

SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 208 Revision 1 (FGE.208Rev1): Consideration of genotoxicity data on representatives for 10 alicyclic aldehydes with the α,β -unsaturation in ring / side-chain and precursors from chemical subgroup 2.2 of FGE.19¹

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)^{2,3}

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ABSTRACT

The EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids was requested to evaluate the genotoxic potential of flavouring substances from subgroup 2.2 of FGE.19 in the Flavouring Group Evaluation 208 Revision 1 (FGE.208Rev1). The Flavour Industry has provided additional genotoxicity studies on *p*-mentha-1,8-dien-7-al [FL-no: 05.117], the representative substance for FGE.19 subgroup 2.2. This substance was tested *in vivo* in a combined micronucleus assay in bone marrow and Comet assay in liver and duodenum. It did not induce any increase in micronucleated polychromatic erythrocytes of the bone marrow of male rats in the micronucleus test and it did not induce DNA damage in duodenum of the same animals as analysed by the Comet assay. The Comet assay performed in liver shows a positive result and therefore the Panel concluded that *p*-mentha-1,8-dien-7-al [FL-no: 05.117] is genotoxic *in vivo* and that, accordingly, there is a safety concern for its use as flavouring substance. Since *p*-mentha-1,8-dien-7-al [FL-no: 05.117] is representative for the nine remaining substances of this subgroup 2.2 (*p*-mentha-1,8-dien-7-ol [FL-no: 02.060], myrtenol [FL-no: 02.091], myrtenal [FL-no: 05.106], 2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde [FL-no: 05.121], myrtenyl formate [FL-no: 09.272], *p*-mentha-1,8-dien-7-yl acetate [FL-no: 09.278], myrtenyl acetate [FL-no: 09.302], myrtenyl-2-methylbutyrate [FL-no: 09.899] and myrtenyl-3-methylbutyrate [FL-no: 09.900]), there is a potential safety concern for these substances.

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KEY WORDS

FGE.19, subgroup 2.2, alicyclic aldehydes, α,β -unsaturated

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SUMMARY

Following a request from the European Commission, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) was asked to deliver a scientific opinion on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Panel was asked to evaluate flavouring substances using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000 (hereafter 'the Procedure').

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

The Flavouring Group Evaluation 208 (FGE.208), corresponding to subgroup 2.2 of FGE.19, concerns three alicyclic aldehydes with the α,β -unsaturation in ring / side-chain and seven precursors for such. The α,β -unsaturated aldehyde structure, which is a structural alert for genotoxicity and the data on genotoxicity previously available for these 10 substances, did not rule out the concern for genotoxicity.

Among the 10 flavouring substances in FGE.19 subgroup 2.2, the Panel identified *p*-mentha-1,8-dien-7-al [FL-no: 05.117], for which appropriate genotoxicity data could be used for reading across to the other substances in the subgroup; therefore genotoxicity data have been requested for *p*-mentha-1,8-dien-7-al [FL-no: 05.117] according to the testing strategy worked out by the Panel.

In 2012, the Flavour Industry submitted new genotoxicity data: a bacterial reverse mutation assay, a hypoxanthine-guanine phosphoribosyl transferase (HPRT) assay and an *in vitro* micronucleus assay. These new data were evaluated in FGE.208 where the Panel concluded that the available data still gave rise to concern for the genotoxic potential of *p*-mentha-1,8-dien-7-al [FL-no: 05.117]. Therefore, the Panel asked to provide an *in vivo* Comet assay performed on the first site of contact (e.g. stomach or duodenum) and on liver.

The Flavour Industry has submitted a combined study in rats: a bone marrow micronucleus test and Comet assay in liver and duodenum. This study is evaluated in the present Revision 1 of FGE.208 (FGE.208Rev1). *p*-Mentha-1,8-dien-7-al [FL-no: 05.117] was administered to rats by oral gavage at three dose levels: 175, 350 and 700 mg/kg bw/day (which is an estimate of the maximum tolerated dose). The results of the micronucleus assay suggest that *p*-mentha-1,8-dien-7-al [FL-no: 05.117] did not induce any increase in micronucleated polychromatic erythrocytes.

In the same animals, results of the Comet assay suggest that *p*-mentha-1,8-dien-7-al [FL-no: 05.117] did not induce any DNA damage in duodenum, but a statistically significant increase in DNA strand breaks was observed in the liver at the highest dose tested (700 mg/kg bw/day).

The Panel noted that the results observed at the highest dose were more than 3-fold higher than the concurrent negative control value and statistically significant different from the negative control value, and a statistically significant positive linear trend was observed. The Panel considered that since there was a wide range of historical control data with an overlap of the positive and negative historical control values, the historical control data could not be used as a criterion to interpret the data.

Overall, the Panel concluded that *p*-mentha-1,8-dien-7-al [FL-no: 05.117] is genotoxic *in vivo* and that, accordingly, there is a safety concern for its use as flavouring substance.

Since *p*-mentha-1,8-dien-7-al [FL-no: 05.117] is representative for the nine remaining substances of this subgroup 2.2 (*p*-mentha-1,8-dien-7-ol [FL-no: 02.060], myrtenol [FL-no: 02.091], myrtenal [FL-no: 05.106], 2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde [FL-no: 05.121], myrtenyl formate [FL-no: 09.272], *p*-mentha-1,8-dien-7-yl acetate [FL-no: 09.278], myrtenyl acetate [FL-no: 09.302],

myrtenyl-2-methylbutyrate [FL-no: 09.899] and myrtenyl-3-methylbutyrate [FL-no: 09.900]), there is a potential safety concern for these substances.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The use of flavouring is regulated under Regulation (EC) No 1334/2008⁴ of the European Parliament and Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods. On the basis of Article 9(a) of this Regulation, an evaluation and approval are required for flavouring substances.

The Union List of flavourings and source materials was established by Commission Implementing Regulation (EC) No 872/2012⁵. The list contains flavouring substances for which the scientific evaluation should be completed in accordance with Commission Regulation (EC) No 1565/2000⁶.

The flavouring substances which belong to FGE.19, subgroup 2.2 are: *p*-mentha-1,8-dien-7-ol [FL-no: 02.060], myrtenol [FL-no: 02.091], myrtenal [FL-no: 05.106], *p*-mentha-1,8-dien-7-al [FL-no: 05.117], 2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde [FL-no: 05.121], myrtenyl formate [FL-no: 09.272], *p*-mentha-1,8-dien-7-yl acetate [FL-no: 09.278], myrtenyl acetate [FL-no: 09.302], myrtenyl-2-methylbutyrate [FL-no: 09.899] and myrtenyl-3-methylbutyrate [FL-no: 09.900].

p-Mentha-1,8-dien-7-al [FL-no: 05.117] was selected as a representative substance for the subgroup 2.2 (The EFSA Journal (2008) 910, 1-5).

These substances were included in the Union List with footnote 2, pending the submission of additional data.

The applicant submitted additional data and on 19 March 2013, the EFSA Panel on Food Contact materials, Enzymes, flavourings and Processing Aids adopted an opinion on Flavouring Group Evaluation 208 (FGE.208): consideration of genotoxicity data on representatives for 10 alicyclic aldehydes with the α,β -unsaturation in ring / side-chain and precursors from chemical subgroup 2.2 of FGE.19 by EFSA⁷.

The Panel concluded that “*the presently available data raise some concern for the genotoxic potential of p-mentha-1,8-dien-7-al [FL-no: 05.117]. In order to clarify the genotoxic potential of this substance, the Panel considered that further in vivo testing should be performed. To address this, an in vivo Comet assay, considering the first site of contact (e.g. stomach or duodenum) and liver, should be carried out according to the Scientific Report of EFSA on Minimum Criteria for the acceptance of in vivo alkaline Comet Assay Reports (EFSA, 2012)*”.

On 15 January and 25 March 2014, the applicant has submitted additional data in response to this EFSA evaluation.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The European Commission requests the European Food Safety Authority (EFSA) to evaluate this new information and, depending on the outcome, proceed to the full evaluation on this flavouring substance in accordance with Commission Regulation (EC) N° 1565/2000.

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

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

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

⁷ EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), 2013. Scientific Opinion on Flavouring Group Evaluation 208 (FGE.208): Consideration of genotoxicity data on representatives for 10 alicyclic aldehydes with the α,β -unsaturation in ring / side-chain and precursors from chemical subgroup 2.2 of FGE.19 by EFSA. EFSA Journal 2013;11(4):3151. [25 pp.] doi:10.2903/j.efsa.2013.3151.

METHODOLOGIES AND DATA

HISTORY OF THE EVALUATION OF FGE.19 SUBSTANCES

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

The α,β -unsaturated aldehyde and ketone structures are structural alerts for genotoxicity (EFSA, 2008a). 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 α,β -unsaturated carbonyls were subdivided into subgroups on the basis of structural similarity (EFSA, 2008a). 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 and Netzeva, 2007a; Benigni and 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. Based on these data the Panel decided that 15 subgroups (1.1.1, 1.2.1, 1.2.2, 1.2.3, 2.1, 2.2, 2.3, 2.5, 3.2, 4.3, 4.5, 4.6, 5.1, 5.2 and 5.3) (EFSA, 2008a) could not be evaluated through the Procedure due to concern with respect to genotoxicity. Corresponding to these subgroups, 15 Flavouring Group Evaluations (FGEs) were established: FGE.200, 204, 205, 206, 207, 208, 209, 211, 215, 219, 221, 222, 223, 224 and 225.

For 11 subgroups the Panel decided, based on the available genotoxicity data and (Q)SAR predictions, that a further scrutiny of the data should take place before requesting additional data from the Flavouring Industry on genotoxicity. These subgroups were evaluated in FGE.201, 202, 203, 210, 212, 213, 214, 216, 217, 218 and 220. For the substances in FGE.202, 214 and 218 it was concluded that a genotoxic potential could be ruled out and accordingly these substances will be evaluated using the Procedure. For all or some of the substances in the remaining FGEs, FGE.201, 203, 210, 212, 213, 216, 217 and 220 the genotoxic potential could not be ruled out.

To ease the data retrieval of the large number of structurally related α,β -unsaturated substances in the different subgroups for which additional data are requested, EFSA worked out a list of representative substances for each subgroup (EFSA, 2008c). Likewise an EFSA genotoxicity expert group has worked out a test strategy to be followed in the data retrieval for these substances (EFSA, 2008b).

The Flavouring Industry has been requested to submit additional genotoxicity data according to the list of representative substances and test strategy for each subgroup.

DATA

The Flavouring Industry has now submitted additional data and the present FGE concerns the evaluation of these data requested on genotoxicity.

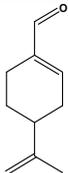
ASSESSMENT

1. History of the evaluation of the substances in subgroup 2.2

Subgroup 2.2 was one of the FGE.19 subgroups for which the Panel concluded that, based on the available data, additional genotoxicity data were necessary to perform the risk assessment for these substances (EFSA, 2008a).

The Panel identified one substance in subgroup 2.2 of FGE.19, *p*-mentha-1,8-dien-7-al [FL-no: 05.117], which will represent the other nine substances in this subgroup (EFSA, 2008c). For this substance genotoxicity data according to the test strategy (EFSA, 2008b) have been requested. The representative substance is shown in Table 1.

Table 1: Representative substance for subgroup 2.2 of FGE.19 (EFSA, 2008c)

FL-no JECFA-no	Subgroup	EU Register name	Structural formula	FEMA no CoE no CAS no
05.117 973	2.2	<i>p</i> -Mentha-1,8-dien-7-al		3557 11788 2111-75-3

In 2012, the Industry submitted new genotoxicity data: a bacterial gene mutation assay (Ames test), a gene mutation assay in mammalian cells (hypoxanthine-guanine phosphoribosyl transferase (HPRT) assay) and an *in vitro* micronucleus assay. These data were evaluated in FGE.208 (EFSA CEF Panel, 2013), where the Panel concluded that the available data still gave rise to concern for the genotoxic potential of *p*-mentha-1,8-dien-7-al [FL-no: 05.117]. Therefore, the Panel asked to provide an *in vivo* Comet assay performed on the first site of contact (e.g. stomach or duodenum) and on liver.

FGE	Adopted by EFSA	Link	No. of Substances
FGE.208	19 March 2013	http://www.efsa.europa.eu/en/efsajournal/pub/3151.htm	10
FGE.208Rev1	24 June 2015	http://www.efsa.europa.eu/en/efsajournal/pub/4173.htm	10

The present Revision 1 of FGE.208 (FGE.208Rev1), concerns the evaluation of a combined bone marrow micronucleus test and Comet assay in the liver and duodenum of rats. These data have been submitted by Industry (Bevers, 2014a, b) in response to the requested genotoxicity data in FGE.208 on the representative substance for subgroup 2.2, *p*-mentha-1,8-dien-7-al [FL-no: 05.117].

Sections from 2 to 6 of this opinion report the same information that was presented in FGE.208. Section 7 reports the evaluation of the new data submitted by Industry.

2. Presentation of the substances belonging to FGE.208Rev1

The present Flavouring Group Evaluation 208, Revision 1 (FGE.208Rev1), corresponding to subgroup 2.2 of FGE.19, concerns three alicyclic aldehydes with the α,β -unsaturation in ring / side-chain and seven precursors for such aldehydes. The 10 substances under consideration in FGE.208Rev1 are listed in Table 2.

Eight of the flavouring substances have been previously evaluated by the JECFA (JECFA, 2002a). A summary of their current evaluation status by the JECFA and the outcome of this consideration is presented in Table 3.

The α,β -unsaturated aldehyde structure is a structural alert for genotoxicity (EFSA, 2008a) and the data on genotoxicity previously available did not rule out the concern for genotoxicity for these 10 flavouring substances.

Table 2: Specification Summary of the Substances in the Present Group Evaluation (JECFA, 2002b)

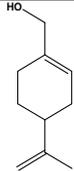
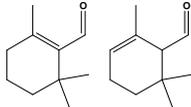
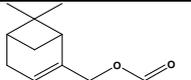
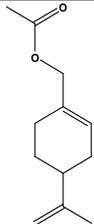
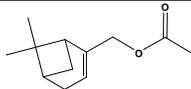
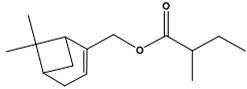
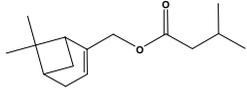
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol. formula Mol. weight	Solubility ^(a) Solubility in ethanol ^(b)	Boiling point, °C ^(c) Melting point, °C ID test Assay minimum	Refrac. Index ^(d) Spec. gravity ^(e)
02.060 974	<i>p</i> -Mentha-1,8-dien-7-ol		2664 2024 536-59-4	Liquid C ₁₀ H ₁₆ O 152.24	Slightly soluble Miscible	119 (14 hPa) – NMR 96 %	1.495-1.505 0.956-0.963
02.091 981	Myrtenol		3439 10285 515-00-4	Liquid C ₁₀ H ₁₆ O 152.24	Insoluble Miscible	221 – IR NMR 95 %	1.490-1.500 0.976-0.983
05.106 980	Myrtenal		3395 10379 564-94-3	Liquid C ₁₀ H ₁₄ O 150.22	Insoluble Miscible	220 – NMR 98 %	1.496-1.507 0.984-0.990
05.117 973	<i>p</i> -Mentha-1,8-dien-7-al		3557 11788 2111-75-3	Liquid C ₁₀ H ₁₄ O 150.22	Insoluble Miscible	104 (13 hPa) – NMR 97 %	1.504-1.513 0.948-0.956
05.121 979	2,6,6-Trimethyl-1-cyclohexen-1-carboxaldehyde		3639 2133 432-25-7	Liquid C ₁₀ H ₁₆ O 152.23	Insoluble Miscible	62 (4 hPa) – IR 99 %	1.476-1.483 0.950-0.957
09.272 983	Myrtenyl formate		3405 10858 72928-52-0	Liquid C ₁₁ H ₁₆ O ₂ 180.25	Insoluble Miscible	127-130 (52hPa) – NMR 96 %	1.477-1.483 1.004-1.010 (20°)

Table 2: Specification Summary of the Substances in the Present Group Evaluation (JECFA, 2002b)

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol. formula Mol. weight	Solubility ^(a) Solubility in ethanol ^(b)	Boiling point, °C ^(c) Melting point, °C ID test Assay minimum	Refrac. Index ^(d) Spec. gravity ^(e)
09.278 975	<i>p</i> -Mentha-1,8-dien-7-yl acetate		3561 10742 15111-96-3	Liquid C ₁₂ H ₁₈ O ₂ 194.27	Insoluble Miscible	218-223 – NMR 97 %	1.476-1.487 0.972-0.980
09.302 982	Myrtenyl acetate		3765 10887 1079-01-2	Liquid C ₁₂ H ₁₈ O ₂ 194.28	Miscible	134 (49 hPa) – IR NMR MS 98 %	1.470-1.477 0.987-0.996
09.899	Myrtenyl-2-methylbutyrate		– – 138530-44-6	Liquid C ₁₅ H ₂₄ O ₂ 236.35	Practically insoluble or insoluble Freely soluble	345 – MS 95 %	1.466-1.470 0.964-0.970
09.900	Myrtenyl-3-methylbutyrate		– – 33900-84-4	Liquid C ₁₅ H ₂₄ O ₂ 236.35	Practically insoluble or insoluble Freely soluble	98 (1 hPa) – MS 95 %	1.470-1.476 0.967-0.973

(a): Solubility in water, if not otherwise stated.

(b): Solubility in 95 % ethanol, if not otherwise stated.

(c): At 1013.25 hPa, if not otherwise stated.

(d): At 20°C, if not otherwise stated.

(e): At 25°C, if not otherwise stated.

– : not reported

3. Additional genotoxicity data evaluated by the Panel in FGE.208⁸

The Industry has submitted additional data concerning genotoxicity studies for the representative substance *p*-mentha-1,8-dien-7-al [FL-no: 05.117] for this subgroup (EFFA, 2012). The data for *p*-mentha-1,8-dien-7-al are one *in vitro* test in bacteria and two *in vitro* tests in mammalian cell systems.

3.1. *In vitro* data

3.1.1. Bacterial reverse mutation assay

An Ames assay was conducted in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 to assess the mutagenicity of *p*-mentha-1,8-dien-7-al, both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver post mitochondrial fraction (S9-mix) in three experiments (Bowen, 2011). A batch of 93.1 % purity was used for the first and second experiment, while a batch of 91.9 % purity was used for the third experiment. An initial toxicity range finding experiment was carried out using the plate incorporation method in the presence and absence of S9-mix for the TA100 strain only at concentrations of 1.6, 8, 40, 200, 1000 and 5000 µg/plate, plus negative (solvent) and positive controls. Evidence of toxicity in the form of complete killing of the background lawn was observed at 5000 µg/plate in the absence and presence of S9-mix. Precipitation was also seen at this concentration. As valid mutation data were available from five different test concentrations, the data from these treatments were considered to be acceptable for mutation analysis as part of the first main experiment. This GLP study complies with OECD Guideline 471.

In the first experiment, treatments of all the remaining tester strains were performed in the absence and presence of S9-mix at concentrations of 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate, plus negative (solvent) and positive controls. Evidence of toxicity was observed in all strains in the absence and presence of S9-mix at 5000 µg/plate, and in some strains also at 1000 µg/plate. Precipitation was also seen at 5000 µg/plate. Valid mutation data were obtained from five or six different test concentrations in each strain. Following experiment 1 treatments a statistically significant and concentration related increase in revertant numbers was observed in strain TA98 at 200 µg/plate (1.8-fold increase) and 1000 µg/plate (3.2-fold increase) in the absence of S9-mix, when data were analysed at the 1 % level using Dunnett's test.

In a second experiment, treatments of the strains assayed in experiment 1 were performed in the absence and presence of S9-mix at 8.192, 20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate. Treatments in the presence of S9-mix were further modified by the inclusion of a pre-incubation step (60 minutes). Evidence of toxicity ranging from a marked reduction in revertant numbers and/or slight thinning of the bacterial lawn to a complete killing of the test bacteria was observed at 320, 800, and/or 2000 µg/plate and above in most of the strains in the absence and presence of S9-mix. Precipitation was again seen at 5000 µg/plate, particularly in the presence of S9-mix. However, valid mutation data were obtained from at least five test concentrations in each strain. Following experiment 2 treatments, a statistically significant and concentration related increase in revertant numbers was again observed in strain TA98 in the absence of S9-mix at 320 µg/plate (2.3-fold increase) and 800 µg/plate (2.9-fold increase), when data were analysed at 1 % level using Dunnett's test.

Following the treatments in experiments 1 and 2, *p*-mentha-1,8-dien-7-al increased the frequency of revertants in strain TA98 by at least 2-fold in the absence of S9-mix activation. These results were in contrast with what had been observed for *p*-mentha-1,8-dien-7-al in previous Ames assays described further below. One possible explanation for the varying pattern of behaviour was that the material tested (93.1 % purity) in experiments 1 and 2, due to impurities, gave positive results. A third experiment was conducted in strain TA98, with a different batch of the test article (91.9 % purity), but with the same treatment conditions as in experiment 1. In the absence of S9-mix toxicity was observed

⁸ The data presented in Sections 3 and 4 are cited from the first version of FGE.208. These data are the basis for the conclusions in FGE.208 requesting additional genotoxicity data.

at 5000 µg/plate, while in the presence of S9-mix toxicity was observed at all concentrations tested. Additionally, while precipitation was observed on all test plates at 5000 µg/plate in experiments 1 and 2, no precipitation was observed at this concentration in experiment 3. Following the treatments in experiment 3, statistically significant and concentration related increases in revertant numbers for strain TA98 in the absence of S9-mix were observed at 8 µg/plate and above when the data were analysed at 1 % level using Dunnett's test. Therefore, the increases observed in strain TA98 were reproduced and are considered to be evidence of mutagenic activity in this strain. No other statistically significant increases in revertant numbers were observed in all other strains when the data were analysed at the 1 % level using Dunnett's test (Table 4).

3.1.2. Hypoxanthine-guanine phosphoribosyl transferase (*hprt*) assay

To assess mutagenic potential in a mammalian system, eukaryotic mouse lymphoma L5178Y cells were treated with *p*-mentha-1,8-dien-7-al in the absence and presence of S9-mix to study the induction of forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (*hprt*) locus (Lloyd, 2012). A batch of 92.5 % purity was used. Across 3 different experiments, treatments were carried out for 3 hours in the absence of S9-mix, 3 hours in the presence of S9-mix and 24 hours in the absence of S9-mix, and each treatment regime was independently repeated. Concentrations for the main experiments were established in preliminary range finding cytotoxicity experiments. This GLP study complies with OECD Guideline 476.

In the first mutation experiment, cells were treated with *p*-mentha-1,8-dien-7-al for 3 hours at 10, 20, 40, 60, 70, 80, 90 and 100 µg/ml in the absence of S9-mix and at 40, 60, 80, 100, 120, 140, 160 and 180 µg/ml in the presence of S9-mix. Per cent relative survival (% RS) decreased to 13 % at 100 µg/ml in the absence of S9-mix and to 16 % at 180 µg/ml in the presence of S9-mix. Negative control mutant frequencies were normal, and were significantly increased by treatment with the positive control. No significant increases in mutation frequency were observed at any concentration analysed in the presence or absence of S9-mix in this experiment, and no statistically significant linear trends were observed.

In a second experiment, cultures were treated with *p*-mentha-1,8-dien-7-al for 3 hours at 20, 40, 50, 60, 70, 80, 90, 100 and 120 µg/ml in the absence of S9-mix and at 25, 50, 75, 100, 120, 140, 160, 170 and 180 µg/ml in the presence of S9-mix. Per cent RS decreased to 7 % at 120 µg/ml in the absence and to 10 % at 180 µg/ml in the presence of S9-mix. Also in this experiment, 24-hour treatments were carried out with *p*-mentha-1,8-dien-7-al in the absence of S9-mix at 4, 8, 12, 15, 18 and 21 µg/ml of *p*-mentha-1,8-dien-7-al. Per cent RS decreased to 9 % at the highest concentration. Negative control mutant frequencies were normal and were significantly increased by treatment with the positive control. In the absence and presence of S9-mix, there were no statistically significant increases in mutant frequency relative to control at any concentration analysed, although in the absence of S9-mix (both 3- and 24-hour treatments), there were statistically significant linear trends.

In a third experiment, cultures were treated with *p*-mentha-1,8-dien-7-al for 24 hours at 4, 8, 12, 14, 16, 18 and 20 µg/ml in the absence of S9-mix. Per cent RS decreased to 14 % at the highest concentration. Negative control mutant frequencies were normal, and were significantly increased by treatment with the positive control. There were no significant or dose-related increases in mutant frequency following *p*-mentha-1,8-dien-7-al treatments. The observations made with the 24-hour treatments in the second experiment were not reproduced at similar concentrations and extents of toxicity and were considered not to be biologically relevant by the authors (Lloyd, 2012).

However, it is not clear why the 3-hour treatment was not repeated. Overall, the results in the HPRT assay in the absence of S9-mix should be considered, differently from the authors' opinion, as equivocal instead of negative, based on the statistically significant trends in both 3- and 24-hour treatments in the second experiment (Table 4).

3.1.3. *In vitro* micronucleus assays

p-Mentha-1,8-dien-7-al (94.9 % purity) was assayed for the induction of chromosome damage in mammalian cells *in vitro* by examining its effect on the frequency of micronuclei (MN) in cultured human peripheral blood lymphocytes (whole blood cultures pooled from 2 healthy male volunteers) treated in the absence and presence of S9-mix (Lloyd, 2009). *p*-Mentha-1,8-dien-7-al was added at 48 hours following culture initiation (stimulation by phytohaemagglutinin) either for 3 hours in the absence or presence of S9-mix followed by 21 hours recovery, or for 24 hours in the absence of S9-mix. Cytochalasin B (6 µg/ml) was added either at the start of treatment (24-hour treatments) or at the start of recovery (following 3 hour treatments) in order to block cytokinesis and generate binucleate cells for analysis. It remained in the cultures until they were harvested 24 hours after the start of treatment. A range-finding experiment had been conducted with and without S9-mix treatment in order to provide toxicity information (reduction in replication index, RI) that could be used as a basis for choosing a range of concentrations to be evaluated in the main micronucleus analysis (Table 4).

In the main assay, micronuclei were analysed from at least three concentrations for each treatment condition. For 3-hour treatment without S9-mix the concentrations were 80, 100, 110 and 120 µg/ml, for 3-hour treatment with S9-mix the concentrations were 100, 120 and 140 µg/ml and for 24-hour treatment without S9-mix the concentrations were 20, 25 and 35 µg/ml. The levels of cytotoxicity (reduction in RI) at the top concentrations reached 58 and 45 % in the 3-hour treatment in the absence and presence of S9-mix and 58 % in the 24-hour treatment in the absence of S9-mix, respectively. These levels of cytotoxicity therefore reached, or were very close to, the recommended (50-60 %) range of cytotoxicity. One thousand binucleate cells per culture from 2 replicate cultures per concentration were scored for micronuclei. This GLP study complies with OECD Test Guideline 487.

The frequencies of micronucleated binucleate (MNBN) cells in negative control cultures were normal, and were significantly increased by treatment with positive control chemicals. Treatment of cells with *p*-mentha-1,8-dien-7-al in the absence and presence of S9-mix under all treatment conditions resulted in frequencies of MNBN cells that were similar to and not significantly different from those observed in concurrent vehicle controls for all concentrations analysed. The MNBN cell frequency of all *p*-mentha-1,8-dien-7-al treated cultures fell within (or slightly below) normal ranges. It was concluded that *p*-mentha-1,8-dien-7-al did not induce micronuclei in cultured human peripheral blood lymphocytes when tested at toxic concentrations in both the absence and presence of S9-mix (Lloyd, 2009).

4. Previously available data

4.1. *In vitro* data

Several *in vitro* mutagenicity/genotoxicity tests have been performed on the FGE.19 subgroup 2.2 representative substance *p*-mentha-1,8-dien-7-al [FL-no: 05.117]. The quality of most of them could not be adequately evaluated, either because they are in Japanese and therefore details are difficult to obtain or because of limitations in the experimental design. Negative results were reported by Ishidate et al. (1984) for an Ames test in which *Salmonella typhimurium* strains TA92, TA1535, TA100, TA1537, TA94 and TA98 were used. Duplicate plates were used for each of the six concentrations up to 1000 µg/plate with S9-mix. The sample used had the same purity (93.1 %) of the batch used by Bowen (2011b). The results were only reported as – or + (a + would be given if revertant numbers exceeded 2x concurrent control) and therefore weaker responses may have been observed but cannot be verified. Fujita et al. (Fujita et al., 1994) also reported negative results for an Ames assay in strains TA97 and TA102 performed both with and without S9-mix. The top concentration of *p*-mentha-1,8-dien-7-al was less than in the Ishidate study, namely 100 µg/plate. Negative results were reported in mutation tests in which *p*-mentha-1,8-dien-7-al was incubated with *Escherichia coli* WP2 cells at 50 to 400 µg/plate (Yoo, 1986). Few details can be obtained from the paper, but it appears that the maximum increase in revertants was 1.3-fold, which is considered negative. However, only one result was given, so the test was probably only conducted in the absence of S9-mix.

p-Mentha-1,8-dien-7-al was considered to be weakly positive in the *rec*-assay with *Bacillus subtilis* strains M45 and H17 at a concentration of 2.5 µl *p*-mentha-1,8-dien-7-al/disk, probably equivalent to 2500 µg/disk (Yoo, 1986). This study is a very short paper, with very few details. Another study using the same strains reported negative results for *p*-mentha-1,8-dien-7-al at concentrations between 0.16 and 0.63 µl/plate (corresponding to 0.15 and 0.6 µg/plate) and positive results at higher concentrations of 1.25 and 2.5 µl/plate (1.2 and 2.4 µg/plate) (Kuroda et al., 1984). It should be noted that these DNA damage assays in bacteria do not detect mutation, are non-standard and not requested by regulatory agencies. The results cannot therefore be considered to carry as much weight as results from recommended, standard assays.

In a study by Eder et al. (1993) *p*-mentha-1,8-dien-7-al gave negative results in a SOS-Chromotest with genetically engineered *Escherichia coli*. The maximum induction factor (I_{max}) with *p*-mentha-1,8-dien-7-al was calculated to be 1.0. Positive results are considered to be significant if the I_{max} is at least 1.5. The SOS-Chromotest is also not a mutation test. It measures induction of the SOS repair system, and this is interpreted as indicating DNA damage. The results cannot therefore be considered to carry as much weight as results from recommended standard assays.

Standard chromosomal aberration (CA) assays for *p*-mentha-1,8-dien-7-al have yielded positive results. In a CA study by Ishidate et al (1984), Chinese hamster fibroblasts (CHL) were only treated in the absence of S9-mix for 24 or 48 hours with a batch of 93.1 % purity. There were no treatments in the presence of S9-mix. Concentrations for the main CA test were selected from a preliminary experiment in which cell density (a crude and subjective measure) on the culture dishes was assessed, but there was no concurrent measure of cytotoxicity in the CA test. Only single cultures of CHL cells were treated with each of 3 concentrations, and therefore only 100 cells/concentration were scored for CA. CA (including gaps) frequencies of 4.9 % or less were considered negative, 5.0-9.9 % were equivocal, and 10 % or higher were considered positive. *p*-Mentha-1,8-dien-7-al gave a strong positive response (39 % cells with CA, and also an increase in polyploid cells to 31 %) at 50 µg/ml. In particular, structural chromosome aberrations were detected at 40 µg/ml at 24 hours (20.0 %) and at 48 hours (28.0 %); the strongest effect was observed at 50 µg/ml at 24 hour. An increase in polyploidy cells was also detected at 40 µg/ml (15 %) and 50 µg/ml (31 %) after 48 hours. As there was no concurrent measure of cytotoxicity, and the results at the other concentrations tested were not given, these results should be considered with caution; however, they cannot be completely dismissed. In the CA study of Tayama et al. (Tayama et al., 1990) in CHO-K1 cells, a significant increase in CA at 150 µg/ml in the absence of S9-mix was associated with no detectable cell division. This result can probably be dismissed as likely to be an artefact of high levels of cell killing. However, a significant increase in CA at 300 µg/ml in the presence of S9-mix was associated with 62 % proliferating cells, which does not indicate excessive toxicity. Most of the chromosome aberrations were chromatid exchanges. These results are clearly in contrast to the negative micronucleus results obtained in human lymphocytes in the recent GLP study (Lloyd, 2009). The reasons of such discrepancy are unclear.

A sister chromatid exchange (SCE) assay was performed with and without metabolic activation in CHO-K1 cells at concentrations up to 300 µg *p*-mentha-1,8-dien-7-al/ml (Tayama et al., 1990). Cytotoxicity was determined by the percentage of cells that showed differentially stained chromatids, i.e. had divided. A doubling of SCE/cell would usually be considered biologically relevant, and in the absence of S9-mix there was a doubling of SCE/cell at 150 µg/ml, where there was little toxicity, whereas in the presence of S9-mix there was a doubling of SCE/cell at all concentrations from 100-300 µg/ml, where there was low or moderate toxicity. However, SCE assays also only provide limited information for assessment of genotoxicity. The mechanism of induction of SCE, and its relevance for mutation and cancer is not understood.

Studies for induction of ouabain resistant mutants conducted in human fetus cells (Rsa) at concentrations of 0, 0.010, 0.015, 0.020 or 0.025 µg/ml gave negative results for *p*-mentha-1,8-dien-7-al at the lowest concentration, positive results (8 - 16 fold increases) for concentrations ranging from 0.015 to 0.02 µg/ml (where toxicity was slight to moderate), and showed *p*-mentha-1,8-dien-7-al to be cytotoxic at the highest concentration (Suzuki et al., 1990). In another mutagenicity study with Rsa

cells (Suzuki and Suzuki, 1994), induction of ouabain resistance was reported at concentrations above 10 ng *p*-mentha-1,8-dien-7-al/ml with apparent cytotoxicity at 20 ng/ml or higher. Also in this study, mutagenicity was detected (K-ras codons) at concentrations of 2–200 ng/ml. Human fetal (Rsa) cells are not routinely used for genotoxicity testing, so evaluation of the quality of the data is difficult. The concentrations used in these tests are much lower than in other mammalian cell tests, and possible reasons for the discrepancy are not clear. Sasaki et al. (1990) tested *p*-mentha-1,8-dien-7-al for induction of ouabain-resistant mutants in CHO-K1 cells. The mutant frequency at the only concentration of *p*-mentha-1,8-dien-7-al tested (10 µg/ml, which reduced survival to 83.5 % of controls) appears to be low (0.7 mutants per 10⁶ cells, compared to zero in controls) and the result would probably be considered negative. The study of ouabain resistance in all of these studies makes interpretation difficult. Ouabain resistance is generally considered not to be a sensitive mutagenic target (spontaneous frequencies very low; frame-shift mutations not detected), and it is difficult to conclude negative results when there is a zero incidence of effects in controls. The biological significance of large increase in ouabain resistant mutants at very low concentrations is equally difficult to interpret. This endpoint is no longer used in regulatory testing.

The *in vitro* studies described above are listed in Table 4.

4.2. *In vivo* data

In vivo mutagenicity/genotoxicity testing has been performed on the FGE.19 subgroup 2.2 representative substance *p*-mentha-1,8-dien-7-al (Table 5). Eight-week-old male ddY mice were administered a single intraperitoneal injection of *p*-mentha-1,8-dien-7-al [FL-no: 05.117] at doses of 75, 150, 300, or 600 mg/kg bw for a mouse micronucleus assay (six mice/group). The dosing regimen and the maximum dose was based on a pilot experiment with 2 mice/group. In the main experiment, after 24 hours the mice were killed and femoral bone marrow cells were collected, fixed and stained with Giemsa. One thousand polychromatic erythrocytes were scored per mouse. No indication of micronucleus induction was reported at any dose level (Hayashi et al., 1988). However, the study does not comply with current guidelines, because, after a single administration, groups of animals should be sacrificed 24 and 48 hours later. Also only 1000 PCE were scored per animal whereas the current recommendation is for 2000 PCE/animal.

5. Discussion

EFFA has submitted three valid, new *in vitro* studies, one in bacteria (Ames test) and two in mammalian cells (MN in human lymphocytes, HPRT in mouse lymphoma cells). The Ames test resulted positive, in the absence of metabolic activation with strain TA98, able to detect gene mutations of frameshift type (insertions/deletions). Equivocal results were reported in the HPRT assay (negative according to the authors) and negative results were reported in the MN test. Equivocal or negative results in the HPRT assay cannot dismiss the positive findings in the new Ames test, positive in the TA98 strain. The different results may be due to a different sensitivity of the two tests to detect frameshift mutations. In this respect the Panel noted that the molecular analysis of mutational spectra at the *hprt* locus show a prevalence of GC to AT transitions and AT to CG transversions among spontaneous mutants, with less than 10 % of frameshifts (Chen et al, 2002). Thus, given the prevailing contribution of mutations different from frameshift to the baseline incidence of *hprt* mutant colonies, it is expected that a many-fold increase in frameshift mutations is needed to give raise to an overall increase in mutation frequency which is detectable and significant on statistical grounds. The Ames test is generally considered as the most sensitive *in vitro* test for the prediction of genotoxic carcinogens and “false positive results” are rare; in this case, the positivity in the TA98 cannot be considered as a “false positive” without any explanation.

Negativity in mammalian cells “per se” cannot be considered more relevant than positivity in bacteria, simply on the basis of the complexity of cells. Among the previously supplied data, several *in vitro* and one *in vivo* mutagenicity/genotoxicity published studies are available. For most of them, performed not in compliance with current guidelines, the quality of data was limited. Negative results were reported in a study with the Ames test; however, the results were only reported as + or - , and

therefore could not be verified. Both positive and negative results were reported for induction of ouabain gene mutations in mammalian cells, in limited studies. Ouabain resistance is generally considered of low sensitivity, compared with other gene mutation assays and is unable to detect mutations of frameshift type; it is no longer routinely used for regulatory purposes. Strong clastogenic effects in the absence of S9-mix were reported in Chinese hamster cell lines in two papers. Notwithstanding some limitations of the study, these positive results cannot be completely dismissed by the negative results in the new *in vitro* MN assay. The different types of cells used (Chinese hamster cell lines and human lymphocytes) and the different concentrations used can only partially explain the different results, which remain unclear. Negative results were reported in a mouse MN assay, in a study of limited validity for inadequate experimental design and insufficient presentation of data. Other published results, both positive and negative for DNA-damage/repair (rec-assay) in bacteria, negative for SOS and positive for SCE in mammalian cells, are not considered as relevant for the assessment of the genotoxic potential of *p*-mentha-1,8-dien-7-al.

6. Conclusion

Overall, the presently available data raise some concern for the genotoxic potential of *p*-mentha-1,8-dien-7-al [FL-no: 05.117]. In order to clarify the genotoxic potential of this substance, the Panel considered that further *in vivo* testing should be performed. To address this, an *in vivo* Comet assay, considering the first site of contact (e.g. stomach or duodenum) and liver, should be carried out according to the Scientific Report of EFSA on Minimum Criteria for the acceptance of *in vivo* alkaline Comet Assay Reports (EFSA, 2012).

7. Additional genotoxicity data evaluated by the Panel in FGE.208Rev1

In response to the EFSA request, in FGE.208, to provide *in vivo* genotoxicity data for the representative substance *p*-mentha-1,8-dien-7-al [FL-no: 05.117], Industry has submitted a combined *in vivo* bone marrow micronucleus test and Comet assay with scoring in the liver and duodenum.

p-Mentha-1,8-dien-7-al [FL-no: 05.117] (purity 94.2 %) was tested for its ability to induce micronuclei in the polychromatic erythrocytes (PCEs) of the bone marrow of treated rats and the potential to induce DNA damage in the liver and duodenum of the same animals in a combined *in vivo* micronucleus and Comet assay (Beevers, 2014a, b).

Based on results from a range finding study, where no substantial inter-sex differences in toxicity were observed in rats, a dose of 700 mg/kg bw/day was considered as the maximum tolerated dose (MTD). Groups of six male out-bred Han Wistar rats were administered doses of 175, 350 and 700 mg/kg bw of *p*-mentha-1,8-dien-7-al by oral gavage at time 0, 24 and 45 hours. All doses were administered at a dose volume of 10 ml/kg. Rats were sacrificed and sampled at 48 hours post the initial dose. Negative (corn oil) and positive control groups (ethyl methanesulfonate (EMS) 150 mg/kg, dosed at 0, 24 and 45 hours) were included in the main study.

Clinical signs of toxicity were limited to animals dosed at 700 mg/kg bw/day, where reduced levels of activity were observed in 5/6 animals dosed with *p*-mentha-1,8-dien-7-al. In addition, one animal displayed symptoms of ataxia and one animal had piloerection. Dose related decreases in body weight gain, or weight loss were observed at all dose levels. No clinical signs of toxicity were seen in the vehicle or the positive control (EMS).

During clinical chemistry assessment of blood samples it was noted that a high number of samples were lipaemic. This was attributed to the corn oil used as vehicle control and for test article formulation, which was administered just three hours prior to blood sampling. As a consequence many samples were deemed unsuitable for the analysis of certain parameters and the data were interpreted with caution. There was a slight increase in aspartate aminotransferase and alanine aminotransferase at the 700 mg/kg bw/day dose.

The anatomical pathology examination showed that there were no gross lesions in tissues of exposed animals related to administration of *p*-mentha-1,8-dien-7-al, however, histopathology revealed hepatocyte vacuolation at the dose of 700 mg/kg bw/day.

In line with the requirement of the OECD guideline 474 the plasma samples were collected. However, analysis of these samples was not conducted since in this case, this is not relevant for the interpretation of the study.

7.1. Micronucleus assay

An *in vitro* micronucleus assay in human peripheral blood lymphocytes (Lloyd, 2009) was evaluated by the Panel as negative in FGE.208. Although not requested, the applicant has submitted an *in vivo* micronucleus assay in bone marrow of rats (Beevers, 2014a). In this *in vivo* study the proportion of immature among total (immature + mature) erythrocytes was determined for each animal by counting a total of at least 500 cells and then at least 2000 immature erythrocytes per animal were scored for the incidence of micronucleated polychromatic erythrocytes (MNPCE). Rats treated with *p*-mentha-1,8-dien-7-al exhibited % PCE values that were similar to the concurrent vehicle control group and which were within the laboratory's historical negative control data, thus indicating that the test substance was not toxic to the bone marrow. Rats treated with *p*-mentha-1,8-dien-7-al exhibited group mean frequencies of MNPCE that were similar to and not statistically different (chi-square calculation) from those observed in concurrent vehicle controls for all dose groups and were also within the historical control values (Beevers, 2014a).

The Panel concluded that in this study *p*-mentha-1,8-dien-7-al did not induce micronucleated erythrocytes in rat bone-marrow cells following administration by oral gavage at the test conditions performed. There was no indication that the test substance reached the target organ. Negative results were observed in an *in vitro* micronucleus test (Lloyd, 2009). Therefore, there is no need to validate the negative result of the *in vivo* micronucleus assay and to investigate the target tissue exposure.

7.2. Comet assay

Duodenum analysis

There was no dose-related increase in % clouds in duodenum cells following treatment with *p*-mentha-1,8-dien-7-al, thus demonstrating that treatment did not cause excessive DNA damage that could have interfered with Comet analysis. Measurements of tail intensity (% DNA in tail) and tail moment were obtained from 150 cells/animal.

Group mean tail intensity and tail moment values for all groups of animals treated with *p*-mentha-1,8-dien-7-al at 175, 350 and 700 mg/kg bw/day were comparable with the group mean vehicle control data. There were no marked differences in tail intensity between treated and control groups. All individual animal data at all dose levels were consistent with the vehicle control animal data (Beevers, 2014b).

The Panel concluded that *p*-mentha-1,8-dien-7-al did not induce DNA damage in the duodenum of treated male rats under the test conditions performed.

Liver analysis

There was no dose-related increase in % clouds or % cells with halos in liver cells following treatment with *p*-mentha-1,8-dien-7-al, thus demonstrating that treatment did not cause excessive DNA damage that could have interfered with Comet analysis. However, clinical chemistry of blood showed a slight increase of aspartate aminotransferase and alanine aminotransferase at the highest dose tested, indicating that the liver was exposed to the test substance.

Measurements of tail intensity (% DNA in tail) and tail moment were obtained from 150 cells/animal.

Group mean % tail intensity and tail moment values for animals treated with *p*-mentha-1,8-dien-7-al at the low and medium dose (175 and 350 mg/kg bw/day, respectively) were comparable with the group mean vehicle control data and there were no statistically significant differences in % tail intensity between treated and control groups. In groups treated with the low and medium dose, all individual animal data were consistent with the values of the vehicle control animals and fell within the laboratory's historical control data.

At the highest dose (animals exposed to 700 mg/kg bw/day) a 3.4-fold and statistically significant increase in tail intensity was observed. A statistically significant linear trend was also apparent. Five out of the six animals treated with the highest dose had tail intensities that exceeded the values observed in the concurrent vehicle control animals, however, the tail intensity values for all animals fell within the laboratory's historical vehicle control values (Bevers, 2014a).

The Panel noted that the range for both the negative and positive historical control values were extremely wide for this test laboratory. In addition, there was an overlap of the negative (95 % range: 0.02–11.39) and positive (95 % range: 7.15–65.07) control values.

The Comet arm of this study indicates that *p*-mentha-1,8-dien-7-al induces DNA damage in liver.

CONCLUSION

The data submitted by the applicant were considered to be in accordance with the data requested by the Panel in FGE.208. Industry submitted a Comet assay on the liver and duodenum and in addition (although not requested) a micronucleus assay in the bone marrow of the same animals (combined bone marrow micronucleus test and Comet assay).

p-Mentha-1,8-dien-7-al [FL-no: 05.117] did not induce any increase in micronucleated polychromatic erythrocytes of the bone marrow of male rats following oral gavage administration up to 700 mg/kg bw/day (an estimate of the maximum tolerated dose for this study). There was no indication in the study that the test substance reached the bone marrow. Negative results were observed in an *in vitro* micronucleus assay on human peripheral blood lymphocytes performed according to OECD test guideline 487. Therefore, there is no need to validate the negative result of the *in vivo* micronucleus assay and to investigate the target tissue exposure.

p-Mentha-1,8-dien-7-al did not induce DNA damage in duodenum of the same animals as analysed by the Comet assay.

In the same animals a statistically significant increase in DNA strand breaks was observed in the liver at the highest tested dose (700 mg/kg bw/day). The observed values for tail intensity (2.20 ± 0.6) and tail moment (0.24 ± 0.07) fell within the test laboratories historical vehicle control range values for tail intensity (0.02–11.39) and tail moment (0.01–1.45), however, five of the six high dose animals had tail intensities that exceeded the values of the concurrent vehicle control animals.

The Panel noted that the results observed at the highest dose were more than 3-fold higher than the concurrent negative control value and statistically significant different from the negative control value. In addition, a statistically significant positive linear trend was observed. The Panel considered that, since there was a wide range of historical control data with an overlap of the positive and negative historical control values, the historical control data could not be used as a criterion to interpret the data.

Overall, the Panel concluded that *p*-mentha-1,8-dien-7-al [FL-no: 05.117] is genotoxic *in vivo* and that, accordingly, there is a safety concern for the use of *p*-mentha-1,8-dien-7-al [FL-no: 05.117] as a flavouring substance.

Since *p*-mentha-1,8-dien-7-al [FL-no: 05.117] is representative for the nine remaining substances of this subgroup 2.2 (*p*-mentha-1,8-dien-7-ol [FL-no: 02.060], myrtenol [FL-no: 02.091], myrtenal [FL-no: 05.106], 2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde [FL-no: 05.121], myrtenyl formate [FL-no: 09.272], *p*-mentha-1,8-dien-7-yl acetate [FL-no: 09.278], myrtenyl acetate [FL-no: 09.302], myrtenyl-2-methylbutyrate [FL-no: 09.899] and myrtenyl-3-methylbutyrate [FL-no: 09.900]), there is a potential safety concern for these substances.

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SUMMARY OF SAFETY EVALUATION APPLYING THE PROCEDURE

Table 3: Summary of Safety Evaluation of the JECFA Substances in the Present Group (JECFA, 2002a)

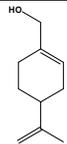
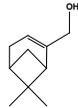
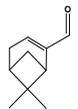
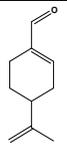
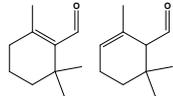
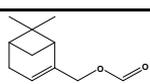
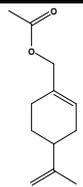
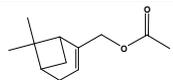
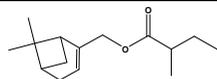
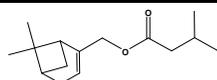
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI ^(a) US MSDI ($\mu\text{g/capita/day}$)	Class ^(b) Evaluation procedure path ^(c)	Outcome on the named compound [^(d) or ^(e)]	EFSA conclusion on the named compound (genotoxicity)
02.060 974	<i>p</i> -Mentha-1,8-dien-7-ol		1.6 1	Class I A3: Intake below threshold	d	Evaluated in FGE.208Rev1, as of potential genotoxicity concern based on the experimental data of the representative substance
02.091 981	Myrtenol		0.37 0.03	Class I A3: Intake below threshold	d	Evaluated in FGE.208Rev1, as of potential genotoxicity concern based on the experimental data of the representative substance
05.106 980	Myrtenal		4 7	Class I A3: Intake below threshold	d	Evaluated in FGE.208Rev1, as of potential genotoxicity concern based on the experimental data of the representative substance
05.117 973	<i>p</i> -Mentha-1,8-dien-7-al		2.1 2	Class I A3: Intake below threshold	d	Evaluated in FGE.208Rev1, as of genotoxicity concern
05.121 979	2,6,6-Trimethyl-1-cyclohexen-1-carboxaldehyde		0.37 ND	Class I A3: Intake below threshold	d	Evaluated in FGE.208Rev1, as of potential genotoxicity concern based on the experimental data of the representative substance
09.272 983	Myrtenyl formate		0.3 ND	Class I A3: Intake below threshold	d	Evaluated in FGE.208Rev1, as of potential genotoxicity concern based on the experimental data of the representative substance

Table 3: Summary of Safety Evaluation of the JECFA Substances in the Present Group (JECFA, 2002a)

FL-no JECFA-no	EU Register name	Structural formula	EU MSDI ^(a) US MSDI ($\mu\text{g/capita/day}$)	Class ^(b) Evaluation procedure path ^(c)	Outcome on the named compound [^(d) or ^(e)]	EFSA conclusion on the named compound (genotoxicity)
09.278 975	<i>p</i> -Mentha-1,8-dien-7-yl acetate		0.35 0.07	Class I A3: Intake below threshold	d	Evaluated in FGE.208Rev1, as of potential genotoxicity concern based on the experimental data of the representative substance
09.302 982	Myrtenyl acetate		0.37 0.04	Class I A3: Intake below threshold	d	Evaluated in FGE.208Rev1, as of potential genotoxicity concern based on the experimental data of the representative substance
09.899	Myrtenyl-2-methylbutyrate		0.012	Class I No evaluation	Not evaluated by JECFA	Evaluated in FGE.208Rev1, as of potential genotoxicity concern based on the experimental data of the representative substance
09.900	Myrtenyl-3-methylbutyrate		0.061	Class I No evaluation	Not evaluated by JECFA	Evaluated in FGE.208Rev1, as of potential genotoxicity concern based on the experimental data of the representative substance

(a): EU MSDI: Amount added to food as flavour in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = $\mu\text{g/capita/day}$.

(b): Thresholds of concern: Class I = 1800 $\mu\text{g/person/day}$, Class II = 540 $\mu\text{g/person/day}$, Class III = 90 $\mu\text{g/person/day}$.

(c): Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.

(d): No safety concern based on intake calculated by the MSDI approach of the named compound.

(e): Data must be available on the substance or closely related substances to perform a safety evaluation.

ND: not determined

GENOTOXICITY DATA CONSIDERED BY THE PANEL IN FGE.208

Table 4: Summary of *in vitro* Genotoxicity Data Evaluated by the Panel in FGE.208

Chemical name [FL-no]	Test system	Test object	Concentration	Results	Reference	Comments
<i>p</i> -Mentha-1,8-dien-7-al [05.117]	Reverse mutation	<i>S. typhimurium</i> TA100	1.6, 8, 40, 200, 1000 and 5000 µg/plate	Negative ^b	Bowen, 2011	Reliable without restriction. GLP study in compliance with OECD Guideline 471.
		<i>S. typhimurium</i> TA98, TA102, TA1535, TA1537	0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate	Positive ^b		All strains were negative except TA98 without S9-mix treatment.
		<i>S. typhimurium</i> TA98, TA102, TA1535, TA1537	8.192, 20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate	Positive ^{a, c}		All strains were negative except TA98 without S9-mix treatment.
		<i>S. typhimurium</i> TA98	0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate	Positive ^b		A different batch of test article was used and positive results in TA98 without S9- mix were confirmed.
	Reverse mutation ^a	<i>S. typhimurium</i> TA97, TA102	Up to 100 µg/plate	Negative ^b	Fujita et al., 1994	Not assignable. Low concentrations; only two strains used, one of which (TA97) not routinely used.
	Reverse mutation ^a	<i>S. typhimurium</i> TA92, TA1535, TA100, TA1537, TA94, TA98,	Up to 1000 µg/plate	Negative ^a	Ishidate et al., 1984	Reliable with restriction. Results reported as - or +.
	Mutagenicity	<i>E. coli</i> WP2	Up to 0.4 mg/plate	Negative ^c	Yoo, 1986	Not assignable. Probably only performed in the absence of S9-mix. Low concentrations tested; only few details available.
	DNA damage	<i>B. subtilis</i> M45 and H17	2.5 µl/disk (probably equivalent to 2500 µg/disk)	Weak positive		Not assignable. Details difficult to obtain. Endpoint not relevant.
	DNA damage	<i>B. subtilis</i> M45 and H17	0.16-0.63 µl/plate (0.15-0.6 µg/plate)	Negative	Kuroda et al., 1984	
			1.25 and 2.5 µl/plate (1.2 and 2.4 µg/plate)	Positive		
DNA repair	<i>E. coli</i> PQ37	Not reported	Negative	Eder et al., 1993	SOS Chromotest. Endpoint not relevant.	
Sister chromatid exchange	Chinese hamster ovary cells	150 µg/ml	Positive ^c	Tayama et al., 1990	Reliable with restriction; genetic endpoint of limited relevance.	
		100-300 µg/ml	Positive ^e			

Table 4: Summary of *in vitro* Genotoxicity Data Evaluated by the Panel in FGE.208

Chemical name [FL-no]	Test system	Test object	Concentration	Results	Reference	Comments
	Chromosomal aberration	Chinese hamster fibroblasts	Up to 50 µg/ml	Positive ^c	Ishidate et al., 1984	Reliable with restriction. No concurrent measure of cytotoxicity. Performed only in the absence of S9.
	Chromosomal aberration	Chinese hamster ovary cells	300 µg/ml	Positive ^{e, f}	Tayama et al., 1990	Reliable with restriction. Moderate toxicity at 300 µg/ml (+S9).
			150 µg/ml	Negative ^{c, d, f}		Reliable with restriction. No detectable cell division at 150 µg/ml (-S9).
	Mutagenicity	Chinese hamster ovary cells	10 µg/ml	Negative ^{c, g}	Sasaki et al., 1990	Not assignable. Ouabain resistance measured. Only one concentration tested without S9; insufficient details.
	Mutagenicity	Human fetus cells (Rsa)	Up to 0.025 µg/ml	Positive ^h	Suzuki et al., 1990	Unreliable; ouabain resistance measured in Rsa cells not routinely used; insufficient details.
			0.010 µg/ml	Negative ^h		
	Mutagenicity	Human fetus cells (Rsa)	>10 ng/ml	Positive ⁱ	Suzuki and Suzuki, 1994	Japanese paper quoted but not available
	Micronucleus Induction	Primary human lymphocytes	Up to 140 µg/ml ^l	Negative ^b	Lloyd, 2009	Reliable without restriction. Complies with GLP and OECD Guideline 487.
	HPRT assay	Mouse lymphoma L5178Y cells	Up to 180 µg/ml ^k	Equivocal ^b	Lloyd, 2012	Reliable without restriction. Complies with GLP and OECD Guideline 476.

(a): Preincubation with exogenous metabolic system from rat liver.

(b): Assay performed with and without metabolic activation.

(c): Assay performed without metabolic activation.

(d): Cytotoxic at 150 µg/ml.

(e): Assay performed with metabolic activation.

(f): Positive only at cytotoxic concentrations.

(g): Cytotoxic at 12 µg/ml.

(h): Cytotoxic at 0.025 µg/ml.

(i): Cytotoxic at > 20 ng/ml.

(j): Cytotoxic ≥ 160 µg/ml.

(k): Cytotoxic ≥ 180 µg/ml (3 hours treatment in the presence of S9); cytotoxic ≥ 100 µg/ml (3 hours treatment in the absence of S9); cytotoxic ≥ 21 µg/ml (24 hour treatment in the absence of S9).

Table 5: Summary of *in vivo* Genotoxicity Data Evaluated by the Panel in FGE.208

Chemical Name FL-no	Test System <i>in vivo</i>	Test Object	Route	Dose	Result	Reference	Comments
<i>p</i> -Mentha-1,8-dien-7-al [05.117]	Micronucleus Assay	Mouse bone marrow cells	Intraperitoneal	75, 150, 300 or 600 mg/kg bw	Negative	(Hayashi et al., 1988)	Unreliable; sampling time only at 24 hours; only 1000 PCE per animal scored; poor presentation of data.

GENOTOXICITY DATA (*IN VIVO*) CONSIDERED BY THE PANEL IN FGE.208REV1

Table 6: Summary of Additionally *In Vivo* Genotoxicity Data Submitted for FGE.208Rev1

Chemical Name FL-no	Test System <i>in vivo</i>	Test Object	Route	Dose	Result	Reference	Comments
<i>p</i> -Mentha-1,8-dien-7-al [05.117]	Micronucleus Assay	Male Han Wistar Rats	Gavage	175, 350 and 700 mg/kg bw/day	Negative	(Beevers, 2014a, b)	Reliable with restriction. Complies with GLP and mainly with OECD 474 (not clear if the bone marrow was exposed).
	Comet assay	Male Han Wistar Rats	Gavage		Positive		Reliable without restriction. Complies with GLP. The study was performed shortly before publication of OECD guideline 489, however, it is consistent with this guideline. Positive in liver, negative in duodenum.

ABBREVIATIONS

CA	Chromosomal Aberration
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CoE	Council of Europe
DNA	Deoxyribonucleic acid
EFFA	European Flavour Association
EMS	Ethyl methanesulfonate
EU	European Union
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
GLP	Good Laboratory Practice
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
ID	Identity
I _{max}	Maximum Induction factor
IR	Infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MN	Micronuclei
MNBN	MicroNucleated BiNucleate cells
MS	Masse spectra
MSDI	Maximised Survey-derived Daily Intake
NMR	Nuclear Magnetic Resonance
No	Number
OECD	Organisation for Economic Co-operation and Development
PCE	Polychromatic erythrocytes
(Q)SAR	(Quantitative) Structure Activity Relationship
RI	Replication Index
RS	Relative Survival
SCE	Sister chromatid exchange
SCF	Scientific Committee on Food
WHO	World Health Organization