Flavouring Group Evaluation 25, (FGE.25)[1]:

Aliphatic and aromatic hydrocarbons from chemical group 31

Scientific Opinion of the Panel on Food Additives,

Flavourings, Processing Aids and Materials in Contact with Food

(EFSA-Q-2003-168)

Adopted on 1 April 2008

SUMMARY

The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (the Panel) is asked to advise the Commission on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Panel is asked to evaluate 32 flavouring substances in the Flavouring Group Evaluation 25 (FGE.25), using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000. These 32 flavouring substances belong to chemical group 31, Annex I of the Commission Regulation (EC) No 1565/2000.

The present Flavouring Group Evaluation deals 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.

Seventeen of the 32 flavouring substances possess chiral centres and 16 can exist as geometrical isomers due to presence and position of double bonds. For eight of these flavouring substances the stereoisomeric composition has not been specified.

Of the 32 flavouring substances 27 are classified into structural class I, two into structural class II, and three into structural class III according to the decision tree approach presented by Cramer et al. (1978).

Twenty-nine of the flavouring substances in the present group have been reported to occur naturally in a wide range of food items.

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[1]   For citation purposes: Scientific Opinion of the Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food on a request from Commission on Flavouring Group Evaluation 87, (FGE.87) bicyclic secondary alcohols, ketones and related esters. The EFSA Journal (2008) 918, 1-109

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 has 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 the 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, the 32 flavouring substances in this group have intakes in Europe from 0.0012 to 2.7 microgram/*capita*/day, which are below the threshold of concern value for structural class I (1800 microgram/person/day), structural class II (540 microgram/person/day) and structural class III (90 microgram/person/day) substances.

Data on the genotoxicity of the flavouring substances in this group are limited and the genotoxicity could not be assessed adequately for these substances. For one candidate substance, 2-methylbuta-1,3-diene (synonym: isoprene) [FL-no: 01.049], there is evidence of an *in vivo* genotoxic potential. Therefore, the Panel concluded that the Procedure could not be applied to this substance. The available data do not preclude evaluating the remaining 31 candidate substances using the Procedure.

The available information on metabolism of the 31 flavouring substances evaluated through the Procedure or on structurally related substances for this FGE was very limited. Overall, only for eight of the flavouring substances it can be concluded that they will be metabolised into innocuous metabolites. For 18 of the flavouring substances the information is too limited and it cannot be assumed that they are metabolised to innocuous metabolites. For the remaining five of the flavouring substances there are data, which show that they may be metabolised to toxic 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 eight flavouring substances which are expected to be metabolised to innocuous substances would not give rise to safety concerns at their estimated intakes arising from their use as flavouring substances based on the MSDI approach. For the remaining 23 flavouring substances, which cannot be anticipated to be metabolised to innocuous products, or for structurally related substances, no adequate No Observed Adverse Effect Level (NOAEL) were available. Therefore, additional toxicological data are required.

The mTAMDI values for the 31 flavouring substances, evaluated using the Procedure, are 3300 microgram/person/day for one substance and 3700 microgram/person/day for each of the remaining 30 flavouring substances. These values are above the threshold for structural class I, II and III of 1800, 540 and 90 microgram/person/day, respectively.

Accordingly, intake estimates according to the mTAMDI for the 31 candidate substances in this flavouring group evaluation exceed the thresholds of concern for the three structural classes, 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 evaluation could be applied to the materials of commerce, it is necessary to consider the available specifications.

Specifications including complete purity criteria and identity tests for the materials of commerce have been provided for 23 flavouring substances. For eight substances [FL-no: 01.027, 01.032, 01.034, 01.035, 01.050, 01.055, 01.056 and 01.060] information on stereoisomerism is missing.

Thus, the final evaluation of the materials of commerce cannot be performed for eight substances [FL-no: 01.027, 01.032, 01.034, 01.035, 01.050, 01.055, 01.056 and 01.060], pending further information on isomerism. For 23 flavouring substances [FL-no: 01.022, 01.023, 01.030, 01.031, 01.032, 01.035, 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, 01.064, 01.066 and 01.067] additional toxicity data are required. For six flavouring substances [FL-no: 01.028, 01.033, 01.038, 01.039, 01.054 and 01.057] the Panel concluded that they would present no safety concern at the level of intake estimated on the basis of the MSDI approach. One flavouring substance, 2-methylbuta-1,3-diene [FL-no: 01.049], is genotoxic *in vitro* and *in vivo* and carcinogenic in experimental animals. Accordingly, the use as chemically defined flavouring substance is toxicologically not acceptable for this substance.

Keywords

Hydrocarbons, aliphatic, alicyclic, aromatic, flavourings, safety.

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BACKGROUND

Regulation (EC) No 2232/96 of the European Parliament and the Council (EC, 1996) lays down a Procedure for the establishment of a list of flavouring substances, the use of which will be authorised to the exclusion of all other substances in the EU. In application of that Regulation, a Register of flavouring substances used in or on foodstuffs in the Member States was adopted by Commission Decision 1999/217/EC (EC, 1999a), as last amended by Commission Decision 2006/252/EC (EC, 2006). Each flavouring substance is attributed a FLAVIS-number (FL-number) and all substances are divided into 34 chemical groups. Substances within a group should have some metabolic and biological behaviour in common.

Substances which are listed in the Register are to be evaluated according to the evaluation programme laid down in Commission Regulation (EC) No 1565/2000 (EC, 2000a), which is broadly based on the Opinion of the Scientific Committee on Food (SCF, 1999). For the submission of data by the manufacturer, deadlines have been established by Commission Regulation (EC) No 622/2002 (EC, 2002b).

After the completion of the evaluation programme the positive list of flavouring substances for use in or on foods in the EU shall be adopted (Article 5 (1) of Regulation (EC) No 2232/96) (EC, 1996).

TERMS OF REFERENCE

The European Food Safety Authority (EFSA) is requested to carry out a risk assessment on flavouring substances prior to their authorisation and inclusion in a positive list according to Commission Regulation (EC) No 1565/2000 (EC, 2000a).

ASSESSMENT

1. Presentation of the Substances in the Flavouring Group Evaluation 25

1.1. Description

The present Flavouring Group Evaluation 25, FGE.25, using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000 (EC, 2000a) (The Procedure – shown in schematic form in Annex I), deals with 32 aliphatic and aromatic hydrocarbons (candidate substances) from chemical group 31, Annex I of Commission Regulation (EC) No 1565/2000 (EC, 2000a). The candidate substances in the group have been divided into eight subgroups: I) acyclic alkanes, II) acyclic alkenes, III) cyclohexene hydrocarbons, IVa) benzene hydrocarbons, IVb) naphthalene hydrocarbons, IVc) diphenylmethane, V) bi- and tricyclic, non-aromatic hydrocarbons and VI) macrocyclic, non-aromatic hydrocarbons.

The 32 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 and Table 2.

The 32 candidate substances are closely related structurally to 19 flavouring substances (supporting substances) evaluated at the 63rd JECFA meeting (JECFA, 2005c) in the groups of "Aliphatic and alicyclic hydrocarbons" and "Aromatic hydrocarbons". The supporting substances are listed in Table 3.

1.2. 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 variation of 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 (Chemical Abstract Service number (CAS number), FLAVIS number, etc.).

Seventeen of the 32 candidate substances possess chiral centres. For 10 of these chiral substances the chemical names and CAS numbers specify the stereoisomers. For six of the substances [FL-no: 01.027, 01.034, 01.050, 01.055, 01.056 and 01.060] the stereoisomeric composition has not been specified, and for one substance [FL-no: 01.067] no CAS number has been provided.

Due to the presence and the position of double bonds, 16 of the 32 candidate substances can exist as geometrical isomers. In three cases [FL-no: 01.027, 01.032 and 01.035] no indication of the configuration of the double bond is given.

1.3. Natural Occurrence in Food

Twenty-nine out of the 32 candidate substances have been reported to occur in various types of alcoholic beverages, chicken, egg (boiled), fish (raw), shrimps, guinea hen, lamb fat, cheese, milk, butter, various herbs, various fruits and vegetables, tea, mace, and wort. Quantitative data on the natural occurrence in foods have been reported for 23 of these substances (TNO, 2000).

These reports include:

- alpha-Cedrene [FL-no: 01.022]: 0.0007 mg/kg in artichoke.

- 1(5),11-Guaiadiene [FL-no: 01.023]: 1.5 mg/kg in mango, trace amounts in basil and in pepper.

- beta-Bisabolene [FL-no: 01.028]: 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.

- beta-Cubebene [FL-no: 01.030]: 0.08 mg/kg in grapefruit juice, up to 0.08 mg/kg in guava fruit, trace amounts in mace and in nutmeg.

- 1,2-Dihydro-1,1,6- trimethylnaphthalene [FL-no: 01.031]: 0.25 mg/kg in rum, up to 0.042 mg/kg in white wine.

- 2,2-Dimethylhexane [FL-no: 01.033]: up to 0.9 mg/kg in tea.

- 2,4-Dimethylhexane [FL-no: 01.034]: up to 2 mg/kg in tea.

- 2,6-Dimethylocta-2,4,6-triene [FL-no: 01.035]: 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.

- Dodec-1-ene [FL-no: 01.037]: up to 0.4 mg/kg in butter, up to 0.01 mg/kg in loquat, up to 0.01 mg/kg in passiflora.

- Dodecane [FL-no: 01.038]: 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.

- delta-Elemene [FL-no: 01.039]: trace amounts in mandarin juice.

- Germacra-1(10),4(14),5-triene [FL-no: 01.042]: 0.001 mg/kg in mandarin juice.

- 3,7,10-Humulatriene [FL-no: 01.043]: up to 1.3 mg/kg in mango, up to 1.2 mg/kg in tea, up to 1.05 mg/kg in guava, 0.12 mg/kg in carrot, up to 0.12 in cheese, 0.2 mg/kg in grapefruit juice, up to 0.09 mg/kg in blackcurrants, 0.02 mg/kg in tamarind, up to 0.01 mg/kg in orange juice, up to 0.0002 mg/kg in artichoke, 0.0004 mg/kg in mandarin, trace amounts in nutmeg, in raspberry and in celery.

- Longifolene [FL-no: 01.047]: 0.0003 mg/kg in artichoke.

- 2-Methylbuta-1,3-diene [FL-no: 01.049]: 0.004 mg/kg in beer.

- 2-Methylnaphthalene [FL-no: 01.051]: 0.1 mg/kg in tea, 0.08 mg/kg in capsicum, up to 0.05 mg/kg in raspberry, up to 0.01 mg/kg in papaya, up to 0.01 mg/kg in passiflora, trace amounts in port wine.

- alpha-Muurolene [FL-no: 01.052]: 7.9 mg/kg in green tea, up to 0.009 mg/kg in mango, 0.005 mg/kg in bilberry, trace amounts in nutmeg.

- Naphthalene [FL-no: 01.053]: 0.1 mg/kg in tea, up to 0.06 mg/kg in capsicum annum (sweet pepper), 0.05 mg/kg in cocoa, up to 0.035 mg/kg in cheese, 0.02 mg/kg in raw fish, 0.02 mg/kg in beer, 0.01 mg/kg in grapefruit juice, up to 0.01 mg/kg in passiflora, up to 0.01 mg/kg in papaya, 0.008 mg/kg in soybean, up to 0.007 mg/kg in plumcot, 0.005 mg/kg in peanut, 0.005 mg/kg in peas, up to 0.005 mg/kg in plum, 0.005 mg/kg in bilberry, up to 0.005 mg/kg in raspberry, 0.004 mg/kg in water buffalo milk, 0.003 mg/kg in beans, 0.0029 mg/kg in skim milk powder, 0.001 mg/kg in kiwifruit, up to 0.001 mg/kg in mango, up to 0.0005 mg/kg in chicken, 0.0008 mg/kg in shrimps, trace amounts in peach, trace amounts in port wine.

- Pentadecane [FL-no: 01.054]: 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.

- beta-Phellandrene [FL-no: 01.055]: up to 85 mg/kg in dill, up to 0.66 mg/kg in tea, up to 0.5 mg/kg in black currants, up to 0.4 mg/kg in mango, up to 0.1 mg/kg in papaya, 0.025 mg/kg in grapefruit juice, up to 0.01 mg/kg in passiflora, up to 0.005 mg/kg in plumcot, up to 0.002 mg/kg in redcurrants, trace amounts in cinnamomum, trace amounts in nectarine.

- Tetradecane [FL-no: 01.057]: 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.

- 1,1,7-Trimethyltricyclo[2.2.1.0(2,6)]heptane [FL-no: 01.060]: up to 50 mg/kg in pepper, trace amounts in cardamom.

- cis-3,7-Dimethyl-1,3,6-octatriene [FL-no: 01.064]: 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.

According to TNO three of the substances, bisabola-1,8,12-triene [FL-no: 01.027], 2-cedrene [FL-no: 01.066], and 8(14)-cedrene [FL-no: 01.067] have not been reported to occur naturally in any food items (TNO, 2000).

2. Specifications

Purity criteria for the 32 substances have been provided by the Flavour Industry (EFFA, 2005a) (Table 1).

Judged against the requirements in Annex II of Commission Regulation (EC) No 1565/2000 (EC, 2000), the information is adequate for the 32 candidate substances. However, information on geometrical stereoisomerism and chirality is needed for three [FL-no: 01.027, 01.032 and 01.035] and six candidate substance [FL-no: 01.027, 01.034, 01.050, 01.055, 01.056 and 01.060], respectively (see Section 1.2 and Table 1).

3. 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 in 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, 2000a). 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).

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

The Maximised Survey-derived Daily Intake (MSDI) (SCF, 1999) 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, 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[2] (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.25 the total annual production volume of the 32 candidate substances from use as flavouring substances in Europe was reported to be approximately 90 kg (EFFA, 2005b). For the 19 supporting substances the total annual volume of production in Europe is approximately 390000 kg (*d*-limonene [FL-no: 01.045] accounts for 280000 kg and 106000 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, 2005c).

On the basis of the annual volumes of production reported for the 32 candidate substances, the MSDI values for each of these flavourings have been estimated (Table 2).

Approximately 90 % of the total annual volume of production for the candidate substances is accounted for by eight substances [FL-no: 01.023, 01.028, 01.033, 01.034, 01.036, 01.043, 01.054, 01.066]. The estimated combined daily *per capita* intake of these eight candidate substances from use as flavouring substance is 10 microgram. The daily *per capita* intakes for each of the remaining substances are less than 0.24 microgram (Table 2).

3.2.   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 the present evaluation of the 32 candidate substances, information on food categories and normal and maximum use levels[3,4] were submitted by the Flavour Industry (EFFA, 2005a; EFFA, 2007a). The 32 candidate substances are used in flavoured food products divided into the food categories, outlined in Annex III of the Commission Regulation (EC) No 1565/2000 (EC, 2000a), as shown in Table 3.1. For the present calculation of mTAMDI, the reported normal use levels were

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[2] EU figure 375 millions (Eurostat, 1998). 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.

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

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

used. In the case where different use levels were reported for different food categories the highest reported normal use level was used.

Table 3.1 Use of Candidate Substances

Food category	Description	Flavourings used
Category 1	Dairy products, excluding products of category 2	All 32
Category 2	Fats and oils, and fat emulsions (type water-in-oil)	All 32
Category 3	Edible ices, including sherbet and sorbet	All 32
Category 4.1	Processed fruits	All 32
Category 4.2	Processed vegetables (incl. mushrooms & fungi, roots & tubers, pulses and legumes), and nuts & seeds	None
Category 5	Confectionery	All 32
Category 6	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery	All 32
Category 7	Bakery wares	All 32
Category 8	Meat and meat products, including poultry and game	All 32
Category 9	Fish and fish products, including molluscs, crustaceans and echinoderms	All 32
Category 10	Eggs and egg products	None
Category 11	Sweeteners, including honey	None
Category 12	Salts, spices, soups, sauces, salads, protein products etc.	All 32
Category 13	Foodstuffs intended for particular nutritional uses	All 32
Category 14.1	Non-alcoholic ("soft") beverages, excl. dairy products	All 32
Category 14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts	None
Category 15	Ready-to-eat savouries	All except for [FL-no: 01.044]
Category 16	Composite foods (e.g. casseroles, meat pies, mincemeat) - foods that could not be placed in categories 1 – 15	All 32

According to the Flavour Industry the normal use levels for the 32 candidate substances are in the range of 2 - 20 mg/kg food, and the maximum use levels are in the range of 10 - 100 mg/kg (EFFA, 2002i; EFFA, 2005a; EFFA, 2007a), Table II.1.2, Annex II.

The mTAMDI values for the 27 candidate substances from structural class I (see Section 5) are for one substance [FL-no: 01.044] 3300 microgram/person/day and 3700 microgram/person/day for each of the remaining 26 candidate substances. For each of the two candidate substances from structural class II and the three candidate substances from structural class III (see Section 5) the mTAMDI is also 3700 microgram/person/day.

For detailed information on use levels and intake estimations based on the mTAMDI approach, see Section 6 and Annex II.

4. Absorption, Distribution, Metabolism and Elimination

Generally, the available data indicate that the aliphatic and aromatic 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. For the naphthalene hydrocarbons, in addition to epoxidation, formation of reactive quinones can be expected. The aromatic hydrocarbons that do not contain alkyl substituents can undergo ring hydroxylation to form phenolic metabolites that are subsequently conjugated with sulphate or glucuronic acid and excreted in the urine. 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 eight subgroups (see Section 1.1 and Annex III) 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 4.1 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.

For a more extensive discussion, see Annex III.

Table 4.1 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
01.050	3-Methylhexane	No (potential formation of neurotoxic gamma-diketone)
01.054	Pentadecane	Yes
01.057	Tetradecane	Yes
Subgroup II: ACYCLIC ALKENES		
01.032	2,3-Dihydrofarnesene	No (lack of supporting data)
01.035	2,6-Dimethylocta-2,4,6-triene	No (lack of supporting data)
01.037	Dodec-1-ene	No (presence of terminal double bond which may give rise to reactive metabolites without counteracting metabolic options)
01.049	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)
Subgroup III: CYCLOHEXENE HYDROCARBONS		
01.055	beta-Phellandrene	No (lack of supporting data)
01.027	Bisabola-1,8,12-triene	Yes
01.028	beta-Bisabolene	Yes

	Table 4.1 Can innocuous metabolites be expected to be formed based on available data?	
FL–no:	**Substance name**	**Innocuous metabolites?**
01.039	delta-Elemene	Yes
Subgroup IV: AROMATIC HYDROCARBONS		
Subgroup IVa: BENZENE HYDROCARBONS		
01.031	1,2-Dihydro-1,1,6-trimethylnaphthalene	No (lack of supporting data)
01.058	1,2,3,4-Tetrahydro-1,1,6-trimethylnaphthalene	No (lack of supporting dat)
Subgroup IVb: NAPHTHALENE HYDROCARBONS		
01.051	2-Methylnaphthalene	No (known metabolism to toxic metabolites)
01.053	Naphthalene	No (known metabolism to toxic metabolites)
Subgroup IVc: DIPHENYLMETHANE		
01.036	Diphenylmethane	No (lack of supporting data)
Subgroup V: BI- and TRICYCLIC, NON-AROMATIC HYDROCARBONS		
01.022	alpha-Cedrene	No (lack of supporting data)
01.023	1(5),11-Guaiadiene	No (lack of supporting data)
01.030	beta-Cubebene	No (lack of supporting data)
01.044	Isolongifolene	No (lack of supporting data)
01.047	Longifolene	No (lack of supporting data)
01.052	alpha-Muurolene	No (lack of supporting data)
01.056	alpha-Santalene	No (lack of supporting data)
01.060	1,1,7-Trimethyltricyclo[2.2.1.0.(2.6)]heptane	No (lack of supporting data)
01.066	2-Cedrene	No (lack of supporting data)
01.067	8(14)-Cedrene	No (lack of supporting data)
Subgroup VI: MACROCYCLIC, NON-AROMATIC HYDROCARBONS		
01.042	Germacra-1(10),4(14),5-triene	No (lack of supporting data)
01.043	3,7,10-Humulatriene	No (lack of supporting data)

5. 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 6.

The candidate substance 2-methyl-1,3-butadiene (isoprene) [FL-no: 01.049] showed genotoxic potential *in vivo* and carcinogenic effects in experimental animals. Therefore, the Panel concluded that the Procedure could not be applied to this flavouring substance.

For the safety evaluation of the remaining 31 candidate substances from chemical group 31, the Procedure as outlined in Annex I was applied, based on the MSDI approach. The stepwise evaluations of the 31 substances are summarised in Table 2.

Step 1

Twenty-six of the 31 candidate substances evaluated using the Procedure are classified into structural class I [FL-no: 01.022, 01.023, 01.027, 01.028, 01.030, 01.032, 01.033, 01.034, 01.035, 01.037, 01.038, 01.039, 01.042, 01.043, 01.044, 01.047, 01.050, 01.052, 01.054, 01.055, 01.056, 01.057, 01.060, 01.064, 01.066 and 01.067], two into structural class II [FL-no: 01.031 and 01.058], and three into structural class III, [FL-no: 01.036, 01.051 and 01.053], according to the decision tree approach presented by Cramer et al. (1978).

Step 2

On the basis of the metabolism information available, five of the six candidate substances of subgroup I [FL-no: 01.033, 01.034, 01.038, 01.054 and 01.057] and three of the four candidate substances of subgroup III [FL-no: 01.027, 01.028 and 01.039] may be predicted to be metabolised to innocuous products at the estimated levels of intake based on the MSDI approach, and accordingly their evaluation proceeds along the A-side of the Procedure.

One candidate substance from subgroup I [FL-no: 01.050] may be biotransformed to a neurotoxic gamma-diketone. Two candidate substances from subgroup II [FL-no: 01.037 and 01.064] contain terminal double bonds in the absence of other functional groups that may provide alternative routes of detoxication. For the two candidate substances from subgroup IVb [FL-no: 01.051 and 01.053] it has been shown that they may be converted to toxic metabolites. Therefore, for these five substances it cannot be concluded that they will be metabolised to innocuous products, and accordingly they proceed along the B-side of the Procedure.

For the remaining 18 candidate substances [FL-no: 01.022, 01.023, 01.030, 01.031, 01.032, 01.035, 01.036, 01.042, 01.043, 01.044, 01.047, 01.052, 01.055, 01.056, 01.058, 01.060, 01.066 and 01.067] 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.

Step A3

The five candidate substances from subgroup I [FL-no: 01.033, 01.034, 01.038, 01.054 and 01.057] and the 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 microgram (Table 6.1). These intakes are below the threshold of concern of 1800 microgram/person/day for structural class I. Accordingly, 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.

Step B3

The 18 candidate substances [FL-no: 01.022, 01.023, 01.030, 01.032, 01.035, 01.037, 01.042, 01.043, 01.044, 01.047, 01.050, 01.052, 01.055, 01.056, 01.060, 01.064, 01.066 and 01.067] proceeding via the B-side and which have been assigned to structural class I have estimated European daily *per capita* intakes between 0.012 and 1.2 microgram (Table 6.1). These intakes are below the threshold of concern of 1800 microgram/person/day for structural class I. Two of the candidate substances proceeding along the B-side have been assigned to structural class II [FL-no: 01.031 and 01.058]. These two substances have estimated European daily *per capita* intakes of 0.0012 and 0.12 micrograms, respectively. These intakes are below the threshold of concern of 540 microgram/person/day for structural class II. Three candidates proceeding along the B-side [FL-no: 01.036, 01.051 and 01.053] have been assigned to structural class III have European daily *per*

capita intakes ranging between 0.0012 and 1.2 microgram. These are below the threshold of concern for structural class III of 90 microgram/person/day. Accordingly, these 23 substances all proceed to step B4 of the Procedure.

Step B4

No adequate data on No Observed Adverse Effect Levels (NOAELs) are available for any of these 23 candidate substances [FL-no: 01.022, 01.023, 01.030, 01.031, 01.032, 01.035, 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, 01.064, 01.066 and 01.067] or have been submitted for any structurally related substances. Therefore, the Panel concluded that additional data are required for these substances.

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

The mTAMDI values for the 26 candidate substances from structural class I and evaluated using the Procedure are 3300 microgram/person/day for one candidate substance and 3700 microgram/person/day for each of the remaining 25 candidate substances. For each of the two candidate substances from structural class II and the three candidate substances from structural class III the mTAMDI is also 3700 microgram/person/day. These values are all above the thresholds for structural class I, II and III of 1800, 540 and 90 microgram/person/day, respectively.

For comparison of the intake estimates based on the MSDI approach and the mTAMDI approach, see Table 6.1.

For all 31 candidate substances evaluated using the Procedure further information is required. This would include more reliable intake data and then, if required, additional toxicological data.

Table 6.1 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)
01.022	alpha-Cedrene	0.012	3700	Class I	1800
01.023	1(5),11-Guaiadiene	1.2	3700	Class I	1800
01.027	Bisabola-1,8,12-triene	0.024	3700	Class I	1800
01.028	beta-Bisabolene	2.7	3700	Class I	1800
01.030	beta-Cubebene	0.012	3700	Class I	1800
01.032	2,3-Dihydrofarnesene	0.12	3700	Class I	1800
01.033	2,2-Dimethylhexane	1.2	3700	Class I	1800
01.034	2,4-Dimethylhexane	1.2	3700	Class I	1800
01.035	2,6-Dimethylocta-2,4,6-triene	0.013	3700	Class I	1800
01.037	Dodec-1-ene	0.024	3700	Class I	1800
01.038	Dodecane	0.012	3700	Class I	1800
01.039	delta-Elemene	0.012	3700	Class I	1800
01.042	Germacra-1(10),4(14),5-triene	0.012	3700	Class I	1800
01.043	3,7,10-Humulatriene	1.2	3700	Class I	1800
01.044	Isolongifolene	0.012	3300	Class I	1800
01.047	Longifolene	0.17	3700	Class I	1800
01.049	2-Methylbuta-1,3-diene	0.061	3700	Class I	1800
01.050	3-Methylhexane	0.061	3700	Class I	1800
01.052	alpha-Muurolene	0.24	3700	Class I	1800
01.054	Pentadecane	0.61	3700	Class I	1800
01.055	beta-Phellandrene	0.012	3700	Class I	1800
01.056	alpha-Santalene	0.012	3700	Class I	1800

FL-no	EU Register name	MSDI (µg/*capita*/day)	mTAMDI (µg/person/day)	Structural class	Threshold of concern (µg/person/day)
01.057	Tetradecane	0.012	3700	Class I	1800
01.060	1,1,7-Trimethyltricyclo[2.2.1.0.(2.6)]heptane	0.012	3700	Class I	1800
01.064	cis-3,7-Dimethyl-1,3,6-octatriene	0.049	3700	Class I	1800
01.066	2-Cedrene	0.97	3700	Class I	1800
01.067	8(14)-Cedrene	0.012	3700	Class I	1800
01.031	1,2-Dihydro-1,1,6-trimethylnaphthalene	0.0012	3700	Class II	540
01.058	1,2,3,4-Tetrahydro-1,1,6-trimethylnaphthalene	0.12	3700	Class II	540
01.036	Diphenylmethane	1.2	3700	Class III	90
01.051	2-Methylnaphthalene	0.0012	3700	Class III	90
01.053	Naphthalene	0.013	3700	Class III	90

Table 6.1 Estimated intakes based on the MSDI approach and the mTAMDI approach

7. 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 Flavouring Group Evaluation 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 candidate substance 2-methylbuta-1,3-diene [FL-no: 01.049] is genotoxic *in vitro* and *in vivo* and carcinogenic in experimental animals and accordingly its use as flavouring substance is toxicologically not acceptable. Therefore, this substance [FL-no: 01.049] is not included in the calculation of the combined intake estimate. The 23 candidate substances, for which additional data are requested, will also not be included. The combined intakes have been calculated for the remaining candidate and supporting substances (only for subgroup I (acyclic alkanes) and subgroup III (cyclohexene hydrocarbons)).

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

Subgroup I (acyclic alkanes): The combined intake of the five candidate substances, all from structural class I, is 3.0 microgram/*capita*/day, which does not exceed the threshold of 1800 microgram/person/day. There are no supporting substances in subgroup I.

Subgroup III (cyclohexene hydrocarbons): The combined intake of the three candidate substances, all from structural class I, is 2.7 microgram/*capita*/day, which does not exceed the threshold of 1800 microgram/person/day. The total combined intake of the candidate and six supporting substances (also from structural class I) is approximately 35500 microgram/*capita*/day. This intake exceeds the threshold of 1800 microgram/person/day for a structural class I substance. However, *d*-limonene (supporting substance [FL-no: 01.045]) accounts for approximately 34000 microgram/*capita*/day.

As JECFA has established an acceptable daily intake (ADI) "not specified" for *d*-limonene (JECFA, 2005c) the Panel concluded that there is no safety concern for this total combined intake.

8. Toxicity

8.1. Acute Toxicity

Data are available for eight candidate and 17 supporting substances. Oral LD50 values in rat and mouse ranged from 500 – 13000 mg/kg body weight (bw).

The acute toxicity data are summarised in Annex IV, Table IV.1.

8.2. Subacute, Subchronic, Chronic and Carcinogenicity Studies

Data are available for three candidate substances, 2-methylbuta-1,3-diene (isoprene) [FL-no: 01.049] (*subgroup II*), naphthalene [FL-no: 01.053] and 2-methylnaphthalene [FL-no: 01.051] (*subgroup IVb*), and for five supporting substances.

Subgroup II

For the candidate substance 2-methylbuta-1,3-diene (isoprene) [FL-no: 01.049] it has been concluded that there is sufficient evidence of carcinogenicity in experimental animals and that the substance is "possibly carcinogenic to humans" (group 2B) (IARC, 1999a). The structurally related substance 1,3-butadiene has been concluded to be genotoxic and carcinogenic, and to be "probably carcinogenic to humans" (group 2A) (IARC, 1999b).

The Panel agrees that 2-methylbuta-1,3-diene [FL-no: 01.049] is of concern with respect to carcinogenicity (and genotoxicity) and accordingly cannot be evaluated using the Procedure.

Subgroup IVb

Naphthalene:

For naphthalene [FL-no: 01.053] two unpublished 13-week oral studies on rats and mice were only available as summaries, referred to in US EPA (1998). Since the original data were not available it was not possible to evaluate the quality of the study. Groups of 20 rats, male and female, were given doses of 0, 25, 50, 100, 200 or 400 mg naphthalene/kg bw by gavage five days a week for 13 weeks. The No Observed Adverse Effect Level (NOAEL) in this study was 100 mg/kg body weight (bw) based on mean terminal bw decreases. Other findings were low incidence of lesions in exposed male kidneys and female thymuses. In the mouse, study groups of 10 male and 10 female mice were given doses of 0, 12.5, 25, 50, 100 or 200 mg/kg bw following the same design as the rat study. Transient signs of toxicity occurred in the 200 mg/kg groups. No exposure-related lesions were observed in any organs examined. The authors considered the highest dose level to be a NOAEL, whereas the EPA judged the highest dose level to be a Low Observed Adverse Effect Level (LOAEL) for transient signs of toxicity.

Naphthalene was given six times per week in food to 28 rats at a dose level varying between 10 and 20 mg/rat/day. Treatment was stopped after 700 days after reaching a total dose of 10 g/rat. Rats were observed until spontaneous death. The conclusion by the author was that naphthalene did not cause cancer in the study. It is however not possible to draw conclusions on carcinogenicity or chronic toxicity from the study since it is deficient in design and poorly reported (Schmähl, 1955).

Groups of male (76-112) and female (40-76) mice were given naphthalene by gavage at dose levels of 0, 5.3, 53 or 133 mg/kg bw/day for 90 consecutive days. The study identified a LOAEL of 133

mg/kg bw/day and a NOAEL of 53 mg/kg bw/day based upon the significant decreases in absolute weight of brain, liver, and spleen and relative weight of spleen in female mice. Histopathology was not conducted in this study (Shopp et al., 1984).

Naphthalene [FL-no: 01.053] was studied in a two-year NTP inhalation study in B6C3F mice. The result showed no evidence of carcinogenic activity in male mice exposed to naphthalene, but some evidence of carcinogenic activity in female mice. Groups of male and female mice were exposed to 0 (75/group), 10 (75/group) or 30 ppm (150/group) naphthalene for six hours daily, five days/week for 105 weeks. No increased tumour incidence related to exposure was observed in male mice, but in females the incidence of pulmonary alveolar/bronchiolar adenomas was significantly greater in the high-dose group than in controls. In both male and female mice exposure to naphthalene caused increased incidences and severity of chronic inflammation, metaplasia of the olfactory epithelium and hyperplasia of the respiratory epithelium in the nose and chronic inflammation in the lungs (NTP, 1992g).

In a two-year NTP inhalation study in F344/N rats on the toxicology and carcinogenesis of naphthalene [FL-no: 01.053] it was concluded by NTP that there was clear evidence of carcinogenic activity based on increased incidences of respiratory epithelial adenoma and olfactory epithelial neuroblastoma of the nose. Groups of 49 male and female rats were exposed to naphthalene by inhalation at concentrations of 0, 10, 30 or 60 ppm for six hours plus 12 minutes per day five days per week for 105 weeks. The highest exposure concentration is the maximum without naphthalene condensation. Additional groups of nine male and female rats were exposed to 10, 30 or 60 ppm for up to 18 months for evaluation of toxicokinetic parameters. At the exposure concentrations in the study approximately 20-30 % of the inhaled dose was metabolised by the rats. Naphthalene that was not absorbed during exposure was presumed to be exhaled.The daily doses were estimated to be 0, 3.6-3.9, 10.7-11.4 and 20.1-20.6 mg/kg bw. Survival of all exposed groups was similar to that of controls. Mean body weights of exposed animals were less than those of controls. Incidences of adenoma of the respiratory epithelium were associated with exposure and increased with dose, as did incidences of neuroblastoma of the olfactory epithelium. Increased incidences of non-neoplastic lesions of the nose associated with exposure to naphtalene included atypical hyperplasia, atrophy, chronic inflammation, hyaline degradation of epithelium, hyperplasia, squamous metaplasia, goblet hyperplasia of respiratory epithelium and glandular hyperplasia and squamous metaplasia. There were no ocular abnormalities in the rats, although naphthalene is a known ocular irritant. Unlike mice, naphthalene was not carcinogenic in the rat lung. Higher rates of metabolism in the mouse lung compared to the rat lung may have been a contributing factor to this species difference in response (NTP, 2000d).

Conclusion on carcinogenicity for naphthalene

The Panel agreed with the European Union Risk Assessment Report (EU-RAR, 2003) in its evaluation of the long-term carcinogenicity studies on naphthalene. It was concluded that overall the balance of evidence indicates that naphthalene is not genotoxic. In the two-year rat study, inhalation of naphthalene produced an increase in the incidence of respiratory epithelial adenomas and olfactory epithelial neuroblastomas in both males and females. All these tumours occurred at sites where dose-related non-neoplastic inflammatory changes in the nose and respiratory epithelium also occurred. In view of the negative results obtained in the *in vivo* genotoxicity studies, these tumours are considered to arise via a non-genotoxic, thresholded mechanism. In mice an increase in the incidence of benign alveolar/bronchiolar adenomas in females was observed after inhalation of naphthalene. These tumours are similarly considered to arise via a non-genotoxic procedure, and are unlikely to be of relevance to human health due to the well-known species differences in pulmonary metabolism, mouse lung preparations being able to metabolise

naphthalene at a substantially greater rate (up to 100-fold) than those from hamster, rat or monkey. Although the significance of these inhalation studies for the evaluation of naphthalene used as a flavouring substance in food is questionable, the Panel noted that they did not provide a NOAEL for the non-neoplastic toxic effects on the olfactory and respiratory tissues preceding the tumour formation.

2-Methylnaphthalene:

In a valid study by Murata et al. (1997) groups of 50 male and 50 female mice were fed diets containing 0, 0.075 % or 0.15 % 2-methylnaphthalene for 81 weeks. The doses were calculated to correspond to 50-53 and 108-114 mg/kg bw/day, respectively. Pulmonary alveolar proteinosis developed in all treated animals differing significantly from controls. No tumours were seen. No NOAEL could be derived from this study (Murata et al., 1997).

Cutaneous application of methylnaphthalene, a mixture of 1-methylnaphthalene and 2-methylnaphthalene in acetone, at doses of 29.7 or 118.8 mg/kg bw painted on the shaved backs of B6C3F$_1$ mice twice a week for life was followed by lesions in the lungs. Control mice received acetone in the same manner. The lesions were diagnosed as endogenous lipid pneumonia. Frequency of pneumonia was 0/4 control, 3/11 low dose and 31/32 high dose animals (Emi & Konishi, 1985). The results suggest that lipid pneumonia could result from systemic administration, and that the lung may be a site for naphthalene toxicity in mice as is discussed in the NTP studies reported above.

Conclusion on carcinogenicity for 2-methylnaphthalene

The data available, principally from a valid 81-week feeding study in mice, do not indicate that 2-methylnaphthalene is carcinogenic.

The toxicity data are summarised in Annex IV, Table IV.2.

8.3. Developmental / Reproductive Toxicity Studies

Developmental and reproductive studies are available for one candidate substance, naphthalene [FL-no: 01.053] (*subgroup IVb)*. Studies have been performed on mice, rats and rabbits. Pregnant mice were given 0 or 300 mg naphthalene/kg/day on gestation days 7-14. The conclusion was drawn that naphthalene toxicity is manifest before a high level of developmental toxicity occurs. The study does not assess skeletal or visceral foetal development and it is not possible to derive a NOAEL from the study (Booth et al., 1983; Plasterer et al., 1985).

Groups of 25-26 pregnant rats were administered naphthalene by gavage at dose levels of 0, 50, 150 or 450 mg/kg bw on gestational days 6-15. Clinical signs of toxicity were observed in all dams, but they subsided in the two lowest dose groups. Decreased body weight and body weight gain were observed in the two highest dose groups. Foetal growth, viability and morphological development were not significantly affected by treatment. There was no significant treatment related effect on implantation, foetal survival or resorption. The authors concluded that the lowest dose level was a LOAEL for maternal toxicity and that 450 mg/kg bw was a NOAEL for foetal developmental toxicity (Navarro et al., 1991).

Groups of 25-27 pregnant rabbits were administered naphthalene by gavage at dose levels of 0, 20, 80 or 120 mg/kg bw on gestational days 6-19. No treatment-related signs of maternal toxicity were observed. No statistically significant differences were found between control and treated groups in average live litter size, foetal body weight or incidence of malformations. The developmental NOAEL was considered to be 120 mg/kg bw/day in this study.

Groups of 18 pregnant rabbits were given naphthalene by gavage at doses of 0, 40, 200 or 400 mg/kg bw on gestational days 6-18. Treatment-related clinical signs were observed in a dose-related manner. Maternal NOAEL was not determined. No statistically significant changes were apparent in the mean number of corpora lutea, foetal body weights, viable foetuses, total implantations, post-implantational loss or foetal sex distribution when comparing control or treatment groups. No treatment-related effects were found on examining foetuses for visceral and skeletal abnormalities. Foetal developmental NOAEL was determined as 400 mg/kg bw (Naismith & Matthews, 1986).

Conclusion

In the tested animal species, mice, rats and rabbits, clinical symptoms of maternal toxicity appeared before signs of reproductive or developmental toxicity were apparent. Developmental NOAELs varied between 120 mg/kg bw/day (rabbit) and 450 mg/kg bw/day (rat, rabbit).

The developmental/reproductive toxicity data are summarised in Annex IV, Table IV.3.

8.4.   Genotoxicity Studies

Data from *in vitro* tests are available for six candidate substances [*subgroup I*: FL-no: 01.038 and FL-no: 01.057; *subgroup II*: FL-no: 01.037 and 01.049; *subgroup IVb*: FL-no: 01.051 and 01.053], and 10 supporting substances [one from *subgroup II*, three from *subgroup III*, one from *subgroup IVb*, five from *subgroup V*]. Three of these studies on candidate substances [FL-no: 01.049 (*subgroup II*), 01.051 and 01.053 (*subgroup IVb*)] and one on a supporting substance [FL-no: 01.014 (*subgroup IVb*)] are considered valid.

Data from *in vivo* tests are available for two candidate substances [*subgroup II*: FL-no: 01.049; *subgroup IVb*: FL-no: 01.053] and for two supporting substances (one from *subgroup II* and one from *subgroup III*).

There are no data available on substances from subgroups IVa, IVc and VI.

Subgroup I

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

Subgroup II

The available *in vivo* studies on the candidate substance 2-methylbuta-1,3-diene (isoprene) [FL-no: 01.049] 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, 1999a).

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, 1999a). Isoprene has been classified in the EU as a *'Muta. Cat. 3; R68'* and *'Carc. Cat. 2; R45'* (ECB, 2005).

The available data on *in vivo* genotoxicity of 2-methylbuta-1,3-diene (synonym: isoprene) [FL-no: 01.049] indicate a genotoxic potential of the substance. In the light of the evidence of carcinogenic activity of isoprene in rats and mice (NTP, 1999d) 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. The structurally related substance myrcene [FL-no: 01.008] like isoprene has two conjugated, terminal double bonds (but has a longer chain length, with 10 carbon atoms). The genotoxicity data available on myrcene do not give rise to concern with respect to genotoxicity. For the remaining four candidate substances in subgroup II [FL-no: 01.032, 01.035, 01.037 and 01.064] there are, except for one negative bacterial mutagenicity test (Ames test), no genotoxicity data available, but it was noted that these substances do not contain conjugated terminal double bonds. The Panel concluded that, except for 2-methylbuta-1,3-diene [FL-no: 01.049], data available do not preclude to evaluate the candidate substances in subgroup II through the Procedure.

Subgroup IVb

Naphthalene

Naphthalene [FL-no: 01.053], which was negative in all bacterial gene mutation tests (Ames tests, Rec assay, Inductest) and in a unscheduled DNA synthesis (UDS) test in primary rat hepatocytes, gave a weakly positive result in a valid SCE test both in the presence and absence of metabolic activation and a positive result in a valid chromosomal aberration test in the presence of S9. A positive result was also reported in a mammalian cell gene mutation test (mouse lymphoma assay). However, the validity of this study cannot be evaluated.

The available *in vivo* studies on the candidate substance naphthalene [FL-no: 01.053] reported negative results in a valid UDS test and in two micronucleus tests, for one of which the validity cannot be evaluated due to insufficiently reported experimental details.

The genotoxicity of naphthalene [FL-no: 01.053] and 2-methylnaphthalene [FL-no: 01.051] has been evaluated by international expert bodies (WHO, 1998; EU-RAR, 2003; US ATSDR, 2005a). Results of the *in vitro* genotoxicity studies that were evaluated are in line with the data summarised in the present evaluation. Negative results were reported for all the evaluated bacterial gene mutation tests (Ames tests, Rec assays, Inductests/SOS response tests). For *in vitro* mammalian gene mutation, cytogenetic, or DNA damage assays, equally mixed results were reported as in the present evaluation. In addition to the studies cited in the present evaluation, negative results were reported for mutations at the hprt and tk locus in a human B-lymphoblastoid cell line (Sasaki et al., 1997) and for single-strand breaks in an alkaline elution test with rat hepatocytes (Sina et al., 1983) as well as for some other less relevant endpoints (different cell transformation assays). Positive results were reported for naphthalene in a non-standard chromosomal aberration assay (Gollahon, 1991) and for the naphthalene metabolites 1,2- and 1,4-naphthoquinone in a SCE test (Wilson et al., 1996). For *in vivo* genotoxicity, besides the negative results from studies examining commonly accepted endpoints (micronuclei formation in mouse bone marrow, DNA single strand breaks and UDS in rat hepatocytes) as reported in the present evaluation, some positive results were reported for somatic mutations in *D. melanogaster* (Delgado-Rodriques et al., 1995), micronuclei in salamander larvae erythrocytes (Djomo et al., 1995), and DNA fragmentation in liver and brain tissue from mice and rats orally exposed to naphthalene (Bagchi et al., 2000). However, DNA fragmentation *per se* cannot be considered a specific endpoint of genotoxicity, being rather an indicator of cytotoxicity, in this case due to oxidative stress. Therefore, the study by Bagchi et al. (2000) has no relevance for the evaluation of the genotoxic potential of naphthalene. WHO (1998) noted that naphthalene was inactive in all short-term mutagenicity tests evaluated by IARC in 1983

(WHO, 1998). US ATSDR (2005) concluded that the available data suggest that genotoxic action by the naphthalene metabolite, 1,2-naphthoquinone, is plausible and that the mutagenic/genotoxic potential of naphthalene and its metabolites may be weak (US ATSDR, 2005a). In the EU-RAR (2003) it was concluded that overall, the balance of evidence indicates that naphthalene is not genotoxic (EU-RAR, 2003).

Conclusion on genotoxicity for naphthalene

For naphthalene there is indication of *in vitro* genotoxicity especially at chromosome level. However, this genotoxic activity is not expressed in valid *in vivo* assays covering different end-points (e.g. micronucleus, UDS and DNA single strand breaks).

The Panel concluded that the available *in vivo* genotoxicity data on naphthalene do not preclude an evaluation of this substance through the Procedure.

2-Methylnaphthalene

The weak increases of chromosome aberrations (chromatid breaks only at the highest concentration) and of the SCE in cultured human lymphocytes in the presence of S9 are of doubtful relevance (Kulka et al., 1988). According to the authors, these effects do not indicate that 2-methylnaphthalene is a potentially genotoxic substance.

The genotoxicity of 2-methylnaphthalene has been evaluated by international expert bodies (WHO, 1998; EU-RAR, 2003; US ATSDR, 2005a). Results of the *in vitro* genotoxicity studies that were evaluated are in line with the data summarised in the present evaluation. Negative results were reported for all the evaluated bacterial gene mutation tests (Ames tests, Rec assays, Inductests/SOS response tests).

Conclusion on genotoxicity for 2-methylnaphthalene

The available data on 2-methylnaphthalene, limited to the *in vitro* data referred to in the present evaluation, were considered not to give evidence for a genotoxic activity.

The Panel concluded that the available genotoxicity data on 2-methylnaphthalene do not preclude an evaluation of this substance through the Procedure.

Supporting substances

From the various *in vitro* genotoxicity studies carried out with supporting substances, there is no indication of a mutagenic activity of these substances in bacterial cells or of a direct clastogenic effect on mammalian cells. For all substances only negative results were reported in the available studies except for delta-3-carene (*subgroup V*) and 1-methylnaphthalene (*subgroup IVb*) (see Table IV.4). Delta-3-carene was reported to be positive in TA100 and TA102 strains in an insufficiently reported Ames test in the absence of metabolic activation at high concentrations only, while it was negative in the presence of metabolic activation. 1-Methylnaphthalene gave a weak increase, of doubtful biological relevance, of SCE in cultured human lymphocytes in the present of S9 (Kulka et al., 1988). This effect was interpreted by the authors not to be an indication of genotoxic potential. Furthermore, 1-methylnaphthalene gave negative results in two gene mutation tests (Ames tests) (Florin et al., 1980; Oberly et al., 1993a) and in a chromosomal aberration assay in human lymphocytes (Kulka et al., 1988).

The *in vivo* genotoxicity studies available on supporting substances gave all negative results; however, only a micronucleus test in peripheral blood cells with myrcene [FL-no: 01.008] (*subgroup II*) is considered valid.

Genotoxicity data are summarised in Annex IV, Table IV.4 and IV.5.

Overall conclusion on genotoxicity:

Data on the genotoxicity of the flavouring substances in this group are limited and the genotoxicity could not be assessed adequately for these substances. For one candidate substance, 2-methylbuta-1,3-diene (synonym: isoprene) [FL-no: 01.049], there is evidence of an *in vivo* genotoxic potential. Therefore, the Panel concluded that the Procedure could not be applied to this substance. The available data do not preclude evaluating the remaining 31 candidate substances using the Procedure.

9. Conclusions

The 32 candidate substances are aliphatic and aromatic hydrocarbons from chemical group 31, which have been divided into eight subgroups: I) acyclic saturated hydrocarbons, II) acyclic unsaturated hydrocarbons, III) cyclohexene hydrocarbons, IVa) benzene hydrocarbons, IVb) napthalene hydrocarbons, IVc) diphenylmethane derivatives, V) bi- and tricyclic, non-aromatic hydrocarbons and VI) macrocyclic, non-aromatic hydrocarbons.

Seventeen of the 32 candidate substances possess chiral centres and 16 of the 32 candidate substances can exist as geometrical isomers due to presence and the position of double bonds. For eight of these candidate substances the stereoisomeric composition has not been specified [FL-no: 01.027, 01.032, 01.034, 01.035, 01.050, 01.055, 01.056 and 01.060].

Of the 32 candidate substances 27 are classified into structural class I [FL-no: 01.022, 01.023, 01.027, 01.028, 01.030, 01.032, 01.033, 01.034, 01.035, 01.037, 01.038, 01.039, 01.042, 01.043, 01.044, 01.047, 01.049, 01.050, 01.052, 01.054, 01.055, 01.056, 01.057, 01.060, 01.064, 01.066 and 01.067], two into structural class II [FL-no: 01.031 and 01.058], and three into structural class III [FL-no: 01.036, 01.051 and 01.053], according to the decision tree approach presented by Cramer et al. (1978).

Twenty-nine out of the 32 candidate substances have been reported to occur naturally in a wide range of food items.

According to the default MSDI approach, the 32 flavouring substances in this group have intakes in Europe from 0.0012 to 2.7 microgram/*capita*/day, which are below the threshold of concern value for structural class I (1800 microgram/person/day), structural class II (540 microgram/person/day) and structural class III (90 microgram/person/day) substances.

Combined intakes can bee calculated for the substances in subgroup I (acyclic alkanes) and subgroup III (cyclohexene hydrocarbons).

Subgroup I (acyclic alkanes): The combined intake of the five candidate substances, all from structural class I, is 3.0 microgram/*capita*/day, which does not exceed the threshold of 1800 microgram/person/day. The are no supporting substances in subgroup I.

Subgroup III (cyclohexene hydrocarbons): The combined intake of the three candidate substances, all from structural class I, is 2.7 microgram/*capita*/day, which does not exceed the threshold of 1800 microgram/person/day. The total combined intake of the candidate and six supporting substances (also from structural class I) is 35500 microgram/*capita*/day. This intake exceeds the threshold of 1800 microgram/person/day for a structural class I substance. However, *d*-limonene (supporting substance [FL-no: 01.045]) accounts for approximately 34000 microgram/*capita*/day. As JECFA has established an ADI "not specified" for *d*-limonene the Panel has concluded there is not safety concern due to this combined intake.

Data on the genotoxicity of the flavouring substances in this group are limited and the genotoxicity could not be assessed adequately for these substances. For one candidate substance, 2-methylbuta-1,3-diene [FL-no: 01.049], there is evidence of an *in vivo* genotoxic and carcinogenic potential. Therefore, the Panel concluded that the Procedure could not be applied to this substance. The available data do not preclude an evaluation of the remaining 31 candidate substances using the Procedure.

The available information on metabolism of the 31 candidate substances evaluated through the Procedure or the supporting substances for this FGE was very limited. Overall, only for the following eight 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: 01.027, 01.028 and 01.039] from subgroup III. For 19 candidate substances [FL-no: 01.022, 01.023, 01.030, 01.031, 01.032, 01.035, 01.036, 01.042, 01.043, 01.044, 01.047, 01.052, 01.055, 01.056, 01.058, 01.060, 01.064, 01.066 and 01.067], the information is too limited and it cannot be assumed that they are metabolised to innocuous metabolites. For the remaining five candidate substances there are data which show that they may be metabolised to toxic metabolites [FL-no: 01.037, 01.049, 01.050, 01.051 and 01.053].

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 eight 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: 01.027, 01.028 and 01.039] 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 the remaining 23 candidate substances, [FL-no: 01.022, 01.023, 01.030, 01.031, 01.032, 01.035, 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, 01.064, 01.066 and 01.067], evaluated through the Procedure, or structurally related supporting substances, no adequate NOAEL were available. Therefore, additional toxicological data are required.

The mTAMDI values for the 26 candidate substances from structural class I and evaluated using the Procedure are 3300 microgram/person/day for one candidate substance and 3700 microgram/person/day for each of the remaining 25 candidate substances. For each of the two candidate substances from structural class II and the three candidate substances from structural class III the mTAMDI is also 3700 microgram/person/day. These values are above the threshold for structural class I, II and III of 1800, 540 and 90 microgram/person/day, respectively.

Accordingly, intake estimates according to the mTAMDI for the 31 candidate substances in this flavouring group evaluation exceed the thresholds of concern for the three structural classes, 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.
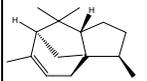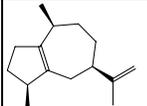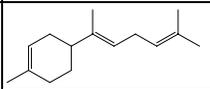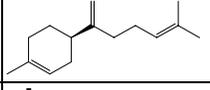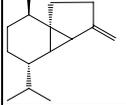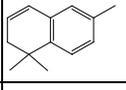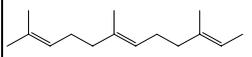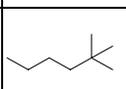
In order to determine whether this conclusion could be applied to the materials of commerce, it is necessary to consider the available specifications:
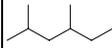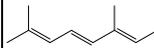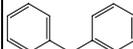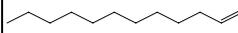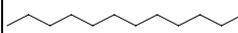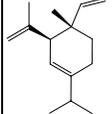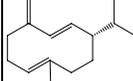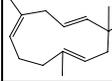
Specifications including complete purity criteria and identity tests for the materials of commerce have been provided for 23 flavouring substances. For eight substances [FL-no: 01.027, 01.032, 01.034, 01.035, 01.050, 01.055, 01.056 and 01.060] information on stereoisomerism is missing.
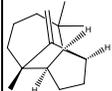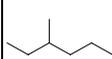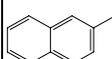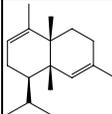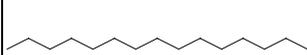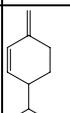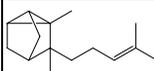
Thus, the final evaluation of the materials of commerce cannot be performed for eight substances [FL-no: 01.027, 01.032, 01.034, 01.035, 01.050, 01.055, 01.056 and 01.060], pending further
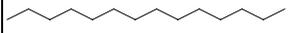
information on isomerism. For 23 candidate substances [FL-no: 01.022, 01.023, 01.030, 01.031, 01.032, 01.035, 01.036, 01.037, 01.042, 01.043, 01.044, 01.047, 01.050, 01.051, 01.052, 01.053, 01.055, 01.056, 01.058, 01.060, 01.064, 01.066 and 01.067] additional toxicity data are required. For six candidate substances [FL-no: 01.028, 01.033, 01.038, 01.039, 01.054 and 01.057] the Panel concluded that they would present no safety concern at their levels of intake estimated on the basis of the MSDI approach. One flavouring substance, 2-methylbuta-1,3-diene [FL-no: 01.049], is genotoxic *in vitro* and *in vivo* and carcinogenic in experimental animals. Accordingly, the use as chemically defined flavouring substance is toxicologically not acceptable for this substance.

TABLE 1: SPECIFICATION SUMMARY OF THE SUBSTANCES IN THE FLAVOURING GROUP EVALUATION 25

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

FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)	Specification comments
01.022	alpha-Cedrene		10985 469-61-4	Liquid C$_{15}$H$_{24}$ 204.35	Practically insoluble or insoluble 1 ml in 1 ml	119 (13 hPa) MS 95 %	1.500-1.506 0.932-0.938	
01.023	1(5),11-Guaiadiene		11003 3691-12-1	Liquid C$_{15}$H$_{24}$ 204.35	Practically insoluble or insoluble 1 ml in 1 ml	78 (3 hPa) MS 95 %	1.493-1.499 0.897-0.903	
01.027	Bisabola-1,8,12-triene 6)		17627-44-0	Liquid C$_{15}$H$_{24}$ 204.35	Practically insoluble or insoluble 1 ml in 1 ml	99 (5 hPa) MS 95 %	1.483-1.489 0.912-0.918	CASrn in Register refers to the racemate, (Z)- or (E)-isomer not specified.
01.028	beta-Bisabolene		495-61-4	Liquid C$_{15}$H$_{24}$ 204.35	Practically insoluble or insoluble 1 ml in 1 ml	130 (13 hPa) MS 95 %	1.489-1.495 0.879-0.885	
01.030	beta-Cubebene		10989 13744-15-5	Solid C$_{15}$H$_{24}$ 204.35	Practically insoluble or insoluble 1 ml in 1 ml	284 60 MS 95 %	n.a. n.a.	
01.031	1,2-Dihydro-1,1,6-trimethylnaphthalene		30364-38-6	Liquid C$_{13}$H$_{16}$ 172.27	Practically insoluble or insoluble 1 ml in 1 ml	115 (24 hPa) MS 95 %	1.542-1.548 0.942-0.948	
01.032	2,3-Dihydrofarnesene 6)		7681-88-1	Liquid C$_{15}$H$_{26}$ 206.37	Practically insoluble or insoluble 1 ml in 1 ml	130 (15 hPa) NMR 95 %	1.468-1.474 0.817-0.823	CASrn in Register does not specify configuration of double bond.
01.033	2,2-Dimethylhexane		590-73-8	Liquid C$_{8}$H$_{18}$ 114.23	Practically insoluble or insoluble 1 ml in 1 ml	107 MS 95 %	1.390-1.396 0.693-0.699	

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

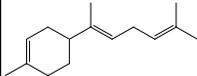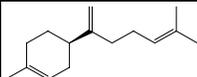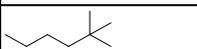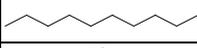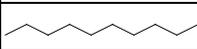| FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)	Specification comments
01.034	2,4-Dimethylhexane 6)		589-43-5	Liquid C₈H₁₈ 114.23	Practically insoluble or insoluble 1 ml in 1 ml	109 MS 95 %	1.390-1.396 0.697-0.703	CASrn in Register refers to the racemate.
01.035	2,6-Dimethylocta-2,4,6-triene 6)		673-84-7	Liquid C₁₀H₁₆ 136.24	Practically insoluble or insoluble 1 ml in 1 ml	75 (13 hPa) MS 95 %	1.539-1.545 0.809-0.815	CASrn in Register does not specify configuration of double bonds.
01.036	Diphenylmethane		11847 101-81-5	Solid C₁₃H₁₂ 168.24	Practically insoluble or insoluble 1 ml in 1 ml	262 27 MS 95 %	n.a. n.a.	
01.037	Dodec-1-ene		10992 112-41-4	Liquid C₁₂H₂₄ 168.23	Practically insoluble or insoluble 1 ml in 1 ml	213 MS 95 %	1.425-1.431 0.755-0.761	
01.038	Dodecane		112-40-3	Liquid C₁₂H₂₆ 170.34	Practically insoluble or insoluble 1 ml in 1 ml	216 MS 95 %	1.417-1.423 0.746-0.752	
01.039	delta-Elemene		10996 20307-84-0	Liquid C₁₅H₂₄ 204.35	Practically insoluble or insoluble 1 ml in 1 ml	107 (13 hPa) MS 95 %	1.480-1.486 0.856-0.862	
01.042	Germacra-1(10),4(14),5-triene		23986-74-5	Liquid C₁₅H₂₄ 204.35	Practically insoluble or insoluble 1 ml in 1 ml	308 MS 95 %	1.507-1.513 0.896-0.892	
01.043	3,7,10-Humulatriene		11004 6753-98-6	Liquid C₁₅H₂₄ 204.35	Practically insoluble or insoluble 1 ml in 1 ml	123 (13 hPa) MS 95 %	1.499-1.505 0.889-0.895	
01.044	Isolongifolene		1135-66-6	Liquid C₁₅H₂₄ 204.35	Practically insoluble or insoluble 1 ml in 1 ml	120 (16 hPa) MS 95 %	1.495-1.501 0.926-0.932	

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

FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)	Specification comments
01.047	Longifolene		475-20-7	Liquid C$_{15}$H$_{24}$ 204.35	Practically insoluble or insoluble 1 ml in 1 ml	115 (13 hPa) MS 95 %	1.498-1.504 0.929-0.935	
01.049	2-Methylbuta-1,3-diene		78-79-5	Liquid C$_5$H$_8$ 68.12	Practically insoluble or insoluble 1 ml in 1 ml	34 MS 95 %	1.415-1.421 0.677-0.683	
01.050	3-Methylhexane 6)		589-34-4	Liquid C$_7$H$_{16}$ 100.20	Practically insoluble or insoluble 1 ml in 1 ml	92 MS 95 %	1.385-1.391 0.684-0.690	CASrn in Register refers to the racemate.
01.051	2-Methylnaphthalene		11010 91-57-6	Solid C$_{11}$H$_{10}$ 142.20	Practically insoluble or insoluble 1 ml in 1 ml	241 35 MS 95 %	n.a. n.a.	
01.052	alpha-Muurolene		11011 10208-80-7	Liquid C$_{15}$H$_{24}$ 204.35	Practically insoluble or insoluble 1 ml in 1 ml	118 (17 hPa) MS 95 %	1.502-1.508 0.911-0.917	
01.053	Naphthalene		11014 91-20-3	Solid C$_{10}$H$_8$ 128.17	Practically insoluble or insoluble 1 ml in 1 ml	218 80 MS 95 %	n.a. n.a.	
01.054	Pentadecane		629-62-9	Liquid C$_{15}$H$_{32}$ 212.42	Practically insoluble or insoluble 1 ml in 1 ml	270 10 MS 95 %	1.428-1.434 0.765-0.771	
01.055	beta-Phellandrene 6)		11017 555-10-2	Liquid C$_{10}$H$_{16}$ 136.24	Practically insoluble or insoluble 1 ml in 1 ml	174 MS 95 %	1.476-1.482 0.839-0.845	CASrn in Register refers to the racemate.
01.056	alpha-Santalene 6)		512-61-8	Liquid C$_{15}$H$_{24}$ 204.35	Practically insoluble or insoluble 1 ml in 1 ml	112 (9 hPa) MS 95 %	1.480-1.486 0.895-0.901	CASrn in Register refers to the (-)-enantiomer.

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

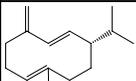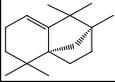| FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)	Specification comments
01.057	Tetradecane		629-59-4	Liquid $C_{14}H_{30}$ 198.39	Practically insoluble or insoluble 1 m l in 1 ml	252 5 MS 95 %	1.422-1.428 0.759-0.765	
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 1 ml in 1 ml	245 MS 95 %	1.519-1.525 0.932-0.938	
01.060	1,1,7-Trimethyltricyclo[2.2.1.0.(2.6)]heptane 6)		508-32-7	Solid $C_{10}H_{16}$ 136.24	Practically insoluble or insoluble 1 ml in 1 ml	152 66 MS 95 %	n.a. n.a.	CASrn in Register refers to the racemate.
01.064	cis-3,7-Dimethyl-1,3,6-octatriene		3338-55-4	Liquid $C_{10}H_{16}$ 136.24	Practically insoluble or insoluble 1 ml 1in 1 ml	177 MS 95 %	1.483-1.489 0.796-0.802	Synonym: cis-beta-ocimene.
01.066	2-Cedrene			Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble 1 ml in 1 ml	82 (11 hPa) MS 95 %	1.495-1.501 0.928-0.934	CASrn 35944-22-0 to be introduced in the Register.
01.067	8(14)-Cedrene			Liquid $C_{15}H_{24}$ 204.35	Practically insoluble or insoluble 1 ml in 1 ml	118 (13 hPa) MS 95 %	1.498-1.504 0.930-0.936	CASrn is lacking.
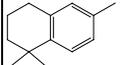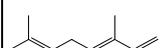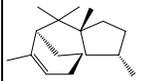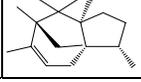
1) Solubility in water, if not otherwise stated.

2) Solubility in 95% ethanol, if not otherwise stated.

3) At 1013.25 hPa, if not otherwise stated.

4) At 20°C, if not otherwise stated.

5) At 25°C, if not otherwise stated.

6) Stereoisomeric composition not specified.

TABLE 2: SUMMARY OF SAFETY EVALUATION APPLYING THE PROCEDURE (BASED ON INTAKES CALCULATED BY THE MSDI APPROACH)

Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

FL-no	EU Register name	Structural formula	MSDI [1) (μg/*capita*/day)	Class [2) Evaluation procedure path [3)	Outcome on the named compound [4) or 5)]	Outcome on the material of commerce [6), 7), or 8)]	Evaluation remarks
01.027	Bisabola-1,8,12-triene		0.024	Class I A3: Intake below threshold	4)	7)	
01.028	beta-Bisabolene		2.7	Class I A3: Intake below threshold	4)	6)	
01.033	2,2-Dimethylhexane		1.2	Class I A3: Intake below threshold	4)	6)	
01.034	2,4-Dimethylhexane		1.2	Class I A3: Intake below threshold	4)	7)	
01.038	Dodecane		0.012	Class I A3: Intake below threshold	4)	6)	
01.039	delta-Elemene		0.012	Class I A3: Intake below threshold	4)	6)	
01.054	Pentadecane		0.61	Class I A3: Intake below threshold	4)	6)	
01.057	Tetradecane		0.012	Class I A3: Intake below threshold	4)	6)	
01.022	alpha-Cedrene		0.012	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.023	1(5),11-Guaiadiene		1.2	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
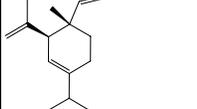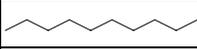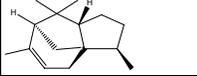
Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

FL-no	EU Register name	Structural formula	MSDI 1) (μg/*capita*/day)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]	Outcome on the material of commerce [6), 7), or 8)]	Evaluation remarks
01.030	beta-Cubebene		0.012	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.032	2,3-Dihydrofarnesene		0.12	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.035	2,6-Dimethylocta-2,4,6-triene		0.013	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.037	Dodec-1-ene		0.024	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.042	Germacra-1(10),4(14),5-triene		0.012	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.043	3,7,10-Humulatriene		1.2	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.044	Isolongifolene		0.012	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.047	Longifolene		0.17	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.050	3-Methylhexane		0.061	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.052	alpha-Muurolene		0.24	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
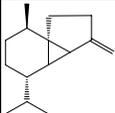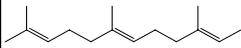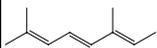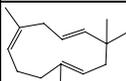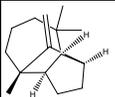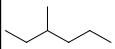
Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach)

FL-no	EU Register name	Structural formula	MSDI 1) (μg/*capita*/day)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5)]	Outcome on the material of commerce [6), 7), or 8)]	Evaluation remarks
01.055	beta-Phellandrene		0.012	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.056	alpha-Santalene		0.012	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.060	1,1,7-Trimethyltricyclo[2.2.1.0.(2.6)]heptane		0.012	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.064	cis-3,7-Dimethyl-1,3,6-octatriene		0.049	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.066	2-Cedrene		0.97	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.067	8(14)-Cedrene		0.012	Class I B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.049	2-Methylbuta-1,3-diene		0.061	Class I No evaluation			a)
01.031	1,2-Dihydro-1,1,6-trimethylnaphthalene		0.0012	Class II B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.058	1,2,3,4-Tetrahydro-1,1,6-trimethylnaphthalene		0.12	Class II B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.036	Diphenylmethane		1.2	Class III B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.051	2-Methylnaphthalene		0.0012	Class III B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
01.053	Naphthalene		0.013	Class III B3: Intake below threshold, B4: No adequate NOAEL	Additional data required		
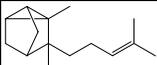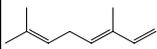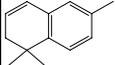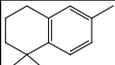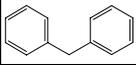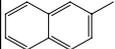
1) *EU MSDI: Amount added to food as flavour in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = μg/capita/day*

2)   *Thresholds of concern: Class I = 1800, Class II = 540, Class III = 90 µg/person/day.*

3)   *Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.*

4)   *No safety concern based on intake calculated by the MSDI approach of the named compound.*

5)   *Data must be available on the substance or closely related substances to perform a safety evaluation.*

6)   *No safety concern at estimated level of intake of the material of commerce meeting the specification of Table 1 (based on intake calculated by the MSDI approach).*

7)   *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.*

8)   *No conclusion can be drawn due to lack of information on the purity of the material of commerce.*

a)   *Due to genotoxic concern the Procedure cannot be applied.*

TABLE 3: SUPPORTING SUBSTANCES SUMMARY (JECFA, 2006A)

Table 3: Supporting Substances Summary (JECFA, 2006a)							
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	JECFA no Specification available	MSDI (EU) 1) (µg/capita/day)	SCF status 2) JECFA status 3) CoE status 4)	Comments
01.003	Pin-2(10)-ene		2903 2114 127-91-3	1330 JECFA specification (JECFA, 2005b)	1323	No safety concern a) Category B b)	
01.004	Pin-2(3)-ene		2902 2113 80-56-8	1329 JECFA specification (JECFA, 2005b)	1836	No safety concern a) Category B b)	
01.005	Terpinolene		3046 2115 586-62-9	1331 JECFA specification (JECFA, 2005b)	656	No safety concern a) Category B b)	
01.006	alpha-Phellandrene		2856 2117 99-83-2	1328 JECFA specification (JECFA, 2005b)	79	No safety concern a) Category B b)	
01.007	beta-Caryophyllene		2252 2118 87-44-5	1324 JECFA specification (JECFA, 2005b)	332	No safety concern a) Category B b)	
01.008	Myrcene		2762 2197 123-35-3	1327 JECFA specification (JECFA, 2005b)	7100	No safety concern a) Category B b)	
01.009	Camphene		2229 2227 79-92-5	1323 JECFA specification (JECFA, 2005b)	14	No safety concern a) Category B b)	
01.014	1-Methylnaphthalene		3193 11009 90-12-0	1335 JECFA specification (JECFA, 2005b)	0.77	No safety concern a)	
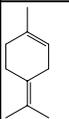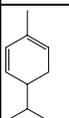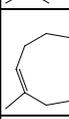
Table 3: Supporting Substances Summary (JECFA, 2006a)							
FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	JECFA no Specification available	MSDI (EU) 1) (µg/capita/day)	SCF status 2) JECFA status 3) CoE status 4)	Comments
01.016	1,4(8),12-Bisabolatriene		3331 10979 495-62-5	1336 JECFA specification (JECFA, 2005b)	13	No safety concern a)	
01.017	Valencene		3443 11030 4630-07-3	1337 JECFA specification (JECFA, 2005b)	53	No safety concern a)	
01.018	beta-Ocimene		3539 11015 13877-91-3	1338 JECFA specification (JECFA, 2005b)	55	No safety concern a)	
01.019	alpha-Terpinene		3558 11023 99-86-5	1339 JECFA specification (JECFA, 2005b)	27	No safety concern a)	
01.020	gamma-Terpinene		3559 11025 99-85-4	1340 JECFA specification (JECFA, 2005b)	1171	No safety concern a)	
01.024	beta-Bourbonene		11931 5208-59-3	1345 JECFA specification (JECFA, 2005b)	ND	No safety concern a)	
01.026	1(5),7(11)-Guaiadiene		88-84-6	1347 JECFA specification (JECFA, 2005b)	0.012	No safety concern a)	
01.029	delta-3-Carene		3821 10983 13466-78-9	1342 JECFA specification (JECFA, 2005b)	ND	No safety concern a)	
01.040	alpha-Farnesene		3839 10998 502-61-4	1343 JECFA specification (JECFA, 2005b)	ND	No safety concern a)	
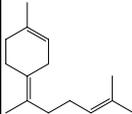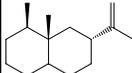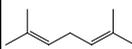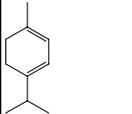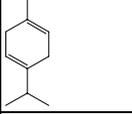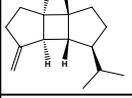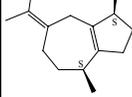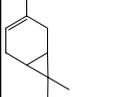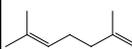
Table 3: Supporting Substances Summary (JECFA, 2006a)

FL-no	EU Register name	Structural formula	FEMA no CoE no CAS no	JECFA no Specification available	MSDI (EU) 1) (μg/capita/day)	SCF status 2) JECFA status 3) CoE status 4)	Comments
01.045	d-Limonene		2633 491 5989-27-5	1326 JECFA specification (JECFA, 2005b)	33542	No safety concern a)	ADI not specified (JECFA, 2006a).
01.061	Undeca-1,3,5-triene		3795 16356-11-9	1341 JECFA specification (JECFA, 2005b)	0.17	No safety concern a)	
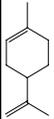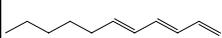
ND) No intake data reported.

1) 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/day.

2) 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.

3) No safety concern at estimated levels of intake.

4) Category A: Flavouring substance, which may be used in foodstuffs, Category B: Flavouring substance which can be used provisionally in foodstuffs.

a) (JECFA, 2005c).

b) (CoE, 1992).

ANNEX I: 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, 2000a), named the "Procedure", is shown in schematic form in Figure I.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, 1996a; JECFA, 1997a; JECFA, 1999b).

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 microgram/person/day, respectively are derived from a large database containing data on subchronic and chronic animal studies (JECFA, 1996a).

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[5] (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[6] (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.

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[5] *"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).*

[6] *"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).*

Procedure for Safety Evaluation of Chemically Defined Flavouring Substances

Step 1.

Decision tree structural class

Step 2.

Can the substance be predicted to be metabolised to innocuous products?

Step A3.   **Step B3.**

Yes   No

Do the conditions of use result in an intake greater than the threshold of concern for the structural class?	Data must be available on the substance or closely related substances to perform a safety evaluation	Do the conditions of use result in an intake greater than the threshold of concern for the structural class?

Yes   Yes

Step A4.   **Step B4.**

Yes   No   No

Is the substance or are its metabolites endogenous?	Substance would not be expected to be of safety concern	Does a NOAEL exist for the substance which provides an adequate margin of safety under conditions of intended use, or does a NOAEL exist for structurally related substances which is high enough to accommodate any perceived difference in toxicity between the substance and the related substances?

Yes   Yes

Step A5.

No   Yes   No

Does a NOAEL exist for the substance which provides an adequate margin of safety under conditions of intended use, or does a NOAEL exist for structurally related substances which is high enough to accommodate any perceived difference in toxicity between the substance and the related substances?	Additional data required

No

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

ANNEX II: USE LEVELS / MTAMDI

II.1 Normal and maximum use levels

For each of the 18 Food categories (Table II.1.1) in which the candidate substances are used, Flavour Industry reports a "normal use level" and a "maximum use level" (EC, 2000a). 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, 2002i). The normal and maximum use levels in different food categories (EC, 2000a) have been extrapolated from figures derived from 12 model flavouring substances (EFFA, 2004e).

Table II.1.1 Food categories according to Commission Regulation (EC) No 1565/2000 (EC, 2000a)	
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 32 candidate substances in the present flavouring group (Table II.1.2).

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

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
01.022	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.023	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.027	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.028	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.030	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.031	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.032	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.033	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.034	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.035	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.036	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.037	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5

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

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
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.038	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.039	7	5	10	7	-	10	4	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.042	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.043	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.044	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	-	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	-	25
01.047	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.049	7	5	10	7	-	10	4	10	2	-	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.050	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.051	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.052	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.053	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.054	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.055	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.056	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.057	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.058	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.060	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.064	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.066	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5	-	20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25	-	100	25
01.067	7	5	10	7	-	10	5	10	2	2	-	-	5	10	5		20	5
	35	25	50	35	-	50	25	50	10	10	-	-	25	50	25		100	25

II.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 consumes the amount of flavourable foods and beverages listed in Table II.2.1. These consumption estimates are then multiplied by the reported use levels in the different food categories and summed up.

Table II.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/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, 2000a) and reported by the Industry in the following way (see Table II.2.2):

- Beverages (SCF, 1995) correspond to food category 14.1 (EC, 2000a)
- 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, 2000a)
- Exception a (SCF, 1995) corresponds to food category 5 and 11 (EC, 2000a)
- Exception b (SCF, 1995) corresponds to food category 15 (EC, 2000a)
- Exception c (SCF, 1995) corresponds to food category 14.2 (EC, 2000a)
- Exception d (SCF, 1995) corresponds to food category 12 (EC, 2000a)
- Exception e (SCF, 1995) corresponds to others, e.g. chewing gum.

Table II.2.2 Distribution of the 18 food categories listed in Commission Regulation (EC) No 1565/2000 (EC, 2000a) 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	Dairy products, excluding products of category 02.0	Food		
02	Fats and oils, and fat emulsions (type water-in-oil)	Food		
03	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	Confectionery			Exception a
06	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery	Food		
07	Bakery wares	Food		
08	Meat and meat products, including poultry and game	Food		
09	Fish and fish products, including molluscs, crustaceans and echinoderms	Food		
10	Eggs and egg products	Food		
11	Sweeteners, including honey			Exception a
12	Salts, spices, soups, sauces, salads, protein products, etc.			Exception d
13	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	Ready-to-eat savouries			Exception b
16	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 II.2.3) are presented for each of the 32 flavouring substances in the present flavouring group, for which Industry has provided use and use levels (EFFA, 2005a; EFFA, 2007a). The mTAMDI values are only given for highest reported normal use levels (see Table II.2.3).

Table II.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)
01.022	alpha-Cedrene	3700	Class I	1800
01.023	1(5),11-Guaiadiene	3700	Class I	1800
01.027	Bisabola-1,8,12-triene	3700	Class I	1800
01.028	beta-Bisabolene	3700	Class I	1800
01.030	beta-Cubebene	3700	Class I	1800
01.032	2,3-Dihydrofarnesene	3700	Class I	1800
01.033	2,2-Dimethylhexane	3700	Class I	1800
01.034	2,4-Dimethylhexane	3700	Class I	1800

Table II.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)
01.035	2,6-Dimethylocta-2,4,6-triene	3700	Class I	1800
01.037	Dodec-1-ene	3700	Class I	1800
01.038	Dodecane	3700	Class I	1800
01.039	delta-Elemene	3700	Class I	1800
01.042	Germacra-1(10),4(14),5-triene	3700	Class I	1800
01.043	3,7,10-Humulatriene	3700	Class I	1800
01.044	Isolongifolene	3300	Class I	1800
01.047	Longifolene	3700	Class I	1800
01.049	2-Methylbuta-1,3-diene	3700	Class I	1800
01.050	3-Methylhexane	3700	Class I	1800
01.052	alpha-Muurolene	3700	Class I	1800
01.054	Pentadecane	3700	Class I	1800
01.055	beta-Phellandrene	3700	Class I	1800
01.056	alpha-Santalene	3700	Class I	1800
01.057	Tetradecane	3700	Class I	1800
01.060	1,1,7-Trimethyltricyclo[2.2.1.0.(2.6)]heptane	3700	Class I	1800
01.064	cis-3,7-Dimethyl-1,3,6-octatriene	3700	Class I	1800
01.066	2-Cedrene	3700	Class I	1800
01.067	8(14)-Cedrene	3700	Class I	1800
01.031	1,2-Dihydro-1,1,6-trimethylnaphthalene	3700	Class II	540
01.058	1,2,3,4-Tetrahydro-1,1,6-trimethylnaphthalene	3700	Class II	540
01.036	Diphenylmethane	3700	Class III	90
01.051	2-Methylnaphthalene	3700	Class III	90
01.053	Naphthalene	3700	Class III	90

ANNEX III: METABOLISM

Table of content

III.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 eight subgroups: I) acyclic saturated hydrocarbons [FL-no: 01.033, 01.034, 01.038, 01.050, 01.054 and 01.057], II) acyclic unsaturated hydrocarbons [FL-no: 01.032, 01.035, 01.037, 01.049 and 01.064], III) cyclohexene hydrocarbons [FL-no: 01.027, 01.028, 01.039 and 01.055], IVa) benzene hydrocarbons [FL-no: 01.031 and 01.058], IVb) naphthalene hydrocarbons [FL-no: 01.051 and 01.053], IVc) diphenylmethane [FL-no: 01.036], V) bi- and tricyclic, non-aromatic hydrocarbons [FL-no: 01.022, 01.023, 01.030, 01.044, 01.047, 01.052, 01.056, 01.060, 01.066 and 01.067] and VI) macrocyclic, non-aromatic hydrocarbons [FL-no: 01.042 and 01.043]. From the evaluation of flavouring substances as carried out by 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 III.1.

Table III.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
01.050	3-Methylhexane		I
01.054	Pentadecane		I
01.057	Tetradecane		I
II: ACYCLIC ALKENES			
01.032	2,3-Dihydrofarnesene		I
01.035	2,6-Dimethylocta-2,4,6-triene		I
01.037	Dodec-1-ene		I
01.049	2-Methylbuta-1,3-diene		I
01.064	cis-3,7-Dimethyl-1,3,6-octatriene		I
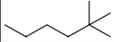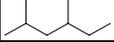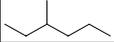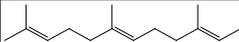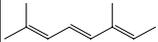
Table III.1 Subgroups. The supporting substances are listed in brackets.

FL-no	EU Register name	Structural formula	Structural class
(01.008)	(Myrcene)		I
(01.018)	(beta-Ocimene)		I
(01.040)	(alpha-Farnesene)		I
(01.061)	(Undeca-1,3,5-triene)		I
III: CYCLOHEXENE HYDROCARBONS			
01.055	beta-Phellandrene		I
01.027	Bisabola-1,8,12-triene		I
01.028	beta-Bisabolene		I
01.039	delta-Elemene		I
(01.005)	(Terpinolene)		I
(01.006)	(alpha-Phellandrene)		I
(01.016)	(1,4(8),12-Bisabolatriene)		I
(01.019)	(alpha-Terpinene)		I
(01.020)	(gamma-Terpinene)		I
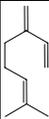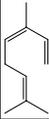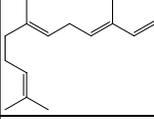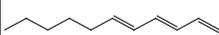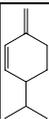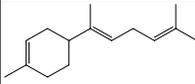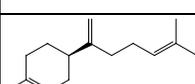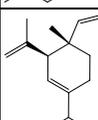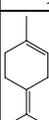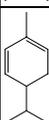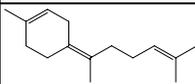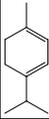
Table III.1 Subgroups. The supporting substances are listed in brackets.			
FL-no	**EU Register name**	**Structural formula**	**Structural class**
(01.045)	(d-Limonene)		I
IV: AROMATIC HYDROCARBONS			
IVa: BENZENE HYDROCARBONS			
01.031	1,2-Dihydro-1,1,6-trimethylnaphthalene		I
01.058	1,2,3,4-Tetrahydro-1,1,6-trimethylnaphthalene		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			
01.022	alpha-Cedrene		I
01.023	1(5),11-Guaiadiene		I
01.030	beta-Cubebene		I
01.044	Isolongifolene		I
01.047	Longifolene		I
01.052	alpha-Muurolene		I
01.056	alpha-Santalene		I
01.060	1,1,7-Trimethyltricyclo[2.2.1.0.(2.6)]heptane		I
01.066	2-Cedrene		I
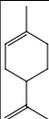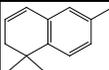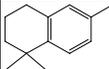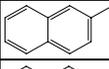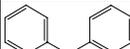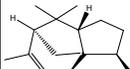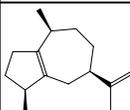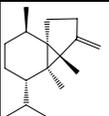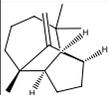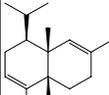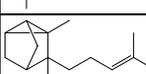
Table III.1 Subgroups. The supporting substances are listed in brackets.

FL-no	EU Register name	Structural formula	Structural class
01.067	8(14)-Cedrene		I
(01.003)	(Pin-2(10)-ene)		I
(01.004)	(Pin-2(3)-ene)		I
(01.007)	(beta-Caryophyllene)		I
(01.009)	(Camphene)		I
(01.017)	(Valencene)		I
(01.024)	(beta-Bourbonene)		I
(01.026)	(1(5),7(11)-Guaiadiene)		I
(01.029)	(delta-3-Carene)		I
VI: MACROCYCLIC, NON-AROMATIC HYDROCARBONS			
01.042	Germacra-1(10),4(14),5-triene		I
01.043	3,7,10-Humulatriene		I
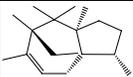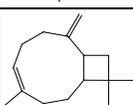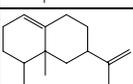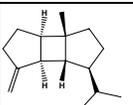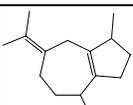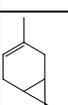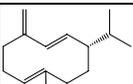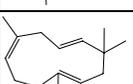
For the majority of the substances in this group no information of biotransformation had been submitted. Also data on structural analogues were scarce. Therefore, an additional search was carried out. Some more papers were retrieved, the information from which has been included as far as relevant in the following text.

III.2   Absorption, Distribution and Excretion

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 (Holmberg et al., 1977; Perbellini et al., 1982; Bahima et al., 1984; Perbellini et al., 1986; Fedtke & Bolt, 1987), 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 et al. (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 Perbellini et al. (1982); Bahima et al., (1984), Perbellini et al. (1986), 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. (1995), but again no quantitative data were provided.

Groups of three or four male F344 rats were exposed to [4-^{14}C] or [5-^{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) 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 %[7]. 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).

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[7] 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

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 B6C3F1 mice, the majority of radioactivity ($\approx$ 54 and 47 %, respectively) was excreted unchanged in the expired air or as urinary metabolites ($\approx$ 32 and 33 %, respectively) over the 24-hour 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 has high as the concentration in blood. Total percentage of the dose recovered was $\approx$ 91 % for both rats and mice (Buckley et al., 1999).

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 two 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)

This subgroup is further divided into three groups, one with benzene derivatives (di- and tetrahydronaphthalenes; IVa), one with naphthalene derivatives (IVb) and one with one candidate substance: diphenylmethane (IVc). For the candidates of subgroup IVb only one supporting substance has been submitted (1-methylnaphthalene), but for both naphthalene and 2-methylnaphthalene many toxicity data are available, and extensive reviews on these substances have been published recently (Shelby & Witt, 1995; EU-RAR, 2003).

Subgroup IVa (benzene derivatives):

No data were available on any of the candidate substances in this group. No supporting substances were suggested.

Subgroup IVb (naphthalene derivatives):

Naphthalene [FL-no: 01.053]:

Bock et al. (1979) reported that naphthalene was rapidly absorbed from isolated rat intestinal loops *in situ*, predominantly unchanged, into the portal blood. Approximately 84 % of the naphthalene related substances in the blood was naphthalene, the remainder were metabolites; mainly naphthalene-1,2-dihydrodiol and 1-naphthol, 40 % of which was recovered as conjugates; predominantly with glucuronic acid (Bock et al., 1979).

Three groups of Sprague-Dawley rats were treated orally with a single administration of 2 mg ^{14}C-naphthalene. The dose per unit body weight was not stated (Bakke et al., 1985). One group of 16 animals was bile duct cannulated, the second group of four germ-free animals was uncannulated, and the third group of 13 animals was standard uncannulated rats. Urine, faeces and bile were collected for 72 hours after administration of naphthalene. In the standard uncannulated rats 75.6 % of the radioactivity was recovered in the urine in 24 hours. At 72 hours, approximately 83 % of the radioactivity had been recovered in the urine, 6 % in the faeces and 4 % remained in the carcass with no account made for the remaining radioactivity. In the cannulated rats the 24-hour urine and bile contained 30 % and 66.8 % of the ^{14}C dose, respectively (faecal data not presented for 24-hour time period). At 72 hours, approximately 30 % of the radioactivity had been recovered in the urine, 68 % was contained in the bile, less than 1 % was excreted in the faeces and 0.2 % remained in the carcass.

When a single dose of 20 mg/kg bw of naphthalene-[ring-U-^{3}H] was administered intraperitoneally in olive oil to outbred male IMP:Wist rats (n = 54), urine and faeces were the main routes of

elimination of the radioactivity with more than 88 % of the radioactivity excreted during the first 72 hours. The maximum level of radioactivity in plasma was observed at two hours post-dosing followed by biphasic clearance ($t_{1/2}$ = 0.8 and 99 hours for phase I and phase II, respectively). The highest concentrations during the first hour following dose administration were detected in fat, liver and kidneys, with a gradual decline of radioactivity in all tissues examined (Kilanowicz et al., 1999).

Male Sprague-Dawley rats were given [1-^{14}C] naphthalene (100 mg/kg) via i.p. injection. Of the administered dose, 20-30 % was excreted in the 0-24-hour urine, while 3-11 % was found in the 24-48-hour urine. Unconjugated metabolites accounted for 5-20 % of the metabolites excreted, while 80-95 % of the metabolites were excreted, as acidic conjugates (Horning et al., 1980).

Urinary excretion of premercapturic acids and mercapturic acids represents a major excretory pathway for naphthalene in mice and rats (Stillwell et al., 1978; Chen & Dorough, 1979; Pakenham et al., 2002). Urine was collected for 24 hours after intraperitoneal administration of 0, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 mg/kg radiolabelled [^{14}C]-naphthalene dissolved in corn oil to male Sprague-Dawley rats and Swiss Webster mice, four animals in each group. The percentage of the administered dose excreted as mercapturic acids in the urine for mice (25–34 %) and rats (24–35 %) did not differ with increasing dose (Pakenham et al., 2002). Studies with chimpanzees (Summer et al., 1979b) and with rhesus monkeys (Rozman et al., 1982) indicated that urinary excretion of mercapturic acids is probably less important in primates. Boyland and Sims reported that only traces of mercapturic acids were detected in the urine of three human subjects after oral administration of 500 mg naphthalene, while for rats, mice, hamsters and rabbits, 1-naphthyl-(pre)mercapturic acid was abundantly present in the urine (Boyland & Sims, 1958).

2-Methylnaphthalene [FL-no: 01.051]:

Following intraperitoneal injection of C57BL/6J mice with a single 400 mg/kg dose of [8-^{14}C]-2-methylnaphthalene, the majority of radioactivity was distributed in fat, liver, kidney and lung, in order of decreasing concentration. Maximal plasma and tissue levels were achieved one (liver), two (plasma; fat) or four (kidneys) hours after dose administration and the $t_{1/2}$ of elimination of radioactivity from blood was approximately three hours (Griffin et al., 1982).

When 10 mg/kg bw 2-[1-^{3}H]-methylnaphthalene was orally administered to guinea pigs, the highest concentrations of radioactivity were found in the gall bladder, kidneys and liver three hours after dosing. Lower concentrations were observed in the blood, brain, heart, lung and spleen. At that time the total gastrointestinal tract contained 27 % of the dose while the urine contained 23 %. At 24 hours only some retention was observed in the liver, gall bladder and kidney, with 11 % in faeces and 79 % in the urine. After 48hours 12 % was recovered from faeces and 72 % from the urine, and 1 % of the dose was recovered from the tissues investigated (Teshima et al., 1983).

Subgroup IVc (diphenylmethane):

After intraperitoneal administration of the candidate flavouring substance diphenylmethane [FL-no: 01.036], metabolites have been shown in urine and faeces. Only a small fraction of the dose (up to 6.8 %) was recovered after 24 hours (Deloach et al., 1978; Stocklinski et al., 1979). The studies were too incomplete to estimate if total elimination is slow, or that this apparently slow elimination is indicated because quantitatively important metabolites were not studied.

Bi- and Tricyclic 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 the candidate substance longifolene [FL-no: 01.047] and on several supporting substances including alpha- and beta-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 (+)-alpha-pinene, (-)-alpha-pinene, (±)-alpha-pinene, (-)-beta-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 alpha-pinene [FL-no: 01.004], beta-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 alpha-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 (-)-alpha-pinene or delta-3-carene in air at 225 and 450 mg/m^3 for 2 hours while doing light physical exercise confirmed that uptake was rapid and high for these agents (58-60 % for (+)- and (-)-alpha-pinene and 70 % for delta-3-carene), and that they were metabolised rapidly. Elimination followed a triphasic pattern, with (+)- and (-)-alpha-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 alpha-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 alpha-pinene or delta-3-carene was eliminated unchanged in the urine, while 7.5-7.8 % and 3 % of the inhaled amount of the alpha-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 alpha-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 alpha-pinene was 0.775 mg/m^3. The mean pre-exposure blood concentration of alpha-pinene of 0.035 microg/l increased to an average concentration of 1.9 microg/l during the 4-hour exposure (50-240 minutes). Thereafter (330-450 minutes), the mean blood concentration decreased to 0.15 microg/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-hour exposure. Plasma elimination for alpha-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 & Prah, 1997).

In the urine of sawmill workers exposed to an atmosphere containing 31-210 mg/m3 alpha-pinene [FL-no: 01.004], 2-17 mg/m^3 beta-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 alpha-pinene (Eriksson & Levin, 1990). Analysis of urinary metabolites eliminated by human volunteers within 4 hours following a 2-hour exposure to 10 - 450 mg alpha-

pinene/m^3 revealed that alpha-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 8-9 hours work shift or from chamber-exposed individuals. Following hydrolysis of glucuronic acid conjugates, several pinene biotransformation products were found (Eriksson & Levin, 1996).

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

No data were submitted on any of the two candidate substances in this subgroup [FL-no: 01.042 and 01.043]. No supporting substances were identified.

III.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 beta-myrcene [FL-no: 01.008] and 2-methylbuta-1,3-diene [FL-no: 01.049]. 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 (beta-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 beta-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 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 beta-phellandrene, beta-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.

No data are available on the absorption, distribution of elimination of any candidate or supporting substance in subgroup IVa (benzene hydrocarbons). There are several data on the candidate substances in subgroup IVb (naphthalene hydrocarbons). The data show that naphthalene [FL-no: 01.053] will be absorbed after oral administration and will be eliminated predominantly via the urine, in the form of various metabolites, conjugated with e.g. glucuronic acid or with glutathione. In bile duct cannulated animals, considerable elimination into the bile was also observed, but as in intact animals urinary elimination exceeds by far faecal excretion, the material eliminated via the bile is reabsorbed. The glucuronide conjugate of 1-naphthol represents a significant urinary metabolite of naphthalene. Glutathione conjugates of naphthalene are catabolised to (pre)mercapturic and mercapturic acids, particularly in rodents, before excretion in bile or urine. In primates and humans, conjugation with glutathione seems to be less important. Less data are available for 2-methylnaphthalene [FL-no: 01.051], but the available data show that 2-methylnaphthalene is also absorbed from the gastrointestinal tract and eliminated predominantly via the urine. For both substances the data indicate that 85–89 % of the dose is excreted within three days after dosing. With respect to the one candidate substance (diphenylmethane [FL-no: 01.036]) in subgroup IVc, it may be concluded that the metabolites of this substance will be eliminated from the body after intraperitoneal injection. No conclusions can be made on absorption from the gastrointestinal tract and the rate of elimination as any oral ADME studies and mass-balance data are available.

For the substances in subgroup V (bi- and tricyclic non-aromatic hydrocarbons), the available data from oral studies are few. These studies have only addressed the excretion of some supporting and one candidate 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 alpha- and beta-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.

No data were submitted on candidate or supporting substances in subgroup VI (macrocyclic non-aromatic hydrocarbons). The two substances in this group have a molecular weight of ~ 204. They are also rather lipophilic (estimated log Kow are 4.6 and 7.0 for [FL-no: 01.042] and [FL-no: 01.043], respectively). Based on these data it may be assumed that these two candidate substances will be absorbed from the gastrointestinal tract. For humulatriene, given the rather high Kow, the extent of absorption may be relatively low.

III.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/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 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/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) in the urine 2- and 3-heptanol were found. 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*, 1600-2000 g) were subjected to single inhalatory treatments of 6, 12 and 24 hours to 5000 ppm (~ 18 000 mg/m^3) *n*-hexane. 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 ten 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 & 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 & Bolt, 1987).

Groups of three or four male F344 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 Annex. 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 1 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 beta-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, 2004e). 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 is 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 [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 beta-myrcene ([FL-no: 01.008]; a supporting substance) and candidate substance 2-methylbuta-1,3-diene [FL-no: 01.049]. Some additional information on the related industrial chemical 1,3-butadiene has also been added. One of the substances in this group (dodec-1-ene; [FL-no: 01.037]) bears a terminal double bond without any other structural features (e.g. hydroxyl groups, methyl substituents). No further data for this or similar substances are available. The metabolic aspects of terminal double bond substances have been discussed in FGE.07 and FGE.18 (EFSA, 2004e; EFSA, 2006b). The relevant parts of these FGEs will also be included in the current discussion.

Beta-Myrcene [FL-no: 01.008]

In the urine of rabbits, orally administered beta-myrcene via gavage (670 mg/kg bw/d 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 beta-myrcene metabolites has been presented in Figure III.1.
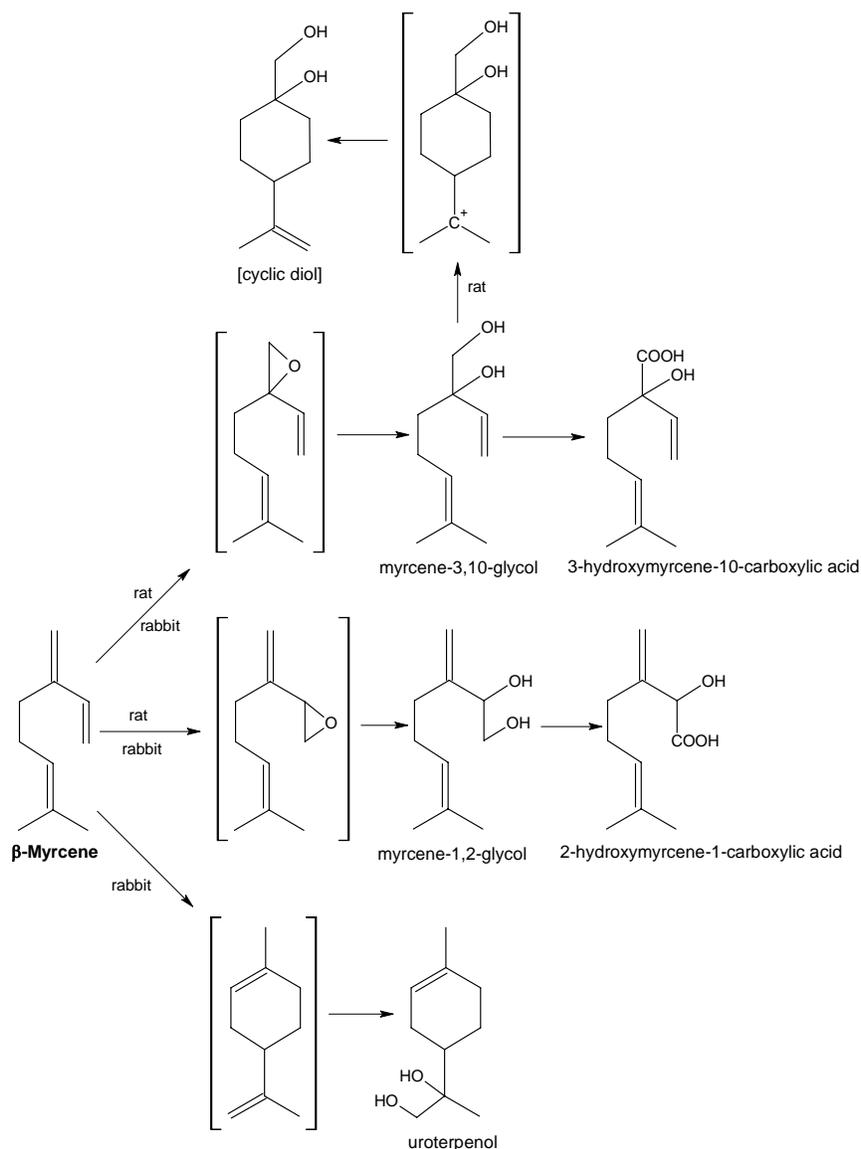
Figure III.1. *Metabolism of beta-myrcene in rats and rabbits.*

When rats were administered 800 mg/kg bw per day of beta-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 & Srivatsan, 1987). It was demonstrated that the biotransformation of beta-myrcene was cytochrome P450 (CYP)-mediated and that it could be enhanced by pretreatment of animals with phenobarbital (Madyastha & 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/d orally for three days), especially those from the CYP2B (phenobarbital-inducible) subfamily (De-Oliveira et al., 1997a). At lower dose levels (40 mg/kg bw/d; intraperitoneally for three days) such induction was not observed (Austin et al., 1988).

2-Methylbuta-1,3-diene [FL- no: 01.049]

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/h 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/(h × kg) in rats and 0.4 micromol/(h × 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/(h × kg) in rats and 0.3 micromol/(h × 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 III.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 3,4-epoxy-3-methyl-1-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/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-hour 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 III.2) in the rat (Buckley et al., 1999).

Following intraperitoneal injection of 64 mg [4-^{14}C]-2-methylbuta-1,3-diene /kg to B6C3F1 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-hour 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.
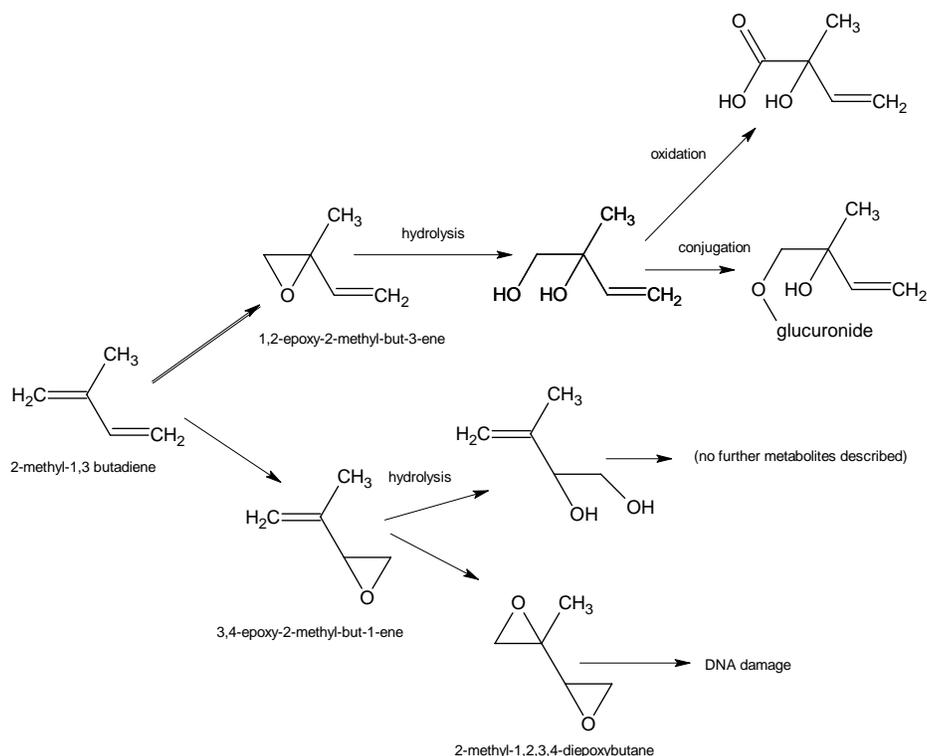
Figure III.2. *Major metabolic pathways for 2-methylbuta-1,3-diene (after Del Monte et al. (1985) and Buckley et al. (1999)).*

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 substance dodec-1-ene [FL-no: 01.037].

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

Conclusions on the metabolism of subgroup II substances

The data available on the metabolism of one supporting and one candidate 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 beta-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 beta-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 beta-myrcene. With beta-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 candidate 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 alpha-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 beta-myrcene and 2-methylbuta-1,3-diene, terminal double bonds appear also in candidate substances [FL-no: 01.037 and 01.064]. 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, 01.049 and 01.064] 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-beta-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 III.3.

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., 1994a). 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 4-6 hours after administration, with the exception of limonene-8,9-diol, which reached its peak level one hour after administration (Poon et al., 1996). 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., 1996).
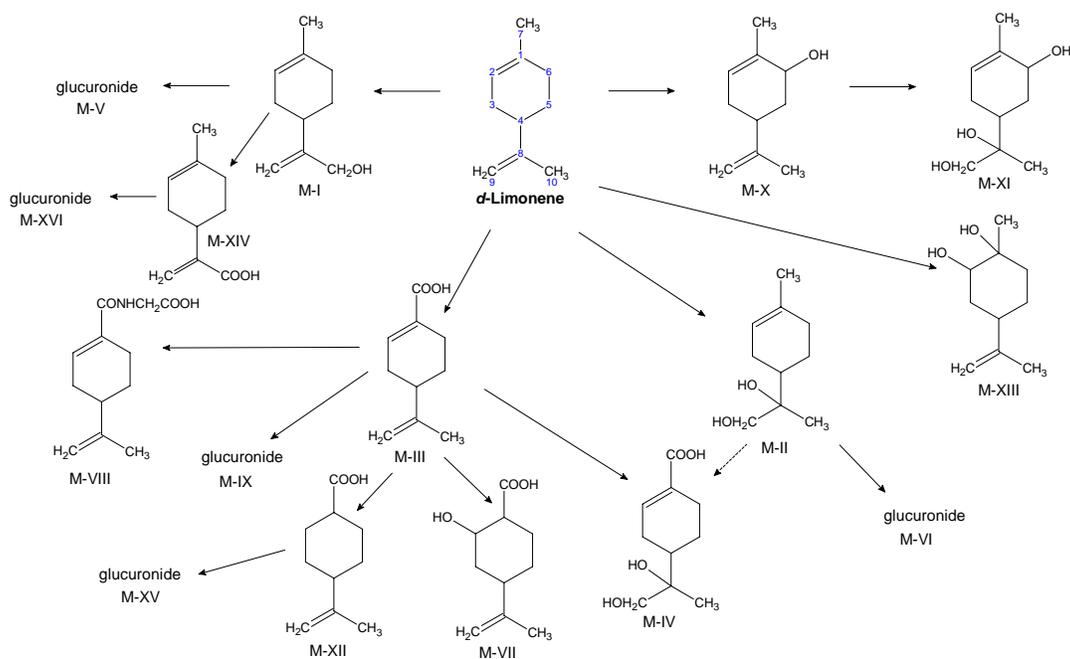
M-I	limonene-10-ol	M-IX	glucuronide conjugate of M-III
M-II	limonene-8,9-diol	M-X	limonene-6-ol
M-III	perillic acid	M-XI	*p*-menth-1-ene-6,8,9-triol
M-IV	perillic acid-8,9-diol	M-XII	dihydroperillic acid

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 III.3. *Metabolism of d-limonene.*

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-1-ene 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*-limonene into 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*-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 (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, resulting in alcohols and/or carboxylic acids, which are, at least partly, conjugated and eliminated with the urine. It is anticipated that three out of the four 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 beta-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)

Subgroup IVa: Benzene Hydrocarbons

No data are available for the candidate substances in this group. Some information was available on the biotransformation of *p*-cymene ([FL-no: 01.002]; *p*-isopropyl toluene). Given the structure of this substance, which deviates considerably from the candidate substances in this subgroup, the information provided was considered to be only of limited relevance for the evaluation of the benzene hydrocarbons in this group. In general, the studies indicate that in various animal species *p*-cymene undergoes extensive oxidation of the methyl and isopropyl side chain to yield polar oxygenated metabolites. These metabolites are either excreted unchanged in the urine or undergo phase II conjugation with glucuronic acid and/or glycine, followed by excretion in the urine (Bakke & Scheline, 1970; Walde et al., 1983; Matsumoto et al., 1992; Boyle et al., 1999). Only in the guinea pig ring hydroxylation products (carvacrol and hydroxycarvacrol) were found in minute amounts. Ring hydroxylation in guinea pigs only occurred *ortho* to the methyl group (Walde et al., 1983).

Subgroup IVb: Naphthalene Hydrocarbons

Naphthalene [FL-no: 01.053]

Metabolism of naphthalene (0.031, 0.062, 0.125, 0.25, 0.50 and 1.0 mM) was studied in lung and liver microsomal preparations from Swiss Webster mice, Sprague-Dawley rats, Sprague-Dawley hamsters and rhesus macaque monkeys. Naphthalene metabolism to glutathione conjugates and diols was 4 to 8-fold higher in mouse lung microsomal incubations than in corresponding incubations from hamster and rat lung. Mice lung had a high degree of stereoselectivity in metabolism of naphthalene to naphthalene epoxide, where the ratio of 1*R*,2*S*- to 1*S*,2*R*-naphthalene epoxide was 11 to 14:1. Little or no stereoselectivity in the metabolism of naphthalene was found in the mouse liver or in the lung and liver from the rat, hamster and monkey. The mouse liver metabolises more of the naphthalene to diol than the mouse lung, 12 % and 5 % of the total metabolites, respectively. Naphthalene is metabolised in lung tissue microsomal incubations of rhesus monkeys at a rate 100-fold less than mice and 10-fold less than rats. Naphthalene is metabolised in lung tissue microsomal incubations of rhesus monkeys at a rate 100-fold less than mice and 10-fold less than rats. In the primate liver microsomes naphthalene diols comprise 80 % of the total metabolites produced (Buckpitt et al., 1992). The olfactory epithelium contained the greatest amount of CYP protein of all tissues investigated in the rat. However, CYP2F expression levels in primate nasal tissues demonstrate that only the nasal ethmoturbinates contained quantifiable amounts of CYP2F with the levels in primates roughly 10- and 20-fold less than the corresponding tissues in rats and mice, respectively (Baldwin et al., 2004). Clearly, humans would produce less naphthalene-1,2-oxide or produce it at a slower rate than rats or mice in respiratory tract tissues. An overview of naphthalene metabolism is presented in Figure III.4.

Three groups of Sprague-Dawley rats were treated orally with a single administration of 2 mg ^{14}C-naphthalene. The dose/unit body weight was not stated (Bakke et al., 1985). One group of 16 animals were bile duct cannulated, the second group of four germ-free animals were uncannulated, and the third group of 13 animals were standard uncannulated rats. Urine, faeces and bile were collected for 72 hours after administration of naphthalene and the metabolites were identified and quantified. In the standard uncannulated rats the urinary metabolites identified at 24 hours were an N-acetyl cysteinyl naphthalene conjugate (38.1 % of the administered radioactivity), 1,2-dihydro 1,2-dihydroxynaphthalene (dihydrodiol) glucuronide (23.9 %), dihydroxynaphthalene (4.9 %),

naphthols and naphthol glucuronides (4.6 %) and 1,2-dihydro-1-hydroxy-2-thionaphthalene glucuronide [CH_3S-metabolite] (4.6 %). At 24 hours in cannulated rats, 14.4 % of the administered dose was present in the urine as N-acetyl cysteinyl naphthalene conjugate and 14.5 % as the dihydroxynaphthalene glucuronide conjugate. Naphthols, thionaphthols, CH_3S-metabolites or their respective glucuronides and sulphates were not detected in either the 24-hour urine or bile. The major urinary metabolites of germ-free rats were N-acetyl cysteinyl naphthalene (89 %) and dihydroxynaphthalene glucuronide (4 %). Only trace amounts of naphthols and no CH_3S-metabolites could be detected indicating that intestinal microflora are probably involved in production of these metabolites followed by enterohepatic recirculation. *In vitro* experiments showed that 1,2-dihydroxy-1,2-dihydronaphthalene may be transformed by isolated rat liver dihydrodiol dehydrogenase to form the 1,2-naphthoquinone, which reacts rapidly with glutathione or cysteine (Smithgall et al., 1986; Smithgall et al., 1988). Naphthalene-1,2-dihydrodiol may be oxidised further by CYP enzymes, resulting in an epoxide, 1,2-dihydroxy-3,4-epoxy-1,2,3,4-tetrahydronaphthalene. Several urinary metabolites, including a number of trihydroxytetrahydromethylthio-derivatives (Horning et al., 1980) and trihydroxytetrahydromercapturic acid (Pakenham et al., 2002) provide support for the *in vivo* formation of 1,2-dihydroxy-3,4-epoxy-1,2,3,4-tetrahydronaphthalene. Initially, naphthalene metabolism involves epoxidation of the aromatic ring by cytochrome (CYP) enzymes yielding naphthalene-1,2-oxide. Several studies have shown that a number of CYP isozymes catalyse this reaction, including CYP2E1 (Wilson et al., 1996) and CYP2F (Shultz et al., 1999; Buckpitt et al., 2002).

Limited information is available regarding the metabolism of naphthalene in humans. Incubation of human lung microsomal fractions demonstrated naphthalene metabolism to naphthalene dihydrodiol and glutathione conjugates (Buckpitt & Bahnson, 1986). Human and mouse liver microsomes exposed to naphthalene produced the 1-naphthol and naphthalene-1,2-dihydrodiol metabolites in the ratio 8.6 and 0.4 in human and in phenobarbital-induced mouse microsomes, respectively (Tingle et al., 1993). In human liver microsomes naphthalene-1,2-dihydrodiol is formed following epoxidation and hydrolysis, whereas rearrangement of the 1,2-epoxide leads to the formation of 1-naphthol (Tingle et al., 1993). Preferential formation of the 1*S*,2*R*-naphthalene oxide enantiomer in human and rat microsomes provides evidence that human metabolism of naphthalene may be more similar to rats than mice (Lanza et al., 1999). Naphthalene metabolising enzymes have been demonstrated in nasal respiratory epithelial and olfactory epithelial tissue from rodents and humans (Buckpitt et al., 1992; Plopper et al., 1992; Thornton-Manning & Dahl, 1997; Buckpitt et al., 2002).

Waidyanatha et al. (2002) have studied covalent binding of naphthalene metabolites to haemoglobin and albumin *in vivo*. They measured cysteinyl adducts of naphthalene-1,2-oxide and 1,2- and 1,4-naphthoquinone in rats, 24 hours after dosing them with 100 - 800 mg naphthalene/kg bw. Cysteinyl adducts with all three metabolites were produced in a dose-dependent manner. Naphthalene-1,2-oxide produced adducts with both proteins and at dose levels > 200 mg/kg bw the formation of adducts became saturated. For 1,2-naphthoquinone-albumin adducts were more abundant than for 1,4-naphthoquinone. Differences for the haemoglobin adducts were much less pronounced. Total naphthoquinone adducts with albumin were also produced in larger amounts than with haemoglobin. Naphthoquinone but not naphthalene-1,2-oxide adducts could also be detected in albumin and heamoglobin of control animals (Waidyanatha et al., 2002).

Urine samples were collected from an infant hospitalised because of haemolytic anaemia following ingestion of unknown quantities of naphthalene mothballs. 1- and 2-naphthol and 1,2- and 1,4-naphthoquinone were identified in the samples for up to eight days of hospitalisation. 1-Naphthol was present in the largest, unspecified quantities (Mackell et al., 1951, as cited in EU-RAR, 2003).

It can be concluded that naphthalene [FL-no: 01.53] is metabolised by cytochrome P450 enzymes, yielding naphthalene-1,2-oxide. This epoxide may be metabolised by glutathione S-transferase enzymes to a glutathione conjugate that is subsequently converted into a mercapturic acid conjugate and excreted in the urine. Naphthalene-1,2-oxide may also rearrange to yield 1- or 2-naphthol, which are excreted as glucuronide or sulphate conjugates in the urine. In addition, 1-naphthol may be further metabolised by CYP enzymes to 1,2- or 1,4-naphthoquinone, and 2-naphthol may be further oxidised to give also 1,2-naphthoquinone. Naphthalene-1,2-oxide may be hydrated by epoxide hydrolase, yielding 1,2-dihydroxy-1,2-dihydronaphthalene, which is oxidised by dihydrodiol dehydrogenase also to form 1,2-naphthoquinone. CYP enzymes may oxidise naphthalene-1,2-dihydrodiol to yield 1,2-dihydroxy-3,4-epoxy-1,2,3,4- tetrahydronaphthalene, which can be further metabolised by a number of conjugation pathways.
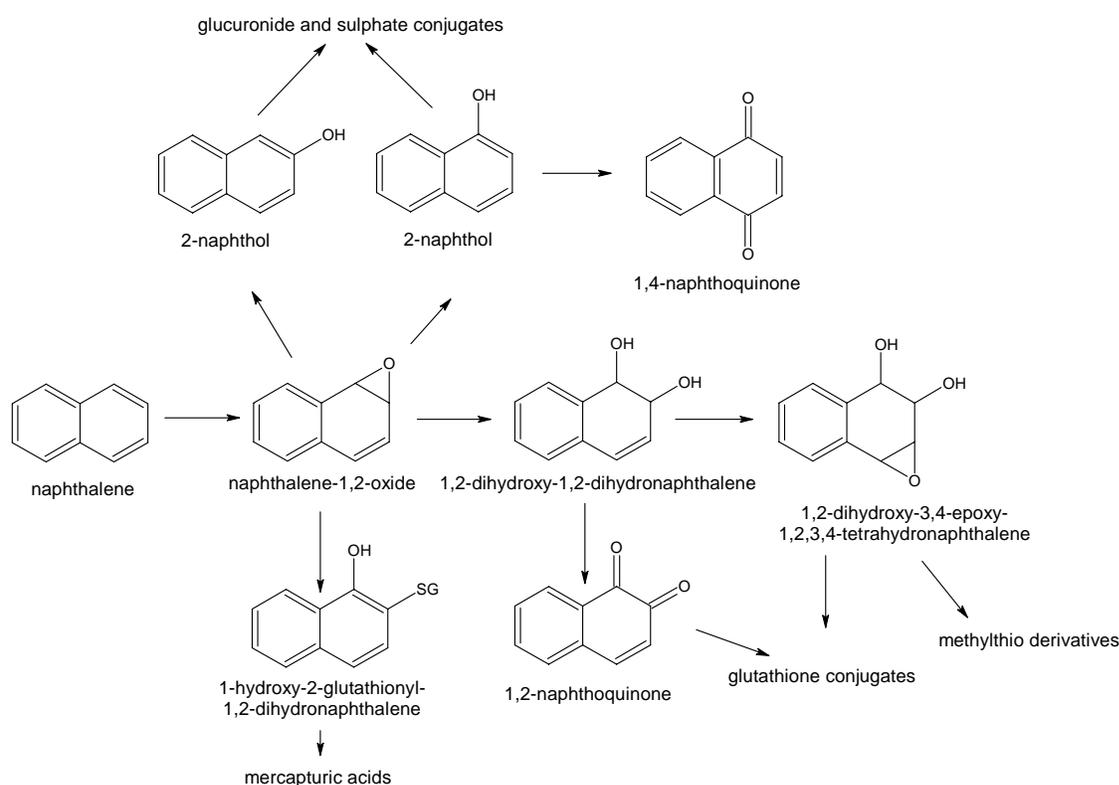
Figure III.4. *Pathways for metabolism of naphthalene (adapted from ATSDR, 2005).*

2-methylnaphthalene [FL-no: 01.051]

The oxidative metabolism of 2-methylnaphthalene was investigated using rat liver microsome suspensions. One monohydroxylated (2-hydroxymethylnaphthalene) and three isomeric dihydrodiol (3,4-dihydrodiol, 5,6-dihydrodiol and 7,8-dihydrodiol) metabolites of 2-methylnaphthalene were identified (Breger et al., 1983). Melancon et al. (1985) confirmed that one 2-hydroxymethyl- and three isomeric dihydrodiol metabolites of 2-methylnaphthalene are formed *in vitro* by rat hepatic microsomes and purified cytochromes (Melancon et al., 1985).

Four female rats were dosed with two subcutaneous injections of 300 mg 2-methyl-[8-^{14}C]-naphthalene/kg bw and urine was collected for three days. Of the radioactivity injected, 55 % was found in the urine and only 3-5 % (*ca.* 2.2 % of the dose) appeared to be unchanged parent compound. Three isomeric dihydrodiol metabolites of 2-methylnaphthalene represented 6-8 % of urine [14]C (*ca.* 3.8 % of the dose). More polar metabolites were identified as 2-naphthoic acid and 2-naphthoylglycine, the latter of which accounted for 30-35 % of urine [14]C (*ca.* 17.5 % of the dose). The remainder of the urinary radioactivity was not fully elucidated but 6-8 % were other conjugates of naphthoic acid (*ca* 3.8 % of the dose), and some were identified as hydrolysable with beta-glucuronidase (not yielding naphthoic acid) (Melancon et al., 1982).

In guinea pigs, when 10 mg/kg bw 2-[1-^{3}H]-methylnaphthalene was orally administered the major metabolites excreted in the urine at 24 h (in total 23 % of the dose) were oxidation products of the methyl group (i.e. 2-naphthoic acid (4 %), 2-naphthoylglucuronic acid (11 %), and 2-naphthoylglycine (61 %)), which accounted for 76 % of the total urinary radioactivity. Dihydrodiols could not be detected, but S-(7-methyl-1-naphthyl)cysteine (10 %), and glucuronic acid and sulphate conjugates of 7-methyl-1-naphthol (each 4 %) were also identified as minor metabolites accounting together for 18 % of the total urinary radioactivity. In incubations of 2-methylnaphthalene with guinea pig liver S9, formation of S-(7-methyl-1-naphthyl)-glutathione could be demonstrated, and 4 hours after oral administration of 500 mg 2-methylnaphthalene/kg bw GSH depletion in the liver was observed, but no mercapturic acids could be found in the urine. It was speculated that the cysteine conjugate might be an artificial breakdown product of mercapturic acid derivatives (Teshima et al., 1983). Other authors have also demonstrated that 2-methylnaphthalene can deplete liver GSH stores (e.g. Griffin et al., 1982). This depletion has been brought into connection with the toxicity of 2-methylnaphthalene (Shelby & Witt, 1995).

Griffin et al. (1982) have studied the relationship between 2-methylnaphthalene (400 mg/kg bw intraperitoneally) -induced lung toxicity and GSH depletion in the lung, liver and kidneys in the mouse in relationship to 2-methylnaphthalene covalent tissue binding and metabolic conversion into dihydrodiol metabolites, using modifiers of cytochrome P450 activity. Both phenobarbital (PB) and 3-methylcholanthrene (3MC) reduced lung toxicity of 2-methylnaphthalene, but only PB reduced pulmonary covalent binding. In the liver, neither PB nor 3MC had an effect on covalent binding of 2-methylnaphthalene. In liver microsomes, PB increased formation of dihydrodiols, but it had no effect in the lung, whereas 3MC reduced formation of dihydrodiols in lung but not in liver microsomes. Piperonyl butoxide decreased covalent binding in the lung, liver and kidney, but did not affect lung toxicity. SKF-525A had also no effect on pulmonary necrosis. Diethyl maleate had no effect on binding in the liver, but it decreased binding in the lung and kidney. It also did not affect the severity of the pulmonary toxicity, but it enhanced the lethality of 2-methylnaphthalene. Pretreatment of mice with piperonyl butoxide or diethyl maleate did not affect the hepatic or pulmonary microsomal conversion of 2-methylnaphthalene into dihydrodiols. It was also shown that 2-methylnaphthalene strongly decrease liver GSH levels, but not GSH levels in the lung or kidney. The authors concluded that the pulmonary toxicity of 2-methylnaphthalene may be related to metabolism, but that the role of the dihydrodiols or covalent binding as indicators of activity of epoxide intermediates *in vivo* is as yet uncertain (Griffin et al., 1982).

Subgroup IVc: Diphenylmethane

The biotransformation of diphenylmethane [FL-no: 01.036] was studied in microsomal fractions prepared from rat liver homogenate. It was determined that diphenylmethane is hydroxylated at both the aromatic (para-) and aliphatic positions of the molecule. If hydroxylation of the methylene group occurs, the resulting diphenylmethanol is further oxidised to diphenylketone (Sípal & Zelingerová, 1978).

Four male rats were administered 900 mg/kg bw of diphenylmethane via intraperitoneal injection. Animals were housed in individual metabolism cages and provided food and water *ad libitum*. Urine and faecal samples were collected after 24 hours and analysed immediately. The urine and faecal samples indicated that both free and conjugated forms of *p*-hydroxydiphenylmethane are eliminated with 88 % of the recovered material (in total *ca.* 6.8 % of the dose) being found in the urine (Deloach et al., 1978). The major part of the urinary *p*-hydroxyphenylmethane was excreted as unconjugated material. In a subsequent experiment by the same investigators, six male rats were administered 500 mg/kg bw of diphenylmethane via intraperitoneal injection. Animals were housed in individual metabolism cages and provided food and water *ad libitum*. Urine samples were collected after 24 hours and analysed immediately. The urine samples indicated that conjugated benzhydrol (= diphenyl methanol) is eliminated by rats at 0.6% of the total dose of diphenylmethane administered free benzhydrol is not eliminated. The authors indicated that others have found that benzhydrol is excreted as glucuronide (Stocklinski et al., 1979).

Conclusions on the metabolism of subgroup IV substances

No data are available for the two substances in subgroup IVa [FL-no: 01.031 and 01.058]. Based on information available on *p*-cymene, it may be anticipated that these substances may be oxidised at the ring-substituent methyl groups (resulting in the formation of hydroxymethyl groups). These products may subsequently be excreted after conjugation. Also epoxidation of the 3,4-double bond in [FL-no: 01.031] may be expected. Oxidative attack on the aromatic ring seems less likely. Nevertheless, due to the lack of experimental data it would be impossible to conclude that these substances are metabolised into innocuous products.

Several data are available on the biotransformation of the naphthalene [FL-no: 01.053] and its 2-methyl derivative [FL-no: 01.051] in subgroup IVb. With naphthalene, the primary products resulting from epoxidation can be further metabolised to naphtoquinones and other metabolites, which have been brought into connection with the toxicity of this substance. Whereas epoxidation is the main metabolic option for naphthalene, methyl group oxidation is the major metabolic pathway in the case of 2-methylnaphthalene. However, also with 2-methylnaphthalene, epoxidation may occur to some extent, and 2-methylnaphthalene-induced depletion of glutathione stores, possibly as a result of conjugation, has been suggested to be related to epoxidation reactions. However, although biotransformation does play a role in the toxicity of 2-methylnaphthalene, the exact mechanism of toxicity and the metabolites which are involved have not been clearly identified. Hence, neither for naphthalene [FL-no: 01.053] nor for 2-methylnaphthalene [FL-no: 01.051] can it be concluded that these are metabolised into innocuous substances.

For the one candidate substance in subgroup IVc (diphenylmethane [FL-no: 01.036]) only a very limited amount of metabolites have been identified in the urine. One of these is an aromatic hydroxylation product (i.e. a phenol derivative), which may be generated from epoxidation. Because of lack of further data it cannot be concluded that diphenylmethane [FL-no: 01.036] is metabolised to innocuous products.

Bi- and Tricyclic 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 (+)-alpha-pinene/m^3 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 hour

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 III.5) (Eriksson & Levin, 1990).

Analysis of the urinary metabolites of a patient attempting suicide with 400 – 500 ml pine oil containing 57 % alpha-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 (+)-alpha-pinene, (-)-alpha-pinene, (+/-)-alpha-pinene, (-)-beta-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 alpha stereochemical forms of pinene was *trans-*verbenol (see Figure III.5). As a minor pathway, allylic oxidation of the exocyclic methyl group to yield myrtenol was observed for all three alpha-pinene stereoisomers, with also myrtenic acid as minor metabolite (Ishida et al., 1981).

The presence of an exocyclic alkene function in (-)-beta-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 (-)-alpha-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 alpha-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).
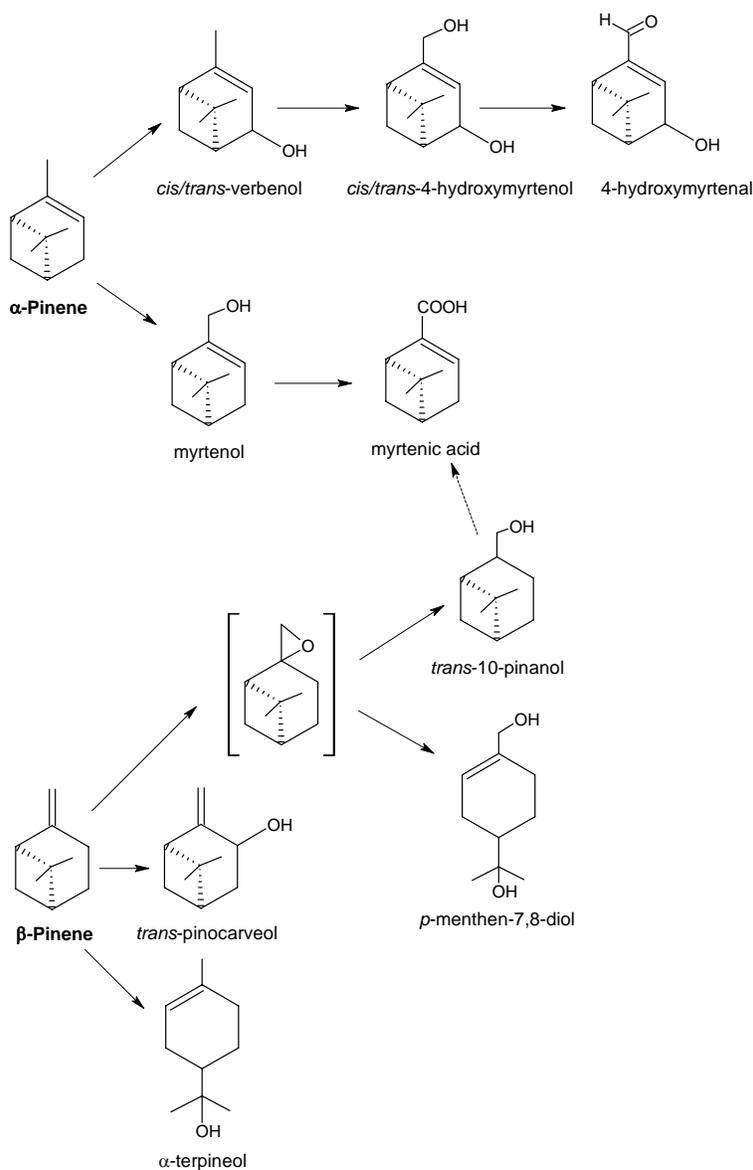
Figure III.5. *Metabolism of α-pinene and β-pinene in animals.*

Trans-verbenol and myrtenic acid have also been found in faeces or urine of brushtail possum fed alpha-pinene. When the same species was fed beta-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, alpha- and beta-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, 1978c).

Delta-3-Carene 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[8] (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, alpha-terpineol, trans-sobrerol, trans-carveol, and verbenol (some of the structures have been shown in Figure 5). 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, beta-caryophyllene 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[9]. Ishida et al. (1979) administered *dl*-camphene 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 beta-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., 1979a).

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 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 beta-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

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[8]
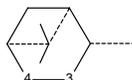
Pinane: Carane:
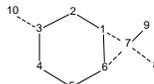
[9] camphene: 3,3-dimethyl-2-methylenenenorbornane
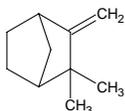
metabolite fraction). It was concluded that (+)-longifolene is metabolised at two sites in two subsequent steps: 1) oxidation of the *exo*-methylene group to form its epoxide with subsequent isomerisation to form a stable aldehyde, and 2) hydroxylation of the *gem*-dimethyl group to form a primary alcohol (Ishida et al., 1982; Asakawa et al., 1986; Ford et al., 1992b).

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

alpha-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 (alpha-pinene) (Austin et al., 1988; Hiroi et al., 1995).

Conclusions on the metabolism of subgroup V substances

Metabolism data for subgroup V are available for some supporting substances (pinenes, camphene, caryophyllene, delta-3-carene, pinane and carane) and the candidate substance longifolene [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 beta-pinene resulting in the formation of monocyclic terpenoid derivatives like alpha-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 except longifolene for which the mass balance is incomplete, it cannot be concluded that the candidate substances in this subgroup [FL-no: 01.022, 01.023, 01.030, 01.044, 01.047, 01.052, 01.056, 01.060, 01.066 and 01.067] can be metabolised to innocuous products.

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

No data are available for the two substances in subgroup VI [FL-no: 01.042 and 01.043] or for any supporting substance. Due to the lack of experimental data it cannot be concluded that these substances are metabolised to innocuous products.

III.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.

III.5.1.   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 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 2, the acyclic alkenes, data were only available for beta-myrcene [FL-no: 01.008] and 2-methylbuta-1,3-diene [FL-no: 01.049]. Given the narrow range of molecular weights of the candidate substances (between ~ 68 and 206 D) and their lipophilic nature (estimated or meassured Log Kow values e.g. 2.4 (2-methylbuta-1,3-diene), 6.1 (dodec-1-ene), 4.17 (beta-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 beta-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 beta-phellandrene, beta-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

No data are available on the absorption, distribution of elimination of any candidate or supporting substance in subgroup IVa (benzene hydrocarbons). But there are several data available on the candidate substances in subgroup IVb (naphthalene hydrocarbons). The data show that naphthalene [FL-no: 01.053] will be absorbed after oral administration and will be eliminated predominantly via the urine, in the form of various metabolites, conjugated with e.g. glucuronic acid or with glutathione. In bile duct cannulated animals, considerable elimination into the bile was also observed, but as in intact animals urinary elimination exceeds by far faecal excretion, the material eliminated via the bile is reabsorbed. The glucuronide conjugate of 1-naphthol represents a significant urinary metabolite of naphthalene. Glutathione conjugates of naphthalene are catabolised to (pre)mercapturic and mercapturic acids, particularly in rodents, before excretion in bile or urine. In primates and humans, conjugation with glutathione seems to be less important. Less data are available for 2-methylnaphthalene [FL-no: 01.051], but the available data show that 2-methylnaphthalene is also absorbed from the gastrointestinal tract and eliminated predominantly via the urine. For both substances the data indicate that 85–89 % of the dose is excreted within three

days after dosing. With respect to the one candidate substance (diphenylmethane [FL-no: 01.036]) in subgroup IVc, it may be concluded that the metabolites of this substance will be eliminated from the body after intraperitoneal injection. No conclusions can be made on absorption from the gastrointestinal tract and the rate of elimination as no oral ADME studies and mass-balance data are available.

Subgroup V

For the substances in subgroup V (bi- and tricyclic non-aromatic hydrocarbons), the available data from oral studies are incomplete. These studies have only addressed the excretion of some supporting and one candidate 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 alpha- and beta-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

No data were available on candidate or supporting substances in subgroup VI (macrocyclic non-aromatic hydrocarbons). The two substances in this group have a molecular weight of ~ 204. They are also rather lipophilic (estimated log Kow are 4.6 and 7.0 for [FL-no: 01.042] and [FL-no: 01.043], respectively). Based on these data it may be assumed that these two candidate chemicals will be absorbed from the gastrointestinal tract. For humulatriene, given the rather high Kow, the extent of absorption may be relatively low.

III.5.2. Metabolism

Subgroup I

The substances in subgroup 1 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 beta-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, 2004e). 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. Already 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 [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 candidate substance for 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 beta-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 beta-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 beta-myrcene. With beta-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 candidate 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. For candidate substance dodec-1-ene [FL-no: 01.037], diol- and alpha-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 1).

Apart from beta-myrcene and 2-methylbuta-1,3-diene, terminal double bonds appear also in candidate substances [FL-no: 01.037 and 01.064]. 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: 01.032, 01.035, 01.037, 01.049 and 01.064] will be metabolised to innocuous products.

Subgroup III

No data are available on the metabolism of the candidate substances in subgroup III. 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. In addition, based on the data available for one of the candidate substances, beta-phellandrene [FL-no: 01.055], with a double bond directly on the ring and in conjugation with double bond in the cyclohexene ring, it cannot be anticipated that it can be metabolised to innocuous products.

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 the candidate substances because of the structural dissimilarities and the absence of any further data on molecules with closer resemblance to the candidate substances. However, for bisabola-1,8,12-triene [FL-no: 01.027], beta-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 alpha2u-microglobulin accumulation in male kidneys, an event known to be associated with male rat specific nephropathy[10], and irrelevant for human toxicological risk assessment.

Subgroup IV

No data are available for the two substances in subgroup IVa [FL-no: 01.031 and 01.058]. Based on information available on *p*-cymene, it may be anticipated that these substances may be oxidised at the ring-substituent methyl groups (resulting in the formation of hydroxymethyl groups). These metabolites may subsequently be excreted after conjugation. Also epoxidation of the 3,4-double bond in [FL-no: 01.031] may be expected. Oxidative attack on the aromatic ring seems less likely. Due to the lack of experimental data it cannot be concluded that these substances are metabolised into innocuous products.

Data are available on the biotransformation of naphthalene [FL-no: 01.053] and its 2-methyl derivative [FL-no: 01.051] in subgroup IVb. With naphthalene, the primary products resulting from epoxidation can be further metabolised to naphtoquinones and other metabolites, which have been brought into connection with the toxicity of this substance. Whereas epoxidation is the main metabolic option for naphthalene, methyl group oxidation is the major metabolic pathway in the case of 2-methylnaphthalene. However, also with 2-methylnaphthalene, epoxidation may occur to some extent, and 2-methylnaphthalene-induced depletion of glutathione stores, possibly as a result of conjugation, has been suggested to be related to epoxidation reactions. However, although

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

biotransformation does play a role in the toxicity of 2-methylnaphthalene, the exact mechanism of toxicity and the metabolites which are involved have not been clearly identified. Hence, neither for naphthalene [FL-no: 01.053] nor for 2-methylnaphthalene [FL-no: 01.051], can it be concluded that they are metabolised into innocuous substances.

For the one candidate substance in subgroup IVc (diphenylmethane [FL-no: 01.036]) only a very limited amount of metabolites have been identified in the urine. One of these is an aromatic hydroxylation product, which may be generated from epoxidation. Because of lack of further data it cannot be concluded that diphenylmethane [FL-no: 01.036] is metabolised to innocuous products.

Subgroup V

Metabolism data for subgroup V are available for the supporting substances pinenes, camphene, caryophyllene, delta-3-carene, pinane and carane and the candidate substance longifolene [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 beta-pinene resulting in the formation of monocyclic terpenoid derivatives like alpha-terpineol. Hydroxylated metabolites or further metabolised 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 except longifolene for which the mass balance is incomplete, it cannot be concluded that the candidate substances in this subgroup [FL-no: 01.022, 01.023, 01.030, 01.044, 01.047, 01.052, 01.056, 01.060, 01.066 and 01.067] can be metabolised to innocuous products.

Subgroup VI

No data are available for the two candidate substances in subgroup VI [FL-no: 01.042 and 01.043] or for any supporting substance. Due to the lack of experimental data it cannot be concluded that these substances are metabolised to innocuous products.

III.6   Overal 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 aromatic hydrocarbons that do not contain alkyl substituents may undergo ring hydroxylation to form phenolic metabolites that are subsequently conjugated with sulphate or glucuronic acid and excreted in the urine. 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 III.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 alpha2u-microglobulin accumulation in male kidneys, an event known to be associated with male rat specific nephropathy, and irrelevant for human toxicological risk assessment.

Table III.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
01.050	3-Methylhexane	No (potential formation of neurotoxic gamma-diketone)
01.054	Pentadecane	Yes
01.057	Tetradecane	Yes
Subgroup II: ACYCLIC ALKENES		
01.032	2,3-Dihydrofarnesene	No (lack of supporting data)
01.035	2,6-Dimethylocta-2,4,6-triene	No (lack of supporting data)
01.037	Dodec-1-ene	No (presence of terminal double bond which may give rise to reactive metabolites without counteracting metabolic options)
01.049	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)
Subgroup III: CYCLOHEXENE HYDROCARBONS		
01.055	beta-Phellandrene	No (lack of supporting data)
01.027	Bisabola-1,8,12-triene	Yes
01.028	beta-Bisabolene	Yes
01.039	delta-Elemene	Yes
Subgroup IV: AROMATIC HYDROCARBONS		
Subgroup IVa: BENZENE HYDROCARBONS		
01.031	1,2-Dihydro-1,1,6-trimethylnaphthalene	No (lack of supporting data)
01.058	1,2,3,4-Tetrahydro-1,1,6-trimethylnaphthalene	No (lack of supporting dat)
Subgroup IVb: NAPHTHALENE HYDROCARBONS		
01.051	2-Methylnaphthalene	No (known metabolism to toxic metabolites)
01.053	Naphthalene	No (known metabolism to toxic metabolites)
Subgroup IVc: DIPHENYLMETHANE		
01.036	Diphenylmethane	No (lack of supporting data)
Subgroup V: BI- and TRICYCLIC, NON-AROMATIC HYDROCARBONS		
01.022	alpha-Cedrene	No (lack of supporting data)

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

FL–no:	Substance name	Innocuous metabolites?
01.023	1(5),11-Guaiadiene	No (lack of supporting data)
01.030	beta-Cubebene	No (lack of supporting data)
01.044	Isolongifolene	No (lack of supporting data)
01.047	Longifolene	No (lack of supporting data)
01.052	alpha-Muurolene	No (lack of supporting data)
01.056	alpha-Santalene	No (lack of supporting data)
01.060	1,1,7-Trimethyltricyclo[2.2.1.0.(2.6)]heptane	No (lack of supporting data)
01.066	2-Cedrene	No (lack of supporting data)
01.067	8(14)-Cedrene	No (lack of supporting data)
Subgroup VI: MACROCYCLIC, NON-AROMATIC HYDROCARBONS		
01.042	Germacra-1(10),4(14),5-triene	No (lack of supporting data)
01.043	3,7,10-Humulatriene	No (lack of supporting data)

ANNEX IV: TOXICITY

Oral acute toxicity data are available for eight candidate substances of the present flavouring group evaluation from chemical group 31, and for 17 supporting substances evaluated by JECFA at the 63rd meeting. The supporting substances are listed in brackets.

TABLE IV.1: ACUTE TOXICITY

Table IV.1: 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, 1973c)	Data not possible to interprete
	Mouse	M, F	Oral	2000 - 4000	(Pellmont, 1973c)	Data not possible to interprete
Dodec-1-ene [01.037]	Rat	NR	Oral	> 3200	(Jones, 1969)	No information given on study design
	Rat	NR	Oral	> 1000	(Ethyl Corporation, 1973)	Study not available
2-Methylbuta-1,3-diene [01.049]	Rat	M, F	Gavage	2043 – 2210	(Kimmerle & Solmecke, 1972)	Study not available
(Myrcene [01.008])	Rat	M	Oral	> 5000	(Moreno, 1972j)	Identity of compound not stated as other than code number
(beta-Ocimene [01.018])	Rat	NR	Oral	5000	(Moreno, 1976z)	One dose tested
(d-Limonene [01.045])	Rat	M	Oral	> 5000	(Moreno, 1972m)	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, 1975o)	Observation period not given.
(alpha-Terpinene [01.019])	Rat	NR	Oral	1680	(Moreno, 1973ah)	
(gamma-Terpinene [01.020])	Rat	M, F	Oral	3650	(Moreno, 1973ag)	
(alpha–Phellandrene [01.006])	Rat	M, F	Oral	> 5700	(Moreno, 1972k)	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
beta-Bisabolene [01.028]	Rat	NR	Oral	> 5000	(Moreno, 1974l)	One dose tested
	Mouse	M, F	Oral	> 13,360	(Hoffman-LaRoche, Inc., 1967b)	No of dose levels not given.
(delta-3-Carene [01.029])	Rat	M	Oral	4800	(Moreno, 1972l)	Identity of compound not stated
(Pin-2(3)-ene [01.004])	Rat	M, F	Oral	3700	(Moreno, 1972n)	Identity of compound not stated
(Pin-2(10)-ene [01.003])	Rat	NR	Oral	> 5000	(Moreno, 1975q)	One dose tested.
(Camphene [01.009])	Rat	NR	Oral	> 5000	(Moreno, 1974m)	One dose tested
(delta-3-Carene [01.029])	Rat	NR	Oral	> 5000	(Keating, 1972a)	Mixture of isomers. Only one dose tested
(Valencene [01.017])	Rat	M	Oral	> 5000	(Moreno, 1980m)	One dose tested
(beta–Caryophyllene [01.007])	Rat	M, F	Oral	> 5000	(Hart & Wong, 1971)	One dose tested

Flavouring Group Evaluation 25 (FGE.25)
Aliphatic and aromatic hydrocarbons from chemical group 31

Table IV.1: ACUTE TOXICITY

Chemical Name [FL-no]	Species	Sex	Route	LD$_{50}$ (mg/kg bw)	Reference	Comments
(1(5),7(11)-Guaiadiene [01.026])	Rat	NR	Oral	> 5000	(Moreno, 1976aa)	One dose tested
Longifolene [01.047]	Rat	NR	Oral	> 5000	(Moreno, 1977ai)	One dose tested
Diphenylmethane [01.036]	Rat	NR	Oral	2250	(Moreno, 1973ai)	
Naphthalene [01.053]	Rat	NR	Oral	> 2000	(Hazleton France, 1990a; Hazleton France, 1990b)	Study not available
	Rat	NR	Oral	> 2000	(Vaughn & Keeler, 1976)	No information on study design
	Rat	M, F	Oral	2400	(Birch, 1978a)	5 animals per dose group
	Rat	M, F	Oral	2800	(Birch, 1978b)	5 animals per dose group
	Rat	M, F	Oral	1900[4]	(Birch, 1978c)	5 animals per dose group
	Rat	NR	Oral	2000	(Hazleton France, 1990c)	Study not available
	Rat	F	Gavage	9430	(Carpenter et al., 1949)	
	Mouse	M, F	Gavage	M: 533 F: 710	(Shopp et al., 1984)	
(1-Methylnaphthalene [01.014])	Rat	NR	Oral	1840	(Izmerov et al., 1982)	Study not available
2-Methylnaphthalene [01.051]	Rat	NR	Oral	1630	(Izmerov et al., 1982)	Study not available

[1] *Administered as a 25 % solution in olive oil.*

[2] *Dowtherm A, which was a mixture of biphenyl (26 %) and diphenyl oxide (72 %), was tested*

[3] *Administered as a 20 % solution in corn oil.*

[4] *Administered as a 10 % solution in corn oil.*

Subacute / subchronic / chronic / carcinogenic toxicity data are available for two candidate substance of the present flavouring group evaluation from chemical group 31 and for five supporting substances evaluated by JECFA at the 63rd meeting. The supporting substances are listed in brackets.

TABLE IV.2: SUBACUTE / SUBCHRONIC / CHRONIC / CARCINOGENICITY STUDIES

Table IV.2: Subacute / Subchronic / Chronic / Carcinogenicity Studies

Chemical Name [FL-no]	Species; Sex No./Group	Route	Dose levels	Duration	NOAEL (mg/kg bw/day)	Reference	Comments
(Undeca-1,3,5-triene [01.061])	Rat; M, F 10	Diet	Calculated to provide 10 mg Galbelica/kg bw/day corresponding to 2 mg undecatriene, 8 mg pinene and the rest dodecene.	14 days	Corresponding to 2 mg undecatriene	(Shapiro, 1988)	Not valid. Study is on Galbelica, a formulation containing 80 % pinene and the rest a mixture of undecatriene and dodcecene.
(d-Limonene [01.045])	Rat; M 5	Gavage	0, 75, 150 or 300 mg/kg bw/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/day, 5 days/week	13 weeks	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/day corresponding to 0, 340, 1000 or 3000 mg/kg bw/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/day corresponding to 0, 100 or 1000 mg/kg bw/day.	180 days	100	(Webb et al., 1990)	
	Mouse; M, F 10	Gavage	0, 413, 825, 1650, 3300 or 6600 mg/kg bw/day for 12 days over 16-day period	16 days	1650	(NTP, 1990e)	
	Mouse; M, F 20	Gavage	0, 125, 250, 500, 1000 or 2000 mg/kg bw/day 5 days per week	13 weeks	M: 500 F: 500	(NTP, 1990e)	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/day F: 0, 500, 1000 mg/kg bw/day 5 days per week	103 weeks	M: 250 F: 500	(NTP, 1990e)	
	Rat; M, F 10	Gavage	0, 413, 825, 1650, 3300 or 6600 mg/kg bw/day for 12 days over 16-day period	16 days	1650	(NTP, 1990e)	
	Rat; M, F 20	Gavage	0, 150, 300, 600, 1200 or 2400 mg/kg bw/day 5 days per week	13 weeks	M: None F: 1200	(NTP, 1990e)	Dose-dependent nephropathy in all treated males.
	Rat; M, F M:50 F:50	Gavage	M: 0, 75 or 150 mg/kg bw/day F: 0, 300 or 600 mg/kg bw/day 5 days per week	103 weeks	M: None F: 300	(NTP, 1990e)	
(Pin-2(10)-ene [01.003])	Rat; M, F 10	Diet	Calculated to provide 10 mg Galbelica/kg bw/day corresponding to 8 mg pin-2(10)-ene	14 days	Corresponding to 8 mg pin-2(10)-ene	(Shapiro, 1988)	Study is on Galbelica, a formulation containing 80 % pinene and a mixture of undecatriene and dodcecene.
(Camphene [01.009])	Rat; M, F 10	Gavage	0, 62.5, 250, 1000 mg/ kg bw/day	28 days	M: None F: 250	(Hoechst, 1991b)	Study report very limited in details. Study according to OECD Guideline 407.
Naphthalene [01.053]	Rat; M, F	Gavage	0, 25, 50, 100, 200 or 400 mg/kg	13 weeks	100	(Battelle Columbus	Unpublished study that is not submitted. It

Table IV.2: Subacute / Subchronic / Chronic / Carcinogenicity Studies

Chemical Name [FL-no]	Species; Sex No./Group	Route	Dose levels	Duration	NOAEL (mg/kg bw/day)	Reference	Comments
	20		bw/day, 5 days/week		(LOAEL 200)	Laboratory, 1980a)	is therefore not possible to assess the quality of the study. Study is referred to in US EPA 1998.
Naphthalene [01.053] cont.	Rat; NR 28	Diet	10-20 mg/kg bw/day	700 days	42[5]	(Schmähl, 1955)	Study not considered valid.
	Mouse; M, F 116 – 156	Gavage	0, 5.3, 53, 133 mg/kg bw/day	90 days	53	(Shopp et al., 1984)	No histopathology was conducted, study not considered valid.
	B6C3F1 Mouse, M,F	Inhalation	0, 10, 30 ppm, 5 days/week estimated to be 0, 3.6-3.9, 10.7-11.4 mg/kg bw/day	105 weeks	None	(NTP, 1992g)	Valid study.
	F344/N rat, M, F	Inhalation	0, 10, 30, 60 ppm, 5 days/week estimated to be 0, 3.6-3.9, 10.7-11.4, 20.1-20.7 mg/kg bw/day	105 weeks	None	(NTP, 2000d)	Valid study.
	Mouse; M, F 20	Gavage	0, 12.5, 25, 50, 100 or 200 mg/kg bw/day, 5 days/week	13 weeks	(LOAEL 200)	(Battelle Columbus Laboratory, 1980b)	Unpublished study that is not submitted. It is therefore not possible to assess the quality of the study. Study is referred to in US EPA 1998.
(1-Methylnaphthalene [01.014])	Mouse; M, F 20	Diet	0, 0,0163, 0.049, 0.147, 0.44 or 1.33 % in diet calculated to correspond to 0, 16, 47, 142, 425 or 1259 mg/kg bw/day	13 weeks	0.147 % (142)	(Murata et al., 1993)	Dose-finding study.
	Mouse; M, F 100	Diet	0, 0.075 or 0.15 % in diet, average daily doses calculated to correspond to 0, 73 or 142 mg/kg bw/day	81 weeks	None	(Murata et al., 1993)	Exposure related increase in incidence of pulmonary alveolar proteinosis.
2-Methylnaphthalene [01.051]	Mouse; M, F 100	Diet	0, 0.075 or 0.15 %. Average daily doses calculated by the authors to correspond to 50.3-54.3 resp. 107.6-113.8 mg/kg bw/day	81 weeks	No NOAEL derived.	(Murata et al., 1997)	Study considered valid. Pulmonary alveolar proteinosis developed in all treated animals.
Mixture of (1-Methylnaphthalene [01.014]) and 2-Methylnaphthalene [01.051]	B6C3F1 Mouse; F 4, 11, 32	Cutaneous application	0, 2.7, 118.8 mg/kg bw twice a week	61 weeks	No NOAEL derived.	(Emi & Konishi, 1985)	The lesions were diagnosed as endogenous lipid pneumonia. Frequency of animals with these lesions were 0/4 control, 3/11 low dose and 31/32 high dose animals.

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

[2]This study was performed at either a single dose level or multiple dose levels.

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

[4] Study evaluated the carcinogenicity of 1-methylnaphthalene. The results were negative in the females. The males in both treatment groups showed a statistically significant increase in the number of lung adenomas from the control group; however, it may be concluded that because of a lack of tumours in females and at other sites (particularly the liver), it is unlikely that 1-methylnaphthalene is a carcinogen or promoter.

[5]True NOEL not determined. Only a single dose level was selected for a lifetime carcinogenicity study. Naphthalene was considered not to be carcinogenic.

[6] Study evaluated the carcinogenicity of 2-methylnaphthalene. The results were negative, with the exception of the males in the 0.075 %-dose group. It was concluded that although the total number of lung adenomas and adenocarcinomas was statistically significant in the 0.075 %-dose group, they were in the range of spontaneous tumours.

Developmental and reproductive toxicity data are available for one candidate substance of the present flavouring group evaluation from chemical group 31 and for three supporting substance evaluated by JECFA at the 63rd meeting. Supporting substance listed in brackets.

TABLE IV.3: DEVELOPMENTAL AND REPRODUCTIVE TOXICITY STUDIES

Table IV.3: Developmental and Reproductive Toxicity Studies

Chemical Name [FL-no]	Study type Durations	Species/Sex No / group	Route	Dose levels	NOAEL (mg/kg bw/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/day	Maternal: 500[2] 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/day	Maternal: 500[3] 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/day	Maternal/paternal: 500 mg/kg bw/day Foetal: 300 mg/kg bw/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/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/day	Maternal: 591 Foetal: 591	(Kodama et al., 1977b)	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/day	Maternal: 250 Foetal: 1000	(Kodama et al., 1977c)	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 & 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[4] Foetal: 1000	(Leuschner, 1992; LPT Research, 1992)	Study not available.
Naphthalene [01.053]	Developmental toxicity: Gestation Days 6 - 15	Rat; F 25 - 26	Gavage	0, 50, 150 or 450 mg/kg bw/day	Maternal LOAEL: 50 Foetal: 450	(Navarro et al., 1991)	Study considered valid.
	Developmental toxicity: Gestation days 7 - 14	Mouse; F 33	Gavage	0 or 300 mg/kg/day	Not possible to derive a NOAEL[1]	(Booth et al., 1983; Plasterer et al., 1985)	Only one dosage. No assessment of visceral or skeletal foetal development.
	Developmental toxicity: Gestation Days 6 - 18	Rabbit; F 18	Gavage	0, 40, 200 or 400 mg/kg bw/day	Maternal: Not determined Foetal: 400	(Naismith & Matthews, 1986)	Study is considered valid, although only code number is given as identity of test substance.
	Developmental toxicity: Gestation days 6 - 19	Rabbit; F 25 - 27	Gavage	0, 20, 80 or 120 mg/kg bw/day	Maternal: 120 Foetal: 120	(Navarro et al., 1992)	Study considered valid.

[1] NOEL not determined; only a single dose level was tested. Naphthalene decreased the number of surviving dams, maternal body weight, number of live pups and litter weights, but not the weight per pup. However, it was concluded by the authors that maternal toxicity would manifest before high levels of developmental toxicity would occur.

[2] Test substance was beta-myrcene.

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

[4]*Test substance was 78 % pure.*

In vitro mutagenicity/genotoxicity data are available for six candidate substances of the present flavouring group evaluation from chemical group 31 and for ten supporting substances evaluated by JECFA at the 63rd meeting. Supporting substances are listed in brackets.

TABLE IV.4: GENOTOXICITY (*IN VITRO*)

Table IV.4: GENOTOXICITY (*in vitro*)						
Chemical Name [FL-no]	**Test System**	**Test Object**	**Concentration**	**Result**	**Reference**	**Comments**
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.
	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]	(Conner et al., 1985)	Published non-GLP study with insufficient report of some details of method and results.

Table IV.4: GENOTOXICITY (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
						Thus, the validity of the study cannot be evaluated. Cytotoxicity not reported.
Dodec-1-ene [01.037]	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538 *E. coli* WP2uvrA	0.2 to 2000 µg/plate	Negative[1]	(Dean, 1980)	Unpublished GLP-study. IUCLID abstract available only. Details of study design and results are not reported. Thus, the validity of the study cannot be evaluated.
2-Methylbuta-1,3-diene [01.049]	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)	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.
	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)	[4]

Table IV.4: GENOTOXICITY (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
	Mammalian cell gene mutation assay	Chinese hamster ovary V79 cells	1000 μg/ml	Negative[1]	(Kauderer et al., 1991)	[4]
	Sister chromatid exchange test	Human lymphocytes	1000 μg/ml	Negative[1]	(Kauderer et al., 1991)	[4]
	Sister chromatid exchange test	Chinese hamster ovary cells and hepatic tumour cell line	500 μg/ml	Negative[1]	(Röscheisen et al., 1991)	[4]
(*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)	[4]
	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	150,000 μg/plate	Negative[1]	(Heck et al., 1989)	[4]
	Ames test	*S. typhimurium* TA102	5000 μg/plate	Negative[1]	(Müller et al., 1993)	[4]
	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537	3333 μg/plate	Negative[1]	(Haworth et al., 1983)	[4]
	Ames test (preincubation method)	*S. typhimurium* TA98; TA100, UTH8414 and UTH8413	0, 10 to 500 μg/plate (5 concentrations)	Negative[1]	(Conner et al., 1985)	[4]
	Forward mutation assay	L5178Y Mouse lymphoma	100 μg/ml	Negative[1]	(Heck et al., 1989)	[4]
	Forward mutation assay	L5178Y Mouse Lymphoma	100 nl/ml (84.1 μg/ml)	Negative[1]	(Myhr et al., 1990)	[4]
	Chromosomal aberration assay	Chinese hamster ovary cells	500 μg/ml	Negative[1]	(Anderson et al., 1990)	[4]
	Sister chromatid exchange test	Chinese hamster ovary cells	162 μg/ml	Negative[1]	(Anderson et al., 1990)	[4]
	Sister chromatid exchange test	Chinese hamster ovary cells	1000 μM (136.2 μg/ml)	Negative	(Sasaki et al., 1989)	[4]
(gamma-Terpinene [01.020])	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	50,000 μg/plate	Negative[1]	(Heck et al., 1989)	[4]
	Unscheduled DNA synthesis	Rat hepatocytes	30 μg/ml	Negative	(Heck et al., 1989)	[4]
(alpha–Phellandrene [01.006])	Sister chromatid exchange test	Chinese hamster ovary cells	1000 μM(136.2 μg/ml)	Negative	(Sasaki et al., 1989)	[4]
(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 & Raw, 1979)	[4]
	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)	[4]
	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	25000 μg/plate	Negative[1]	(Heck et al., 1989)	[4]
	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	25 μl/plate (21,450 μg/ plate)	Negative[1]	(Jagannath, 1984)	[4]
	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	5 μl/plate (4290 μg/plate)	Negative[1]	(DeGraff, 1983a)	[4]

Table IV.4: GENOTOXICITY (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
	Ames test	*S. typhimurium* TA98; TA100; UTH8414; UTH8413	0, 10 to 500 µg/plate (5 concentrations)	Negative[1]	(Conner et al., 1985)	[4]
	Unscheduled DNA synthesis	Rat hepatocytes	10000 µg/ml	Negative	(Heck et al., 1989)	[4]
(Pin-2(10)-ene [01.003])	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	5000 µg/plate	Negative[1]	(Heck et al., 1989)	[4]
	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)	[4]
	Sister chromatid exchange	Chinese hamster ovary cells	1000 µM (136.2 µg/ml)	Negative	(Sasaki et al., 1989)	[4]
(Camphene [01.009])	Ames test	*S. typhimurium* TA98; TA100	100 µl/plate (84,500 µg/ plate)	Negative[2]	(Rockwell & Raw, 1979)	[4]
	Ames test	*S. typhimurium* TA98; TA100; UTH8414; UTH8413	0, 10 to 1000 µg/plate (5 concentrations)	Negative[1]	(Conner et al., 1985)	[4]
	Sister chromatid exchange test	Chinese hamster ovary cells	1000 µM (136.2 µg/ml)	Negative	(Sasaki et al., 1989)	[4]
(beta–Caryophyllene [01.007])	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	150,000 µg/plate	Negative[1]	(Heck et al., 1989)	[4]
	Ames test	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	150 µl/plate	Negative[1]	(Lorillard, 1984)	[4]
	Ames test (plate incorporation method)	*S. typhimurium* TA98; TA100; TA102; TA1535; TA1537	10,000 µg/plate	Negative[1]	(Longfellow, 1998)	[4]
	Sister chromatid exchange test	Chinese hamster ovary cells	1000 µM (204.4 µg/ml)	Negative	(Sasaki et al., 1989)	[4]
Naphthalene [01.053]	Ames test (preincubation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537	0.03, 0.3, 3, 30 µM/plate (3.9, 39, 385, 3850 g/plate)	Negative[1]	(Florin et al., 1980)	Published non-GLP study. Part of a larger mutagenicity screening study evaluating 239 compounds. Due to the limited report of experimental details and results the validity of the study cannot be evaluated. Cytotoxicity observed at doses >3 µM/plate.
	Ames test (plate incorporation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	0, 3, 10, 30, 100, 300 µg/plate	Negative[1]	(Godek et al., 1985)	Unpublished GLP study carried out according to OECD guideline 471.
	Ames test (plate incorporation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	0, 3, 10, 30, 100, 300 µg/plate	Negative[1]	(Stankowski, 1987)	Unpublished GLP study carried out according to OECD guideline 471. Repeat confirmation of Ames test by Godek et al, 1985.
	Ames test (preincubation method)	*S. typhimurium* TM677	0, 1, 2 mM (0, 128, 256 µg/ml)	Negative[2]	(Kaden et al., 1979)	Published non-GLP study of limited validity (only one strain, concentrations used were cytotoxic, insufficient report of experimental details and results).
	Ames test (plate incorporation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537	up to 100 µg/plate	Negative[2]	(McCann et al., 1975)	Published summary report of a large study evaluating the mutagenic potential of 300 chemicals. Due to the limited report of experimental details and results the validity of the study cannot be evaluated.
	Ames test	*S. typhimurium*	0, (0.3,) 1, 3.3, 10, 33, 100	Negative[1]	(Mortelmans et al., 1986)	Published summary report including detailed

Table IV.4: GENOTOXICITY (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
	(preincubation method)	TA98; TA100; TA1535; TA1537	µg/plate		(NTP, 1992g)	results from studies on 270 compounds tested in various laboratories within the NTP to a large extent in accordance with OECD guideline 471. Study design and detailed results on naphthalene also included in NTP, 1992g. In the absence of metabolic activation the concentration of 100 microgram/plate was completely toxic and not tested any more in the second trial when 0.3 microgram/plate was used as additional concentration. In the presence of metabolic activation the highest concentration was slightly toxic.
Naphthalene [01.053] cont.	Ames test (plate incorporation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	0, 5, 10, 25, 50, 100, 300 µg/plate (-S9) 0, 10, 25, 50, 100, 300, 900 µg/plate (+ S9)	Negative[1]	(Lawlor, 1994)	Unpublished GLP study carried out in accordance with OECD guideline 471. Cytotoxicity was observed in a preliminary study at 66.7 microgram/plate and above in the absence of S9 mix and at 333 microgram/plate and above in the presence of S9 mix.
	Ames test (preincubation method)	*S. typhimurium* TA98; TA100; UTH8414; UTH8413	0, 100, 500, 1000, 2000 µg/plate	Negative[1]	(Conner 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.
	Ames test (plate incorporation method)	*S. typhimurium* TA97; TA98; TA100	0, 5, 10, 50, 250 µg/plate	Negative[1]	(Sakai et al., 1985)	Published non-GLP study of acceptable quality. Cytotoxicity was observed at the highest concentration with complete toxicity in TA97 (+/-S9) and in TA100 (-S9) and a reduced number of mutants in TA100 (+S9) and in TA98 (+/-S9).
	Ames test (plate incorporation)	*S. typhimurium* TA98; TA100; TA1535; TA1537; TA1538	0, 3.3 to 10,000 µg/plate	Negative[1]	(Longfellow, 1991)	Only summary from CCRIS database available from. Validity of the study cannot be evaluated.
	Ames test (preincubation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537 *E. coli* WP2uvrA	1 to 100 µg/plate 10 to 10,000 µg/plate	Negative[1]	(Japan Chemical Industry)	Only summary from CCRIS database available from. Validity of the study cannot be evaluated.
	Rec assay	*E. coli* WP2 and WP100 *uvrA⁻ recA⁻*	0 to 2000 µg/plate (≥ 4 concentrations)	Negative[2]	(Mamber et al., 1984)	Published non-GLP study with adequate study design, however, deficient in the report of some details on method and results (no single doses, no detailed results, no data on cytotoxicity reported).
	Inductest (prophage induction test)	*E. coli* GY5027 *envA⁻ uvrB⁻* (λ); GY4015 amp^R	0 to 2000 µg/plate (≥ 4 concentrations)	Negative[2]	(Mamber et al., 1984)	Published non-GLP study with adequate study design, however, deficient in the report of some details on method and results (no single doses, no detailed results, no data on cytotoxicity reported).
	Unscheduled DNA synthesis test	Primary rat hepatocytes	0, 0.16, 0.5, 1.6, 5, 16, 50,	Negative	(Barfknecht et al., 1985)	Unpublished GLP-study according to OECD

Table IV.4: GENOTOXICITY (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
			166, 500, 1666, 5000 µg/ml			guideline 482. Excessive cytotoxicity observed at 50 to 5000 microgram/ml.
Naphthalene [01.053] cont.	Sister chromatid exchange test	Chinese hamster ovary cells	0, 2.7, 9, 27, 45, 90 µg/ml (-S9) 0, 9. 15, 27, 90 µg/ml (+S9)	Positive[1]	(NTP, 1992g)	Valid study in accordance with OECD guideline 479. No data on cytotoxicity reported. According to the study protocol the highest dose chosen was limited by cytotoxicity. Significant dose-related increase in frequency of SCE at concentrations from 27 - 90 µg/ml (without metabolic activation) and 15 - 27 µg/ml (with metabolic activation). Maximum values for the percent increase in SCEs/chromosome in cultures exposed to naphthalene relative to those exposed to solvent of 40 and 50 % were reached at the highest dose tested in the presence and absence of S9, respectively, whereas values of 360-640 % were reached with the positive control mitomycin C. Result is considered positive by NTP since the increase over solvent control observed is ≥20 % (NTP, 1992g; Galloway et al., 1987). Results would be considered negative by UK HSE as the increase in SCEs per cell does not reach the required minimum of at least 100 %).(EU RAR, 2003).
	Sister chromatid exchange test	Human peripheral mononuclear leukocytes	100 µM (13 µg/ml)	Negative[1]	(Tingle et al., 1993; Wilson et al., 1995)	Published non-GLP study of limited validity (only one concentration tested). Naphthalene was not cytotoxic to the dividing lymphocytes with and without metabolic activation at the concentration tested.
	Mammalian cell gene mutation test (Mouse lymphoma assay)	Mouse lymphocytes L5178Y tk^+/tk^-	0, 22 to 87 µg/ml (-S9) 0, 8 to 30 µg/ml (+S9)	Negative[3] Positive[2]	(Longfellow, 1991)	Only summary from CCRIS database available. Validity of the study cannot be evaluated.
	Chromosomal aberration assay	Chinese hamster ovary cells	15 to 75 µg/ml (-S9) 30 to 67.5 µg/ml (+S9)	Negative[3] Positive[2]	(NTP, 1992g)	Study carried out in accordance with OECD guideline 473, except that data on cytotoxicity are not reported. According to the study protocol the highest dose chosen was limited by cytotoxicity. Study is considered valid. The structural aberrations did not include gaps. In the presence of S9 the percent of cells with structural aberrations was significantly (p≤0.05) elevated at all concentrations tested compared to controls and the increase was significantly dose-related (p≤0.001). A maximum of 32 % of cells with aberrations

Table IV.4: GENOTOXICITY (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
						was reached at the highest concentration vs. 0-1.5 % in negative and up to 52 % in positive controls. Result is considered positive by NTP since a statistically significant difference is observed for two or more doses (Galloway et al., 1987).
(1-Methylnaphthalene [01.014])	Ames test (preincubation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537	0.03, 0.3, 3, 30 µM/plate (4.3, 43, 427, 4266 µg/plate)	Negative[1]	(Florin et al., 1980)	[4]
	Ames test (preincubation method)	*S. typhimurium* TM677	0, 0.7, 3.5, 7 mM (0, 498, 995 µg/ml)	Negative[2]	(Kaden et al., 1979)	[4]
(1-Methylnaphthalene [01.014]) cont.	Chromosomal aberration assay	Human lymphocytes	0, 1, 2 mM (0, 142, 284 µg/ml) (-S9)\n\n0, 0.25, 0.5, 1, 2 mM (0, 36, 71, 142, 284 µg/ml) (+S9)	Negative[1]	(Kulka et al., 1988)	[4], Published non-GLP study largely in accordance with OECD guideline 473. Even if cytotoxicity data are not reported, the study is considered acceptable. The highest dose did not impair cell proliferation. No evidence for differences in the incidences of structural chromosomal aberrations (chromatid breaks, no exchanges seen) and gaps between treated and untreated cells (p≤0.05). 0.5 to 2.0 % of treated cells showed aberrations (gaps excluded) vs. 1 % of control cells.
	Sister chromatid exchange test	Human lymphocytes	0, 1, 2 mM (0, 142, 284 µg/ml) (-S9)\n\n0, 0.25, 0.5, 1, 2 mM (0, 36, 71, 142, 284 µg/ml) (+S9)	Negative[3]\n\nPositive[2] (limited evidence)	(Kulka et al., 1988)	Published non-GLP study largely in accordance with OECD guideline 479. Cytotoxicity data not reported. The highest dose did not impair cell proliferation. In the presence of S9 the SCE frequency was significantly increased at each dose. An increase of 43 % was reported at the highest dose compared to the control. The effect was dose-related, but with a less marked increase at higher doses (saturation). According to OECD and NTP criteria the result in the presence of S9 is considered positive (significant increase, dose-relation, increase ≥20 % over solvent control). The authors of the study refer to the UK HSE guidelines on mutagenicity testing that require at least a doubling in SCE frequency for a positive response.
2-Methylnaphthalene [01.051]	Ames test (preincubation method)	*S. typhimurium* TA98; TA100; TA1535; TA1537	0.03, 0.3, 3, 30 µM/plate (4.3, 43, 427, 4266 µg/plate)	Negative[1]	(Florin et al., 1980)	Published non-GLP study. Part of a larger mutagenicity screening study evaluating 239 compounds. Due to the limited report of experimental details and results the validity of the study cannot be evaluated. Bacterial toxicity observed at doses >3 µM/plate.

Table IV.4: GENOTOXICITY (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
2-Methylnaphthalene [01.051] cont.	Chromosomal aberration assay	Human lymphocytes	0, 2.0, 4.0 mM (0, 284, 569 microgram/ml) (-S9) 0, 0.25, 0.5, 1.0, 2.0, 4.0 mM (0, 35.6, 71.1, 142, 284 and 569 microgram/ml) (+S9)	Negative[3] Positive[2] (limited evidence)	(Kulka et al., 1988)	Published non-GLP study largely in accordance with OECD guideline 473. Even if cytotoxicity data are not reported, the study is considered acceptable. The highest dose did not impair cell proliferation. In the presence of S9 a statistically significant but weak increase (6.5-fold above control) of structural aberrations (chromatid breaks, no exchanges seen) was observed at the highest tolerated dose (4 mM). The percent of cells with structural aberrations (excluding gaps) showed a dose-related increase (up to 5 % vs. 1.0 % in negative and 40 % in positive controls), however, the increase was not reported as statistically significant. A dose-dependent weak increase of gaps was also noted over the concentration range tested in the presence of S9. According to OECD and NTP criteria there is a weak evidence for a positive response since a statistically significant difference for structural aberrations per cell is observed for one dose (NTP, 1992g; Galloway et al., 1987) and the effect was dose-related (OECD guideline 473).
	Sister chromatid exchange test	Human lymphocytes	0, 2.0, 4.0 mM (0, 284, 569 microgram/ml) (-S9) 0, 0.25, 0.5, 1.0, 2.0, 4.0 mM (0, 36, 71, 142, 284 and 569 microgram/ml) (+S9)	Negative[3] Positive[2] (limited evidence)	(Kulka et al., 1988)	Published non-GLP study largely in accordance with OECD guideline 479. Even if cytotoxicity data are not reported, the study is considered acceptable. The highest dose did not impair cell proliferation. In the presence of S9 the SCE frequency was significantly increased at each dose. An increase of 80 % was reported at the highest dose compared to the control. The effect was dose-related. According to OECD and NTP criteria the result in the presence of S9 is considered positive (significant increase, dose-relation, increase ≥20 % over solvent control). The authors of the study refer to the UK HSE guidelines on mutagenicity testing that require at least a doubling in SCE frequency for a positive response.

NR: Not Reported

[1] *With and without S9 metabolic activation.*

[2] *With metabolic activation.*

[3] *Without metabolic activation*

[4] *Abstracted by JECFA at their 63rd meeting (JECFA, 2006a)*

In vivo mutagenicity/genotoxicity data are available for two candidate substances of the present flavouring group evaluation from chemical group 31 and for two supporting substances evaluated by JECFA at the 63rd meeting. Supporting substances are listed in brackets.

TABLE IV.5: GENOTOXICITY (*IN VIVO*)

Table IV.5: GENOTOXICITY (*in vivo*)

Chemical Name [FL-no]	Test System	Test Object	Route	Dose	Result	Reference	Comments
2-Methylbuta-1,3-diene [01.049]	*In vivo* Chromosomal aberration assay	Mouse (B6C3F1) bone marrow (male mice)	Inhalation	0, 438, 1750, 7000 ppm for 6 hours/day for 12 exposures over a period of 16 days (Trial 1) 0, 70, 220, 700 ppm for 6 hours/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/day for 12 exposures over a period of 16 days (Trial 1) 0, 70, 220, 700 ppm for 6 hours/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 sponsored 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/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.
	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 & 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)	[1]
	In vivo Micronucleus test	Mouse (B6C3F1) peripheral blood cells	Gavage	0, 250, 500, 1000, 2000 mg/kg bw (single exposure)	Negative	(NTP, 2003c)	[1]
(*d*-Limonene [01.045])	*In vivo* Comet assay	Mouse (ddY) / Rat (Wistar).	Oral	0, 2000 mg/kg	Negative	(Sekihashi et al., 2002)	[1]

Table IV.5: GENOTOXICITY (*in vivo*)

Chemical Name [FL-no]	Test System	Test Object	Route	Dose	Result	Reference	Comments
	In vivo Mammalian spot test	Mouse embryos from C57BL/6JHan x T stocks	Intraperitoneal injection	215 mg/kg bw	Negative	(Fahrig, 1984)	[1]
Naphthalene [01.053]	*In vivo* Unscheduled DNA synthesis	Rat hepatocytes	Gavage	0, 600, 1000, 1600 mg/kg bw	Negative	(Research Toxicology Center, 1999)	Summarised report of unpublished study carried out in accordance with OECD guideline 486. Although some minor details of the results are not reported (viability of cells, individual slide values for nuclear grains and cytoplasmic grains) the study is considered valid.
	In vivo Micronucleus test	Mouse (Swiss ICR) bone marrow	Gavage	50, 250, 500 mg/kg bw (single exposure)	Negative	(Harper et al., 1984)	Published non-GLP study not fully in accordance with OECD guideline 474 (only males tested, sampling time not indicated, effect on PCE/NCE ratio not reported). Due to the limited report of experimental details and results the validity of the study cannot be evaluated. At the dose of 500 mg/kg bw two of ten animals died. The dose of 1500 mg/kg bw was toxic (lethal) to all animals. Induction of micronuclei in benzene-treated mice was significantly enhanced by co-treatment with naphthalene at 50 and 250 mg/kg bw.
	In vivo Micronucleus test	Mouse (CD-1) bone marrow	Intraperitoneal injection	250 mg/kg bw (single exposure)	Negative	(Sorg et al., 1985)	Unpublished valid GLP-study carried in accordance with OECD guideline 474. Naphthalene was negative in the micronucleus test at the dose of 250 mg/kg bw at all of the time intervals tested. A harvest-time dependent depression in the PCE/NCE ratio was observed in animals treated with the test substance, which was statistically significant ($p \leq 0.05$) at sacrifice time of 72 hrs.

[1] *Abstracted by JECFA at their 63rd meeting (JECFA, 2006a)*

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