EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF); Scientific Opinion on Flavouring Group Evaluation 212 Rev1 (FGE.212 Rev1):alpha,beta-Unsaturated alicyclic ketones and precursors from chemical subgroup 2.6 of FGE.19

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SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 212 Revision 1 (FGE.212Rev1):

alpha,beta-Unsaturated alicyclic ketones and precursors from chemical subgroup 2.6 of FGE.19.[1]

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

European Food Safety Authority (EFSA), Parma, Italy

SUMMARY

The European Food Safety Authority (EFSA) asked the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (the Panel) to provide scientific advice to 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 was requested to evaluate flavouring substances using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000.

In the present revision of FGE.212, FGE.212Rev1, there has been a reassessment of [FL-no: 02.083, 02.101, 07.033, 07.035, 07.094, 07.098, 07.112, 07.129, 07.140, 07.172, 07.175, 07.196, 07.202 and 07.255] for which there were a request for genotoxicity data in FGE.212.

Flavouring Group Evaluation 212 (FGE.212) concerns 23 substances. The 23 substances correspond to subgroup 2.6 of FGE.19. Fifteen of these substances are alpha,beta-unsaturated alicyclic ketones [FL-no: 07.033, 07.035, 07.094, 07.098, 07.112, 07.126, 07.129, 07.140, 7.146, 07.147, 07.172, 07.175, 07.196, 07.202 and 07.255] and eight are precursors for such ketones [FL-no: 02.062, 02.083, 02.101, 02.214, 09.143, 09.215, 09.821 and 09.870].

In the previous version of this Opinion, FGE.212, the Panel had expressed the following view.

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1 On request from the Commission, Question No EFSA-Q-2010-01251, adopted on 25 November 2010.

2 Panel members Arturo Anadon, Mona-Lise Binderup, Wilfried Bursch, Laurence Castle, Riccardo Crebelli, Karl-Heinz Engel, Roland Franz, Nathalie Gontard, Thomas Haertle, Trine Husøy, Klaus-Dieter Jany, Catherine Leclercq, Jean Claude Lhuguenot, Wim Mennes, Maria Rosaria Milana, Karla Pfaff, Kettil Svensson, Fidel Toldra, Rosemary Waring, Detlef Wölfle. Correspondence: cef-unit@efsa.europa.eu

3 Acknowledgement: The Panel wishes to thank the members of the Working Groups on Flavourings for the preparation of this Opinion: Ulla Beckman Sundh, Vibe Beltoft, Wilfried Bursch, Angelo Carere, Karl-Heinz Engel, Henrik Frandsen, Rainer Gürtler, Frances Hill, Trine Husøy, John Christian Larsen, Pia Lund, Wim Mennes, Gerard Mulder, Karin Nørby, Gerard Pascal, Iona Pratt, Gerrit Speijers, Harriet Wallin and EFSA's staff member Kim Rygaard Nielsen for the preparatory work on this scientific Opinion.

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d-Carvone [FL-no: 07.146] was found genotoxic *in vitro*. However, d-carvone was not carcinogenic in mice. Therefore, the Panel concluded that this substance together with the structurally related l-carvone as well as carveol and the carvyl derivatives [FL-no: 02.062, 07.147, 09.143, 09.215 and 09.870] could be evaluated through the Procedure.

Isophorone [FL-no: 07.126 (3,5,5-trimethylcyclohex-2-en-1-one)] is genotoxic *in vitro* and since there is some evidence of carcinogenicity in male rats and equivocal evidence of carcinogenicity in male mice and since a non-threshold mechanism could not be excluded based on the data currently available, the Panel concluded that additional data are required for isophorone in order to clarify whether genotoxicity occurs *in vivo* and whether there is a threshold for the effects observed in the target organs in the long-term bioassays. Therefore, an *in vivo* Comet assay in F344/N rats covering these target organs is required in addition to an *in vivo* bone marrow assay with oral application.

Due to structural similarities and lack of data at the time of evaluation in previous version of FGE.212, the remaining substances could not be evaluated through the Procedure [FL-no: 02.083, 02.101, 02.214, 07.033, 07.035, 07.094, 07.098, 07.112, 07.129, 07.140, 07.172, 07.175, 07.196, 07.202, 07.255 and 09.821]. Additional data on genotoxicity are requested for representative substances of this subgroup according to the Opinion of the Panel on Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19.

In response to the Panel request expressed in FGE.212, the Flavouring Industry has submitted additional genotoxicity data. Based on these data the Panel could rule out the genotoxicity concern for isophorone and the substances structurally related to isophorone [FL-no: 02.083, 02.101, 07.035, 07.098, 07.126, 07.129, 07.172, 07.175, 07.196, 07.202 and 07.255]. On the other hand, the Panel could not agree in the Industry argument that isophorone can be representative for the remaining substances for which the Panel requested additional genotoxicity data in FGE.212 [FL-no: 02.214, 07.033, 07.094, 07.112, 07.140 and 09.821]. For these substances additional data are still requested.

KEYWORDS

alpha,beta-Unsaturated ketones, alicyclic ketones, flavouring substances, safety evaluation.

TABLE OF CONTENTS

BACKGROUND

Regulation (EC) No 2232/96 of the European Parliament and the Council (EC, 1996a) 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 flavouring 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 2009/163/EC (EC, 2009a). 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, 1999a). 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 Union 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, 1996a).

Flavouring Group Evaluation 19 (FGE.19) contains 360 flavouring substances from the EU Register being alpha,beta-unsaturated aldehydes or ketones and precursors which could give rise to such carbonyl substances via hydrolysis and/or oxidation (EFSA, 2008b).

The alpha,beta-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity. The Panel noted that there were limited genotoxicity data on these flavouring substances but that positive genotoxicity studies were identified for some substances in the group.

The alpha, beta-unsaturated carbonyls were subdivided into 28 subgroups on the basis of structural similarity (EFSA, 2008b). In an attempt to decide which of the substances could go through the Procedure, a (quantitative) structure-activity relationship (Q)SAR prediction of the genotoxicity of these substances was undertaken considering a number of models (DEREKfW, TOPKAT, DTU-NFI-MultiCASE Models and ISS-Local Models, (Gry et al., 2007)).

The Panel noted that for most of these models internal and external validation has been performed, but considered that the outcome of these validations was not always extensive enough to appreciate the validity of the predictions of these models for these alpha,beta-unsaturated carbonyls. Therefore, the Panel considered it inappropriate to totally rely on (Q)SAR predictions at this point in time and decided not to take substances through the Procedure based on negative (Q)SAR predictions only.

The Panel took note of the (Q)SAR predictions by using two ISS Local Models (Benigni & Netzeva, 2007a; Benigni & Netzeva, 2007b) and four DTU-NFI MultiCASE Models (Gry et al., 2007; Nikolov et al., 2007) and the fact that there are available data on genotoxicity, *in vitro* and *in vivo*, as well as data on carcinogenicity for several substances. The Panel decided that 11 subgroups (1.1.2, 1.1.3, 1.1.4, 2.4, 2.6, 2.7, 3.1, 3.3, 4.1, 4.2 and 4.4) (EFSA, 2008b) should be further examined to determine whether evaluation through the Procedure is feasible. Corresponding to these 11 subgroups 11 Flavouring Group Evaluations (FGEs) were established, FGE.201, 202, 203, 210, 212, 213, 214, 216, 217, 218 and 220. If the Panel concludes for any substances in these 11 FGEs that they cannot be evaluated using the Procedure then it has to be decided if there is a safety concern for certain substances or if additional data are required in order to finalise the evaluation. If the Panel concludes that a genotoxic potential can be ruled out for the substances they will be merged with structurally related substances in other FGEs and evaluated using the Procedure.

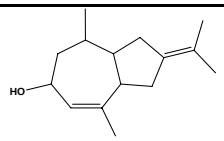
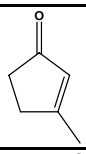
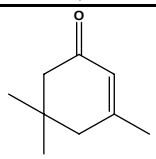
HISTORY OF EVALUATION

In FGE.212 the Panel concluded that additional genotoxicity data were required for isophorone [FL-no: 07.126] and due to structural similarities and lack of data, the following substances could presently not be evaluated through the Procedure [FL-no: 02.083, 02.101, 02.214, 07.033, 07.035, 07.094, 07.098, 07.112, 07.129, 07.140, 07.172, 07.175, 07.196, 07.202, 07.255 and 09.821].

FGE	Adopted by EFSA	Link	No. of Substances
FGE.212	27 November 2008	http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902780085.htm	23
FGE.212Rev1	November 2010		23

Additional data on genotoxicity were requested for representative substances (EFSA, 2008bc) of this subgroup according to the Opinion of the Panel on the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19 (EFSA, 2008bb).

Representative substances for subgroup 2.6 of FGE.19 (EFSA, 2008bc)

FL-no JECFA-no	Subgroup	EU Register name	Structural formula	Comments
02.214 1866	2.6	Vetiverol		Representative: 2,6-Dimethyl-9-(1-methylethylidene)-bicyclo[5.3.0]dec-2-en-4-one (not in register) or its precursor [02.214].
07.112 1105	2.6	3-Methyl-2-cyclopenten-1-one		Representative
07.126 1112	2.6	3,5,5-Trimethylcyclohex-2-en-1-one		Representative An *in vivo* Comet assay in F344/N rats covering the target organs in the carcinogenicity studies on [07.126] and an *in vivo* bone marrow assay with oral application

The present Flavouring Group Evaluation 212, Revision 1 (FGE.212Rev1) includes the assessment of additional genotoxicity data submitted by Industry in reply to data request presented in FGE.212 for isophorone and structurally related substances. These new data are described and evaluated in Section 4 in the present FGE. Sections 1-3 report the same information that was present in the earlier version of FGE.212.

TERMS OF REFERENCE

The European Commission requests the European Food Safety Authority to carry out risk assessment on the following 15 substances: p-Menth-1-en-3-ol [FL-no: 02.083], pin-2-en-4-ol [FL-no: 02.101], isojasmone [FL-no: 07.033], tetramethyl ethylcyclohexenone [FL-no: 07.035], 3-methyl-2-(pent-2(cis)-enyl)cyclopent-2-en-1-one [FL-no: 07.094], 3-methylcyclohex-2-en-1-one [FL-no: 07.098], 3-methyl-2-cyclopenten-1-one [FL-no: 07.112], isophorone (3,5,5-trimethylcyclohex-2-en-1-one) [FL-no: 07.126], 3-methyl-5-propylcyclohex-2-en-1-one [FL-no: 07.129], 3-methyl-2-pentylcyclopent-2-en-1-one [FL-no: 07.140], 4-isopropylcyclohex-2-en-1-one [FL-no: 07.172], p-menth-1-en-3-one [FL-

no: 07.175], pin-2-en-4-one [FL-no: 07.196], 2,6,6-trimethylcyclohex-2-en-1-one [FL-no: 07.202] and 1-piperitone [FL-no: 07.255], in accordance with Commission Regulation (EC) No 1565/2000 by end of 2010.

ASSESSMENT

1. Presentation of the Substances in the Flavouring Group Evaluation 212 Revision 1

1.1. Description

The present Flavouring Group Evaluation 212, Revision 1 (FGE.212Rev1) concerns 23 substances, which are presented in Table 1. The 23 substances correspond to subgroup 2.6 of FGE.19 (EFSA, 2008b). Fifteen of these substances are alpha,beta-unsaturated alicyclic ketones (alpha,beta-unsaturation in the side chain) [FL-no: 07.033, 07.035, 07.094, 07.098, 07.112, 07.126, 07.129, 07.140, 7.146, 07.147, 07.172, 07.175, 07.196, 07.202 and 07.255] and eight are precursors for such ketones [FL-no: 02.062, 02.083, 02.101, 02.214, 09.143, 09.215, 09.821 and 09.870].

A summary of their current evaluation status by the JECFA is given in Table 2 (JECFA, 1999a; JECFA, 2003a; JECFA, 2006a).

The alpha,beta-unsaturated aldehyde and ketone structures are considered by the Panel to be structural alerts for genotoxicity (EFSA, 2008b). Accordingly the available data on genotoxic or carcinogenic activity for the 15 ketones in FGE.212 [FL-no: 07.033, 07.035, 07.094, 07.098, 07.112, 07.126, 07.129, 07.140, 7.146, 07.147, 07.172, 07.175, 07.196, 07.202 and 07.255] and one ketone [2,6-dimethyl-9-(1-methylethylidene)-bicyclo[5.3.0]dec-2-en-4-one (non Register substance)] corresponding to the 23 substances in FGE.212, will be considered in this FGE.

The Panel also noted that for one substance [FL-no: 07.033], the CASrn, name and chemical structure were not consistent (Table 1). Therefore a clarification is needed.

The Panel has also taken into consideration the outcome of the predictions from five selected (Q)SAR models (Benigni & Netzeva, 2007a; Gry et al., 2007; Nikolov et al., 2007) on the 15 ketones FL-no: 07.033, 07.035, 07.094, 07.098, 07.112, 07.126, 07.129, 07.140, 7.146, 07.147, 07.172, 07.175, 07.196, 07.202 and 07.255] and the one non-register ketone [2,6-dimethyl-9-(1-methylethylidene)-bicyclo[5.3.0]dec-2-en-4-one in this FGE. The 15 ketones and the one non-Register ketone as well as their (Q)SAR predictions are shown in Table 3.

2. Toxicity

2.1. (Q)SAR Predictions

In Table 3 the outcomes of the (Q)SAR predictions for possible genotoxic activity in five *in vitro* (Q)SAR models (ISS Local Model-Ames test, DTU-NFI MultiCASE-Ames test, -Chromosomal aberration test in Chinese hamster ovary cells (CHO), -Chromosomal aberration test in Chinese hamster lung cells (CHL), and -Mouse lymphoma test) are presented.

Positive predictions have been obtained for six substances with the MultiCASE Mouse lymphoma model and for one of these substances also with the MultiCASE model on chromosomal aberrations. For the other substances, the predictions of the MultiCASE models were negative, equivocal or the substances were out of domain. All substances were out of domain in the ISS model.

2.2. Carcinogenicity Studies[4]

Groups of 50 male and 50 female F344/N rats were administered isophorone (3,5,5-trimethylcyclohex-2-en-1-one [FL-no: 07.126]) in corn oil by gavage at dose levels of 0 (controls), 250 or 500 mg/kg body weight (bw)/day, five times per week for 103 weeks. During the study the body weights of the high-dose male and female rats were slightly lower than those of the vehicle controls. The survival of high-dose male rats was significantly lower than that of the vehicle controls after week 96. Dosed male rats showed a variety of proliferative lesions of the kidney (tubular cell hyperplasia, 0/50, 1/50, 4/50; tubular cell adenoma, 0/50, 0/50, 2/50; tubular cell adenocarcinoma, 0/50, 3/50, 0/50; epithelial hyperplasia of the renal pelvis, 0/50, 5/50, 5/50). Dosed male rats also exhibited increased mineralisation of the medullary collecting ducts (1/50, 31/50, 20/50) and low-dose male rats showed a more severe nephropathy than is commonly seen in aging F344/N rats. Carcinomas of the preputial gland were significantly increased ($P < 0.03$) in high-dose male rats (0/50, 0/50, 5/50). With the exception of a moderate increase in nephropathy (21/50, 39/50, 32/50), female rats did not show chemically related increased incidences of neoplastic or non-neoplastic lesions (NTP, 1986d).

Groups of 50 male and 50 female $B6C3F_1$ mice were administered isophorone (3,5,5-trimethylcyclohex-2-en-1-one [FL-no: 07.126]) in corn oil by gavage at dose levels of 0 (controls), 250 or 500 mg/kg bw/day, five times per week for 103 weeks. During the study the body weights of the high-dose female mice were slightly lower than those of the vehicle controls. The survival of male mice was low, whereas there was a significant trend toward increased survival of dosed female mice relative to that of the vehicle controls. In high-dose male mice, isophorone exposure was associated with an increased incidence of hepatocellular adenomas and carcinomas (18/48, 18/50, 29/50) and of mesenchymal tumors of the integumentary system (fibroma, fibrosarcoma, neurofibrosarcoma, or sarcoma, 6/48, 7/50, 14/50). An increased incidence of lymphomas or leukemias was noted in low-dose male mice (8/48, 18/50, 5/50). Coagulative necrosis (3/48, 10/50, 11/50) and hepatocytomegaly (23/48, 39/50, 37/50) were observed more frequently in the livers of dosed male mice than in vehicle controls. No compound-related neoplastic or non-neoplastic lesions associated with isophorone exposure were seen in female mice (NTP, 1986d).

The Panel concluded that isophorone increased the incidences of renal tubular cell adenomas and adenocarcinomas and of carcinomas of the preputial gland in male rats but not in female rats. In male mice, but not in females, it produced increased incidences of hepatocellular adenomas and carcinomas, mesenchymal tumors in the integumentary system, and malignant lymphomas.

The Panel agrees with the authors of the NTP report who concluded that "under the conditions of these 2-year gavage studies, there was some evidence of carcinogenicity of isophorone in male F344/N rats as shown by the occurrence of renal tubular cell adenomas and adenocarcinomas in animals given 250 or 500 mg/kg bw per day; carcinomas of the preputial gland were also observed at increased incidence in male rats given 500 mg/kg bw. There was no evidence of carcinogenicity in female F344/N rats given 250 or 500 mg/kg bw per day. For male $B6C3F_1$ mice, there was equivocal evidence of carcinogenicity of isophorone as shown by an increased incidence of hepatocellular adenomas or carcinomas (combined) and of mesenchymal tumors in the integumentary system in animals given 500 mg/kg bw per day and by an increase in malignant lymphomas in animals given 250 mg/kg bw per day. There was no evidence of carcinogenicity of isophorone in female $B6C3F_1$ mice given 250 or 500 mg/kg bw per day."

Groups of 50 male and 50 female $B6C3F_1$ mice (7-week old) were administered 0, 375 or 750 mg/kg bw *d*-carvone [FL-no: 07.146] in corn oil by gavage, five days per week for 103 weeks. The mean body weights of dosed and control male and female mice were similar throughout most of the study. The survival of both the low-dose and the high-dose females were significantly greater than that of the controls. No differences in survival were observed between any groups of male mice. Atrophy of the

[4] The data presented in Section 2.2 and Section 2.3 is cited from the previous version of the present FGE.212. These data are the basis for the conclusions in FGE.212 requesting additional genotoxicity data.

olfactory epithelium and hyperplasia of the underlying Bowman's glands occurred together with high incidence in either sex in both dosed groups. This effect was found due to a local effect of *d*-carvone caused by reflux of the gavage material when the gavage needle was withdrawn. No increases in tumour incidences were seen in mice administered *d*-carvone. The incidences of male mice with primary neoplasms and the total numbers of primary neoplasms were significantly lower in the dosed groups than in the vehicle controls (NTP, 1990b).

The Panel concluded that *d*-carvone was not carcinogenic in mice under the study conditions. It agrees with the authors of the NTP report who concluded that "under the conditions of these 2-year gavage studies, there was no evidence of carcinogenic activity of d-carvone for male or female $B6C3F_1$ mice administered 375 or 750 mg/kg, 5 days per week for 2 years."

Study validation and results are presented in Table 4.

2.3. Genotoxicity Studies[4]

In subgroup 2.6 there are studies available for four substances. For tetramethyl ethylcyclohexenone (mixture of isomers [FL-no: 07.035] one *in vitro* and one *in vivo* study have been evaluated.

Seven *in vitro* and three *in vivo* studies are available for 3,5,5 trimethylcyclohex-2-en-1-one [FL-no: 07.126] (isophorone).

Three *in vitro* studies are available concerning d-carvone [FL-no: 07.146] and two *in vitro* studies concerning l-carvone [FL-no: 07.147].

Study validation and results are presented in Table 5 and 6.

3,5,5 Trimethylcyclohex-2-en-1-one [FL-no: 07.126] (isophorone) did not induce gene mutations in bacteria but it induced mutations in mammalian cells in a mouse lymphoma TK assay in the absence of metabolic activation (it was not tested in the presence of metabolic activation) (NTP, 1986d). No mutations in the MLTK assay were observed in a study of O'Donoghue et al. (O'Donoghue et al., 1988) at comparable concentrations. Isophorone induced chromosomal aberrations in Chinese hamster lung fibroblasts with and without metabolic activation (Matsuoka et al., 1996) and sister chromatid exchanges (SCE) in CHO cells without metabolic activation (Gulati et al., 1989). Chromosomal aberrations have not been observed in two other studies (Gulati et al., 1989; NTP, 1986d); however, the validity of the results was limited because the types of aberrations were not reported. Isophorone did not induce unscheduled DNA synthesis (UDS) in rat hepatocytes *in vitro*. *In vivo*, isophorone was tested negative in a sex-linked recessive lethal mutation assay in *Drosophila* (Foureman et al., 1994) and in two micronucleus assays in mice (McKee et al., 1987; O'Donoghue et al., 1988). However, the *Drosophila* assay has only limited relevance and the micronucleus assays were of limited validity.

Negative results were also observed with tetramethyl ethylcyclohexenone [FL-no: 07.035] in bacteria, in a sex-linked recessive lethal mutation assay in *Drosophila* (Wild et al., 1983) and in a mouse micronucleus assay (Wild et al., 1983); however, there was a mixture of isomers tested and the studies were only of limited validity.

d-Carvone [FL-no: 07.146] was not mutagenic in bacteria but induced SCE and chromosomal aberrations in CHO cells in the presence and absence of metabolic activation, respectively (NTP, 1990b).

2.4. Conclusion on Genotoxicity and Carcinogenicity[5]

The Panel concluded that 3,5,5 trimethylcyclohex-2-en-1-one [FL-no: 07.126] (isophorone) is genotoxic *in vitro* while a final conclusion on the genotoxicity *in vivo* could not be drawn based on the data available. It is carcinogenic in male rats and male mice. It was also predicted to be genotoxic in one of the four MultiCASE models (while it was out of domain in the ISS model).

d-Carvone [FL-no: 07.146] is genotoxic *in vitro* while no *in vivo* data were available. d-Carvone, was not carcinogenic in mice and was predicted to be non-genotoxic in the four MultiCASE models (while it was out of domain in the ISS model). No data are available on l-carvone. However, *in vivo* studies in humans show that the metabolism of ingestion-correlated amounts of d- or l-carvone occurs via a major oxidative pathway of the isopropylene side chain yielding diol and two carboxylic acids, irrespective of the stereochemical difference between the two parent isomers of carvone (Engel, 2001). Accordingly, the results for d-carvone can be used for l-carvone as well.

The negative results reported from *in vivo* studies on the genotoxicity of tetramethyl ethylcyclohexenone [FL-no: 07.035] were only of limited validity.

3. Conclusion[5]

The present Flavouring Group Evaluation 212 (FGE.212) concerns 23 substances. The 23 substances correspond to subgroup 2.6 of FGE.19. Fifteen of these substances are alpha,beta-unsaturated alicyclic ketones [FL-no: 07.033, 07.035, 07.094, 07.098, 07.112, 07.126, 07.129, 07.140, 7.146, 07.147, 07.172, 07.175, 07.196, 07.202 and 07.255] and eight are precursors for such ketones [FL-no: 02.062, 02.083, 02.101, 02.214, 09.143, 09.215, 09.821 and 09.870].

d-Carvone [FL-no: 07.146] was found genotoxic *in vitro.* However, d-carvone was not carcinogenic in mice. Therefore, the Panel concluded that this substance together with the structurally related *l*-carvone as well as carveol and the carvyl derivatives [FL-no: 02.062, 07.147, 09.143, 09.215 and 09.870] could be evaluated through the Procedure.

Isophorone [FL-no: 07.126 (3,5,5-trimethylcyclohex-2-en-1-one)] is genotoxic *in vitro* and since there is some evidence of carcinogenicity in male rats and equivocal evidence of carcinogenicity in male mice and since a non-threshold mechanism could not be excluded based on the data currently available, the Panel concluded that additional data are required for isophorone in order to clarify whether genotoxicity occurs *in vivo* and whether there is a threshold for the effects observed in the target organs in the long-term bioassays. Therefore, an *in vivo* Comet assay in F344/N rats covering these target organs is required in addition to an *in vivo* bone marrow assay with oral application.

Due to structural similarities and lack of data, the remaining substances cannot presently be evaluated through the Procedure [FL-no: 02.083, 02.101, 02.214, 07.033, 07.035, 07.094, 07.098, 07.112, 07.129, 07.140, 07.172, 07.175, 07.196, 07.202, 07.255 and 09.821]. Additional data on genotoxicity are requested for representative substances of this subgroup according to the Opinion of the Panel on the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19 (EFSA, 2008bb).

4. Data submitted from Industry in reply to genotoxicity data requested in FGE.212

Honma *et al.* (Honma et al., 1999a; Honma et al., 1999b) found that isophorone did not clearly induce mutations in the mouse lymphoma assay (MLA) following 3 hours treatments, but observed that it was mutagenic after 24 hours treatments in the absence of S9. Although only graphs are plotted, it seems

[5] The conclusions in Section 2.4 and Section 3 are cited from the previous version of the present FGE, FGE.212. This conclusion is the basis for the request of additional genotoxicity data in FGE.212.

that increases in mutation frequency (MF) that exceeded the Global Evaluation Factor (GEF) occurred at around 1250-1500 µg/ml where toxicity (by relative survival) reached 70-90 %.

The NTP conducted a mouse bone marrow chromosomal aberration (CA) study on isophorone. Groups of 8 male $B6C3F_1$ mice (larger group sizes than required by OECD) were dosed i.p. with isophorone at 125, 250 and 500 mg/kg bw. The standard protocol for *in vivo* CA is not given on the NTP website. However, based on Shelby and Witt (Shelby & Witt, 1995), animals should have been sampled at 17 hours and, if negative, also at 36 hours. The data on the NTP website are only for bone marrow sampled at 36 hours. It is therefore possible that a 17 hours sample was also taken, and found to be negative, but the data have not been posted. Fifty cells per animal were scored for CA and no increases in CA were seen. No measures of toxicity were recorded, but i.p. dosing should have guaranteed systemic exposure. The control CA frequency was normal (2.75 %) and the positive control (dimethylbenzanthracene) produced a significant response in CA frequency.

A DNA binding study was conducted in which F344-rats and $B6C3F_1$-mice (the strains used in the NTP carcinogenicity study) were exposed to isophorone (Thier et al., 1990). Animals of both sexes were dosed once or five times by gavage with 500 mg/kg bw of unlabelled isophorone spiked with [1,3,5-^{14}C]-isophorone (specific activity: 52 mCi per mmol, 1.92 GBq per mmol). An additional group of acute dosed male rats received undiluted ^{14}C-isophorone for increased sensitivity. Rats and mice were maintained for 24 hours in closed metabolic cages. Twenty four hours after exposure, livers and kidneys (the tumour target tissues) were removed from the animals. DNA was isolated through hydroxyapatite chromatography and radioactivity was measured by liquid scintillation counting. No positive controls were included. Also no untreated controls were included, but, except for the liver sample of one mouse in the five times dose group, radioactivity values were within 2σ of background (6 dpm). Radioactivity values therefore did not indicate significant attachment of radioactivity to DNA. From these results it can be concluded that neither isophorone nor its metabolites bind covalently to DNA.

In addition, a report by Morishita *et al.* (Morishita et al., 1997b) submitted to EPA (EPA, 1997), is relevant and appears to have been previously submitted only as an abstract. This study was designed to investigate whether isophorone and/or α2µ-globulin[6] might be involved in the induction of preputial gland tumours in F-344 rats (10/sex/dose group). A series of experiments was performed in order to study several parameters including:

- binding of isophorone to DNA of kidney and preputial gland. Groups of 10 male rats were dosed by gavage with 500 mg/kg of [^{14}C]-isophorone (specific activity 14.65 mCi/mmol; 100 µCi/animal). Positive control animals were dosed with ^{3}H-labeled methyl nitrosourea.

- DNA adduct detection by ^{32}P-postlabeling in young adult male and female rats (7 per group) dosed by gavage with 0, 250 or 500 mg/kg isophorone for five days.

Extraction of preputial gland and kidney DNA from rats treated with single 500 mg/kg labeled doses yielded no evidence of isophorone binding to DNA, whereas the positive control showed significant binding to DNA of preputial gland and kidney. These negative results with isophorone were confirmed in the ^{32}P -postlabeling assays.

In addition Industry has also asked whether the information submitted for isophorone, (cyclohexenyl derivative), could also be applied to evaluate the genotoxic potential of the five-carbon membered ring substances (i.e. cyclopentenyl derivatives) in subgroup 2.6 (letter of EFFA to EFSA, dated 14/4-2010). This request was supported by the argumentation that there is structural resemblance with respect to steric hindrance around the alpha,beta-unsaturated double bond. In addition, Industry argued that the

[6] Since interaction with α2µ-glubulin is not of direct relevance for the evaluation of genotoxic potential, this information is omitted from this study summary.

π-conjugation systems in these molecules is very nearly planar and that therefore the reactivity and genotoxic potentials of the five- and six-membered ring systems would be similar. No further data were provided to substantiate this argumentation.

5. Discussion of the additional data

Conflicting results were reported in two valid studies with the mouse lymphoma assay (MLA): one negative (O'Donoghue et al., 1988) and one positive (NTP, 1986d) at comparable concentrations. Mixed results were also reported in two studies of limited validity: one negative (Honma et al., 1999a) and one positive (Honma et al., 1999b). Another negative result was reported in a study (McKee et al., 1987), the validity of which cannot be evaluated. In the light of the clearly negative results in two valid bacterial gene mutation tests (Ames test) and in a valid Sex Linked Recessive Lethal Mutations test (SLRL) in *Drosophila*, and taking into account the lack of specificity and high sensitivity of the MLA, overall the results presently available are considered of questionable relevance. The Panel agrees that isophorone demonstrates some genotoxic activity *in vitro* but that the new data demonstrate lack of clastogenicity *in vivo*. In addition, the new DNA-binding data from two separate studies provide convincing evidence that isophorone does not induce tumours via a genotoxic mechanism. On the basis of these data it may be argued that there is no need to perform further *in vivo* genotoxicity studies such as the Comet assay or bone marrow micronucleus test. Thus, based on the data available the Panel concluded that there is no concern with respect to genotoxicity of isophorone.

6. Conclusion

Since based on the additional information the concern for the genotoxic potential for isophorone has been alleviated, a genotoxic potential can also be ruled out for the other six-carbon members of subgroup 2.6 related to isophorone [FL-no: 02.083, 02.101, 07.035, 07.098, 07.126, 07.129, 07.172, 07.175, 07.196, 07.202 and 07.255].

The Panel also concluded that isophorone can only be considered as representative for the six-carbon ring members of subgroup 2.6. The argumentation of Industry to expand this conclusion also to the cyclopentenyl derivatives in this subgroup [FL-no: 07.033, 07.094, 07.112 and 07.140] was considered too limited, given the lack of support from experimental data. Therefore, additional genotoxicity tests are still required for the representative substance [FL-no: 07.112] already chosen by the Panel. Alternatively, a more thorough explanation (physico-chemical parameters; experimental underpinning) of the proposed similar reactivity of six- and five-membered ring substances should be provided by Industry. Also for the seven-membered ring substance [FL-no: 02.214] (also covering [FL-no: 09.821]) additional data on genotoxicity are still required.

TABLE 1: SPECIFICATION SUMMARY OF THE SUBSTANCES IN THE FLAVOURING GROUP EVALUATION 212 (JECFA, 1999A; JECFA, 2003A; JECFA, 2006A)

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 212 (JECFA, 1999a; JECFA, 2003a; JECFA, 2006a)

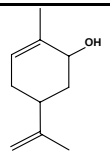
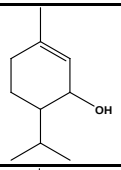
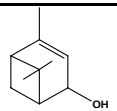
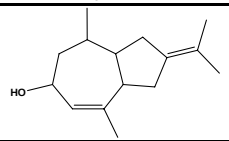
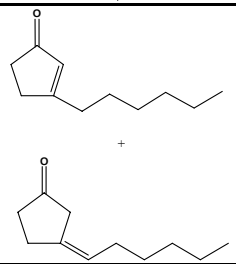
FL-no JECFA- no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
02.062 381	Carveol		2247 2027 99-48-9	Liquid $C_{10}H_{16}O$ 152.24	 Freely soluble	226-227 IR 96 %	1.493-1.497 0.947-0.953
02.083 434	p-Menth-1-en-3-ol		3179 10248 491-04-3	Liquid $C_{10}H_{18}O$ 154.25		232 NMR 97 %	1.4762 (25C) 0.930-0.936
02.101 1404	Pin-2-en-4-ol		3594 10304 473-67-6	Solid $C_{10}H_{16}O$ 152.24	Very slightly soluble Soluble	n.a. 63-67 NMR 95 %	n.a. n.a.
02.214	Vetiverol		4217 10321 89-88-3	Solid $C_{15}H_{24}O$ 220.35	Practically insoluble or insoluble Freely soluble	n.a. 69 NMR 95 %	n.a. n.a.
07.033 1115	Isojasmone 6)		3552 167 11050-62-7	Liquid $C_{11}H_{18}O$ 166.26		144 (13 hPa) NMR 95 %	1.472-1.477 0.917-0.924

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 212 (JECFA, 1999a; JECFA, 2003a; JECFA, 2006a)

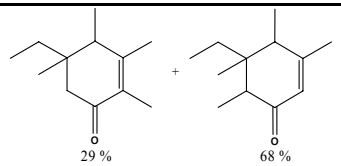
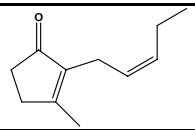
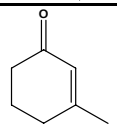
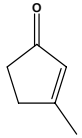
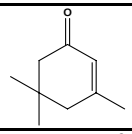
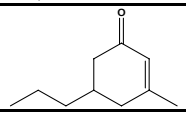
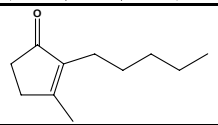
FL-no JECFA- no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
07.035 1111	Tetramethyl ethylcyclohexenone (mixture of isomers)		3061 168 17369-60-7	Liquid $C_{12}H_{20}O$ 180.29		113-115 NMR 97 %	1.485-1.490 0.927-0.934
07.094 1114	3-Methyl-2-(pent-2(cis)-enyl)cyclopent-2-en-1-one		3196 11786 488-10-8	Liquid $C_{11}H_{16}O$ 164.25		248 NMR 98 %	1.495-1.501 0.942-0.948
07.098 1107	3-Methylcyclohex-2-en-1-one		3360 11134 1193-18-6	Liquid $C_7H_{10}O$ 110.16		199-200 NMR 98 %	1.490-1.498 0.967-0.972
07.112 1105	3-Methyl-2-cyclopenten-1-one		3435 11137 2758-18-1	Liquid C_6H_8O 96.12		74 (20 hPa) NMR 98 %	1.485-1.491 0.968-0.975
07.126 1112	3,5,5-Trimethylcyclohex-2-en-1-one		3553 11918 78-59-1	Liquid $C_9H_{14}O$ 138.21		213-215 NMR 97 %	1.474-1.481 0.919-0.927
07.129 1113	3-Methyl-5-propylcyclohex-2-en-1-one 6)		3577 3720-16-9	Liquid $C_{10}H_{16}O$ 152.23		242-244 NMR 95 %	1.481-1.486 0.924-0.928
07.140 1406	3-Methyl-2-pentylcyclopent-2-en-1-one		3763 1128-08-1	Liquid $C_{11}H_{18}O$ 166.26	Very slightly soluble Soluble	79 (0.2 hPa) NMR 99 %	1.676-1.682 0.911-0.917

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 212 (JECFA, 1999a; JECFA, 2003a; JECFA, 2006a)

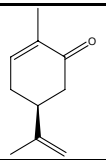
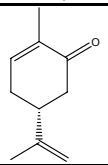
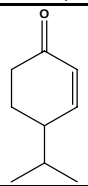
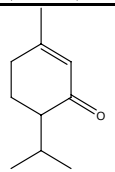
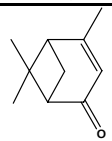
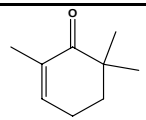
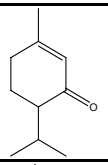
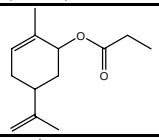
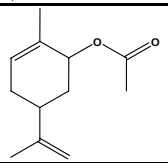
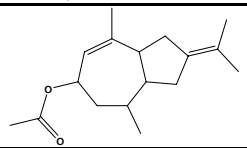
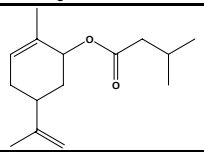
FL-no JECFA- no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
07.146 380.1	d-Carvone		- - 2244-16-8				
07.147 380.2	l-Carvone		- - 6485-40-1				
07.172 1110	4-Isopropylcyclohex-2-en-1-one 6)		3939 11127 500-02-7	Liquid $C_9H_{14}O$ 138.21		198 NMR 97 %	1.481-1.490 0.930-0.950
07.175 435	p-Menth-1-en-3-one		2910 2052 89-81-6	Liquid $C_{10}H_{16}O$ 152.24	Insoluble	233-235 IR 94 %	1.483-1.487 0.929-0.934
07.196	Pin-2-en-4-one		- 11186 80-57-9	Liquid $C_{10}H_{14}O$ 150.22	Freely soluble	90 (16 hPa) 95 %	1.492-1.498 0.975-0.981
07.202	2,6,6-Trimethylcyclohex-2-en-1- one		- - 20013-73-4	Liquid $C_9H_{14}O$ 138.21	Slightly soluble Freely soluble	63 (16 hPa) MS 95 %	1.470-1.476 0.924-0.930

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 212 (JECFA, 1999a; JECFA, 2003a; JECFA, 2006a)

FL-no JECFA- 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)
07.255 1856	l-Piperitone		4200 - 4573-50-6	Liquid $C_{10}H_{16}O$ 152.24	Slightly soluble Freely soluble	246 MS 99 %	1.482-1.488 0.929-0.935
09.143 383	Carvyl propionate		2251 424 97-45-0	Liquid $C_{13}H_{20}O_2$ 208.30	Insoluble	239 IR 98 %	1.469-1.479 0.942-0.962
09.215 382	Carvyl acetate		2250 2063 97-42-7	Liquid $C_{12}H_{18}O_2$ 194.27	Slightly soluble	229 IR 98 %	1.473-1.479 0.964-0.970
09.821	Vetiveryl acetate		- 11887 117-98-6	Solid $C_{17}H_{26}O_2$ 262.39	Freely soluble	406 73 95 %	n.a. n.a.
09.870	Carvyl-3-methylbutyrate		- - 94386-39-7	Liquid $C_{15}H_{24}O_2$ 236.37	Practically insoluble or insoluble Freely soluble	343 MS 95 %	1.462-1.468 0.932-0.938

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) (JECFA, 1999A; JECFA, 2003A; JECFA, 2006A)

Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach) (JECFA, 1999a; JECFA, 2003a; JECFA, 2006a)

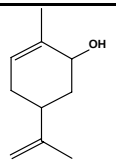
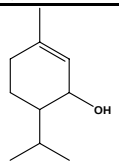
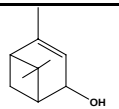
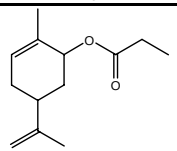
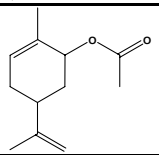
FL-no JECFA-no	EU Register name	Structural formula	MSDI 1) µg/capita/day) EU USA	Class 2) JECFA Evaluation procedure path 3)	Outcome on the named compound [4) or 5]	EFSA comments
02.062 381	Carveol		9.5 140	Class I A3: Intake below threshold	4)	Evaluated in FGE.212, genotoxic concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
02.083 434	p-Menth-1-en-3-ol		0.012 0.02	Class I A3: Intake below threshold	4)	Evaluated in FGE.212Rev1, genotoxic concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
02.101 1404	Pin-2-en-4-ol		0.012 0.2	Class I A3: Intake below threshold	4)	Evaluated in FGE.212Rev1, genotoxic concern could be ruled out.
09.143 383	Carvyl propionate		ND 0.04	Class I A3: Intake below threshold	4)	Evaluated in FGE.212, genotoxic concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
09.215 382	Carvyl acetate		4.0 36	Class I A3: Intake below threshold	4)	Evaluated in FGE.212, genotoxic concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.

Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach) (JECFA, 1999a; JECFA, 2003a; JECFA, 2006a)

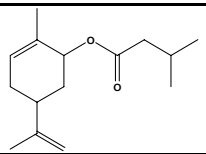
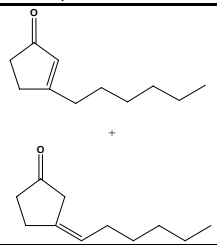
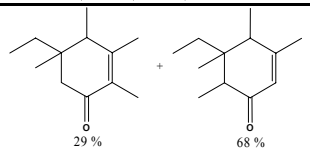
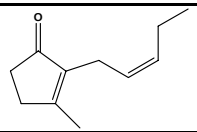
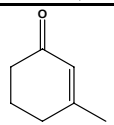
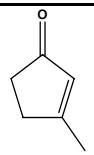
FL-no JECFA-no	EU Register name	Structural formula	MSDI 1) µg/capita/day) EU USA	Class 2) JECFA Evaluation procedure path 3)	Outcome on the named compound [4) or 5]	EFSA comments
09.870	Carvyl-3-methylbutyrate		0.0012	Class I A3: Intake below threshold	4)	Evaluated in FGE.212, genotoxic concern could be ruled out.
07.033 1115	Isojasmone		0.37 0.01	Class II A3: Intake below threshold	4)	Evaluated in FGE.212Rev1, genotoxic concern could not be ruled out. Additional genotoxicity data required for the representative [FL-no: 07.112].
07.035 1111	Tetramethyl ethylcyclohexenone (mixture of isomers)		7.8 0.2	Class II A3: Intake below threshold	4)	Evaluated in FGE.212Rev1, genotoxic concern could be ruled out.
07.094 1114	3-Methyl-2-(pent-2(cis)-enyl)cyclopent-2-en-1-one		13 7.2	Class II A3: Intake below threshold	4)	Evaluated in FGE.212Rev1, genotoxic concern could not be ruled out. Additional genotoxicity data required for the representative [FL-no: 07.112].
07.098 1107	3-Methylcyclohex-2-en-1-one		0.012 0.1	Class II A3: Intake below threshold	4)	Evaluated in FGE.212Rev1, genotoxic concern could be ruled out.
07.112 1105	3-Methyl-2-cyclopenten-1-one		0.06 ND	Class II A3: Intake below threshold	4)	Evaluated in FGE.212Rev1, genotoxic concern could not be ruled out. Additional genotoxicity data required for the representative [FL-no: 07.112].

Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach) (JECFA, 1999a; JECFA, 2003a; JECFA, 2006a)

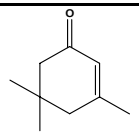
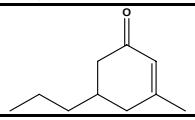
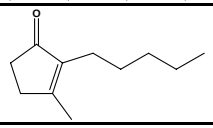
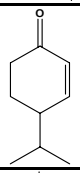
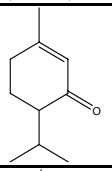
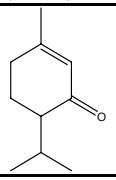
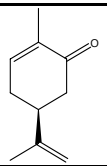
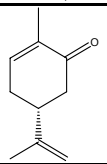
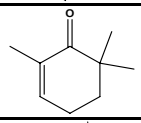
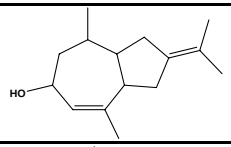
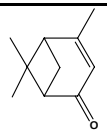
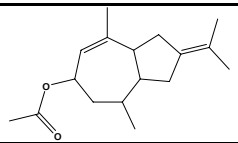
FL-no JECFA-no	EU Register name	Structural formula	MSDI 1) µg/capita/day) EU USA	Class 2) JECFA Evaluation procedure path 3)	Outcome on the named compound [4) or 5]	EFSA comments
07.126 1112	3,5,5-Trimethylcyclohex-2-en-1-one		4.6 0.1	Class II A3: Intake below threshold	4)	Evaluated in FGE.212Rev1, genotoxic concern could be ruled out.
07.129 1113	3-Methyl-5-propylcyclohex-2-en-1-one		0.097 4.1	Class II A3: Intake below threshold	4)	Evaluated in FGE.212Rev1, genotoxic concern could be ruled out.
07.140 1406	3-Methyl-2-pentylcyclopent-2-en-1-one		0.34 0.2	Class II A3: Intake below threshold	4)	Evaluated in FGE.212Rev1, genotoxic concern could not be ruled out. Additional genotoxicity data required for the representative [FL-no: 07.112].
07.172 1110	4-Isopropylcyclohex-2-en-1-one		0.0012 0.001	Class II A3: Intake below threshold	4)	Evaluated in FGE.212Rev1, genotoxic concern could be ruled out.
07.175 435	p-Menth-1-en-3-one		44 10	Class II A3: Intake below threshold	4)	Evaluated in FGE.212Rev1, genotoxic concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.255 1856	l-Piperitone		490 -	Class II A3: Intake below threshold	4)	Evaluated in FGE.212Rev1, genotoxic concern could be ruled out.

Table 2: Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach) (JECFA, 1999a; JECFA, 2003a; JECFA, 2006a)

FL-no JECFA-no	EU Register name	Structural formula	MSDI 1) µg/capita/day) EU USA	Class 2) JECFA Evaluation procedure path 3)	Outcome on the named compound [4) or 5]	EFSA comments
07.146 380.1	d-Carvone		2390 9900	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	4)	Evaluated in FGE.212, genotoxic concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.147 380.2	l-Carvone		2390 9900	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	4)	Evaluated in FGE.212, genotoxic concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.202	2,6,6-Trimethylcyclohex-2-en-1-one		0.12 -	Class II No evaluation	Not evaluated by JECFA	Evaluated in FGE.212, genotoxic concern could be ruled out.
02.214	Vetiverol		0.011 -	Class II No evaluation	Not evaluated by JECFA during 2000 to 2008	Evaluated in FGE.212, additional genotoxicity data required.
07.196	Pin-2-en-4-one		15 -	Class II No evaluation	Not evaluated by JECFA during 2000 to 2008	Evaluated in FGE.212, genotoxic concern could be ruled out.
09.821	Vetiveryl acetate		0.011 -	Class II No evaluation	Not evaluated by JECFA during 2000 to 2008	Evaluated in FGE.212, additional genotoxicity 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 µg/person/day, Class II = 540 µg/person/day, 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.

TABLE 3: QSAR PREDICTIONS ON MUTAGENICITY IN FIVE MODELS FOR 16 KETONES FROM SUBGROUP 2.6

Table 3: QSAR Predictions on Mutagenicity in Five Models for 16 Ketones from Subgroup 2.6

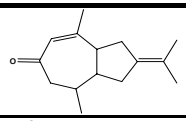
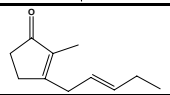
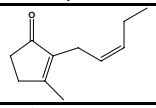
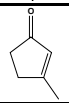
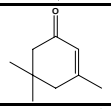
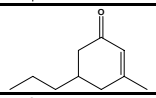
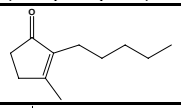
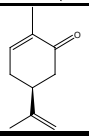
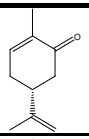
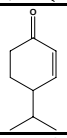
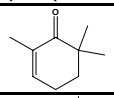
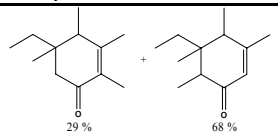
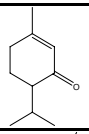
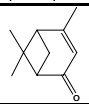
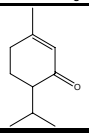
FL-no JECFA-no	Sub-group	EU Register name	Structural formula	FEMA no CoE no CAS no	ISS Local Model Ames Test TA100	MultiCASE Ames test	MultiCASE Mouse lymphoma test	MultiCASE Chromosomal aberration test in CHO	MultiCASE Chromosomal aberration test in CHL
Not in Register	2.6	2,6-Dimethyl-9-(1-methylethylidene)-bicyclo[5.3.0]dec-2-en-4-one		- - -	OD	NEG	NEG	NEG	NEG
07.033 1115	2.6	Isojasmone		3552 167 11050-62-7	OD	NEG	NEG	NEG	NEG
07.094 1114	2.6	3-Methyl-2-(pent-2(cis)-enyl)cyclopent-2-en-1-one		3196 11786 488-10-8	OD	NEG	OD	NEG	NEG
07.098 1107	2.6	3-Methylcyclohex-2-en-1-one		3360 11134 1193-18-6	OD	NEG	POS	NEG	EQU
07.112 1105	2.6	3-Methyl-2-cyclopenten-1-one		3435 11137 2758-18-1	OD	NEG	POS	NEG	EQU
07.126 1112	2.6	3,5,5-Trimethylcyclohex-2-en-1-one		3553 11918 78-59-1	OD	NEG	POS	NEG	EQU
07.129 1113	2.6	3-Methyl-5-propylcyclohex-2-en-1-one		3577 3720-16-9	OD	NEG	POS	NEG	EQU
07.140 1406	2.6	3-Methyl-2-pentylcyclopent-2-en-1-one		3763 - 1128-08-1	OD	NEG	OD	NEG	NEG
07.146 380.1	2.6	d-Carvone		- - 2244-16-8	OD	NEG	NEG	NEG	NEG

Table 3: QSAR Predictions on Mutagenicity in Five Models for 16 Ketones from Subgroup 2.6

FL-no JECFA-no	Sub-group	EU Register name	Structural formula	FEMA no CoE no CAS no	ISS Local Model Ames Test TA100	MultiCASE Ames test	MultiCASE Mouse lymphoma test	MultiCASE Chromosomal aberration test in CHO	MultiCASE Chromosomal aberration test in CHL
07.147 380.2	2.6	l-Carvone		- - 6485-40-1	OD	NEG	NEG	NEG	NEG
07.172 1110	2.6	4-Isopropylcyclohex-2-en-1-one		3939 11127 500-02-7	OD	NEG	NEG	NEG	EQU
07.202	2.6	2,6,6-Trimethylcyclohex-2-en-1-one		- - 20013-73-4	OD	NEG	OD	NEG	NEG
07.035 1111	2.6	Tetramethyl ethylcyclohexenone (mixture of isomers)		3061 168 17369-60-7	OD	NEG	NEG	NEG	NEG
07.255	2.6	l-Piperitone		- - 4573-50-6	OD	NEG	OD	NEG	EQU
07.196 -	2.6	Pin-2-en-4-one		11186 80-57-9	OD	NEG	POS	NEG	POS
07.175	2.6	p-Menth-1-en-3-one		2910 2052 89-81-6	OD	NEG	POS	NEG	OD

Column 2: Structure group 2.6: alpha,beta-unsaturated alicyclic ketones.
Column 6: Local model on aldehydes and ketones, Ames TA100. (NEG: Negative; POS: Positive; OD: Out of domain).
Column 7: MultiCASE Ames test (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
Column 8: MultiCASE Mouse lymphoma test (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
Column 9: MultiCASE Chromosomal aberration in CHO (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
Column 10: MultiCASE Chromosomal aberration in CHL (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
OD, out of applicability domain: not matching the range of conditions where a reliable prediction can be obtained in this model. These conditions may be physicochemical, structural, biological, etc.

TABLE 4: CARCINOGENICITY STUDIES

Table 4: Carcinogenicity Studies

Chemical Name [FL-no]	Species; Sex No./Group	Route	Dose levels	Duration	Results	Reference	Comments*
3,5,5-Trimethylcyclohex-2-en-1-one [07.126]	Rats; Male, Female 50/sex/group	Gavage in corn oil	0 (controls), 250, or 500 mg/kg bw/day, five times per week	103 weeks	Males: Increased incidences of renal tubular cell adenomas and adenocarcinomas and of carcinomas of the preputial gland. Females: No carcinogenic effect.	NTP, 1986d	Valid.
	Mice; Male, Female 50/sex/group	Gavage in corn oil	0 (controls), 250, or 500 mg/kg bw/day, five times per week	103 weeks	Males: Increased incidences of hepatocellular adenomas and carcinomas, mesenchymal tumors in the integumentary system, and malignant lymphomas. Females: No carcinogenic effect.	NTP, 1986d	Valid.
d-Carvone [07.146]	Mice; Male, Female 50/sex/group	Gavage	0, 375 or 750 mg/kg bw/day, five times per week	103 weeks	Males and females: No increases in tumour incidences.	NTP, 1990b	Valid.

* Validity of genotoxicity studies:
Valid.
Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation).
Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system).
Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

TABLE 5: GENOTOXICITY (*IN VITRO*)

Table 5: GENOTOXICITY (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Reported Result	Reference	Comments [e]
Tetramethyl ethylcyclohexenone (mixture of isomers [07.035]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537, TA1538	5 concentrations up to cytotoxicity, or max. 3600 µg/plate	Negative[a]	Wild et al., 1983	Limited validity (no TA 102 or *E. Coli*); possibly slightly low maximal concentration tested.
3,5,5-Trimethylcyclohex-2-en-1-one [07.126]	Reverse mutation	*S. typhimurium* TA97, TA98, TA100, TA1535, TA1537	33–10 000 microg/plate	Negative[a]	Mortelmans et al., 1986	Valid.
	Mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	33–10 000 microg/plate	Negative[a]	NTP, 1986d	NTP study carried out according to standard US-EPA guideline; result is considered as valid.
	Mutation	L5178YTk+/– mouse lymphoma cells	67–810 microg/ml	Negative[b]	McKee et al., 1987	Validity cannot be evaluated (tested with S9; abstract only with very limitred information).
	Mutation	L5178YTk+/– mouse lymphoma cells	130–1300 microg/ml	Negative[c]	McKee et al., 1987	Validity cannot be evaluted (tested without S9; abstract only with very limitred information).
	Mutation	L5178YTk+/– mouse lymphoma cells	0.089–0.89 microl/ml	Negative[c]	O'Donoghue et al., 1988	Valid according to current guidelines.
	Mutation	L5178YTk+/– mouse lymphoma cells	0.13–1.3 microl/ml	Negative[b]	O'Donoghue et al., 1988	Valid according to current guidelines.
	Mutation	L5178YTk+/– mouse lymphoma cells	1200 microg/ml	Positive[b]	NTP, 1986d	NTP study carried out according to standard US-EPA guideline; Not tested with S9. Result is considered as valid.
	Mutation	L5178YTk+/– mouse lymphoma cells	Not reported (however, up to cytotoxic concentrations) for 3 hr exposure.	Negative[a]	Honma et al., 1999a	Limited validity since data were presented in a summarized Table format only (as a result of an international collaborative study).
	Mutation	L5178YTk+/– mouse lymphoma cells	Up to 1500 microg/ml	Positive[b]	Honma et al., 1999b	Limited validity since mutation frequencies were not reported in Table format. Tested only in the absence of S9. Isophorone was mutagenic after 24 hr treatments in the absence of S9. Although only graphs are plotted, it seems that increases in MF that exceeded the Global Evaluation Factor occurred at around 1250-1500 µg/ml where toxicity (by relative survival) reached 70-90 %.
	Chromosomal aberration	Chinese hamster ovary cells	5–1600 microg/ml	Negative[a]	Gulati et al., 1989	Limited validity (not clear if gaps were included in the scores).
	Chromosomal aberration	Chinese hamster ovary cells	250–1600 microg/ml	Negative[a]	NTP, 1986d	NTP study carried out according to standard US-EPA guideline; result is considered as valid.
	Chromosomal aberration	Chinese hamster lung fibroblasts	0-1250[b] µg/ml 0 – 1500[c] µg/ml	Positive[a]	Matsuoka et al., 1996	Valid.

Table 5: GENOTOXICITY (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Reported Result	Reference	Comments [e]
	Chromosomal aberration	Chinese hamster lung fibroblasts	250–1000 mg/ml	Negative[a]	Matsuoka et al., 1996	Valid. Exposed to isophorone without metabolic activation for 24 hrs or 48 hours, cytotoxic at highest concentrations.
	Sister chromatid exchange	Chinese hamster ovary cells	5–1600 mg/ml	Positive[b,d]	Gulati et al., 1989	Valid (pos – S9; neg + S9).
	Sister chromatid exchange	Chinese hamster ovary cells	160–1000 mg/ml	Negative[a]	NTP, 1986d	Valid. NTP study carried out according to Standard US-EPA guideline; result is considered as valid.
	Unscheduled DNA synthesis	Rat hepatocytes	0.005–0.4 µl/ml	Negative	O'Donoghue et al., 1988	Valid according to current guidelines
	Unscheduled DNA synthesis	Rat hepatocytes	5 - 200 µl/ml	Negative[a]	McKee et al., 1987	Validity cannot be evaluated (abstract only with very limited information).
Carvone (isomer not specified)	Gene mutation	*S. typhimurium* TA1535, TA1537, TA98, TA100	3 µmol/plate	Negative	Florin et al., 1980	Insufficient validity (spot test, not according to OECD guideline, methods and results insufficiently reported). Isomer (D or L) not reported.
	Rec assay	*Bacillus subtilis* H17 (rec+) and M45 (rec-)	0.6 ml/disc	Negative	Matsui et al., 1989	The test system used is considered inappropriate.
d-Carvone [07.146]	Gene mutation	*S. typhimurium* TA1535, TA98, TA100, TA1537	333 µg/plate	Negative[a]	NTP, 1990b	Valid.
	Gene mutation (preincubation)	*S. typhimurium* TA1535, TA98, TA100, TA1537	560 µg/plate	Negative	Mortelmans et al., 1986	Valid.
	Sister chromatid exchange	Chinese hamster ovary cells	502 µg/ml	Positive[a]	NTP, 1990b	Valid.
	Chromosomal aberration	Chinese hamster ovary cells	400 µg/ml	Positive[a]	NTP, 1990b	Valid.

a: With and without metabolic activation.
b: Without metabolic activation.
c: With metabolic activation.
d: Cytotoxic at next highest dose tested (1600 mg/ml).
e: Validity of genotoxicity studies:
Valid.
Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation).
Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system).
Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

TABLE 6: GENOTOXICITY (*IN VIVO*)

Table 6: GENOTOXICITY (*in vivo*)

Chemical Name [FL-no]	Test System	Test Object	Route	Dose	Result	Reference	Comments [a]
Tetramethyl ethylcyclohexenone (mixture of isomers [07.035]	Sex-linked recessive lethal mutation	*D. melanogaster*	Feed	10 mM	Negative	Wild et al., 1983	Limited validity (low nr of chromosomes, limited reporting).
	Micronucleus formation	Mouse bone marrow	i.p.	180, 307, 450 mg/kg bw	Negative	Wild et al., 1983	Limited validity. Only analysis at one time point; no PCE/NCE ratio reported.
3,5,5-Trimethylcyclohex-2-en-1-one [07.126]	Sex-linked recessive lethal mutation	*D. melanogaster*		2000[b] and 12500[c] ppm	Negative	Foureman et al., 1994	Valid, however, only limited relevance.
	Micronucleus formation	CD-1 mice	i.p.	540 mg/kg bw (MTD)	Negative	McKee et al., 1987	Validity cannot be evaluated. Abstract only; very limited information nodata on PCE/NCE ratio.
	Micronucleus formation	CD-1 mice	i.p.	0.54 ml/kg bw	Negative	O'Donoghue et al., 1988	Limited validity. Only one dose level tested, this dose level corresponded to the LD20; sample schedule inadequate.
	Chromosomal aberration	B6C3F1 mice	i.p.	125, 250, 500 mg/kg bw	Negative	NTP-Website	Valid. Submitted by Industry in 2009. The standard protocol for *in vivo* CA is not given on the NTP website. However, based on Shelby and Witt (1995), animals should have been sampled at 17 hr and, if negative, also at 36 hr. The data on the NTP website are only for bone marrow sampled at 36 hr. It is therefore possible that a 17 hr sample was also taken, and found to be negative, but the data not posted. Fifty cells per animal were scored for CA and no increases in CA were seen. No measures of toxicity were recorded, but i.p. dosing should have guaranteed systemic exposure.
	DNA binding	F344 rats	Gavage	500 mg unlabelled isophorone/kg bw spiked with ^{14}C-isophorone (0.4 mCi/rat)	Negative	Thier et al., 1990	Limited validity. Submitted by Industry in 2009. No positive controls and no untreated controls used. Liver and kidney were analysed.
	DNA binding	B6C3F1 mice	Gavage	500 mg unlabelled isophorone/kg bw spiked with ^{14}C-isophorone (0.08 mCi/mouse)	Negative	Thier et al., 1990	Limited validity. Submitted by Industry in 2009. No positive controls and no untreated controls used. Liver and kidney were analysed.
	DNA binding	F344 rats (10 males)	Gavage	500 mg/kg bw ^{14}C-isophorone (0.1 mCi/rat)	Negative	Morishita et al., 1997	Valid. Preputial glands and kidneys were analysed.
	DNA adducts (^{32}P-Postlabelling)	F344 rats (7 males and 7 females per dose group)	Gavage	0 and 500 mg/kg/day for 5 days.	Negative	Morishita et al., 1997	Valid. Preputial glands were analysed.

a: Validity of genotoxicity studies:
Valid.
Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation).
Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system).
Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).
b: Oral administration.
c: Injection.

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ABBREVIATIONS

BW	Body Weight
CAS	Chemical Abstract Service
CHL	Chinese hamster lung cell(s)
CHO	Chinese hamster ovary cell(s)
CoE	Council of Europe
DNA	Deoxyribonucleic acid
DTU-NFI	Danish Technical University – National Food Institute
EC	European Commission
EFSA	The European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS	Flavour Information System database
ID	Identity
IP	Intraperitoneal
IR	Infrared spectroscopy
ISS	Istituto Superiore di Sanita
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MS	Mass spectrometry
MSDI	Maximum Survey-derived Daily Intake
NMR	Nuclear magnetic resonance
No	Number
NOAEL	No observed adverse effect level
NTP	National Toxicology Programme
OECD	Organisation for Economic Co-operation and Development
PCE/NCE	Polychromatic erythrocytes/normochromatic erythrocytes
(Q)SAR	(Quantitative) structure-activity relationship
SCE	Sister chromatid exchange
SCF	Scientific Committee on Food
UDS	Unscheduled DNA synthesis
US EPA	United States Environmental Protection Agency
WHO	World Health Organisation