which is derived from the evaluation Procedure developed by the Joint FAO/WHO Expert Committee on Food Additives at its 44th, 46th and 49th meetings (JECFA, 1995; JECFA, 1996; JECFA, 1997; JECFA, 1999).

The Procedure is a stepwise approach that integrates information on intake from current uses, structure-activity relationships, metabolism and, when needed, toxicity. One of the key elements in the Procedure is the subdivision of flavourings into three structural classes (I, II, III) for which thresholds of concern (human exposure thresholds) have been specified. Exposures below these thresholds are not considered to present a safety concern.

Class I contains flavourings that have simple chemical structures and efficient modes of metabolism, which would suggest a low order of oral toxicity. Class II contains flavourings that have structural features that are less innocuous, but are not suggestive of toxicity. Class III comprises flavourings that have structural features that permit no strong initial presumption of safety, or may even suggest significant toxicity (Cramer et al., 1978). The thresholds of concern for these structural classes of 1800, 540 or 90 µg/person per day, respectively, are derived from a large database containing data on subchronic and chronic animal studies (JECFA, 1996).

In Step 1 of the Procedure, the flavourings are assigned to one of the structural classes. The further steps address the following questions:

- can the flavourings be predicted to be metabolised to innocuous products[16] (Step 2)?
- do their exposures exceed the threshold of concern for the structural class (Step A3 and B3)?
- are the flavourings or their metabolites endogenous[17] (Step A4)?
- does a NOAEL exist on the flavourings or on structurally related substances (Step A5 and B4)?

In addition to the data provided for the flavouring substances to be evaluated (candidate substances), toxicological background information available for compounds structurally related to the candidate substances is considered (supporting substances), in order to assure that these data are consistent with the results obtained after application of the Procedure.

The Procedure is not to be applied to flavourings with existing unresolved problems of toxicity. Therefore, the right is reserved to use alternative approaches if data on specific flavourings warranted such actions.

[16] "Innocuous metabolic products": Products that are known or readily predicted to be harmless to humans at the estimated intakes of the flavouring agent" (JECFA, 1997a).

[17] "Endogenous substances": Intermediary metabolites normally present in human tissues and fluids, whether free or conjugated; hormones and other substances with biochemical or physiological regulatory functions are not included (JECFA, 1997a).

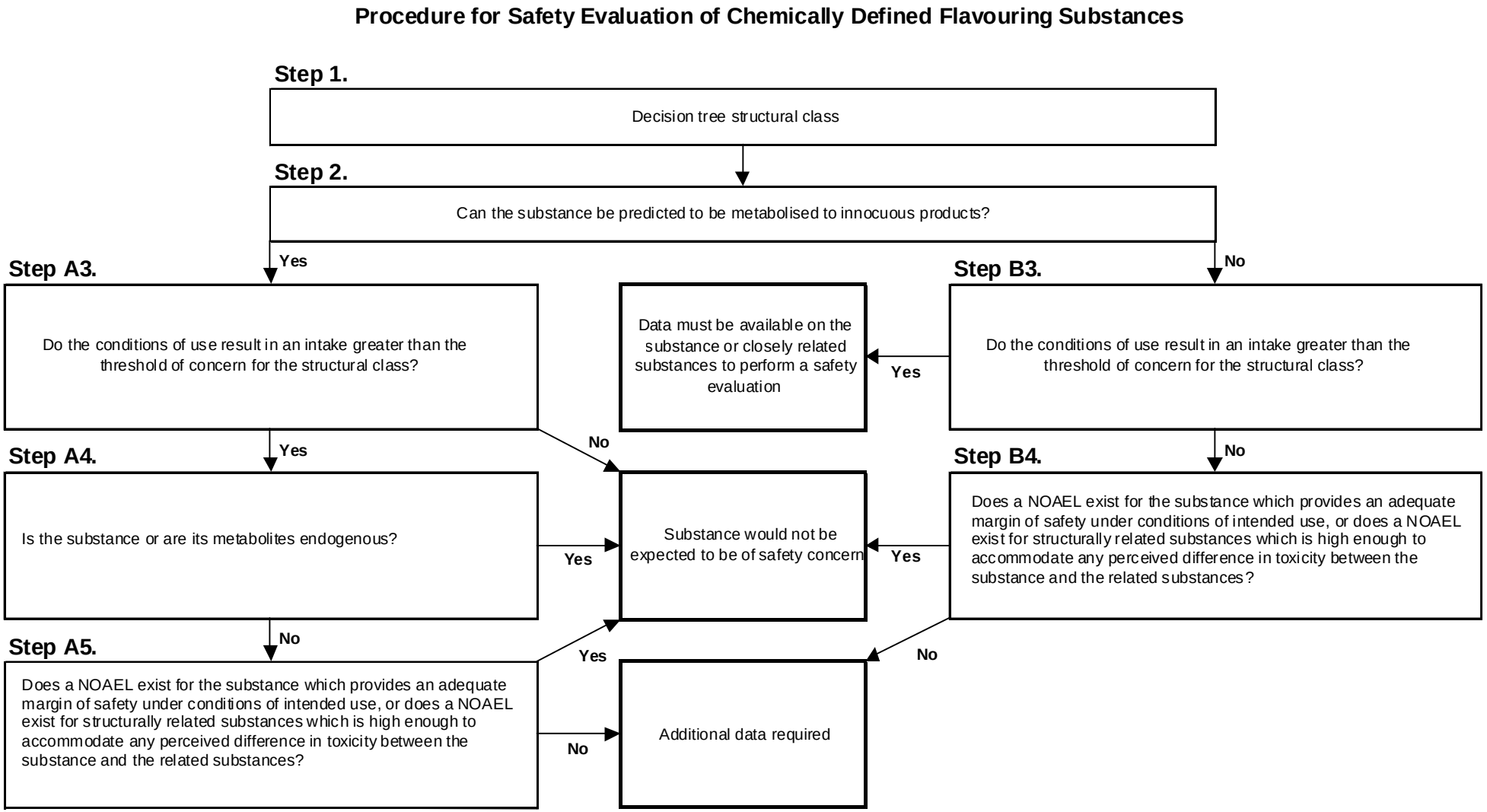


Figure A.1 *Procedure for Safety Evaluation of Chemically Defined Flavouring Substances*

Appendix B. Use Levels / mTAMDI

B.1 Normal and Maximum Use Levels

For each of the 18 Food categories (Table B.1.1) in which the candidate substances are used, Flavour Industry reports a “normal use level” and a “maximum use level” (EC, 2000). According to the Industry the “normal use” is defined as the average of reported usages and “maximum use” is defined as the 95th percentile of reported usages (EFFA, 2002). The normal and maximum use levels in different food categories have been extrapolated from figures derived from 12 model flavouring substances (EFFA, 2004).

Table B.1.1 Food categories according to Commission Regulation (EC) No 1565/2000 (EC, 2000).

Food category	Description
01.0	Dairy products, excluding products of category 02.0
02.0	Fats and oils, and fat emulsions (type water-in-oil)
03.0	Edible ices, including sherbet and sorbet
04.1	Processed fruit
04.2	Processed vegetables (incl. mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds
05.0	Confectionery
06.0	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery
07.0	Bakery wares
08.0	Meat and meat products, including poultry and game
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms
10.0	Eggs and egg products
11.0	Sweeteners, including honey
12.0	Salts, spices, soups, sauces, salads, protein products, etc.
13.0	Foodstuffs intended for particular nutritional uses
14.1	Non-alcoholic ("soft") beverages, excl. dairy products
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts
15.0	Ready-to-eat savouries
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) - foods that could not be placed in categories 01.0 - 15.0

The “normal and maximum use levels” are provided by Industry for the 12 of the 14 candidate substances in the present flavouring group (Table B.1.2).

Table B.1.2. Normal and Maximum use levels (mg/kg) for the candidate substances in FGE.25Rev3 (EFFA, 2005a; EFFA, 2006a; EFFA, 2007).

FL-no	Food Categories																	
	Normal use levels (mg/kg) Maximum use levels (mg/kg)																	
	01.0	**02.0**	**03.0**	**04.1**	**04.2**	**05.0**	**06.0**	**07.0**	**08.0**	**09.0**	**10.0**	**11.0**	**12.0**	**13.0**	**14.1**	**14.2**	**15.0**	**16.0**
01.001	5 25	2 5	5 10	1 1	0 0	10 25	5 10	5 25	1 10	1 5	1 5	1 5	2 20	3 10	2 10	5 10	2 5	1 5
01.027	7 35	5 25	10 50	7 35	- -	10 50	5 25	10 50	2 10	2 10	- -	- -	5 25	10 50	5 25	10 50	20 100	5 25
01.028	7 35	5 25	10 50	7 35	- -	10 50	5 25	10 50	2 10	2 10	- -	- -	5 25	10 50	5 25	10 50	20 100	5 25
01.033	7 35	5 25	10 50	7 35	- -	10 50	5 25	10 50	2 10	2 10	- -	- -	5 25	10 50	5 25	10 50	20 100	5 25
01.034	7 35	5 25	10 50	7 35	- -	10 50	5 25	10 50	2 10	2 10	- -	- -	5 25	10 50	5 25	10 50	20 100	5 25
01.035	7 35	5 25	10 50	7 35	- -	10 50	5 25	10 50	2 10	2 10	- -	- -	5 25	10 50	5 25	10 50	20 100	5 25
01.038	7 35	5 25	10 50	7 35	- -	10 50	5 25	10 50	2 10	2 10	- -	- -	5 25	10 50	5 25	10 50	20 100	5 25
01.039	7 35	5 25	10 50	7 35	- -	10 50	4 25	10 50	2 10	2 10	- -	- -	5 25	10 50	5 25	10 50	20 100	5 25
01.046	3 15	2 5	5 10	1 1	0 0	5 15	2 10	5 20	1 5	1 5	1 5	1 5	2 15	3 10	2 10	4 10	2 5	2 5
01.054	7 35	5 25	10 50	7 35	- -	10 50	5 25	10 50	2 10	2 10	- -	- -	5 25	10 50	5 25	10 50	20 100	5 25
01.057	7 35	5 25	10 50	7 35	- -	10 50	5 25	10 50	2 10	2 10	- -	- -	5 25	10 50	5 25	10 50	20 100	5 25
01.059	3 15	2 10	3 15	2 10	- -	10 50	5 25	10 50	2 10	2 10	- -	- -	5 25	10 50	3 15	5 8	15 75	5 25
01.064	7 35	5 25	10 50	7 35	- -	10 50	5 25	10 50	2 10	2 10	- -	- -	5 25	10 50	5 25	10 50	20 100	5 25
01.070	3 15	2 10	3 15	2 10	0 0	4 20	2 10	5 25	1 5	1 5	1 5	1 5	2 10	3 15	2 10	4 20	5 25	2 10

B.2 mTAMDI Calculations

The method for calculation of modified Theoretical Added Maximum Daily Intake (mTAMDI) values is based on the approach used by SCF up to 1995 (SCF, 1995). The assumption is that a person may consume the amount of flavourable foods and beverages listed in Table B.2.1. These consumption estimates are then multiplied by the reported use levels in the different food categories and summed up.

Table B.2.1 Estimated amount of flavourable foods, beverages, and exceptions assumed to be consumed per person per day (SCF, 1995).

Class of product category	Intake estimate (g per day)
Beverages (non-alcoholic)	324.0
Foods	133.4
Exception a: Candy, confectionery	27.0
Exception b: Condiments, seasonings	20.0
Exception c: Alcoholic beverages	20.0
Exception d: Soups, savouries	20.0
Exception e: Others, e.g. chewing gum	e.g. 2.0 (chewing gum)

The mTAMDI calculations are based on the normal use levels reported by Industry. The seven food categories used in the SCF TAMDI approach (SCF, 1995) correspond to the 18 food categories as outlined in Commission Regulation (EC) No 1565/2000 (EC, 2000) and reported by the Flavour Industry in the following way (see Table B.2.2):

- Beverages (SCF, 1995) correspond to food category 14.1 (EC, 2000)
- Foods (SCF, 1995) correspond to the food categories 1, 2, 3, 4.1, 4.2, 6, 7, 8, 9, 10, 13 and/or 16 (EC, 2000)
- Exception a (SCF, 1995) corresponds to food category 5 and 11 (EC, 2000)
- Exception b (SCF, 1995) corresponds to food category 15 (EC, 2000)
- Exception c (SCF, 1995) corresponds to food category 14.2 (EC, 2000)
- Exception d (SCF, 1995) corresponds to food category 12 (EC, 2000)
- Exception e (SCF, 1995) corresponds to others, e.g. chewing gum.

Table B.2.2 Distribution of the 18 food categories listed in Commission Regulation (EC) No 1565/2000 (EC, 2000) into the seven SCF food categories used for TAMDI calculation (SCF, 1995).

	Food categories according to Commission Regulation 1565/2000	**Distribution of the seven SCF food categories**		
Key	Food category	Food	Beverages	Exceptions
01.0	Dairy products, excluding products of category 02.0	Food		
02.0	Fats and oils, and fat emulsions (type water-in-oil)	Food		
03.0	Edible ices, including sherbet and sorbet	Food		
04.1	Processed fruit	Food		
04.2	Processed vegetables (incl. mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds	Food		
05.0	Confectionery			Exception a
06.0	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery	Food		
07.0	Bakery wares	Food		
08.0	Meat and meat products, including poultry and game	Food		
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms	Food		
10.0	Eggs and egg products	Food		
11.0	Sweeteners, including honey			Exception a
12.0	Salts, spices, soups, sauces, salads, protein products, etc.			Exception d
13.0	Foodstuffs intended for particular nutritional uses	Food		
14.1	Non-alcoholic ("soft") beverages, excl. dairy products		Beverages	
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts			Exception c
15.0	Ready-to-eat savouries			Exception b
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) - foods that could not be placed in categories 01.0 - 15.0	Food		

The mTAMDI values (see Table B.2.3) are presented for each of the 14 flavouring substances in the present flavouring group (EFFA, 2005a; EFFA, 2006a; EFFA, 2007, EFFA, 2015). The mTAMDI values are only given for the highest reported normal use levels.

Table B.2.3 Estimated intakes based on the mTAMDI approach.

FL-no	**EU Register name**	**mTAMDI (μg/person/day)**	**Structural class**	**Threshold of concern (μg/person/day)**
01.027	Bisabola-1,8,12-triene	3900	Class I	1800
01.028	beta-Bisabolene	3900	Class I	1800
01.033	2,2-Dimethylhexane	3900	Class I	1800
01.034	2,4-Dimethylhexane	3900	Class I	1800
01.038	Dodecane	3900	Class I	1800
01.039	delta-Elemene	3900	Class I	1800
01.054	Pentadecane	3900	Class I	1800
01.057	Tetradecane	3900	Class I	1800
01.035	2,6-Dimethylocta-2,4,6-triene	3900	Class I	1800
01.059	4(10)-Thujene	3100	Class I	1800
01.064	cis-3,7-Dimethyl-1,3,6-octatriene	3900	Class I	1800
01.070	1-Octene	1600	Class I	1800
01.001	Limonene	1800	Class I	1800
01.046	l-Limonene	1600	Class I	1800

Appendix C. Metabolism

C.1. Introduction

This group of flavouring substances is very diverse with respect to the chemical structures. In order to facilitate the evaluation of the metabolism aspects of the individual substances, the candidate substances in the group have been divided into four subgroups: I) acyclic saturated hydrocarbons [FL-no: 01.033, 01.034, 01.038, 01.054 and 01.057], II) acyclic unsaturated hydrocarbons [FL-no: 01.001, 01.035, 01.046, 01.064 and 01.070], III) cyclohexene hydrocarbons [FL-no: 01.027, 01.028 and 01.039], V) bicyclic, non-aromatic hydrocarbon [FL-no: 01.059,]. From the evaluation of flavouring substances as carried out by the JECFA in 2004, a group of supporting substances has been identified. These supporting substances have also been allocated to subgroups in the same way as has been indicated for the candidate substances. The allocation of the candidate and supporting substances is shown in Table C.1.

Table C.1 Subgroups. The supporting substances are listed in brackets.

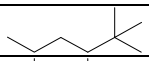
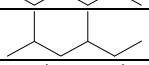
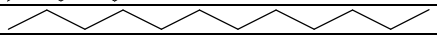
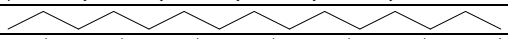
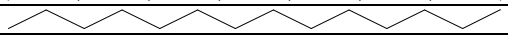
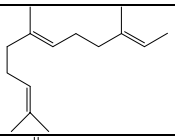
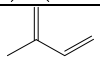
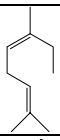
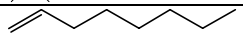
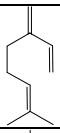
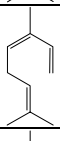
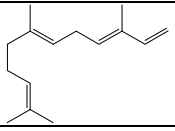
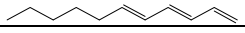
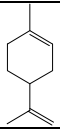
FL-no	EU Register name	Structural formula	Structural class
I: ACYCLIC ALKANES			
01.033	2,2-Dimethylhexane		I
01.034	2,4-Dimethylhexane		I
01.038	Dodecane		I
01.054	Pentadecane		I
01.057	Tetradecane		I
II: ACYCLIC ALKENES			
01.035	2,6-Dimethylocta-2,4,6-triene		I
Deleted from the Register	2-Methylbuta-1,3-diene		I
01.064	cis-3,7-Dimethyl-1,3,6-octatriene		I
01.070	1-Octene		I
(01.008)	(Myrcene)		I
(01.018)	(β-Ocimene)		I
(01.040)	(α-Farnesene)		I
(01.061)	(Undeca-1,3,5-triene)		I
III: CYCLOHEXENE HYDROCARBONS			
01.001	Limonene		I

Table C.1 Subgroups. The supporting substances are listed in brackets.

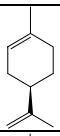
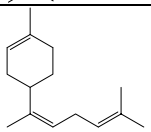
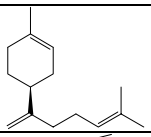
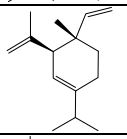
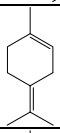
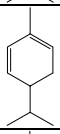
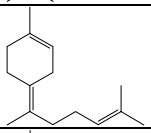
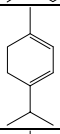
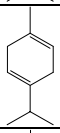
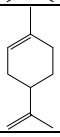
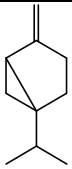
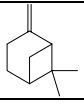
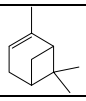
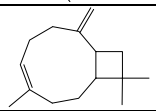
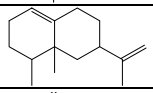
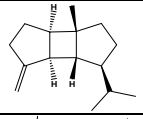
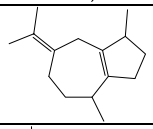
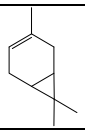
FL-no	EU Register name	Structural formula	Structural class
01.046	l-Limonene		I
01.027	Bisabola-1,8,12-triene		I
01.028	β-Bisabolene		I
01.039	delta-Elemene		I
(01.005)	(Terpinolene)		I
(01.006)	(α-Phellandrene)		I
(01.016)	(1,4(8),12-Bisabolatriene)		I
(01.019)	(α-Terpinene)		I
(01.020)	(gamma-Terpinene)		I
(01.045)	(d-Limonene)		I
IV: AROMATIC HYDROCARBONS			
The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry			
V: BICYCLIC, NON-AROMATIC HYDROCARBONS			
01.059	4(10)-Thujene		I
(01.003)	(Pin-2(10)-ene)		I

Table C.1 Subgroups. The supporting substances are listed in brackets.

FL-no	EU Register name	Structural formula	Structural class
(01.004)	(Pin-2(3)-ene)		I
(01.007)	(β-Caryophyllene)		I
(01.009)	(Camphene)		I
(01.017)	(Valencene)		I
(01.024)	(β-Bourbonene)		I
(01.026)	(1(5),7(11)-Guaiadiene)		I
(01.029)	(delta-3-Carene)		I
VI: MACROCYCLIC, NON-AROMATIC HYDROCARBONS			
The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry			

For the majority of the substances no information of biotransformation had been submitted. Also data on structural analogues were scarce. Therefore, an additional search was carried out. The additional information retrieved has been included in the following text.

C.2. Absorption, Distribution and Elimination

Acyclic Alkanes (Candidate and Structurally Related Supporting Substances from Subgroup I)

No data on absorption, distribution and elimination were submitted for any of the candidate substances in this subgroup. Some studies on saturated alkanes were retrieved from the additional data search, but apart from two oral studies (Olson et al., 1986; Serve et al., 1995) and one subcutaneous study (Manini et al., 1999), in all other studies retrieved, inhalation was the route of exposure. From these studies (Bahima et al., 1984; Perbellini et al., 1986; Perbellini et al., 1982; Fedtke and Bolt, 1987; Holmberg et al., 1977), absorption through the membranes of the inhalatory tract was observed. Absorption rates and distribution ratios were dependent on blood/air and blood/tissue partition coefficients (Imbriani et al., 1985; Nilsen et al., 1988). In addition, one inhalation study by (Dahl, 1989) showed that more branched isomers were less well absorbed than less branched or unbranched isomers. More saturated hydrocarbons were less well absorbed than unsaturated and more volatile substances were less well absorbed than less volatile (reversely correlated to chain length), but this may not be a relevant difference for oral exposure situations. The main purpose of these studies was however to study metabolism, but these inhalation exposure studies do in general not provide appropriate mass balance data. For that reason, apart from the two oral and one inhalation study (Dahl, 1989) which give insight in mass balance, the studies by (Bahima et al., 1984; Perbellini et al., 1986; Perbellini et al., 1982; Fedtke and Bolt, 1987) and (Manini et al., 1999) will be discussed in the section on biotransformation.

Male and female rats were given a dose of 2 ml/kg or 1.4 g/kg *n*-octane by gavage and urine was collected for up to 48 hours. Urine samples were treated with glucuronidase/sulphatase and the liberated metabolites were analysed by gas chromatography. Several oxidised metabolites in the urine could be found, but quantitative data were not given (Olson et al., 1986). A similar study was carried out with n-nonane by Serve *et al*. (Serve et al., 1995), but again no quantitative data were provided.

Groups of three or four male F 344 rats were exposed to [4-^{14}C] or [5-^{14}C]-labelled iso-octane (= 2,2,4-trimethyl pentane (trivial name)) and [1-^{14}C]-octane vapours at approximately 1 and 350 ppm (~ 4.76 or 1700 mg/m^3 for both substances) by the nose for two hours. During the experiment exhalant was drawn through a bubbler train for sampling. Urine and faeces were collected at the same times, except that none were collected at one and two hours. For iso-octane, all rats exposed to 350 ppm were exposed to C4-labeled substance, but three of four low-exposure rats were exposed to C5-labeled iso-octane. Values for exhaled $^{14}CO_2$ were 0.36; 0.31; and 0.52 % of inhaled ^{14}C. For the single rat exposed to C4-labeled iso-octane the corresponding value was 2 %[18]. Values for all rats were averaged regardless of position of label. The validity of conclusions regarding low production of $^{14}CO_2$ from iso-octane would have been enhanced by exclusive use of C5-labeled material.

For both *n*-octane and iso-octane the metabolised fraction was higher at low compared to high inhaled concentrations. For octane the major route of elimination was as carbon dioxide (15 % of the radioactive dose within 70 hours). For iso-octane the major route of excretion was urine. Half of the octane-introduced ^{14}C that was retained at the end of the two-hour exposure period was eliminated 5-10 hours post exposure and the exhalation of radiolabel became undetectable after 30 hours after which 75-85 % of the label was eliminated. For iso-octane the time to eliminate half of the label was 15 hours and was not completed at the end of the observation period. Based on a discussion of papers from other research groups, the study authors suggest that for straight-chain hydrocarbons, breakdown of the carbon skeleton with the release of CO_2 is an important metabolic pathway. The route of excretion for *n*-octane in this study was markedly affected by the concentrations of the inhaled vapour. The ratio of total exhaled $^{14}CO_2$: total ^{14}C in urine was 5:1 after inhalation at 1 ppm but about 1:1 after inhalation of 350 ppm (Dahl, 1989).

Acyclic Alkenes (Candidate and Structurally Related Supporting Substances from Subgroup II)

When given to male Japanese White rabbits by gavage at a dose of 670 mg/kg bw per day for two days, approximately 25 % of the total administered amount of myrcene [FL no: 01.008] (19 g to six rabbits) could be recovered from the urine over a period of three days following administration (Ishida et al., 1981). Only metabolites of myrcene were identified. The fate of the remaining part of the dose is unclear.

Following intraperitoneal injection of 64 mg [4-^{14}C]-2-methylbuta-1,3-diene /kg to F344 rats and B6C3F$_1$ mice, the majority of radioactivity (≈ 54 and 47 %, respectively) was excreted unchanged in the expired air or as urinary metabolites (≈ 32 and 33 %, respectively) over the 24-hours collection period. Less than 2 % of the radioactivity was recovered as CO_2 for both species, and 0.2 and 7.2 % in rats and mice, respectively, of the radioactivity was recovered in the faeces over the same time period. Radioactivity remaining in the carcass and tissues amounted to only 3.1 and 1.7 % in rats and mice, respectively. From the tissues examined, the highest concentration was found in the kidneys but in both species the renal concentration of radioactivity was only twice as high as the concentration in blood. Total percentage of the dose recovered was ≈ 91 % for both rats and mice (Buckley et al., 1999).

[18] In an additional study (not reported in detail), three rats were exposed to C4- or C5-labeled iso-octane at about 350 ppm. More $^{14}CO_2$ was exhaled during exposure to the C4-labeled material (0.07% of the inhaled amount) than after exposure to the C5-labeled material (0.03%). This observation also confirms that C5 labelled iso-octane is less well metabolised to CO_2 than C4 labelled iso-octane

Cyclohexene Derivatives (Candidate and Structurally Related Supporting Substances from Subgroup III)

Data were only available for one supporting substance in subgroup III, namely *d*-limonene [FL-no: 01.045].

Following the oral administration of [9-^{14}C]-*d*-limonene to male Wistar rats by stomach tube at a dose of 800 mg/kg bw, radioactivity was determined in blood, tissues (fat not included), excreta, bile and expired air. The animals were sacrificed at 48 hours post dosing. Radioactivity reached a peak plasma level at two hours post dosing, and after maintaining high levels for 10 hours, declined to negligible levels at 48 hours. In most tissues, peak levels of radioactivity were reached within two hours post dosing indicating rapid distribution. The liver, kidney and adrenals contained the highest levels of radioactivity (higher than blood or serum); other tissues (including brain) contained less than 0.2 % of the administered radioactivity. Hardly any radioactivity could be detected at 48 hours post dosing. Whole body autoradiography confirmed these findings. At 48 hours post-dosing, about 60 % of the administered radioactivity was recovered from the urine, 5 % from faeces and 2 % from exhaled air as CO_2. Approximately 25 % of the administered radioactivity was excreted in the bile during 24 hours after administration. Total recovery of radioactivity was less than 100 % and as there was hardly any radioactivity present in the tissues at 48 hours, this could point to loss of volatile ^{14}C from the excreta or to the elimination of volatile ^{14}C-compounds other than CO_2 (Igimi et al., 1974). When a similar radioactive dose of [9-^{14}C]-*d*-limonene was given to male rabbits, 72 % and 7 % of the radioactivity was excreted in the urine and faeces during 72 hours, respectively (Kodama et al., 1974).

In an additional study with several species (rats, hamsters, guinea pigs, rabbits, dogs and humans) dosed orally with [9-^{14}C]-*d*-limonene, urinary excretion of radioactivity in rodents and rabbits comprised 82-96 % of the dose within 72 hours and faecal excretion 2-9 %. The total excretion rate in dogs was somewhat lower (77 % *via* urine and 9 % *via* faeces within 72 hours), while two human volunteers excreted 55-83 % of the administered dose in the urine. Faecal excretion in humans was not measured, but may have been considerable in the person with lower urinary excretion as this person developed a diarrhoea shortly after administration. In all species, most excretion occurred within the first 24 hours (Kodama et al., 1976).

In vitro, the solubility of *d*-limonene in blood and olive oil was high, but low in water, which suggests a high respiratory uptake and accumulation in adipose tissues (Falk et al., 1990a). Indeed, uptake was rapid and high (68 %) in an experiment in which human volunteers were exposed to *d*-limonene in air at 225 and 450 mg/m^3 for 2 hours while doing light physical exercise. The absorbed *d*-limonene was metabolised rapidly. Elimination followed a triphasic pattern, with a short half-life in blood immediately after exposure (2.6 minutes) but a long half-life during the late elimination phase (12.5 hours), which indicates slow elimination from adipose tissues. Approximately 1 % of the total uptake was eliminated unchanged in expired air, while approximately 0.003 % was eliminated unchanged in urine (Falk-Filipson et al., 1993).

Aromatic Hydrocarbons (Candidate and Structurally Related Supporting Substances from Subgroup IV)

The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry.

Bicyclic, Non-Aromatic Hydrocarbons (Candidate and Structurally Related Supporting Substances from Subgroup V)

No data on absorption on candidate substances in this group were submitted. Some data were retrieved after database search on longifolene [former FL-no: 01.047] and on several supporting substances including α- and β-pinene [FL-no: 01.004 and 01.003], caryophyllene [FL-no: 01.007] and delta-3-carene [FL-no: 01.029].

Asakawa *et al*. (1986) studied the metabolism of (+)-longifolene and caryophyllene in rabbits after gavage dosing. At two days after administration, metabolites of all of these substances could be detected in the urine, from which it can be concluded that these substances are absorbed. As no mass balance data were given, the extent of absorption cannot be assessed (Asakawa et al., 1986).

Male albino rabbits (6/group) were administered single gavage doses of 400 – 700 mg/kg bw of (+)-α-pinene, (-)-α-pinene, (±)-α-pinene, (-)-β-pinene, or delta-3-carene. Urine of individual animals was collected for three days. The animals excreted bicyclic terpene hydrocarbon metabolites as glucuronic acid conjugates or as further oxidised metabolites, notably carboxylic acids (Schreiner, 2003).

In vitro data on the solubility of α-pinene [FL-no: 01.004], β-pinene [FL-no: 01.003] and delta-3-carene [FL-no: 01.029] in blood, olive oil and water suggest a high respiratory uptake and accumulation in adipose tissues. For α-pinene this is supported by a high estimated brain/blood partition coefficient of 18 (Falk et al., 1990a). Experiments in which human volunteers were exposed to (+)- and (-)-α-pinene or delta-3-carene in air at 225 and 450 mg/m^3 for two hours while doing light physical exercise confirmed that uptake was rapid and high for these agents (58-60 % for (+)- and (-)-α-pinene and 70 % for delta-3-carene), and that they were metabolized rapidly. Elimination followed a triphasic pattern, with (+)- and (-)-α-pinene exhibiting a rapid initial (distribution) phase (4.8 and 5.6 minutes, respectively), a rapid second distribution phase (38 and 40 minutes, respectively), and a slow elimination phase (695 and 555 minutes, respectively). Triphasic elimination was also observed for delta-3-carene with half-lives of 4.5, 35 and 1800 minutes for the initial, rapid, and slow phases, respectively. It was estimated that it would require over two or six days to eliminate α-pinene or delta-3-carene, respectively, from the body. The long half-lives indicate slow elimination from adipose tissues. Less than 0.001 % of the total uptake of α-pinene or delta-3-carene was eliminated unchanged in the urine, while 7.5-7.8 % and 3 % of the inhaled amount of the α-pinenes and delta-3-carene were exhaled (Falk et al., 1990b; Falk et al., 1991).

In another study, humans were exposed for four or six hours to atmospheres containing a mixture of volatile organic substances, which included α-pinene, at total concentrations of 12 or 24 mg/m^3. At a concentration of 24 mg/m^3 for the total volatiles, the air concentration of α-pinene was 0.775 mg/m^3. The mean pre-exposure blood concentration of α-pinene of 0.035 µg/l increased to an average concentration of 1.9 µg/l during the 4-hours exposure (50-240 minutes). Thereafter (330-450 minutes), the mean blood concentration decreased to 0.15 µg/l. Changes proportional to those observed at 24 mg/m^3 were recorded at 12 mg/m^3 exposure. Similar results were recorded for the 6-hours exposure. Plasma elimination for α-pinene was best described with a three-exponential curve, with half-lives ranging from 0.22-7.8 minutes, 19-58 minutes and >150 minutes for the initial, mid and terminal phases, respectively (Ashley and Prah, 1997).

In the urine of sawmill workers exposed to an atmosphere containing 31-210 mg/m^3 α-pinene [FL-no: 01.004], 2-17 mg/m^3 β-pinene [FL-no: 01.003] and 6-90 mg/m^3 delta-3-carene [FL-no: 01.029] for three days, *cis* and *trans*-verbenol were identified as metabolites. They were excreted as conjugates, probably with glucuronic acid. The authors suggested that these metabolites were formed by hydroxylation of α-pinene (Eriksson and Levin, 1990). Analysis of urinary metabolites eliminated by human volunteers within four hours following a 2-hours exposure to 10 - 450 mg α-pinene /m^3 revealed that α-pinene is indeed eliminated as cis- and trans-verbenol, in a ratio of 1:10, within 20 hours following exposure (Levin et al., 1992). In a more extensive metabolic study, urine was collected from sawmill workers at the end of an eight - nine hours work shift or from chamber-exposed individuals. Following hydrolysis of glucuronic acid conjugates, several pinene biotransformation products were found (Eriksson and Levin, 1996).

Macrocyclic, Non-Aromatic Hydrocarbons (Candidate and Structurally Related Supporting Substances from Subgroup VI)

The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry.

C.3. Conclusions on Absorption, Distribution and Elimination

Based on the available studies it may be concluded that the candidate alkane substances (subgroup I) will be absorbed after oral exposure. The extent of absorption is not known, but may be high, given their lipophilic character and their low molecular weight. The ease with which these substances cross the membranes of the respiratory tract further supports the assumption that these substances will also be absorbed after oral intake. Data indicate that straight-chain alkanes will predominantly be broken down to carbon dioxide at low dose levels. At high dose levels, biotransformation becomes saturated and other metabolites will be generated, which will be excreted via the urine. As illustrated by for iso-octane, metabolism of (highly) branched alkanes to carbon dioxide is less favourable, and their metabolites will be eliminated more slowly via the urine.

For the candidate or supporting substances in subgroup II (acyclic alkenes), data were only available for the supporting substance myrcene [FL-no: 01.008] and for the structurally related substance 2-methylbuta-1,3-diene (former candidate substance (See "History of the evaluation")). Given the narrow range of molecular weights of the candidate substances (all between ~ 68 and 206 D) and their lipophilic nature (Log Kow values e.g. 2.4 (2-methylbuta-1,3-diene), 6.1 (dodec-1-ene), 4.17 (myrcene) or 4.8 (*cis*-3,7-dimethyl-1,3,6-octatriene)), it must be assumed that these candidate flavouring substances will be absorbed from the gastrointestinal tract, at least to some extent, if not completely. Mass balance data are also incomplete. At least for myrcene it has been shown its metabolites will be excreted via urine in an amount of 25 % of the dose within three days after dosing. For 2-methylbuta-1,3-diene a fairly complete mass balance of elimination has been presented, which shows also elimination via the exhaled air (~ 50 % of the dose), but as this substance was administered via the intraperitoneal dose route, the elimination pattern may be different after oral dosing with a lower fraction of the dose exhaled, e.g. because of more efficient first-pass biotransformation after oral dosing.

For the candidate or supporting substances in subgroup group III (cyclohexene derivatives), data were only available for *d*-limonene [FL-no: 01.045], which is a supporting substance. For this substance, data show a considerable absorption from the gastrointestinal tract. In humans, elimination of the substance followed a triphasic pattern, but still 55-83 % of an oral dose could be found in the urine within 72 hours post-dosing, with the major part excreted within 24 hours. Also in other species urinary elimination was most important accounting for up to 82–96 % of the dose within 72 hours. Hence, it may be argued that monocyclic cyclohexene derivatives, such as *d*-limonene, administered orally are absorbed and distributed throughout the body. Following oral administration to humans, *d*-limonene was distributed preferentially to fatty tissues, as indicated by a high oil-blood partition coefficient and a long half-life during the slow elimination phase. Because of the limited molecular weight of the candidate substances in this group (range ~ 136–204 D) and their lipophilic character (e.g. log Kow values for β-phellandrene, β-bisabolene and *d*-limonene are *ca.* 4.7, 7.1 or 4.6, respectively) it may be assumed that all of the substances in this group will be absorbed to some degree, although the extent of absorption for individual substances cannot be accurately estimated from these physico-chemical properties. For the substances in subgroup III with conjugated double bonds no data are available, but based on the structural similarity with the other substances in subgroup III, it may be anticipated that at the substances will also be absorbed.

The substances previously allocated to the subgroup IV are no longer supported for use as flavouring substances in Europe by Industry.

For the substances in subgroup V (bicyclic, non-aromatic hydrocarbons), the available data from oral studies are few. These studies have only addressed the excretion of some supporting and one related substance (longifolene) in the form of metabolites via the urine. No mass-balance data were provided. So these studies only show that these substances will be absorbed to some extent. The same may be anticipated for the (other) candidate substances in this subgroup. For the supporting substances α- and β-pinene and delta-carene information on kinetics is available from humans exposed via inhalation, in occupational settings. These

studies show that these substances can be absorbed after inhalation exposure and that metabolites will be excreted into the urine e.g. as glucuronide conjugates. The elimination follows a triphasic pattern with rather long terminal half-lives. The absorbed amount will be eliminated within several days. Based on the lipophilic character of these substances it may be anticipated that they will preferentially distribute in the adipose tissues, which is supported by the slow terminal elimination rates.

The substances previously allocated to the subgroup VI are no longer supported for use as flavouring substances in Europe by Industry.

C.4. Metabolism

Acyclic Alkanes (Candidate and Structurally Related Supporting Substances from Subgroup I)

Oral

Male and female rats were given a dose of 1400 mg *n*-octane per kg bw by gavage and urine was collected for up to 48 hours. Urine samples were treated with glucuronidase/sulphatase and the liberated metabolites were analysed by gas chromatography. Compounds found in urine were 2-octanol, 3-octanol, 5-oxohexanoic acid and 6-oxoheptanoic acid. In female rats 2-octanol was found to be the major urinary metabolite and 5-oxohexanoic acid was the major metabolite in males. The authors state that in contrast to *n*-hexane and *n*-heptane, *n*-octane was not metabolised to a ketone, diketone or a diol derivative. The structures of the keto acids that were produced indicate that metabolic oxidation is occurring on both ends of the *n*-octane molecule. A former metabolism study of *n*-heptane did yield *gamma*-valerolactone, which was presumed to have been formed on the GC column from 4-hydroxy-1-pentanoic acid, which would involve a 2 carbon loss. The authors speculate that with *n*-heptane, *n*-octane and perhaps higher straight chain hydrocarbon homologues, a major metabolic pathway involves the formation of acids with loss of carbon (Olson et al., 1986).

The metabolism of n-nonane has been studied in rats after oral administration of 800 mg/kg bw over a 48 hours excretion period. In the urine the following metabolites were detected: 2-, 3- and 4-nonanol, 4-nonanone, 2,5-hexanedione, *gamma*-valerolactone, delta-hexanolactone and delta-heptanolactone and 5-methyl-2-(3-oxobutyl)furane. The authors also reported the formation of 1-heptanol, but no explanation for this metabolite is available. Keto-acids, as observed with n-octane could not be detected after administration on n-nonane (Serve et al., 1995).

Other Routes of Exposure

Rats were exposed to n-heptane in a concentration of 8100 mg/m^3 in the air for six hours per day five days/week for 12 weeks. After exposure the animals were kept in a metabolism cage to collect urine samples during the next 18 hours for the first week of the experiment and subsequently at the end of every five-day exposure period. Primary metabolites of n-heptane in the urine were 1-, 2-, 3,- and 4-heptanol, of which the 2- and to a lesser extent the 3- isomer were quantitatively the most important. Subsequent oxidations of these two secondary alcohols provided 2- and 3-heptanone and the diols 2,6- and 2,5-heptanediol. In follow-up oxidations, final products were 5-hydroxy-2-heptanone, 6-hydroxy-2-heptanone, 2,6-and 2,5-heptanedione and 6-hydroxy-3-heptanone, which was de-acetylated to yield 4-hydroxy-pentanoic acid. This metabolite was detected in the urine as gamma-valerolactone. The alcoholic metabolites were rapidly excreted as sulphate and glucuronide conjugates. Although 2,5-heptanedione is a gamma-diketone, no signs of peripheral neuropathy were observed. The authors speculated that the plasma levels of this ketone did not become high enough to trigger this effect, because of the rapid conjugation of the precursor alcohols which was supported by the minute amounts in which they were present in the urine, although their (conjugated) precursors reached much higher concentrations (Bahima et al., 1984).

In Sprague-Dawley rats exposed to vapours (7680 mg/m^3) of n-heptane for six hours, main urinary metabolites were 2- and 3-heptanol. Other metabolites detected were 2- and 3- heptanone, 4-heptanol, 2,5-heptanedione, gamma-valerolactone, 2,6-dimethyl-2,3-dihydropyrane and 2-ethyl-5-methyl-2,3,-dihydrofurane. In humans, occupationally exposed to n-heptane (5-196 mg/m^3) 2- and 3-heptanol were found in the urine. Other metabolites detected were 2- and 4- heptanone and 2,5-heptanedione. In human urinary samples no ring-closure products could be detected, but it is noted that the human exposure was less than that of the rats. In rat tissues (blood, muscle, kidney, nervous tissue, liver), levels of 2-heptanol were between 0.2 and 2 mg/l. Levels of n-heptane in the same tissues were around 20 mg/l (Perbellini et al., 1986).

After oral administration of n-octane (1400 mg/kg bw) to rats in the urine the following metabolites could be detected within 48 hours after dosing: 2- and 3-octanol and 5-keto-hexanoic and 6-keto-heptanoic acid. In contrast to heptane, n-octane was not converted into mono- or diketones or into diols (Olson et al., 1986). Based on this study and the data for n-heptane, the authors argued that for n-heptane and higher straight-chain alkanes, formation of carboxylic acids and subsequent loss of carbon may be a major metabolic pathway. However, the quantitative contribution of de-carboxylation was not thoroughly studied (e.g. no studies with radioactive material) and no mass balances were provided. It has been proposed that n-octane is also converted into 1-octanol and further into octanoic acid, but no detailed information on these studies was submitted (Low et al., 1987).

Groups of three or six rats (Sprague-Dawley, 300-600g), groups of two male rabbits (New Zealand, 1600-2000g) and one male monkey (*Maccaca mulatta*, 1600-2000g) were subjected to single inhalatory treatments of 5000 ppm (~ 18 000 mg/m^3) *n*-hexane for 6, 12 and 24 hours. After treatment animals were kept in metabolic cages for the following 72 hours. Urine from treated and control animals were collected during treatment and at various intervals thereafter. For rats blood was collected during and after treatment. Urine samples were subjected to enzymatic and then acid hydrolysis and analysed by gas chromatography. In urine of rats 2-hexanol, 3-hexanol, 2,5-dimethylfurane, *gamma*-valerolactone and 2,5-hexanedione along with methyl *n*-butylketone were found. The metabolites detected in urine from rabbit and monkey were 2-hexanol, 3-hexanol, methyl *n*-butylketone and 2,5-hexanedione. 1-hexanol and 2,5-hexanediol were not detectable in any of the species. In rats peaks of 2,5-hexanedione and 2,5-dimethylfurane in urine were preceded by analogous peaks in blood, while blood concentrations of *n*-hexane, methyl *n*-butylketone and 2-hexanol “peaked” immediately after termination of exposure (Perbellini et al., 1982).

Male rats were exposed to *n*-hexane by inhalation at concentrations ranging from approximately 180 to approximately 11000 mg/m^3 for 8 hours in an all-glass exposure system. Urinary kinetics for the metabolites 1-hexanol, 2-hexanol, 3-hexanol, 2 hexanone, 2,5-hexanedione and 4,5-dihydroxy-2-hexanone were

assessed. Urine samples were collected up to 48 hours following the start of exposure. Amounts of metabolites were linearly dependent on the exposure concentrations, up to an exposure of about 1070 mg/m^3. Above this the metabolite excretion indicated saturation kinetics in the metabolism. 2-Hexanol amounted to about twice the excretion of all other metabolites excreted. In quantity 4,5-dihydroxy-2-hexanone was the second metabolite, the amount in urine being about 10 times higher than that of excreted 2,5-hexanedione. 2-Hexanol and 4,5-dihydroxy-2-hexanone accounted for 90 % of the total excretion (Fedtke and Bolt, 1987).

A male volunteer was exposed to a mean *n*-hexane atmosphere of 217 ppm (~ 800 mg/m^3) for 4 hours. The occurrence of 4,5-dihydroxy-2-hexanone as a metabolite in urine of the man was confirmed. Twenty-six hours after exposure the excretion of 4,5-dihydroxy-2-hexanone reached a level that was four times higher than the excretion of 2,5-hexanedione. The authors conclude that formation of 4,5-dihydroxy-2-hexanone may be viewed as a route of detoxification, and also state that it is clear that 4,5-dihydroxy-2-hexanone is responsible for an artificial appearance of 2,5-hexanedione after drastic acid treatment of human urine, presumably via 2,5-dimethylfuran as intermediate (Fedtke and Bolt, 1987).

Groups of three or four male F 344 rats were exposed to [4-^{14}C]-labelled iso-octane (2,2,4-trimethyl pentane) and [1-^{14}C]-octane vapours at approximately 1 and 350 ppm (~ 4.76 or 1700 mg/m^3 for both substances) for two hours. During the experiment exhalant was drawn through a bubbler train for sampling. Urine and faeces were collected at the same times, except that none were collected at one or two hours. Exhalation of $^{14}CO_2$ appeared to be much more important for n-octane than for iso-octane, from which a larger part of the radioactivity is eliminated via urine. Based on this observation and on a discussion of papers from other research groups, the study authors suggest that for straight-chain hydrocarbons, breakdown of the carbon skeleton with the release of CO_2 is an important metabolic pathway. The route of excretion for *n*-octane in this study was markedly affected by the concentrations of the inhaled vapour. The ratio of $^{14}CO_2$: ^{14}C in urine was 5:1 after inhalation at 1 ppm but 1:1 after inhalation of 350 ppm (Dahl, 1989). The radioactivity eliminated via the urine was not identified.

Hexane was given to rats via subcutaneous injections during three consecutive days. Urinary metabolites were various hexanols, 5-hydroxy-2-hexanone, 4,5-dihydroxy-2-hexanone, 2,5-hexanedione, 2-hexanone and the ring closure products *gamma*-valerolactone and 2,5-dimethylfurane. It was noted that most of the alcoholic metabolites were excreted as conjugates (in particular glucuronide) (Manini et al., 1999). Such conjugation has also been reported in the other alkane biotransformation studies discussed in this Appendix. The study authors (Manini et al., 1999) further demonstrated that the cyclization products (i.e. the lactones and furans) may be formed under the acidic conditions during deconjugation procedures for sample treatment.

Conclusions on the metabolism of subgroup I substances

The substances in subgroup I can be expected to be metabolised through omega-oxidation, which will lead to the formation of alcohols, and after oxidation to carboxylic acids, which may be further oxidised via β-oxidation to yield carbon dioxide. Extensive mass balance data are not available, but in some of the studies described above some excretion of carboxylic acids and carbon dioxide has been reported. Formation of keto-acids has also been reported, and these may be formed from omega-oxidation at one end of the molecule and omega-1 or -2 oxidation at the other end. More interest has been put in the identification of metabolites which did not undergo substantial chain shortening. These metabolites will arise from oxidation of the non-terminal carbon atoms, e.g. via omega-1, -2, -3 or even -4 oxidations. The resulting secondary alcohols can be conjugated with e.g. glucuronic acid and excreted via the urine, or can be further oxidised to yield ketones. A subsequent introduction of another secondary hydroxyl group may result in the formation of diketones. With n-hexane, the resulting 2,5-hexanedione (a gamma-diketone) has been demonstrated to be responsible for hexane-induced neurotoxicity. Neurotoxicity is a common feature of gamma-diketones, and for that reason, the candidate substance 3-methylhexane [FL-no: 01.050] cannot be anticipated to be metabolised into innocuous compounds. In addition, it is known that methyl-branching of the carbon chain

potentiates the neurotoxicity of the gamma-diketone (Topping et al., 1994; EFSA, 2004b). The two other hexane derivatives in this group [FL-no: 01.033 and 01.034] cannot be oxidised to gamma-diketone, due to the presence of methyl groups on the C2 and C5 carbon atoms. Therefore, these substances may be metabolised to innocuous metabolites. For the longer chain alkanes in this group, it would require oxidation of the more central carbon atoms in order to be converted into gamma-diketones. Such oxidations are less favourable than omega, omega-1 or -2 oxidations, and therefore it is concluded that oxidation of these higher alkanes will not result in toxicologically relevant levels of gamma-diketones, also because of rapid conjugation of the precursor alcohols. For heptane it was shown that this substance does not result in neurotoxicity (Bahima et al., 1984), although the gamma-diketone itself (2,5-heptanedione) is known to be neurotoxic (Topping et al., 1994). The remaining three candidate flavouring substances in this group i.e. [FL-no: 01.038, 01.054 and 01.057] can be expected to be metabolised to innocuous products.

Acyclic Alkenes (Candidate and Structurally Related Supporting Substances from Subgroup II)

For the substances in this group, information is only available on the biotransformation of myrcene ([FL-no: 01.008]; a supporting substance) and 2-methylbuta-1,3-diene (a structurally related substance). Some additional information on the related industrial chemical 1,3-butadiene has also been added. Two of the substances in this group (dodec-1-ene; [FL-no: 01.037] and 1-octene [FL-no: 01.070) bear a terminal double bond without any other structural features (e.g. hydroxyl groups, methyl substituents). No further data for these or similar substances are available. The metabolic aspects of terminal double bond substances have been discussed in FGE.07 and FGE.18 (EFSA, 2004b; EFSA, 2006). The relevant parts of these FGEs will also be included in the current discussion.

Myrcene [FL-no: 01.008]

In the urine of rabbits, orally administered myrcene via gavage (670 mg/kg bw per day for two days), 25 % of the total amount administered could be recovered from the urine within three days post-dosing, and > 80 % of the myrcene-derived substances were neutral metabolites; the rest were acidic substances. The main metabolites identified were myrcene-3,10-glycol, myrcene-1,2-glycol and uroterpenol (as acetate) (40.7, 20.8 and 11.8 %, respectively, of the neutral metabolites). Additionally, the glycols underwent further oxidation to yield 2-hydroxymyrcene-1-carboxylic acid and 3-hydroxymyrcene-10-carboxylic acid (no quantitative data were given for these acidic metabolites). The authors suggested that uroterpenol (or limonene-8,9-diol) may have been formed from limonene, which is derived from cyclization of myrcene in the acidic conditions of the rabbit stomach (Ishida et al., 1981). A graphic representation of myrcene metabolites has been presented in Figure C.1.

When rats were administered 800 mg/kg bw per day of myrcene orally via gavage for 20 days, the principal metabolites isolated from the urine were 10-hydroxylinalool (or myrcene-3,10-glycol) and, to a lesser extent, 7-methyl-3-methylene-oct-6-ene-1,2-diol (or myrcene-1,2-glycol). Other minor metabolites included the hydroxy- acids of both the 3,10- and 1,2-glycols (10-carboxylinalool (or 3-hydroxymyrcene-10-carboxylic acid) and 2-hydroxy-7-methyl-3-methylene-oct-6-enoic acid (or 2-hydroxymyrcene-1-carboxylic acid), respectively) and a cyclic diol, 1-hydroxymethyl-4-isopropenylcyclohexanol (or *p*-menth-8-ene-1,7-diol), formed by intramolecular cyclization of an open chain metabolite (Madyastha and Srivatsan, 1987). It was demonstrated that the biotransformation of myrcene was cytochrome P450 (CYP)-mediated and that it could be enhanced by pretreatment of animals with phenobarbital (Madyastha and Srivatsan, 1987).

Aside from being a substrate for CYP enzymes, myrcene has also been shown to induce these enzymes at high dose levels (1000 mg/kg bw per day orally for three days), especially those from the CYP2B (phenobarbital-inducible) subfamily (De-Oliveira et al., 1997). At lower dose levels (40 mg/kg bw per day intraperitoneally for three days) such induction was not observed (Austin et al., 1988).

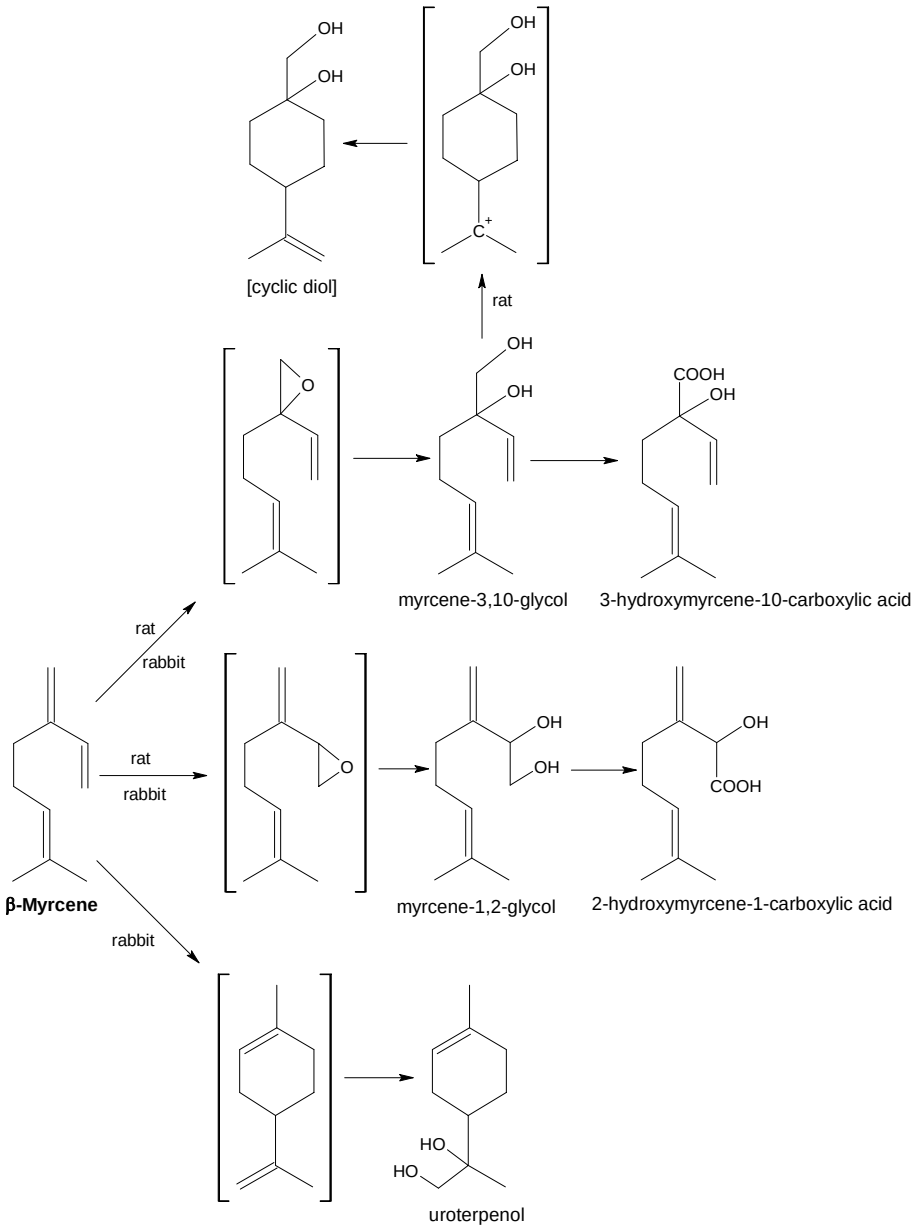


Figure C.1. *Metabolism of myrcene in rats and rabbits*

2-Methylbuta-1,3-diene

In a sequence of inhalation exposure studies in which rats and mice were exposed to 2-methylbuta-1,3-diene in the air in concentration of 13-11 000 mg/m^3, the metabolism of isoprene became saturated at a level of ca. 700 mg/m^3 in the rat and ca. 840 mg/m^3 in the mouse. Maximal rates of metabolism were 130 micromol/kg bw/hour in the rat and 400 micromol/kg bw/h in the mouse. It was demonstrated that 2-methylbuta-1,3-diene is also produced endogenously at rates of 1.9 micromol/(hour × kg) in rats and 0.4 micromol/(hour × kg) in mice. Part of the endogenous 2-methylbuta-1,3-diene is exhaled by animals, but it is metabolised extensively. The rate of metabolism of endogenously produced and systemically available 2-methylbuta-1,3-diene is 1.6 micromol/(hour × kg) in rats and 0.3 micromol/(hour × kg) in mice, respectively (Peter et al., 1987). The authors quoted literature demonstrating that 2-methylbuta-1,3-diene is also endogenously produced in humans (Gelmont *et al*., 1981 as cited in (Peter et al., 1987)).

Liver microsomes from mice, rats, rabbits and hamsters metabolise 2-methylbuta-1,3-diene to the corresponding monoepoxides, 1,2-epoxy-2-methyl-3-butene (major) and 3,4-epoxy-2-methyl-1-butene (minor). Both monoepoxides are hydrolysed to their respective diols (for chemical structures, see Figure C.2). The main metabolite of 2-methylbuta-1,3-diene (i.e. 1,2-epoxy-2-methyl-3-butene) exhibited a half-life of 75 minutes in aqueous environments; whereas the minor metabolite, 3,4-epoxy-2-methyl-1-butene (14 – 25 % with respect to the main metabolite) is more stable (half-life not specified). The kinetic constants for the formation of the major epoxide metabolite (trans-2-methyl-3-butene-1,2-diol) of 2-methylbuta-1,3-diene were determined in the four test species as apparent Km = 0.06 – 0.2 mM and Vmax = 0.24 – 1.79 nmol trans-2-methyl-3-butene-1,2-diol/mg protein × min. The minor metabolite, 3,4-epoxy-2-methyl-1-butene, was further epoxidised to the diepoxide, 2-methyl-1,2:3,4-diepoxybutane, by microsomes of all rodents studied. The authors argued that the latter diepoxide metabolite could be responsible for the genotoxic and carcinogenic activity of 2-methylbuta-1,3-diene, as the two mono-epoxies were not reported to be genotoxic (Del Monte et al., 1985; Longo et al., 1985).

The *in vitro* metabolism of 2-methylbuta-1,3-diene was investigated in rat, mouse and human liver microsomes and in microsomes derived from cell lines expressing eight different human cytochrome P-450 enzymes. Human CYP2E1 showed the highest rates for formation of the monoepoxides, 1,2-epoxy-2-methyl-3-butene and 3,4-epoxy-2-methyl-1-butene, and CYP2B6 showed the second highest rate. Only CYP2E1 catalysed formation of the diepoxide, 2-methyl-1,2:3,4-diepoxybutane. With human liver microsomes in the presence of an epoxide hydrolase inhibitor, the formation of 1,2-epoxy-2-methyl-3-butene was four times faster than the formation of the 3,4-epoxy-2-methyl-1-butene, which is comparable to the results in rats and mice obtained by others (e.g. Del Monte *et al*., 1985; Longo *et al*., 1985). The rates of monoepoxide formation from isoprene and diepoxide formation from either monoepoxide intermediate were strongly correlated with the microsomal activity of CYP2E1, rather than with the activities of the other CYP enzymes, and both monoepoxides were equally good substrates for the formation of the diepoxide.

Also, species differences with regard to the role of epoxide hydrolase were investigated by comparing the epoxidation of 2-methylbuta-1,3-diene by rat, mouse and human liver microsomes. When an epoxide hydrolase inhibitor was used, similar rates of monoepoxide formation in mouse, rat and human liver microsome systems were measured. However, without epoxide hydrolase inhibition, the total amount of 1,2-epoxy-2-methyl-3-butene measured at the end of the incubation period was twice as high for mouse as for rat liver microsomes and 30 times as high for mouse as for human liver microsomes in which formation of this epoxide was reduced to 4 % of the rate in presence of the inhibitor. For the 3,4-epoxy 2-methyl-1-butene metabolite the effect of epoxide hydrolase inhibition was less dramatic. While hardly any effect was observed in mouse or rat microsomes, in the human microsomes, the rate was reduced to approximately 25 %. The effect of epoxide inhibition on the rate of formation of the diepoxide was not studied. The authors concluded that differences in epoxide hydrolase activity between species may be of crucial importance for the toxicity of 2-methylbuta-1,3-diene in various species (Bogaards et al., 1996).

Following intraperitoneal injection of 64 mg [4-^{14}C]-2-methylbuta-1,3-diene per kg to F344 rats, the parent compound was excreted unchanged in the breath (> 50 % of the dose together with < 4 % unidentified material) or via the urine in the form of metabolites (≈ 32 %) over the 24-hours collection period. Only 1.7 % was expired as CO_2 and 0.2 % was eliminated via faeces. 3 % remained in the carcass. In the urine 2-methylbuta-1,3-diene was excreted primarily as 2-hydroxy-2-methyl-3-butenoic acid (53 % of total urinary metabolites excreted), 2-methyl-3-buten-1,2-diol (23 %), and the C-1 glucuronide conjugate of 2-methyl-3-buten-1,2-diol (13 %). A fraction of 7 % of the radioactivity in the urine was (an) unidentified polar material. The principal urinary acidic metabolite forms via oxidation of the corresponding 1,2-diol. These metabolites indicate a preferential epoxidation of the methyl-substituted vinyl group of 2-methylbuta-1,3-diene (see Figure C.2) in the rat (Buckley et al., 1999).

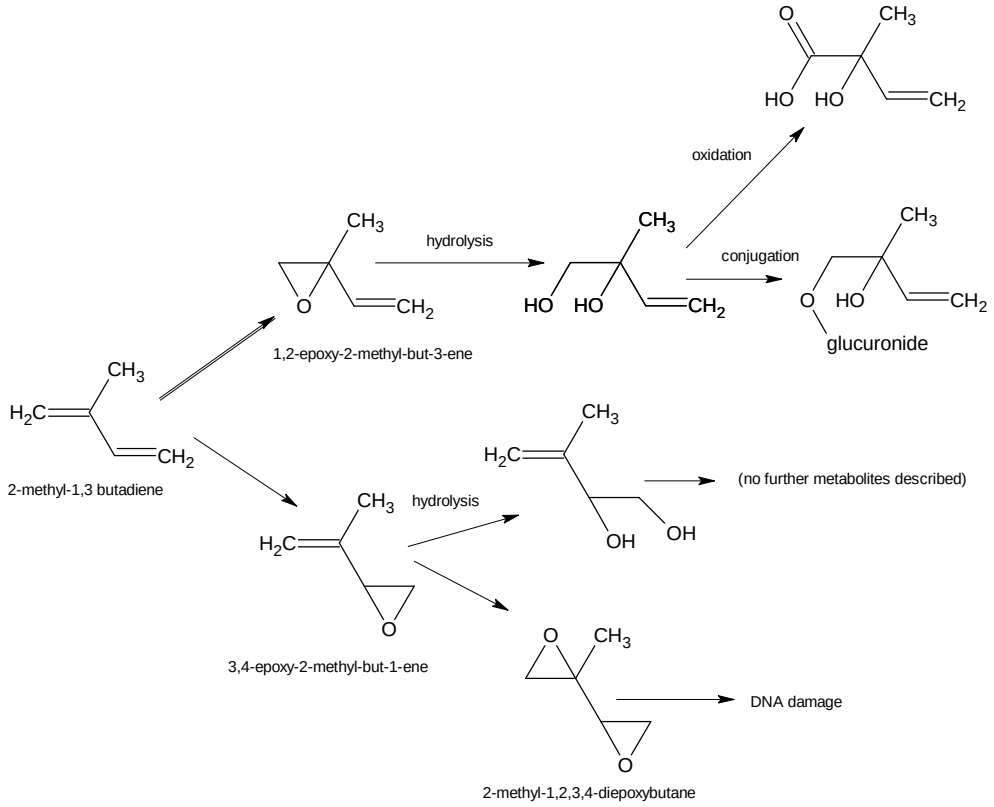


Figure C.2. *Major metabolic pathways for 2-methylbuta-1,3-diene (after Del Monte et al. (1985) and Buckley et al. (1999)).*

Following intraperitoneal injection of 64 mg [4-^{14}C]-2-methylbuta-1,3-diene /kg to B6C3F$_1$ mice, the parent compound was excreted unchanged in the breath (≈ 44 % of the dose and < 3 % unidentified material) or as urinary metabolites (≈ 33 %) over the 24-hours collection period. Only 1.9 % was expired as CO_2 and 7 % was eliminated via faeces. 2 % remained in the carcass. In comparison with rat urine (see above), the urine of the mice contained several other metabolites, including an unidentified polar fraction which comprised 25 % of the total urinary radioactivity as compared to 7 % in rat urine. The major identified metabolite in mouse urine, 2-hydroxy-2-methyl-3-butenoic acid, accounted for ≈ 15 % of the total urinary radioactivity, whereas 2-methyl-3-buten-1,2-diol and its glucuronide conjugate accounted for ≈ 3.5 and 2.5 %, respectively (Buckley et al., 1999). The authors speculated that the unidentified metabolite in the urine of rats and mice could be related to glutathione conjugates. They concluded that if this were the case, in rats glutathione conjugation of 2-methylbuta-1,3-diene is less important than in mice.

Special features of terminal double bond oxidation

Double bonds are usually oxidised by P450 to the corresponding epoxides, which are highly reactive molecules. Due to the large strain associated with the three membered ring structure epoxides easily react with nucleophilic sites of cellular macromolecules; conversely they are readily detoxified either spontaneously or by the action of epoxide hydrolase to diols or conjugated with reduced glutathione by glutathione-S-transferases. 1-Alkenes are metabolised by cytochrome P450, through double bond oxidation to the corresponding epoxide or alternatively allylic oxidation (Chiappe et al., 1998). The rates of the two reactions measured with different cytochrome P450 isoforms indicate that epoxide formation is generally favoured (Chiappe et al., 1998) and this may also apply in particular to the candidate substances dodec-1-ene [FL-no: 01.037] and 1-octene [FL-no: 01.070].

Based on this information and the data available for myrcene and 2-methylbuta-1,3-diene, it cannot be excluded that the candidate substances with these terminal double bonds [FL-no: 01.037, 01.064 and 01.070] may be metabolised to epoxides.

Conclusions on the metabolism of subgroup II substances

The data available on the metabolism of one supporting and one structurally related substance in subgroup II show that metabolic options for the substances in this group are epoxidation of double bonds ultimately resulting in diols, which can be further conjugated. With the supporting substance myrcene also further metabolism of the diols into carboxylic acids has been reported. Both in rats and rabbits, the principal urinary metabolite following gavage administration of myrcene is myrcene-3,10-glycol, formed from the hydration of the epoxide intermediate in both species. Epoxidation of the 3,10-double bond was favoured over epoxidation of the 1,2-double bond.

The studies indicate that the formation of diols from the myrcene-epoxides is very efficient. It is noted, however, that the diols and the related carboxylic acids are all the result of epoxidation of double bonds in which one of the carbon atoms has only hydrogen substituents, but no further carbon chains. In this respect, the candidate substance cis-3,7-dimethyl-1,3,6-octatriene [FL-no: 01.064] is most similar to myrcene. With myrcene no diepoxide metabolites or further reaction products thereof have been reported, but it is noted that mass balance data are incomplete, so some metabolites may have been overlooked.

With the structurally related substance 2-methyl-buta-1,3-diene, epoxidation of the 1,2-double bond is favoured over epoxidation of the 3,4-double bond. While the 1,2-epoxide is readily hydrolysed, the 3,4-epoxide is far more stable. Both metabolites can be converted to the corresponding diols and at least one hydroxy-carboxylic acid (2-hydroxy-2-methyl-3-butenoic acid) has been identified. In addition, in particular the 3,4-epoxide metabolite can be oxidised a second time, resulting in the formation of a diepoxide metabolite, which is known to be reactive and has been suggested to be responsible for 2-methylbuta-1,3-diene-induced DNA damage. For candidate substance dodec-1-ene [FL-no: 01.037], diol- and α-hydroxy-carboxylic acid formation may also be expected. In addition, this substance may also undergo omega and omega-1 or -2 oxidation at the other (saturated) end of the carbon chain (similar to the candidate substances in subgroup I).

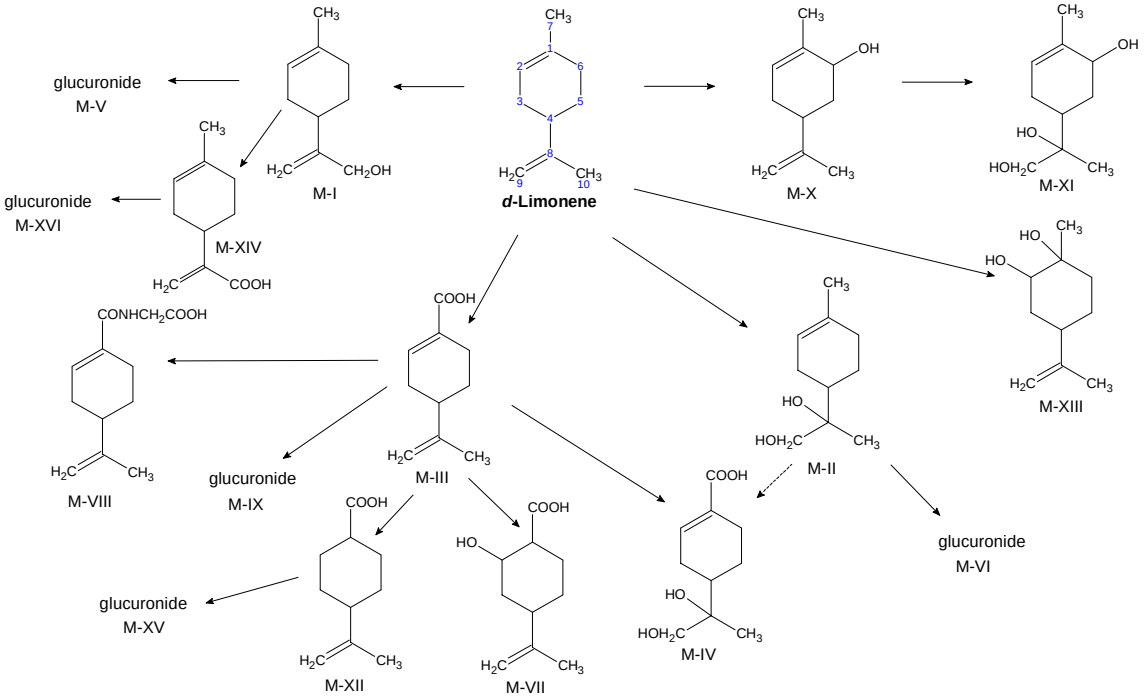
Apart from myrcene and 2-methylbuta-1,3-diene, terminal double bonds appear in candidate substances [FL-no: 01.037, 01.064 and 01.070]. In contrast to corresponding substances in FGE.07 and FGE.18 there is no other functional group in the molecule (e.g. hydroxyl- or keto-group) that could provide a more direct option for detoxication e.g. via conjugation with glucuronide or sulphate. In the two FGEs mentioned above the presence of such groups was an argument to consider that the metabolism of the particular candidate substances would go via innocuous products. It would not be possible to conclude similarly for the candidate substances in FGE.25. The other candidate substances in this subgroup (II) might also be oxidised in the various methyl groups but no data are available to substantiate this. Because of these considerations, it cannot be concluded that the candidate substances in this subgroup [FL-no: 01.032, 01.035, 01.037, 01.064, 01.070 and 01.078] will be metabolised to innocuous products.

Cyclohexene derivatives (Candidate and structurally related supporting substances from subgroup III)

For the candidate substances in this group, data for one supporting substance (*d*-limonene; [FL-no: 01.045]) were submitted.

More than 10 metabolites were found in the urine of rats given an oral gavage dose of 800 mg/kg bw *d*-limonene (or *p*-mentha-1,8-diene). Four of the metabolites were identified as perillic acid, *p*-menth-1-ene-8,9-diol (= limonene-8,9-diol), perillic acid-8,9-diol and 8-hydroxy-*p*-menth-1-en-9-yl-glucuronic acid. The bile of these rats contained three metabolites, the most important of which was 8-hydroxy-*p*-menth-1-en-9-

yl-β-D-glucuronic acid (Igimi et al., 1974). Six metabolites were identified in the urine of rabbits given the same oral dose. In addition to the four metabolites identified in rat urine, the rabbit urine contained *p*-mentha-1,8-dien-10-ol (= limonene-10-ol) and *p*-mentha-1,8-dien-10-yl-glucuronic acid (= *p*-mentha-1,8-dien-10-ol-glucuronide). Although not determined quantitatively, perillic acid, perillic acid-8,9-diol and both glucuronic acid conjugates were the major metabolites in rabbit urine, and no unchanged *d*-limonene was detected (Kodama et al., 1974). The same authors identified five additional metabolites in the urine of rats and dogs treated orally with *d*-limonene. These were characterised as 2-hydroxy-*p*-menth-8-en-7-oic acid, perillylglycine, perillyl-glucuronide, *p*-mentha-1,8-dien-6-ol (or limonene-6-ol) and *p*-menth-1-ene-6,8,9-triol. They also found some species differences in the nature of the major metabolites in urine. Perillic acid-8,9-diol was the main metabolite in rats and rabbits, perillyl-glucuronide in hamsters, limonene-8,9-diol in dogs and limonene-8,9-diol-glucuronide in guinea pigs and humans. It should be noted that the fate of only 40-65 % of the *d*-limonene dose administered orally to these animals and humans was accounted for (Kodama et al., 1976). The metabolites of *d*-limonene are shown in Figure C.3.



M-I	limonene-10-ol	M-IX	glucuronide conjugate of M-III
M-II	limonene-8,9-diol	M-X	limonene-6-ol
M-III	perillic acid	M-XI	*p*-menth-1-ene-6,8,9-triol
M-IV	perillic acid-8,9-diol	M-XII	dihydroperillic acid
M-V	glucuronide conjugate of M-I	M-XIII	limonene-1,2-diol
M-VI	glucuronide conjugate of M-II	M-XIV	*p*-mentha-1,8-diene-10-carboxylic acid
M-VII	2-hydroxy-*p*-menth-8-en-7-oic acid	M-XV	glucuronide conjugate of M-XII
M-VIII	perillylglycine	M-XVI	glucuronide conjugate of M-XIV

Figure C.3. *Metabolism of d-limonene*

Perillic acid, dihydroperillic acid and limonene-1,2-diol were the major metabolites identified in the plasma of humans given an oral dose of *d*-limonene. Minor metabolites were the methyl esters of perillic acid and dihydroperillic acid, and *d*-limonene itself (Crowell et al., 1994). Apart from the parent compound (Poon et al., 1996) and (Vigushin et al., 1998) also identified perillic acid, dihydroperillic acid and limonene-1,2-diol

as major metabolites in human plasma. However, they also found two other metabolites, i.e. *p*-mentha-1,8-diene-10-carboxylic acid and limonene-8,9-diol, whereas they did not detect the methyl esters of perillic acid and dihydroperillic acid. Peak plasma levels for all metabolites were achieved four to six hours after administration, with the exception of limonene-8,9-diol which reached its peak level one hour after administration (Poon et al., 1986). Metabolites in human urine comprised the glucuronic acid conjugates of perillic acid, dihydroperillic acid, *p*-mentha-1,8-diene-10-carboxylic acid, limonene-8,9-diol and limonene-10-ol (Poon et al., 1986).

Experiments with rat liver microsomes have shown that epoxidation of the C8 double bond (in the vinyl substituent) of *d*-limonene is favoured over epoxidation of the C1-double bond (the one in the ring), due to steric hindrance by the 1-methyl group, which was demonstrated by comparison of the metabolism of *d*-limonene with that of 4-vinylcyclohex-1-ene. Upon incubation with rat liver microsomes, the majority of *d*-limonene was converted to the 8,9-epoxide and the 8,9-diol, and to a much lesser extent to the 1,2-epoxide and the 1,2-diol (ratio of 8,9- vs.1,2-epoxidation = 4:1). In contrast, with 4-vinylcyclohex-1-ene the epoxidation rate of the ring double bond was about four times as fast as the epoxidation rate of the vinyl double bond. Because the 1,2-epoxide of *d*-limonene is a very poor substrate for microsomal epoxide hydrolase, the 1,2-diol could not be found in microsomal incubates, whereas the 8,9-diol could be found (Watabe et al., 1981). Both epoxides of *d*-limonene were tested for mutagenicity in several *Salmonella* strains and showed to be inactive. The mono-epoxides of 4-vinylcyclohex-1-ene were inactive in *Salmonella* strain TA100, but were not tested in the other strains. However, the diepoxide of 4-vinylcyclohex-1ene was mutagenic in this bacterial strain, but this diepoxide was not detected in the microsomal metabolism studies with 4-vinylcyclohex-1-ene as starting material (Watabe et al., 1981).

Other *in vitro* experiments have shown that male rats can convert *d*- and *l*-limonene into the corresponding *trans*-perillyl alcohol (by hydroxylation of the methyl group at C7) and carveol (or limonene-6-ol; by ring C6-hydroxylation). These reactions are catalysed by CYP2C11 and, when pretreated with phenobarbital, CYP2B1. In female rats, the activity for conversion to either alcohol is much lower. Apparently, the female-specific CYP2C12 has no activity with respect to *d*- and *l*-limonene hydroxylation. In males, the hydroxylation activities were not detectable with foetal liver microsomes, but they increased after birth, closely related to the developmental increase in CYP2C11. This study also investigated whether the *d*- and *l*-limonene enantiomers are differently metabolised by liver microsomes. Both in liver microsomes from untreated and treated with phenobarbital, approximately the same amounts of carveol and perillyl alcohol were formed from the two limonene enantiomars. Ratios over the two routes of metabolism were carveol/perillyl alcohol: 0.87/1.23 for *d*-limonene and 0.61/1.03 for *l-limonene*. Also the rate of formation of carveol and perillyl alcohol from *d*- and *l*-limonene is similar using either liver microsomes or recombinant P450 enzymes (Miyazawa et al., 2002).

In male rats orally administered 3 mmol/kg (408 mg/kg) of [^{14}C]-*d*-limonene radioactivity was detected in the renal cytosol. Forty percent of the total cytosolic radioactivity was reversibly associated with the protein fraction and further analysis showed that > 97 % of this activity was associated with one single protein, which was identified as α2u-globulin. 1,2-Limonene epoxide, 1,2-limonene-diol and *d*-limonene comprised 82, 5 or 13 %, respectively, of the radioactivity associated with this protein (Lehman-McKeeman et al., 1989).

d-Limonene has been shown to induce P450 enzymes of the CYP2B and CYP2C subfamilies and epoxide hydrolase in rats (Austin et al., 1988; Maltzman et al., 1991).

Conclusions on the metabolism of subgroup III substances

In the subgroup III there are only metabolism data available for one supporting substance, *d*-limonene, which in several animal species and humans has been demonstrated to be oxidised in both side chains and at the cyclohexene ring, resulting in alcohols and/or carboxylic acids. Ring and side chain hydroxylation has also

been described for its structural isomer *l*-limonene (a constituent of candidate substance [FL no: 01.001] and a candidate substance on its own [FL-no: 01.046]) in rat liver microsomes. The metabolites of these limonenes are, at least partly, conjugated and eliminated with the urine. It is anticipated that three out of the six candidate substances [FL-no: 01.027, 01.028 and 01.039] in subgroup III are metabolised in a similar way to innocuous products. It cannot be anticipated based on the data available that β-phellandrene [FL-no: 01.055] with a double bond directly on the ring and in conjugation with double bond in the cyclohexene ring can be metabolised to innocuous products.

Aromatic Hydrocarbons (Candidate and Structurally Related Supporting Substances from Subgroup IV)

The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry.

Bicyclic Non-Aromatic Hydrocarbons (Candidate and Structurally Related Supporting Substances from Subgroup V)

Analysis of urinary metabolites eliminated by human volunteers within four hours following a 2-hour inhalation exposure to 10 – 450 mg (+)-α-pinene/m^3 [FL-no: 01.004] in a pharmacokinetic study (Falk *et al*., 1990b) revealed *cis*- and *trans*-verbenol in a ratio of 1:10, with 3.8 and 1.7 % being eliminated at 10 and 450 mg/m^3, respectively. Most of the verbenols were eliminated within 20 hours. In a more extensive metabolic study, urine was collected from sawmill workers at the end of an 8-9 hours work shift or from chamber-exposed individuals. Following hydrolysis of glucuronic acid conjugates, *cis*- and *trans*-verbenol were identified in the urine along with two diols, *cis*- and *trans*-4-hydroxymyrtenol, formed by methyl group hydroxylation of *cis*- and *trans*-verbenol. *trans*-4-Hydroxymyrtenal was also detected (see Figure C.4) (Eriksson and Levin, 1990).

Analysis of the urinary metabolites of a patient attempting suicide with 400 – 500 ml pine oil containing 57 % α-pinene showed the presence of myrtenol, verbenol, and borneol. Renal excretion reached a peak level five days after ingestion (Koppel et al., 1981).

Male albino rabbits (six/group) administered single gavage doses of 400 – 700 mg/kg bw of (+)-α-pinene, (-)-α-pinene, (+/-)-α-pinene, (-)-β-pinene, or delta-3-carene, excreted bicyclic terpene hydrocarbon metabolites as (glucuronic acid) conjugates or as further oxidised metabolites, notably carboxylic acids. Animals were housed individually and urine was collected daily for three days.

The principal neutral metabolite formed by oxidation at the C_4 position in the alicyclic ring of each of the three α stereochemical forms of pinene was *trans*-verbenol (see Figure C.4). As a minor pathway, allylic oxidation of the exocyclic methyl group to yield myrtenol was observed for all three α-pinene stereoisomers, with also myrtenic acid as minor metabolite (Ishida et al., 1981).

The presence of an exocyclic alkene function in (-)-β-pinene provided additional metabolic options, and four neutral and one acidic metabolites were identified. Allylic oxidation of the methyl group at the C_2 position yields (+)-trans-pinocarveol, while epoxidation of the exocyclic alkene followed by hydration or rearrangement yields (-)-*trans*-10-pinanol and (-)-1-*p*-menthene-7,8-diol, respectively. Ring cleavage yields (-)-α-terpineol. These metabolites comprised 11, 39, 30 or 5 % of the total urinary neutral metabolite fraction, respectively. The acidic metabolite identified was identical to the one identified for the α-pinenes (i.e. myrtenic acid), which was suggested to be formed via double bond epoxidation and subsequent rearrangement to give myrtenol and further oxidation to the carboxylic acid (Ishida et al., 1981).

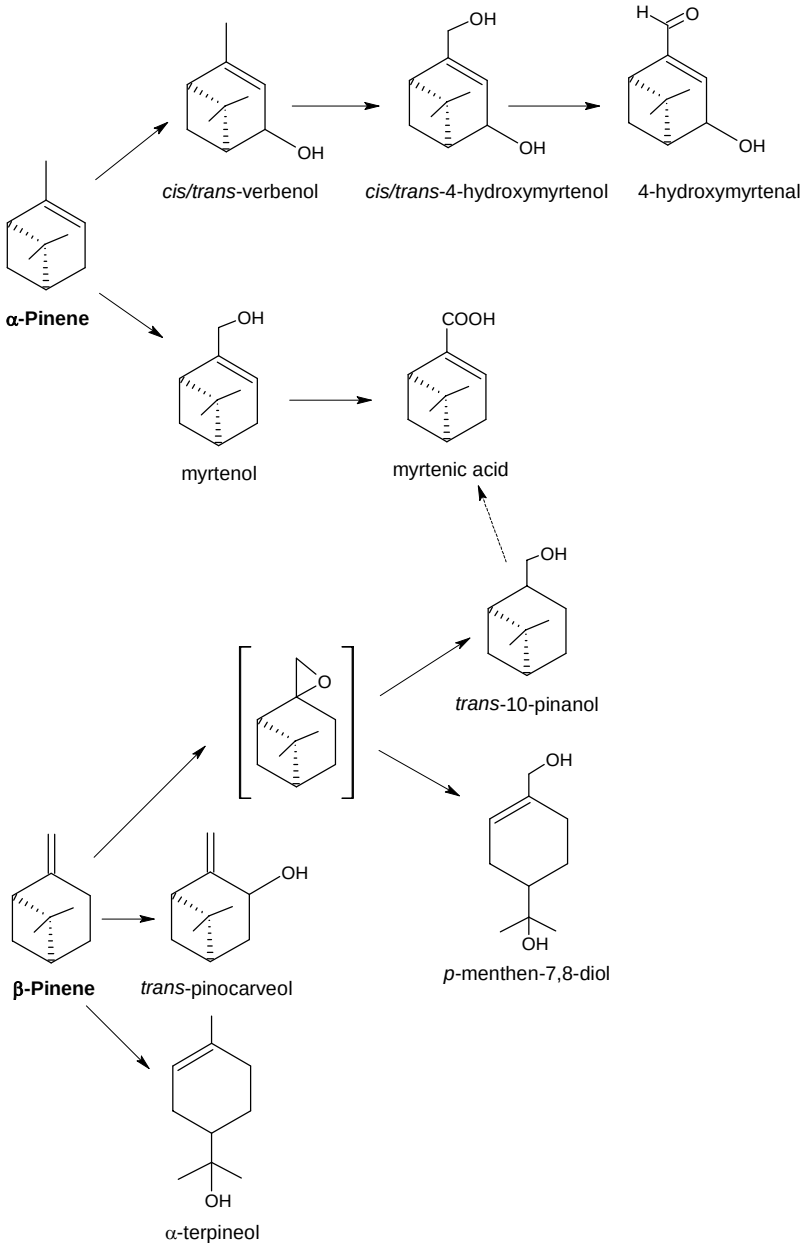


Figure C.4. *Metabolism of α-pinene and β-pinene in animals*

Trans-verbenol and myrtenic acid have also been found in faeces or urine of brushtail possum fed α-pinene. When the same species was fed β-pinene, only myrtenic acid was found in the excreta (Southwell et al., 1980).

It has been stated in a limited review paper that in rabbits, α- and β-pinenes can be excreted as glucuronide conjugates of undetermined nature, which can release cymene upon heating in diluted acids (Williams, 1959 as cited in (Opdyke, 1978)).

Delta-3-Carene [FL-no: 01.029] undergoes stereoselective hydroxylation at the *gem*-methyl group (yielding 3-caren-9-ol) followed by carboxylation, allylic oxidation of the C_{10} methyl group followed by carboxylation or, as the main route, allylic ring opening and hydroxylation at a secondary carbon atom, yielding (-)-*m*-

mentha-4,6-dien-8-ol (72 % of the total urinary neutral metabolite fraction) and *m*-cymen-8-ol (Ishida et al., 1981).

In addition to the terpenoids pinene and carene, the group of Ishida has also studied the metabolism of the saturated analogues pinane and carane[19] (Ishida *et al*., 1981). Only a relatively small part of the dose (in total 18 g given to six rabbits) was identified, among which were 3- and 4-pinalol, α-terpineol, trans-sobrerol, trans-carveol, and verbenol (some of the structures have been shown in Figure C.4). In short, these products result from hydroxylations of secondary carbon atoms in the 6-membered ring, or cleavage of the 4-membered ring in combination with hydroxylation in the remaining 6-membered ring. In addition, desaturation of the 6-membered ring was also observed and hence, some of the metabolites may be considered as cyclohexene derivatives. No hydroxylation of the primary carbon atom (i.e. the methyl ring substituent) in pinane was observed. With carane, however, the hydroxylation of one of the *gem*-methyl groups and hydroxylation of the C10 exocyclic carbon atom was observed, ultimately resulting in the formation of carane-9,10-dicarboxylic acid.

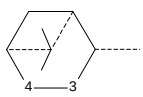
In rabbits, β-caryophyllene [FL-no: 01.007] undergoes epoxidation of the endocylic 5,6-double bond to yield a stable epoxide metabolite and hydroxylation at the *gem*-dimethyl group. The resulting metabolite 14-hydroxycaryophyllene-5,6-epoxide and its C14-acetylated conjugate could be detected in the urine. A second epoxidation of the 5,6-epoxide's exocyclic 2,12-double bond, ultimately resulting in the 14-hydroxycaryophyllene-5,6-epoxide-2,12-diol, was also reported (Asakawa et al., 1981; Asakawa et al., 1986).

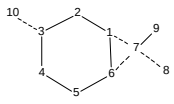
Data were also found on the metabolism of camphene[20]. Ishida *et al*. (1979) administered *dl*-camphene [FL-no: 01.009] to five starved male rabbits at a dose level of 800 mg/kg bw via stomach tube. In total 8 g of the substance was given. Urinary metabolites, collected over three days post-dosing were examined. The following metabolites were identified in urine samples after treatment with β-glucuronidase/sulphatase: camphene-2,10-glycol (after epoxidation of the double bond), 6-*exo*-hydroxycamphene, 7-hydroxycamphene, 10-hydroxytricyclene and 3-hydroxytricyclene. Quantitative information was provided only for the diol which was found in a total amount of 260 mg which corresponds to *ca.* 3 % of the dose (Ishida et al., 1979).

In a human volunteer and in a young pig, camphene was eliminated from the body by exhalation and via bile as unchanged substance or as glucuronide conjugate via the urine (Opdyke, 1975).

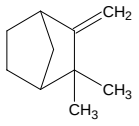
(+)-Longifolene [former FL-no: 01.047] metabolism was studied following the oral administration of about 2 g/animal as a suspension in 0.02 % Tween80 aqueous solution to six male rabbits. Urine samples were collected daily for three days and treated with β-glucuronidase / sulphatase. Due to imprecise description of dose and amounts recovered, it is not possible to indicate the extent of metabolism and excretion as percentage of the dose. Deconjugated metabolites were extracted in a neutral and an acidic fraction. In the neutral fraction (10 % of the dose), many peaks were observed but only one was further characterised to be (2S, 7S)-(+)-14-hydroxyisolongifolaldehyde (35 % of the neutral metabolite fraction). It was concluded that

19

Pinane: 

Carane: 

20 camphene: 3,3-dimethyl-2-methylenenenorbornane



(+)-longifolene is metabolised at two sites in two subsequent steps: 1) oxidation of the *exo*-methylene group to form its epoxide with subsequent isomerisation to form a stable aldehyde, and 2) hydroxylation of the *gem*-dimethyl group to form a primary alcohol (Ishida et al., 1982; Asakawa et al., 1986; Ford et al., 1992).

An *in vitro* study with rat liver microsomes demonstrated the involvement of cytochrome P450 enzymes in the metabolism of α-pinene. Metabolites present were β-pinene and *d*-limonene together with smaller amounts of *trans*-verbenol, myrtenol, verbenone, and pinene oxide (White and Agrosin, 1980).

α-Pinene and cadinene, have been shown to induce cytochrome P450 enzymes, especially those from the CYP2B subfamily, and to a lesser extent also CYP3A2 (cadinene) and CYP4A2 (α-pinene) (Austin et al., 1988; Hiroi et al., 1995). Based on similarity with β-pinene it may be speculated that the candidate substance 4(10)-thujene [FL-no: 01.059] may be hydroxylated to thujyl alcohol, which is known to be conjugated with glucuronic acid and eliminated via urine (Hämäläinen, 1912; EFSA, 2009a). However, based on the same similarity, epoxidation of the exocyclic double bond may also be expected, and it is not clear what other reactions might occur. No indications of the relevance of the various routes are available.

Conclusions on the metabolism of the subgroup V substance

Metabolism data for subgroup V are available for some supporting substances (pinenes, camphene, caryophyllene, delta-3-carene, pinane and carane) and longifolene [former FL-no: 01.047]. In general the metabolic options for these substances include oxidation of methyl ring substituent groups to give the corresponding alcohol and further oxidation products. For the substances studied, double bond epoxidation has also been demonstrated. In addition, ring cleavage has also been observed, e.g. for β-pinene resulting in the formation of monocyclic terpenoid derivatives like α-terpineol. Hydroxylated metabolites (i.e. alcohols) or further metabolic products may be eliminated as conjugates e.g. with glucuronic acid. However, given the diversity of this group, the lack of data on any of the candidate substances, it cannot be concluded that the candidate substance in this subgroup [FL-no: 01.059] can be metabolised to innocuous products.

Macrocyclic, Non-Aromatic Hydrocarbons (Candidate and Structurally Related Supporting Substances from Subgroup VI)

The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry.

C.5. Summary on Absorption, Distribution, Metabolism and Excretion

Given the diverse nature of the chemical structures of the candidate flavouring substances in this FGE, it is impossible to draw conclusions, which are applicable to all substances. The amount on information on kinetics of either candidate or supporting substances is very limited. For these reasons, the available information is presented here in a subgroup-wise manner.

Absorption, Distribution and Elimination

Subgroup I

From the available studies it may be concluded that the candidate alkane substances in subgroup 1 will be absorbed after oral exposure. The extent of absorption is not known, but may be high, given their lipophilic character and their low molecular weight. The ease with which these substances cross the membranes of the respiratory tract further supports the assumption that these substances will also be absorbed after oral intake. Data indicate that straight-chain alkanes will predominantly be broken down to carbon dioxide at low dose levels. At high dose levels, biotransformation becomes saturated and other metabolites will be generated which will be excreted via the urine.

Subgroup II

For the candidate and supporting substances in subgroup II, the acyclic alkenes, data were only available for myrcene [FL-no: 01.008] and 2-methylbuta-1,3-diene (a structurally related substance). Given the narrow range of molecular weights of the candidate substances (between ~ 68 and 206 D) and their lipophilic nature (estimated or measured Log Kow values e.g. 2.4 (2-methylbuta-1,3-diene), 6.1 (dodec-1-ene), 4.17 (myrcene) or 4.8 (cis-3,7-dimethyl-1,3,6-octatriene)), it is assumed that these candidate flavouring substances may be absorbed from the gastrointestinal tract. Mass balance data for myrcene are incomplete. For 2-methylbuta-1,3-diene a mass balance of elimination has been presented, which also shows elimination via the exhaled air (~ 50 % of the dose), but as this substance was administered via the intraperitoneal route, the elimination pattern may be different after oral dosing with a lower fraction of the dose exhaled, e.g. because of more efficient first-pass biotransformation after oral dosing.

Subgroup III

For the candidate and supporting substances in subgroup III (cyclohexene derivatives), data were only available for *d*-limonene [FL-no: 01.045]. For this substance, data show a considerable absorption from the gastrointestinal tract. In humans, elimination of the substance followed a triphasic pattern, but still 55-83 % of an oral dose could be found in the urine within 72 hours post dosing, with the major part excreted within 24 hours. Also in other species urinary elimination was most important accounting for up to 82-96 % of the dose within 72 hours. Hence, it may be anticipated that monocyclic cyclohexene derivatives, such as *d*-limonene, administered orally, are absorbed and distributed throughout the body. Following oral administration to humans, *d*-limonene was distributed preferentially to fatty tissues, as indicated by a high oil-blood partition coefficient and a long half-life during the slow elimination phase. Because of the limited molecular weight of the candidate substances in this group (range ~ 136-204 D) and their lipophilic character (e.g. estimated or measured log Kow values for β-phellandrene, β-bisabolene and *d*-limonene are approximately 4.7, 7.1 or 4.6, respectively) it may be assumed that all of the substances in this group may be absorbed, although the extent of absorption for individual substances cannot be accurately estimated from these physico-chemical properties.

Subgroup IV

The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry.

Subgroup V

For the substances in subgroup V (bicyclic, non-aromatic hydrocarbons), the available data from oral studies are incomplete. These studies have only addressed the excretion of some supporting and longifolene [former FL-no: 01.047] in the form of metabolites via the urine. No mass-balance data were provided. So, these studies only show that these substances will be absorbed to some extent. The same may be anticipated for the other candidate substances in this subgroup. For the supporting substances α- and β-pinene and delta-carene information on kinetics is available from humans exposed via inhalation, in occupational settings. These studies show that these substances can be absorbed after inhalation exposure and that metabolites will be excreted into the urine e.g. as glucuronide conjugates. The elimination follows a triphasic pattern with rather long terminal half-lives and the absorbed amount will be eliminated within several days. Based on the lipophilic character of these substances it may be anticipated that they will preferentially distribute in the adipose tissues, which is supported by the slow terminal elimination rates.

Subgroup VI

The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry.

Metabolism

Subgroup I

The substances in subgroup I can be expected to be metabolised through omega-oxidation which will lead to the formation of alcohols, and after subsequent further oxidation to carboxylic acids, which may be further oxidised via β-oxidation to yield carbon dioxide. Extensive mass balance data are not available, but in some of the available studies excretion of carboxylic acids and carbon dioxide has been reported. Formation of keto-acids has also been reported, and these may be thought to be formed from omega-oxidation at one end of the molecule and omega-1 or -2 oxidation at the other end. More interest has been put in the identification of metabolites, which did not undergo substantial chain shortening. These metabolites will arise from oxidation of the non-terminal carbon atoms, e.g. via omega-1, -2, -3 or even -4 oxidation. The resulting secondary alcohols can be conjugated with e.g. glucuronic acid and excreted via the urine, or can be further oxidised to yield ketones. A subsequent introduction of another secondary hydroxyl group may result in the formation of diketones. With n-hexane, the resulting 2,5-hexanedione (a gamma-diketone) has been demonstrated to be responsible for hexane-induced neurotoxicity. Neurotoxicity is a common feature of gamma-diketones, and for that reason, candidate substance 3-methylhexane [FL-no: 01.050] cannot be anticipated to be metabolised into innocuous compounds. In addition, it is known that methyl-branching of the carbon chain potentiates the neurotoxicity of the gamma-diketone (Topping et al., 1994; EFSA, 2004b). The two other hexane derivatives in this group [FL-no: 01.033 and 01.034] cannot be oxidised to give a gamma-diketone, due to the presence of methyl groups on the C2 and C5 carbon atoms. Therefore, these substances may be considered as being metabolised to innocuous metabolites. For the longer chain alkanes in this group it would require oxidation of the more central carbon atoms in order to be converted into gamma-diketones. Such oxidations are less favourable than omega, omega-1 or -2 oxidations and therefore it is concluded that oxidation of these higher alkanes will not result in toxicologically relevant levels of gamma-diketones, also because of rapid conjugation of the precursor alcohols. For heptane it was shown that this substance does not result in neurotoxicity (Bahima et al., 1984), although the gamma-diketone itself (2,5-heptanedione) is known to be neurotoxic (Topping et al., 1994). The remaining three candidate flavouring substances in this group (i.e. [FL-no: 01.038, 01.054 and 01.057]) can be expected to be metabolised to innocuous products.

Subgroup II

The data available on the metabolism of one supporting and one structurally related substance in subgroup II show that metabolic options for the chemicals in this group are epoxidation of double bonds, ultimately resulting in diols, which can be further conjugated. With the supporting substance myrcene, further metabolism of the diols into carboxylic acids has also been reported. Both in rats and rabbits, the principal urinary metabolite following gavage administration of myrcene is myrcene-3,10-glycol, formed from the hydration of the epoxide intermediate in both species. Epoxidation of the 3,10-double bond was favoured over epoxidation of the 1,2-double bond.

The studies indicate that the formation of diols from the myrcene-epoxides is very efficient. It is noted, however, that the diols and the related carboxylic acids are all the result of epoxidation of double bonds in which one of the carbon atoms has only hydrogen substituents, but no further carbon chains. In this respect, the candidate substance cis-3,7-dimethyl-1,3,6-octatriene [FL-no: 01.064] is most similar to myrcene. With myrcene no diepoxide metabolites or further reaction products thereof have been reported, but it is noted that mass balance data are highly incomplete, so some metabolites may have been overlooked.

With the structurally related substance, 2-methyl-buta-1,3-diene, epoxidation of the 1,2-double bond is favoured over epoxidation of the 3,4-double bond. While the 1,2-epoxide is readily hydrolysed, the 3,4-epoxide is far more stable. Both metabolites can be converted to the corresponding diols and at least one hydroxy-carboxylic acid (2-hydroxy-2-methyl-3-butenoic acid) has been identified. In addition, in particular the 3,4-epoxide metabolite can be oxidised for a second time, resulting in the formation of a diepoxide metabolite, which is known to be reactive and has been suggested to be responsible for 2-methylbuta-1,3-diene-induced DNA damage.

Apart from myrcene and 2-methylbuta-1,3-diene, terminal double bonds appear in candidate substances [FL-no: 01.064 and 01.070]. In contrast to corresponding substances in FGE.07 and FGE.18 there are no other functional groups in the molecule (e.g. hydroxyl- or keto-group) that could provide a more direct option for detoxication e.g. via conjugation with glucuronide or sulphate. In the two FGEs mentioned above, the presence of such groups was an argument to conclude that the metabolism of the particular candidate substances would give rise to innocuous products. It would not be possible to conclude similarly for the candidate substances in FGE.25. The other candidate substances in this subgroup (II) might also be oxidised in the various methyl groups but no data are available to substantiate this. Because of these considerations, it cannot be concluded that the candidate substances in this subgroup [FL-no: 01.035, 01.064 and 01.070] will be metabolised to innocuous products.

Subgroup III

Hardly any data are available on the metabolism of the candidate substances in subgroup III. Only for *l*-limonene a very limited amount of data was available on biotransformation in incubations with rat liver microsomes. In comparison with the structures of the candidate substances in subgroup III, the structure of the one supporting chemical *d*-limonene is comparatively simple. Major differences between *d*-limonene and the candidate substances are the length of the ring substituents and the number of double bonds.

Allylic oxidation is by far the major pathway for metabolism of limonene in humans. Minor pathways in limonene metabolism reported for the rat include epoxidation of either the 1,2- or the 8,9-double bond and subsequent hydrolysis to the diol. Given the many metabolic options for this substance, a myriad of metabolites has been found, including conjugates, and for a change mass balance data are available, which show that in various animal species the substance is completely eliminated within three days, predominantly via the urine. No genotoxicity of limonene epoxides could be detected, and it may well be concluded that *d*-limonene is metabolised to innocuous substances. However, although it is very likely that the candidate substances may undergo the same metabolic conversions (allylic oxidations and double bond epoxidation) the conclusion for *d*-limonene cannot be extrapolated to all of the candidate substances because of the structural dissimilarities and the absence of any further data on molecules with closer resemblance to the candidate substances. The data available for *l*-limonene do not indicate that major differences are to be anticipated between the metabolism of *d*-limonene and *l*-limonene. For bisabola-1,8,12-triene [FL-no: 01.027], β-bisabolene [FL-no: 01.028] and delta-elemene [FL-no: 01.039] it may be concluded that these are metabolised to innocuous products.

It is noted that *d*-limonene (metabolites) causes α2μ-microglobulin accumulation in male kidneys, an event known to be associated with male rat specific nephropathy[21], and irrelevant for human toxicological risk assessment.

Subgroup IV

The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry.

[21] For introduction into this area see e.g. the referred paper by Lehman-McKeeman *et al*. (1989)

Subgroup V

Metabolism data for subgroup V are available for the supporting substances pinenes, camphene, caryophyllene, delta-3-carene, pinane and carane. In general the metabolic options for these substances include oxidation of methyl ring substituent groups to give the corresponding alcohol and further oxidation products. For the substances studied, double bond epoxidation has also been demonstrated. In addition, ring cleavage has also been observed, e.g. for β-pinene resulting in the formation of monocyclic terpenoid derivatives like α-terpineol. Hydroxylated metabolites or further metabolised products may be eliminated as conjugates e.g. with glucuronic acid. However, given the lack of data on the candidate substances it cannot be concluded that the candidate substance in this subgroup [FL-no: 01.059] can be metabolised to innocuous products.

Based on similarity with β-pinene it may be speculated that the candidate substance 4(10)-thujene [FL-no: 01.059] may be hydroxylated to thujyl alcohol, which is known to be conjugated with glucuronic acid and eliminated via urine (Hämäläinen, 1912; EFSA, 2009a). However, based on the same similarity, epoxidation of the exocyclic double bond may also be expected, and it is not clear what other reactions might occur. No indications of the relevance of these various routes is available. Hence, despite the knowledge of the fate of thujyl alcohol, also for thujene it cannot be concluded that it will be metabolised to innocuous products.

Subgroup VI

The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry.

C.6. Overall Conclusion on Absorption, Distribution, Metabolism and Elimination.

Generally, the available data indicate that the aliphatic and aromatic hydrocarbons participate in similar pathways of metabolic detoxication. Being lipophilic and of low molecular weight, these hydrocarbons may be assumed to be absorbed by the gastrointestinal tract. Subsequently, they are oxidised to more polar oxygenated metabolites e.g. by CYP-450 enzymes. The phase I metabolites are then conjugated and excreted mainly in the urine. The candidate substances and supporting substances are oxidised either by side chain oxidation or epoxidation of the exocyclic or endocyclic double bond. Oxidation initially yields hydroxylated metabolites that may be excreted in conjugated form or undergo further oxidation, yielding more polar metabolites that are also excreted. If a double bond is present, epoxide metabolites may form that are further metabolised either by hydrolysis to yield diols or by conjugation with glutathione to yield mercapturic acid derivatives. The saturated alkanes in this group may be metabolised via omega and omega-1, -2, -3 or -4 oxidation. Whereas omega oxidation would ultimately lead to the formation of carboxylic acids, the other oxidations would give rise to secondary alcohols and ketones. The carboxylic acids may be expected to participate in the endogenous fatty acid metabolism. However, for most of the subgroups the information was incomplete and the similarity between supporting and candidate substances was limited. In addition, proper mass balance data were not available. Some mass balance data available indicated slow elimination. For several subgroups no data were available at all. In Table C.2 the final conclusions for each of the candidate substances has been presented, together with a brief explanatory statement, about the conclusion reached. It is noted that the subgroup III supporting substance *d*-limonene causes α2μ-microglobulin accumulation in male kidneys, an event known to be associated with male rat specific nephropathy, and irrelevant for human toxicological risk assessment.

Table C.2 Can innocuous metabolites be expected to be formed based on available data?

FL-no:	Substance name	Innocuous metabolites?
Subgroup I: ACYCLIC ALKANES		
01.033	2,2-Dimethylhexane	Yes
01.034	2,4-Dimethylhexane	Yes
01.038	Dodecane	Yes
01.054	Pentadecane	Yes
01.057	Tetradecane	Yes
Subgroup II: ACYCLIC ALKENES		
01.035	2,6-Dimethylocta-2,4,6-triene	No (lack of supporting data)
Deleted from the Register	2-Methylbuta-1,3-diene	No (known biotransformation to reactive metabolite responsible for toxicity and genotoxicity)
01.064	cis-3,7-Dimethyl-1,3,6-octatriene	No (presence of terminal double bond which may give rise to reactive metabolites without counteracting metabolic options)
01.070	1-Octene	No (presence of terminal double bond which may give rise to reactive metabolites without counteracting metabolic options)
Subgroup III: CYCLOHEXENE HYDROCARBONS		
01.027	Bisabola-1,8,12-triene	Yes
01.028	β-Bisabolene	Yes
01.039	delta-Elemene	Yes
01.001	Limonene	Yes
01.046	l-Limonene	Yes
Subgroup IV: AROMATIC HYDROCARBONS		
The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry.		
Subgroup V: BICYCLIC, NON-AROMATIC HYDROCARBONS		
01.059	4(10)-Thujene	No (but supported by the supporting substance β-caryophyllene [FL-no: 01.007])
Subgroup VI: MACROCYCLIC, NON-AROMATIC HYDROCARBONS		
The substances previously allocated to the group are no longer supported for use as flavouring substances in Europe by Industry.		

Appendix D. Substances no Longer Supported for Use as Flavouring Substances in Europe by Industry

Since the publication of FGE.25Rev2 (EFSA CEF Panel, 2011), the following 23 substances [former FL-no: 01.021, 01.022, 01.023, 01.030, 01.031, 01.032, 01.036, 01.037, 01.042, 01.043, 01.044, 01.047, 01.050, 01.051, 01.052, 01.053, 01.055, 01.056, 01.058, 01.060, 01.066, 01.067 and 01.078] of 37 candidate substances evaluated in FGE.25Rev2 are no longer supported for use as flavouring substances in Europe by Industry and will therefore not be considered any further (DG SANCO, 2012).

The 23 substances were evaluated via the B-side of the Procedure scheme and as they all had estimated European daily *per capita* intakes below the threshold of concern for their respective structural class they all proceeded to step B4 of the Procedure. Here the Panel concluded that additional data were required for these substances as no NOAELs were available for any of these substances or for any structurally related substances. The 23 substances are listed here below together with their specifications.

Table D1: Specification Summary of the Substances no Longer Supported by Industry

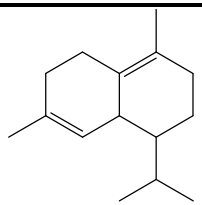
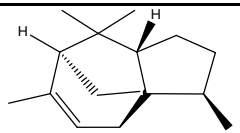
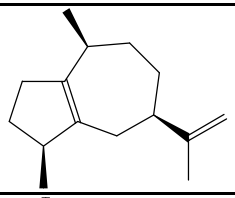
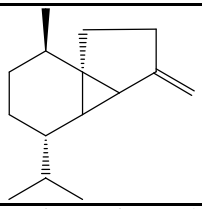
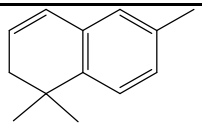
Former FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility[a] Solubility in ethanol[b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index[d] Spec.gravity[e]	Specification comments
01.021	delta-Cadinene		10982 29350-73-0	Liquid $C_{15}H_{24}$ 204.36	Freely soluble	286 MS 95 %	1.497-1.503 0.917-0.923	No longer supported by Industry (DG SANCO, 2012).
01.022	α-Cedrene		10985 469-61-4	Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble Freely soluble	119 (13 hPa) MS 95 %	1.500-1.506 0.932-0.938	No longer supported by Industry (DG SANCO, 2012).
01.023	1(5),11-Guaiadiene		11003 3691-12-1	Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble Freely soluble	78 (3 hPa) MS 95 %	1.493-1.499 0.897-0.903	No longer supported by Industry (DG SANCO, 2012).
01.030	β-Cubebene		10989 13744-15-5	Solid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble Freely soluble	284 60 MS 95 %	n.a. n.a.	No longer supported by Industry (DG SANCO, 2012).
01.031	1,2-Dihydro-1,1,6-trimethylnaphthalene		30364-38-6	Liquid $C_{13}H_{16}$ 172.27	Practically insoluble or insoluble Freely soluble	115 (24 hPa) MS 95 %	1.542-1.548 0.942-0.948	No longer supported by Industry (DG SANCO, 2012).

Table D1: Specification Summary of the Substances no Longer Supported by Industry

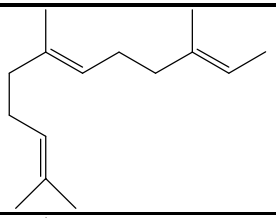
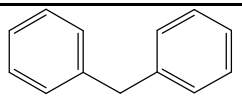
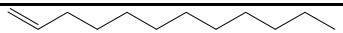
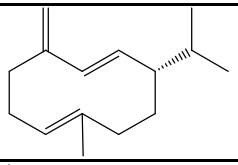
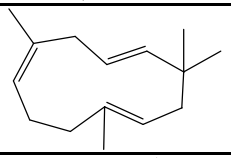
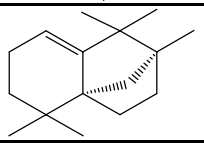
Former FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility[a] Solubility in ethanol[b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index[d] Spec.gravity[e]	Specification comments
01.032	2,3-Dihydrofarnesene		7681-88-1	Liquid $C_{15}H_{26}$ 206.37	Practically insoluble or insoluble Freely soluble	130 (15 hPa) NMR 95 %	1.468-1.474 0.817-0.823	No longer supported by Industry (DG SANCO, 2012).
01.036	Diphenylmethane		11847 101-81-5	Solid $C_{13}H_{12}$ 168.24	Practically insoluble or insoluble Freely soluble	262 27 MS 95 %	n.a. n.a.	No longer supported by Industry (DG SANCO, 2012).
01.037	Dodec-1-ene		10992 112-41-4	Liquid $C_{12}H_{24}$ 168.23	Practically insoluble or insoluble Freely soluble	213 MS 95 %	1.425-1.431 0.755-0.761	No longer supported by Industry (DG SANCO, 2012).
01.042	Germacra-1(10),4(14),5-triene		23986-74-5	Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble Freely soluble	308 MS 95 %	1.507-1.513 0.896-0.892	No longer supported by Industry (DG SANCO, 2012).
01.043	3,7,10-Humulatriene		11004 6753-98-6	Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble Freely soluble	123 (13 hPa) MS 95 %	1.499-1.505 0.889-0.895	No longer supported by Industry (DG SANCO, 2012).
01.044	Isolongifolene		1135-66-6	Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble Freely soluble	120 (16 hPa) MS 95 %	1.495-1.501 0.926-0.932	No longer supported by Industry (DG SANCO, 2012).

Table D1: Specification Summary of the Substances no Longer Supported by Industry

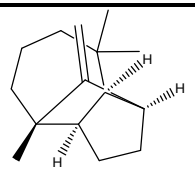
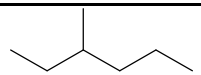
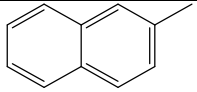
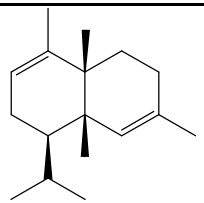
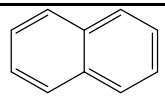
Former FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility[a] Solubility in ethanol[b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index[d] Spec.gravity[e]	Specification comments
01.047	Longifolene		475-20-7	Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble Freely soluble	115 (13 hPa) MS 95 %	1.498-1.504 0.929-0.935	No longer supported by Industry (DG SANCO, 2012).
01.050	3-Methylhexane		589-34-4	Liquid C_7H_{16} 100.20	Practically insoluble or insoluble Freely soluble	92 MS 95 %	1.385-1.391 0.684-0.690	No longer supported by Industry (DG SANCO, 2012).
01.051	2-Methylnaphthalene		11010 91-57-6	Solid $C_{11}H_{10}$ 142.20	Practically insoluble or insoluble Freely soluble	241 35 MS 95 %	n.a. n.a.	No longer supported by Industry (DG SANCO, 2012).
01.052	α-Muurolene		11011 10208-80-7	Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble Freely soluble	118 (17 hPa) MS 95 %	1.502-1.508 0.911-0.917	No longer supported by Industry (DG SANCO, 2012).
01.053	Naphthalene		11014 91-20-3	Solid $C_{10}H_8$ 128.17	Practically insoluble or insoluble Freely soluble	218 80 MS 95 %	n.a. n.a.	No longer supported by Industry (DG SANCO, 2012).

Table D1: Specification Summary of the Substances no Longer Supported by Industry

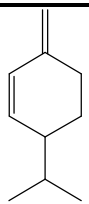
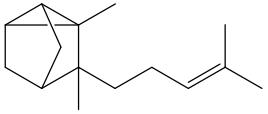
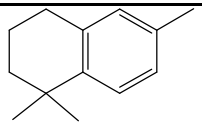
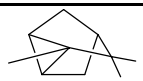
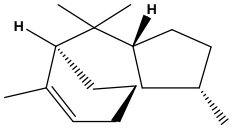
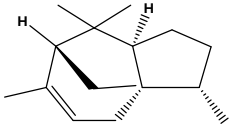
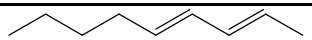
Former FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility[(a)] Solubility in ethanol[(b)]	Boiling point, °C[(c)] Melting point, °C ID test Assay minimum	Refrac. Index[(d)] Spec.gravity[(e)]	Specification comments
01.055	β-Phellandrene		11017 555-10-2	Liquid $C_{10}H_{16}$ 136.24	Practically insoluble or insoluble Freely soluble	174 MS 95 %	1.476-1.482 0.839-0.845	No longer supported by Industry (DG SANCO, 2012).
01.056	α-Santalene		512-61-8	Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble Freely soluble	112 (9 hPa) MS 95 %	1.480-1.486 0.895-0.901	No longer supported by Industry (DG SANCO, 2012).
01.058	1,2,3,4-Tetrahydro-1,1,6-trimethylnaphthalene		475-03-6	Liquid $C_{13}H_{18}$ 174.29	Practically insoluble or insoluble Freely soluble	245 MS 95 %	1.519-1.525 0.932-0.938	No longer supported by Industry (DG SANCO, 2012).
01.060	1,1,7-Trimethyltricyclo[2.2.1.0.(2.6)]heptane		508-32-7	Solid $C_{10}H_{16}$ 136.24	Practically insoluble or insoluble Freely soluble	152 66 MS 95 %	n.a. n.a.	No longer supported by Industry (DG SANCO, 2012).
01.066	2-Cedrene			Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble Freely soluble	82 (11 hPa) MS 95 %	1.495-1.501 0.928-0.934	No longer supported by Industry (DG SANCO, 2012).
01.067	8(14)-Cedrene			Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble Freely soluble	118 (13 hPa) MS 95 %	1.498-1.504 0.930-0.936	No longer supported by Industry (DG SANCO, 2012).
01.078	2,4-Nonadiene		4292 56700-78-8	Liquid C_9H_{16} 124.23	Insoluble Slightly soluble	155 MS	1.446 0.755	No longer supported by

Table D1: Specification Summary of the Substances no Longer Supported by Industry

Former FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility[(a)] Solubility in ethanol[(b)]	Boiling point, °C[(c)] Melting point, °C ID test Assay minimum	Refrac. Index[(d)] Spec.gravity[(e)]	Specification comments
						79 %		Industry (DG SANCO, 2012).

(a): Solubility in water, if not otherwise stated.
(b): Solubility in 95 % ethanol, if not otherwise stated.
(c): At 1013.25 hPa, if not otherwise stated.
(d): At 20°C, if not otherwise stated.
(e): At 25°C, if not otherwise stated.
n.a. Not applicable

The candidate substances in FGE.25Rev2 were allocated to subgroups. The subgrouping of the 23 substances no longer supported by Industry is shown here (Table D2). Subgroups still remaining in FGE.25Rev3 are: I, II, III and V. The Subgroups IVa, IVb, IVc and VI no longer contain any substances.

Table D2: Subgroups of Substances no longer Supported by Industry.

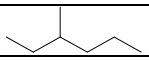
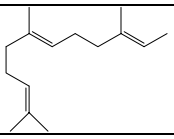
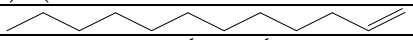
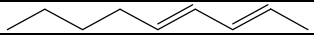
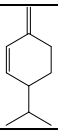
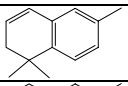
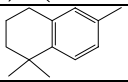
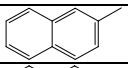
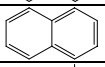
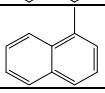
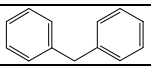
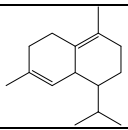
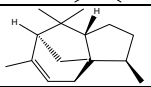
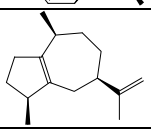
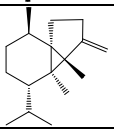
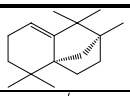
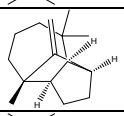
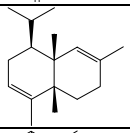
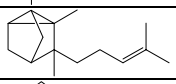
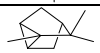
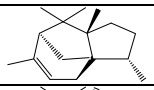
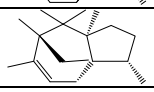
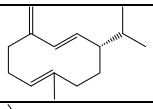
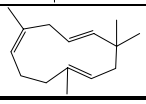
Former FL-no	**Name**	**Structural formula**	**Structural class**
I: ACYCLIC ALKANES			
01.050	3-Methylhexane		I
II: ACYCLIC ALKENES			
01.032	2,3-Dihydrofarnesene		I
01.037	Dodec-1-ene		I
01.078	2,4-Nonadiene		I
III: CYCLOHEXENE HYDROCARBONS			
01.055	β-Phellandrene		I
IV: AROMATIC HYDROCARBONS			
IVa: BENZENE HYDROCARBONS			
01.031	1,2-Dihydro-1,1,6-trimethylnaphthalene		I
01.058	1,2,3,4-Tetrahydro-1,1,6-trimethylnaphthalene		II
IVb: NAPHTHALENE HYDROCARBONS			
01.051	2-Methylnaphthalene		III
01.053	Naphthalene		III
(01.014)	(1-Methylnaphthalene)		III
IVc: DIPHENYLMETHANE			
01.036	Diphenylmethane		III
V: BI- and TRICYCLIC, NON-AROMATIC HYDROCARBONS			
01.021	Delta-Cadinene		III
01.022	α-Cedrene		I
01.023	1(5),11-Guaiadiene		I
01.030	β-Cubebene		I

Table D2: Subgroups of Substances no longer Supported by Industry.

Former FL-no	Name	Structural formula	Structural class
01.044	Isolongifolene		I
01.047	Longifolene		I
01.052	α-Muurolene		I
01.056	α-Santalene		I
01.060	1,1,7-Trimethyltricyclo[2.2.1.0.(2.6)]heptane		I
01.066	2-Cedrene		I
01.067	8(14)-Cedrene		I
VI: MACROCYCLIC, NON-AROMATIC HYDROCARBONS			
01.042	Germacra-1(10),4(14),5-triene		I
01.043	3,7,10-Humulatriene		I

ABBREVIATIONS

ADI	Acceptable Daily Intake
BW	Body weight
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CHO	Chinese hamster ovary (cells)
CoE	Council of Europe
CPN	Chronic progressive nephropathy
DNA	Deoxyribonucleic acid
DTU-NFI	Danish Technical University – National Food Institute
EC	European Commission
EFFA	European Flavour and Fragrance Association
EPA	United States Environmental Protection Agency
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
GLP	Good laboratory practise
ID	Identity
IOFI	International Organization of the Flavor Industry
Ip	Intraperitoneal
IR	Infrared spectroscopy
ISS	Istituto Superiore di Sanita
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOAEL	Lowest Observed Adverse Effect Level
MSDI	Maximised Survey-derived Daily Intake
mTAMDI	Modified Theoretical Added Maximum Daily Intake

NCE	Normochromatic erythrocyte
NOAEL	No observed adverse effect level
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
PCE	Polychromatic erythrocyte
SCE	Sister chromatic exchange
SCF	Scientific Committee on Food
UDS	Unscheduled DNA Synthesis
US EPA	United States Environmental Protection Agency
WHO	World Health Organization