EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF); Scientific Opinion on Flavouring Group Evaluation 204 (FGE.204): Consideration of genotoxicity data on representatives for 18 mono-unsaturated, aliphatic, ,-unsaturated ketones and precursors from chemical subgroup 1.2.1 of FGE.19 by EFSA

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Link to article, DOI:
10.2903/j.efsa.2012.2992

Publication date:
2012

Document Version
Publisher's PDF, also known as Version of record

SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 204 (FGE.204): Consideration of genotoxicity data on representatives for 18 mono-unsaturated, aliphatic, α,β-unsaturated ketones and precursors from chemical subgroup 1.2.1 of FGE.19 by EFSA[1]

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

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids of the European Food Safety Authority was requested to evaluate the genotoxic potential of 18 flavouring substances from subgroup 1.2.1 of FGE.19 in the Flavouring Group Evaluation 204. The Flavour Industry provided additional genotoxicity studies for two representative substances, 4-methylpent-3-en-2-one [FL-no: 07.101] and 7-methyl-3-octenone-2 [FL-no: 07.177], which were evaluated in this FGE.204. Based on these new data, the Panel concluded that the flavouring substance [FL-no: 07.101] does not present a safety concern with respect to genotoxicity and accordingly it can be evaluated using the Procedure. On the contrary, the Panel could not conclude on the *in vivo* genotoxicity of [FL-no: 07.177] and more appropriate *in vivo* genotoxicity tests, considering also first site of contact, should be performed. In addition, the substance 4-methyl-3-hepten-5-one [FL-no: 07.261] was now identified as a substance for which no representative substances could be identified in the present FGE, resulting in a need for additional data on the genotoxic potential of this flavouring substance. However, the Panel noted that the 2-methyl substituted α,β-unsaturated aldehydes in FGE.201Rev1 can be considered as structurally related to [FL-no: 07.261]. Thus, the final conclusion on [FL-no: 07.261] will be drawn based on the outcome of the evaluation of FGE.201Rev1.

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1 On request from the European Commission, Question from No EFSA-Q-2012-00458 to EFSA-Q-2012-00475, adopted on 21 November.

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3 Acknowledgement: The Panel wishes to thank the members of the Working Groups on Flavourings and Genotoxicity for the preparation of this Opinion: Ulla Beckman Sundh, Vibe Beltoft, Mona-Lise Binderup, Wilfried Bursch, Riccardo Crebelli, Angelo Carere, Karl-Heinz Engel, Henrik Frandsen, Rainer Gürtler, Frances Hill, Trine Husøy, John Christian Larsen, Pia Lund, Daniel Marzin, Wim Mennes, Pasquale Mosesso, Gerard Mulder, Karin Nørby, Gerrit Speijers, Harriet Wallin and EFSA's staff members Maria Carfi and Kim Rygaard Nielsen for the support provided to this scientific Opinion.

Suggested citation: EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF); Scientific Opinion on Flavouring Group Evaluation 204 (FGE.204): Consideration of genotoxicity data on representatives for 18 mono-unsaturated, aliphatic, α,β-unsaturated ketones and precursors from chemical subgroup 1.2.1 of FGE.19 by EFSA. EFSA Journal 2012;10(12):2992. [25 pp.]. doi:10.2903/j.efsa.2012.2992. Available online: www.efsa.europa.eu/efsajournal.htm

KEY WORDS

FGE.19; subgroup 1.2.1, aliphatic, mono-unsaturated, α,β-unsaturated ketones.

SUMMARY

The European Food Safety Authority (EFSA) asked the Scientific 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 consider the Joint FAO/WHO Expert Committee on Food Additives (the JECFA) evaluations of flavouring substances assessed since 2000, and to decide whether no further evaluation is necessary, as laid down in Commission Regulation (EC) No 1565/2000. These flavouring substances are listed in the Register, which was adopted by Commission Decision 1999/217/EC and its consecutive amendments.

The present Flavouring Group Evaluation 204 (FGE.204), corresponding to subgroup 1.2.1 of FGE.19, concerns 16 mono-unsaturated, aliphatic, α,β-unsaturated ketones and two precursors for such ketones, oct-3-en-2-ol [FL-no: 02.102], oct-2-en-4-ol [FL-no: 02.193], pent-3-en-2-one [FL-no: 07.044], 4-hexen-3-one [FL-no: 07.048], oct-2-en-4-one [FL-no: 07.082], 4-methylpent-3-en-2-one [FL-no: 07.101], hept-2-en-4-one [FL-no: 07.104], hept-3-en-2-one [FL-no: 07.105], 5-methylhex-3-en-2-one [FL-no: 07.106], oct-3-en-2-one [FL-no: 07.107], dec-3-en-2-one [FL-no: 07.121], 5-methylhept-2-en-4-one [FL-no: 07.139], 7-methyl-3-octenone-2 [FL-no: 07.177], non-2-en-4-one [FL-no: 07.187], non-3-en-2-one [FL-no: 07.188], trans-6-methyl-3-hepten-2-one [FL-no: 07.244], 6-methyl-3-hepten-2-one [FL-no: 07.258] and 4-methyl-3-hepten-5-one [FL-no: 07.261].

The α,β-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity and the data on genotoxicity previously available did not rule out the concern for genotoxicity for these 18 flavouring substances.

The Panel identified two substances in subgroup 1.2.1 which will represent the other substances in this subgroup (4-methylpent-3-en-2-one [FL-no: 07.101] and 7-methyl-3-octenone-2 [FL-no: 07.177]). For these two substances the Panel requested genotoxicity data according to the test strategy worked out by the Panel.

According to the above requirements the Industry has submitted additional genotoxicity studies for 4-methylpent-3-en-2-one and 7-methyl-3-octenone-2.

Based on these new data, the Panel concluded that 4-methylpent-3-en-2-one and 7-methyl-3-octenone-2 do not induce mutations in *Salmonella typhimurium* when tested up to toxic concentrations in the absence and in the presence of metabolic activation.

4-Methylpent-3-en-2-one did not induce micronuclei in cultured human peripheral blood lymphocytes when tested up to toxic concentrations in both the absence and presence of S9-mix metabolism. Contrary, 7-methyl-3-octenone-2 induced micronuclei in human peripheral blood lymphocytes in the absence of S9-mix metabolism following 24-hour treatment.

To clarify whether 7-methyl-3-octenone-2 was acting as a clastogen or an aneugen a fluorescence *in situ* hybridization (FISH) analysis was performed for 24 hours without S9-mix. Results obtained indicated that 7-methyloct-3-en-2-one is acting as a clastogenic compound.

In order to determine whether positive results obtained in *vitro* for 7-methyl-3-octenone-2 could be confirmed *in vivo,* a bone marrow micronucleus test in rats was performed. 7-Methyl-3-octenone-2 did not induce micronuclei in bone marrow polychromatic erythrocytes (PCE). However, this outcome was accompanied by complete absence of clinical signs of toxicity in all animals, in all treatment groups. The observed very weak dose-related decrease in mean % PCE was considered by the Panel

not indicative of bone marrow exposure. Therefore, the absence of induction of micronuclei in bone marrow erythrocytes does not necessarily reflect absence of clastogenicity *in vivo* of the test compound.

On the basis of the available data, the Panel noted that no conclusions can be drawn about *in vivo* genotoxicity of 7-methyl-3-octenone-2 [FL-no: 07.177] and more appropriate *in vivo* genotoxicity tests, considering also first site of contact, should be performed. Alternatively, chemical analysis of the already available blood sample demonstrating target tissue exposure would also be suitable to make the results of the available micronucleus assay acceptable for the assessment of the hazard on genotoxic potential.

For 4-methylpent-3-en-2-one [FL-no: 07.101], the data available showed that it did not induce mutations in bacteria or micronuclei in human peripheral blood lymphocytes, neither in the presence nor in the absence of rat liver S9-mix metabolic activation. Based on these findings, the Panel concluded that 4-methylpent-3-en-2-one does not present a safety concern with respect to genotoxicity and accordingly the flavouring substance can be evaluated using the Procedure. Since this substance is considered to be representative for itself only ("stand-alone"), as it is the only substance with a methyl-substituent on the β-carbon atom of the double bond, this conclusion does not apply to any of the other candidate substances in this FGE.

Subsequent to the publication of the list of representatives (EFSA, 2008bc), the Panel noted that 4-methyl-3-hepten-5-one [FL-no: 07.261] differs structurally from all other substances in this subgroup owing to the presence of a methyl group in α-position of the double bond. Therefore, this substance is considered as a "stand alone" substance. The Panel noted that the 2-methyl substituted α,β-unsaturated aldehydes in FGE.201Rev1 (EFSA, 2012h) can be considered as structurally related to [FL-no: 07.261]. For the substances in FGE.201Rev1, 2-methylpent-2-enal [FL-no 05.090] has been selected as representative and further genotoxicity data were required. Thus, the final conclusion on [FL-no: 07.261] will be drawn based on the outcome of the evaluation of FGE.201Rev1.

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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 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).

The Union list of flavourings and source materials is established in Commission Regulation (EC) No 872/2012 (EC, 2012a).

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, 2008b).

The α,β-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 α,β-unsaturated carbonyls were subdivided into 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 α,β-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, 2008b) 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 the genotoxic potential could not be ruled out (FGE.201. 203, 210, 212, 213, 216, 217 and 220).

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 has worked out a list of representative substances for each subgroup (EFSA, 2008bc). Likewise, an EFSA genotoxicity expert group has worked out a test strategy to be followed in the data retrieval for these substances (EFSA, 2008bb).

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

The Flavouring Industry has now submitted additional data and the present FGE concerns the evaluation of these data requested on genotoxicity for subgroup 1.2.1 of FGE.19.

TERMS OF REFERENCE

The European Commission request the European Food Safety Authority to carry out a safety assessment on the following 18 substances: oct-3-en-2-ol [FL-no: 02.102], oct-2-en-4-ol [FL-no: 02.193], pent-3-en-2-one [FL-no: 07.044], 4-hexen-3-one [FL-no: 07.048], oct-2-en-4-one [FL-no: 07.082], 4-methylpent-3-en-2-one [FL-no: 07.101], hept-2-en-4-one [FL-no: 07.104], hept-3-en-2-one [FL-no: 07.105], 5-methylhex-3-en-2-one [FL-no: 07.106], oct-3-en-2-one [FL-no: 07.107], dec-3-en-2-one [FL-no: 07.121], 5-methylhept-2-en-4-one [FL-no: 07.139], 7-methyl-3-octone-2 [FL-no: 07.177], non-2-en-4-one [FL-no: 07.187], non-3-en-2-one [FL-no: 07.188], trans-6-methyl-3-hepten-2-one [FL-no: 07.244], 6-methyl-3-hepten-2-one [FL-no: 07.258] and 4-methyl-3-hepten-5-one [FL-no: 07.261], in accordance with Commission Regulation (EC) No 1565/2000.

ASSESSMENT

1. Presentation of the substances in the Flavouring Group

1.1. Description

The present Flavouring Group Evaluation 204 (FGE.204), corresponding to subgroup 1.2.1 of FGE.19, concerns 16 mono-unsaturated, aliphatic α,β-unsaturated ketones and two precursors for such ketones. The 18 substances under consideration in the present evaluation are listed in Table 1.

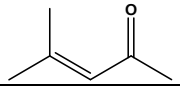
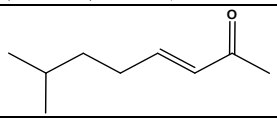
Fifteen of the flavouring substances have previously been evaluated by the JECFA (JECFA, 2002c). A summary of their current evaluation status by the JECFA and the outcome of this consideration is presented in Table 2.

The α,β-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity (EFSA, 2008b) and the data on genotoxicity previously available did not rule out the concern for genotoxicity for these 18 flavouring substances.

1.2. Representative substances for subgroup 1.2.1

In 2008, the Panel has identified two substances in subgroup 1.2.1 (EFSA, 2008bc) for which genotoxicity data according to the test strategy (EFSA, 2008bb) have been requested. These substances are listed in Table 1.1.

TABLE 1.1 SUBSTANCES FOR WHICH GENOTOXICITY DATA HAVE BEEN REQUESTED FOR SUBGROUP 1.2.1 OF FGE.19 (EFSA, 2008BC)

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no
07.101 1131	4-Methylpent-3-en-2-one		3368 11853 141-79-7
07.177 1135	7-Methyl-3-octenone-2 = 7-methyloct-3-en-2-one		3868 - 33046-81-0

Substance [FL-no: 07.101] was considered to be representative for itself only, since it is the only substance with a methyl-substituent on the β carbon atom of the double bond. The other substance, [FL-no: 07.177], was considred representative of the remaining substances of this FGE.

Subsequent to the publication of the list of representative substances (EFSA, 2008bc), the Panel noted that 4-methyl-3-hepten-5-one [FL-no: 07.261] differs structurally from all other substances in this subgroup owing to the presence of a methyl group in α-position of the double bond. Therefore, this substance is considered as a "stand alone" substance. The Panel noted that the 2-methyl substituted α,β-unsaturated aldehydes in FGE.201Rev1 (EFSA, 2012h) can be considered as structurally related to [FL-no: 07.261]. For the substances in FGE.201Rev1, 2-methylpent-2-enal [FL-no 05.090] has been selected as representative and further genotoxicity data were required. Thus, the final conclusion on [FL-no: 07.261] will be drawn based on the outcome of the evaluation of FGE.201Rev1.

2. Additionally submitted genotoxicity data on representative substances of subgroup 1.2.1

Introduction

The Industry has submitted data concerning genotoxicity studies for the two representative substances for this subgroup (EFFA, 2012h)

- 4-Methylpent-3-en-2-one [FL-no: 07.101] *in vitro* tests in bacteria and mammalian cell systems.
- 7-Methyl-3-octenone-2 [FL-no: 07.177] *in vitro* tests in bacteria and mammalian cell systems and *in vivo* micronucleus test in rats.

2.1. *In vitro* data

2.1.1. Bacterial Reverse Mutation Assay

Results of the *in vitro* studies described below are summarised in Table 3.

4-Methylpent-3-en-2-one [FL-no: 07.101]

An Ames assay was conducted in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 to assess the mutagenicity of 4-methylpent-3-en-2-one [FL-no: 07.101], both in the absence and presence of rat liver metabolizing system (S9-mix) in two experiments (Williams, 2009b). A preliminary range-finding cytotoxicity experiment using standard plate-incorporation methodology was conducted in strain TA100 only at concentrations of 1.6, 8, 40, 200, 1000 and 5000 µg/plate in the absence and presence of S9-mix, plus negative (solvent) and positive controls. Evidence of toxicity, in terms of a slight thinning of the background bacterial lawn, was observed only at the top concentration in the presence of S9-mix. The data from the range-finding experiment were considered acceptable for mutation assessment, and therefore, to complete the first experiment, the remaining four strains were tested at the same concentrations both in the presence and absence of S9-mix using the same methodology. No evidence of toxicity was observed in these strains, and no increases in reverse mutants relative to the vehicle control were observed.

In a second experiment, 4-methylpent-3-en-2-one was tested in all five *S. typhimurium* strains with and without S9-mix, using a narrowed concentration range of 156.25, 312.5, 625, 1250, 2500 and 5000 µg/plate. A pre-incubation step was also included when the chemical was tested in the presence of S9-mix. Following these treatments, evidence of toxicity in the form of a slight thinning of the background bacterial lawn was observed at the highest concentrations (2500 and 5000 µg/plate) in all strains in the presence of S9-mix and in strain TA102 in the absence of S9-mix. A small increase (1.5-fold) in TA1535 revertants was seen at the higest concentration in the absence of S9-mix that was significant at $p < 0.05$, but this small increase was not seen in the first experiment at similar concentrations and was considered by the study authors to be due to chance. No increases in revertant numbers were observed for the other strains and treatment conditions.

Based on the above results the Panel concluded that 4-methylpent-3-en-2-one [FL-no: 07.101] did not induce mutations in five strains of *S. typhimurium* when tested up to toxic concentrations in the absence and in the presence of metabolic activation (Williams, 2009b).

7-methyl-3-octenone-2 *[FL-no: 07.177]*

Previously, an Ames assay had been performed with 7-methyl-3-octenone-2 [FL-no: 07.177] in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA1538 with and without S9-mix metabolic activation at concentrations ranging from 15 - 5000 µg/plate. The test compound showed toxicity in terms of reduction of bacterial lawn at dose-levels of 1500 µg/plate and higher, both in the

absence and presence of S9-mix. No significant increases in the frequencies of revertant colonies were observed in any of the tester strains employed, at any dose-level assayed with and without metabolic activation (Thompson, 1996a). To supplement these results and thus provide data for a battery of test strains consistent with the requirements for current regulatory guidelines (OECD TG 471, 1997), a new Ames study (standard plate incorporation method) using the *S. typhimurium* tester strain TA102 at concentrations of 1.6, 8, 40, 200, 1000 and 5000 µg/plate with and without S9-mix was performed with 7-methyl-3-octenone-2 (Ballantyne, 2011b). Evidence of toxicity in the presence and absence of S9-mix was observed at the highest concentration tested. No increase in reverse mutant counts following treatments with 7-methyl-3-octenone-2 compared with the vehicle control were observed at any dose-level assayed. In a second experiment, 7-methyl-3-octenone-2 was assayed in strain TA102 with and without S9-mix and a narrowed concentration range of 51.2, 128, 320, 800, 2000 and 5000 µg/plate. In the presence of S9-mix, a pre-incubation step was also included. Following 7-methyl-3-octenone-2 treatment, evidence of toxicity was observed in the presence and absence of S9-mix, on plates treated at 2000 or 5000 µg/plate. Treatment of strain TA102 with 7-methyl-3-octenone-2, with and without S9-mix, did not induce any statistically significant increase in revertant numbers (data were analysed at the 1 % level using Dunnett's) at the tested concentrations (Ballantyne, 2011b).

Taken together with the data of Thompson (1996), the Panel concluded that 7-methyl-3-octenone-2 [FL-no: 07.177] did not induce mutations in six strains of *S. typhimurium* when tested up to toxic concentrations in the absence and in the presence of metabolic activation (Thompson, 1996a; Ballantyne, 2011b).

2.1.2. *In vitro* micronucleus assays

Results of the studies described below are summarised in Table 3.

4-Methylpent-3-en-2-one [FL-no: 07.101]

4-Methylpent-3-en-2-one was tested for the induction of chromosome damage and potential aneugenic effects in mammalian cells *in vitro* by examining the effect on the frequency of micronuclei in cultured human peripheral blood lymphocytes, treated in the absence and presence of rat liver metabolizing system (S9-mix) (Stone, 2011).

A preliminary range-finding experiment was conducted with and without S9-mix in order to determine the effect of the test substance upon Replication Index (RI), which was used as a basis for choosing a range of concentrations to be evaluated in the main study. 4-Methylpent-3-en-2-one was added to cell cultures after 48 hours from culture initiation (stimulation by phytohaemagglutinin PHA), either for 3 hours in the absence or presence of S9-mix, or for 24 hours in the absence of S9-mix. Micronuclei were analyzed at multiple concentrations for each treatment group. For the 3-hour treatment (3 + 21 hours recovery) the concentrations were 0, 600, 800 and 981.4 µg/ml (without S9-mix) and 0, 200, 400, 800, 981.4 µg/ml (with S9-mix). The levels of cytotoxicity (reduction in RI) induced at the top concentrations were 22 % and 54 % in the absence and presence of S9-mix, respectively. Although the recommended range of toxicity (50 - 60 %) was not reached in the absence of S9-mix, the top concentration of 981.4 µg/ml was equivalent to 10 mM, which is the required upper limit for a non-toxic substance. For 24-hour treatment without S9-mix the concentrations were 0, 100, 200, 275 and 300 µg/ml and the level of cytotoxicity (reduction in RI) at the top concentration reached 62 %, which exceeded the target (50 - 60 %) range. One thousand binucleate cells per culture from two replicate cultures per concentration were scored for micronuclei.

Treatment of cells with 4-methylpent-3-en-2-one for 3 hours in the presence of S9-mix resulted in statistically significant ($p \leq 0.05$) increases in MNBN frequency compared to the concurrent vehicle control at the highest concentration analysed (981.4 µg/mL). However, only one replicate culture in the assay resulted in MNBN cell frequencies outside of the normal range and the authors considered this result as equivocal. Therefore a confirmatory experiment was performed with 4-methylpent-3-en-

2-one at concentrations of 0, 100, 200, 400 and 500 µg/ml for 3 hours with S9-mix. The lower concentrations chosen in the second experiment were on the basis of an unexplained shift in toxicity, but the concentrations selected for analysis in this experiment gave comparable toxicity to those selected in the prior experiment under this treatment condition, and 58 % cytotoxicity (determined as reduction in RI) was achieved at the top concentration. These treatments resulted in frequencies of MNBN cells that were similar to concurrent controls and there were no significant differences.

Considering that the significant increase in MNBN cell frequencies in the first experiment were not reproduced in the second one, the Panel concluded that 4-methylpent-3-en-2-one [FL-no: 07.101] did not induce micronuclei in cultured human peripheral blood lymphocytes when tested up to toxic concentrations in both the absence and presence of S9-mix metabolism (Stone, 2011).

7-Methyl-3-octenone-2 [FL-no: 07.177]

7-Methyl-3-octenone-2 [FL-no: 07.177] was tested in an *in vitro* micronucleus assay in cultured human peripheral blood lymphocytes treated in the absence and presence of rat liver metabolizing system (S9-mix) (Lloyd, 2009d). 7-Methyl-3-octenone-2 [FL-no: 07.177] was added at 48 hours following mitogen stimulation by PHA either for 3 hours in the absence or presence of S9-mix, or for 24 hours in the absence of S9-mix. The test substance concentrations for micronucleus analysis were selected by evaluating the effect of 7-methyl-3-octenone-2 on the Replication Index (RI). In the main experiment, micronuclei were analysed at multiple concentrations for each treatment group.

For 3-hour treatment without S9-mix the concentrations were 5, 10 and 15 µg/ml, for 3-hour treatment with S9-mix the concentrations were 30, 40 and 60 µg/ml, and for 24-hour treatment without S9-mix the concentrations were 2, 4 and 6 µg/ml. The levels of cytotoxicity (reduction in RI) at the top concentrations reached 45 % (in the 3 + 21 hours treatment without S9-mix), 58 % (in the 3 + 21 hours treatment with S9-mix) and 59 % (in the 24 hours treatment without S9-mix). One thousand binucleate cells per culture from two replicate cultures per concentration were scored for micronuclei.

Treatment of cells with 7-methyl-3-octenone-2 for 3 + 21 hours resulted in frequencies of MNBN cells that were significantly higher ($p \leq 0.05$) than those observed in concurrent vehicle controls at the highest concentrations analysed in the absence and presence of S9-mix (15.00 µg/mL and 60.00 µg/mL, respectively). However, the MNBN cell frequencies in all treated cultures in the absence and presence of S9-mix fell within historical range values for vehicle control. These observations were not considered biologically relevant by the authors of the study report.

For the 24-hour treatment, at the two highest concentrations (4.0 and 6.0 µg/mL), the frequencies of MNBN cells were significantly higher ($p \leq 0.05$, $p \leq 0.001$, respectively) than those observed in the concurrent vehicle control (0.2 %). The MNBN cell frequencies in those cultures treated with the highest concentration exceeded the normal ranges and therefore this was considered to be a positive result.

Therefore, the Panel concluded that 7-methyl-3-octenone-2 induced micronuclei in cultured peripheral blood lymphocytes when tested up to toxic concentrations for 24 hours in the absence of S9-mix (Lloyd, 2009d).

In order to determine whether 7-methyl-3-octenone-2 was acting as a clastogen or an aneugen, a follow-up study was performed using fluorescence in situ hybridization (FISH) analysis (Lloyd, 2010c). Micronuclei were analyzed at multiple concentrations separated by narrow intervals, which were based on the toxicity displayed in a preliminary range-finding experiment for 24 hours without S9-mix treatment. The concentrations analyzed were 5.5, 6.0, 7.0 and 8.0 µg/ml. The levels of cytotoxicity (reduction in replication index, RI) was concentration-dependent and, at the top concentration, reached 56 %.

All concentrations of 7-methyl-3-octenone-2 (5.5, 6.0, 7.0 and 8.0 µg/ml) resulted in significant increases of MNBN cells ($p \leq 0.05$, $p \leq 0.01$, $p \leq 0.01$ and $p \leq 0.001$, respectively) relative to vehicle controls. The increase of MNBN cells ranged from 2.3 to 4.7 fold greater than the concurrent control, and exceeded the historical control range at the highest concentration tested (1.4 % mean MNBN cell frequency vs. 0.1 - 0.9 % historical control range).

The FISH analyses, with a fluorochrome-labelled pan-centromeric human DNA probe specific for all human chromosomes, to identify micronuclei containing centromeres, were performed on slides generated from vehicle control, 7-methyl-3-octenone-2 along with a clastogenic positive control (Mitomycin C) and an aneugenic positive control (Vinblastine).

Treatment with 7 and 8 µg/ml 7-methyl-3-octenone-2 resulted in 20 % and 18 % centromere-positive micronuclei, respectively compared to 46 % centromere-positive micronuclei for the vehicle control, 9 % for the reference clastogen and 84 % for the reference aneugen. On this basis, the authors concluded that the induction of increased MNBN cell frequency is primarily a result of chromosome breakage rather than chromosome loss following treatment of cultured peripheral blood lymphocytes with 7-methyl-3-octen-2-one for 24 hours in the absence of S9-mix (Lloyd, 2010c). Based on these results, the Panel agreed that 7-methyl-3-octenone-2 [FL-no: 07.177] is an *in vitro* clastogen for human lymphocytes.

2.2. *In vivo* data

Considering the results from the *in vitro* micronucleus assays, it was concluded that 7-methyl-3-octenone-2 [FL-no: 07.177] was an *in vitro* clastogen for human lymphocytes, and therefore it was decided that it was most appropriate to carry out an *in vivo* micronucleus assay to determine whether the results obtained in the *in vitro* micronucleus assays could be confirmed *in vivo*. Therefore, groups of Han-Wistar rats were administered 7-methyl-3-octenone-2 via oral gavage and the induction of micronuclei in the polychromatic erythrocyte (PCE) of the bone marrow was examined.

As no substantial difference in toxicity was observed between males and females in the range-finding experiment, male rats only were used in the micronucleus experiment. Groups of male (6 animals/group) rats were administered 7-methyl-3-octenone-2 by oral gavage at 500, 1000 and 2000 mg/kg bw/day on 2 occasions 24 hours apart (Table 4). An additional satellite group of animals was dosed at 2000 mg/kg bw/day to facilitate later blood analysis if desired. Animals were sampled 24 hours after the final administration, thus enabling examination of cells exposed to the test article over a period of 24 to 48 hours prior to sampling. No clinical signs of toxicity were observed in any animals in any main treatment groups. In the satellite group, animals showed decreased signs of activity on day 2 at 0.5 and 1.0 hours post dose.

Rats treated with 7-methyl-3-octenone-2 exhibited a very weak but dose-related decrease in mean % polychromatic erythrocytes (PCE) was however comparable with the historical control data for this experiment at the testing laboratory. No statistically significant increase in micronucleus frequency was observed for any of the groups receiving the test substance, compared to the concurrent vehicle control (Table 5). On this basis, the authors concluded that 7-methyl-3-octenone-2 did not induce micronuclei in the polychromatic erythrocytes of the bone marrow of male rats treated up to 2000 mg/kg/day (Henderson, 2011).

Results of the *in vivo* studies described above are summarised in Table 4 and 5.

2.3. Discussion of Mutagenicity/Genotoxicity Data

The two representative substances, 4-methylpent-3-en-2-one [FL-no: 07.101] and 7-methyl-3-octenone-2 [FL-no: 07.177], are both considered negative in the Ames test with *S. typhimurium* tester strains consistent with the requirements for current regulatory guidelines. Statistically significant

increase in the number of revertant colonies observed in tester strain TA1535 in the absence of S9-mix metabolism in one experiment following treatment with 4-methylpent-3-en-2-one are judged not biologically relevant, since they were not reproduced in the second experiment (Williams, 2009b; Ballantyne, 2011b).

Investigations at chromosome and genome levels in mammalian cells *in vitro* showed that 4-methylpent-3-en-2-one induced a small but statistically significant increase in the frequency of micronucleated binucleate cells (MNBN) only in the presence of S9-mix metabolism following a three hour treatment at the highest concentration tested (981.4 µg/ml). However, only one replicate culture fell outside the historical vehicle control range values. Following additional scoring of 2000 erythrocytes, the resulting MNBN frequencies, although still significantly higher than concurrent vehicle control, lied within historical control range values. In a second confirmatory experiment (3-hour treatment in the presence of S9-mix) performed at concentrations lower than concentrations used in the previous experiment, due to an unexplained shift of toxicity (comparable toxicity to those observed in the first experiment, but at lower concentrations), no significant increase in MNBN frequencies was observed. Based on these results the Panel concluded that 4-methylpent-3-en-2-one did not induce micronuclei in human peripheral blood lymphocytes, both in the absence and presence of rat liver S9-mix metabolism. On the contrary, 7-methyl-3-octenone-2 induced statistically significant increases in MNBN following the 3-hour treatment both in the absence and presence of S9-mix at the highest concentrations tested. However, the observed increase of MNBN cell frequencies fell within relevant historical control values. In the 24-hour treatment in the absence of S9-mix, 7-methyl-3-octenone-2 induced statistically significant increase in MNBN cell frequencies at the two highest concentrations, selected for scoring and exceeded the historical control range values (Lloyd 2009d). Therefore, the Panel concluded that 7-methyl-3-octenone-2 induces micronuclei in human peripheral blood lymphocytes *in vitro* in the absence of S9-mix metabolism following 24-hour treatment.

To determine whether 7-methyl-3-octenone-2 was acting as a clastogen or an aneugen, a follow up study was performed, in the absence of S9-mix, following a treatment of 24 hours at similar concentrations used in the previous study (Lloyd 2010c). FISH analysis was performed to identify micronuclei containing centromeres. Results obtained confirmed previous findings and clearly indicated that 7-methyl-3-octenone-2 is acting as a clastogenic compound. In order to determine whether positive results obtained in *vitro* for 7-methyl-3-octenone-2 could be confirmed *in vivo,* a bone marrow micronucleus test in rats treated *in vivo* was performed.

The test substance 7-methyl-3-octenone-2 administered by oral gavage at doses of 500, 1000 and 2000 mg/kg bw on two occasions with 24 hours apart to Han-Wistar rats did not prove to induce micronuclei in bone marrow polychromatic erythrocytes (PCE). However, this outcome was accompanied by complete absence of clinical signs of toxicity in any animal, in any treatment group. The observed very weak dose-related decreases in mean % PCE was considered by the Panel not indicative of bone marrow exposure as suggested by the author of the study report, since group mean values of % PCE fall within observed historical range values at 95 % confidence interval as reported in the study report (Henderson, 2012) and displayed in Table 5. These findings do not allow to conclude a bone marrow exposure and therefore absence of induction of micronuclei in bone marrow erythrocytes does not necessarily reflect absence of clastogenicity *in vivo* of the test substance. Blood was appropriately collected and processed but, as stated in the study report, it was not analysed to measure the plasma level of 7-methyl-3-octenone-2 due to the lack of a bioanalytical method. The direct reactivity of the test substance evinced by clastogenicity induced *in vitro,* (in the absence of S9-mix metabolism) indicates a reduced systemic availability of the test substance.

CONCLUSIONS

On the basis of the available data, the Panel noted that no conclusions can be drawn about *in vivo* genotoxicity of 7-methyl-3-octenone-2 [FL-no: 07.177] and more appropriate *in vivo* genotoxicity

tests, considering also first site of contact, should be performed. Alternatively, chemical analysis of the already available blood samples demonstrating target tissue exposure would also be suitable to make the results of the available micronucleus assay acceptable for the assessment of the genotoxic potential.

For 4-methylpent-3-en-2-one [FL-no: 07.101], the data available showed that it did not induce mutations in bacteria or micronuclei in human peripheral blood lymphocytes, neither in the presence nor in the absence of rat liver S9-mix metabolic activation. Based on these findings, the Panel concluded that 4-methylpent-3-en-2-one does not present a safety concern with respect to genotoxicity and accordingly the flavouring substance can be evaluated using the Procedure. Since this substance is considered to be representative for itself only ("stand-alone"), as it is the only substance with a methyl-substituent on the β-carbon atom of the double bond, this conclusion does not apply to any of the other candidate substances in this FGE.

Subsequent to the publication of the list of representatives (EFSA, 2008bc), the Panel noted that 4-methyl-3-hepten-5-one [FL-no: 07.261] differs structurally from all others in this subgroup owing to the presence of a methyl group in α-position of the double bond. Therefore, this substance is considered as a "stand alone" substance. The Panel noted that the 2-methyl substituted α,β-unsaturated aldehydes in FGE.201Rev1 (EFSA, 2012h) can be considered as structurally related to [FL-no: 07.261]. For the substances in FGE.201Rev1, 2-methylpent-2-enal [FL-no 05.090] has been selected as representative and further genotoxicity data were required. Thus, the final conclusion on [FL-no: 07.261] will be drawn based on the outcome of the evaluation of FGE.201Rev1.

TABLE 1: SPECIFICATION SUMMARY OF THE SUBSTANCES IN THE FLAVOURING GROUP EVALUATION 204 (JECFA, 2002D)

Table 1: Specification Summary of the Substances in the present group (JECFA, 2002d)

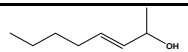
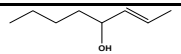
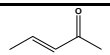
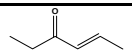
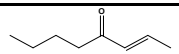
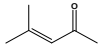
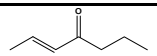
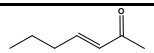
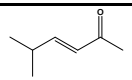
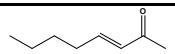
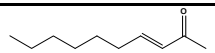
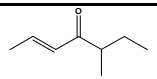
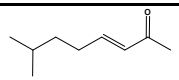
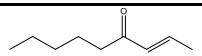
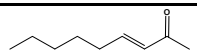
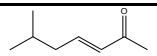
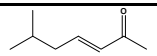
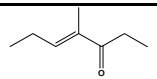
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
02.102 1140	Oct-3-en-2-ol 6)		3602 76649-14-4	Liquid $C_8H_{16}O$ 128.22	Insoluble Miscible	73-76 (13 hPa) IR NMR MS 98 %	1.422-1.428 0.826-0.836
02.193 1141	Oct-2-en-4-ol 6)		3888 4798-61-2	Liquid $C_8H_{16}O$ 128.22	Insoluble 50% Soluble in ethanol	 IR NMR MS 95 %	1.438-1.442 0.830-0.838
07.044 1124	Pent-3-en-2-one 6)		3417 666 625-33-2	Liquid C_5H_8O 84.12	Slightly soluble Miscible	122 NMR 98 %	1.433-1.437 0.860-0.865
07.048 1125	4-Hexen-3-one 6)		3352 718 2497-21-4	Liquid $C_6H_{10}O$ 98.15	Slightly soluble Miscible	93 (195 hPa) NMR 98 %	1.437-1.443 0.855-0.861
07.082 1129	Oct-2-en-4-one 6)		3603 2313 4643-27-0	Liquid $C_8H_{14}O$ 126.20	Insoluble Miscible	81 (26-27 hPa) IR NMR 96 %	1.440-1.446 0.835-0.842
07.101 1131	4-Methylpent-3-en-2-one		3368 11853 141-79-7	Liquid $C_6H_{10}O$ 98.14	Slightly soluble Miscible	126.76 NMR 95 %	1.442-1.447 0.862-0.868
07.104 1126	Hept-2-en-4-one 6)		3399 11093 4643-25-8	Liquid $C_7H_{12}O$ 112.17	Slightly soluble Miscible	156-157 IR NMR 99 %	1.440-1.445 0.845-0.852
07.105 1127	Hept-3-en-2-one 6)		3400 11094 1119-44-4	Liquid $C_7H_{12}O$ 112.17	Slightly soluble Miscible	162 NMR 96 %	1.439-1.448 0.841-0.847
07.106 1132	5-Methylhex-3-en-2-one 6)		3409 11149 5166-53-0	Liquid $C_7H_{12}O$ 112.17	Insoluble Miscible	77.5 (65 hPa) NMR 99 %	1.437-1.441 0.838-0.843
07.107 1128	Oct-3-en-2-one 6)		3416 11170 1669-44-9	Liquid $C_8H_{14}O$ 126.19	Insoluble Miscible	75-79 (26 hPa) NMR 94 %	1.445-1.449 0.834-0.839

Table 1: Specification Summary of the Substances in the present group (JECFA, 2002d)

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)
07.121 1130	Dec-3-en-2-one 6)		3532 11751 10519-33-2	Liquid $C_{10}H_{18}O$ 154.25	Almost insoluble Miscible	125-126 NMR 95 %	1.446-1.452 0.809-0.813
07.139 1133	5-Methylhept-2-en-4-one 6)		3761 81925-81-7	Liquid $C_8H_{14}O$ 126.19	Slightly soluble Miscible	86-87 (78 hPa) NMR 98 %	1.440-1.445 0.845-0.852
07.177 1135	7-Methyl-3-octenone-2 6)		3868 33046-81-0	Liquid $C_9H_{16}O$ 140.2	Slightly soluble Miscible	198 IR NMR MS 94 %	1.446-1.451 0.838-0.847
07.187	Non-2-en-4-one 6)		 11162 32064-72-5	Liquid $C_9H_{16}O$ 140.22	Insoluble Freely soluble	82 (27 hPa) MS 95 %	1.422-1.428 0.823-0.829
07.188 1136	Non-3-en-2-one 6)		3955 11163 14309-57-0	Liquid $C_9H_{16}O$ 140.22	Insoluble Miscible	198 IR MS 95 %	1.443-1.452 0.843-0.846
07.244 1138	trans-6-Methyl-3-hepten-2-one		4001 20859-10-3	Liquid $C_8H_{14}O$ 126.2	Insoluble Miscible	178-170 NMR 96 %	1.438-1.447 0.840-0.850
07.258	6-Methyl-3-hepten-2-one 6)		 2009-74-7	Liquid $C_8H_{14}O$ 126.20	Practically insoluble or insoluble Freely soluble	179 MS 96 %	1.436-1.442 0.842-0.848
07.261	4-Methyl-3-hepten-5-one 6)		 22319-31-9	Liquid $C_8H_{14}O$ 126.20	Insoluble Freely soluble	179 MS 96.12 %	1.442-1.462 0.851-0.871

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: CURRENT SAFETY EVALUATION STATUS APPLYING THE PROCEDURE (BASED ON INTAKES CALCULATED BY THE MSDI APPROACH) (JECFA, 2002C)

Table 2: Summary of Safety Evaluation of the JECFA substances in the present group (JECFA, 2002c)

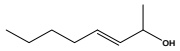
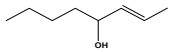
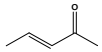
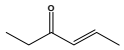
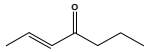
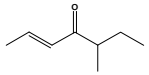
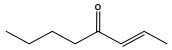
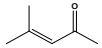
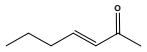
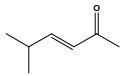
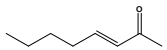
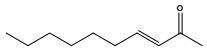
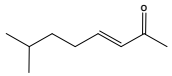
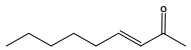
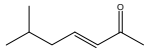
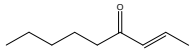
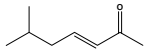
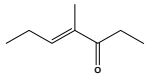
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI 1) US MSDI (µg/*capita*/day)	Class 2) Evaluation procedure path 3)	JECFA Outcome on the named compound [4) or 5)]	EFSA conclusion on the named compound (genotoxicity)
02.102 1140	Oct-3-en-2-ol		1.2 ND	Class I A3: Intake below threshold	4)	Evaluated in FGE.204, additional genotoxicity data required
02.193 1141	Oct-2-en-4-ol		ND ND	Class I A3: Intake below threshold	4)	Evaluated in FGE.204, additional genotoxicity data required
07.044 1124	Pent-3-en-2-one		0.26 ND	Class I A3: Intake below threshold	4)	Evaluated in FGE.204, additional genotoxicity data required
07.048 1125	4-Hexen-3-one		13 1	Class I A3: Intake below threshold	4)	Evaluated in FGE.204, additional genotoxicity data required
07.104 1126	Hept-2-en-4-one		0.012 ND	Class I A3: Intake below threshold	4)	Evaluated in FGE.204, additional genotoxicity data required
07.139 1133	5-Methylhept-2-en-4-one		5.8 1	Class I A3: Intake below threshold	4)	Evaluated in FGE.204, additional genotoxicity data required
07.082 1129	Oct-2-en-4-one		0.85 3	Class II A3: Intake below threshold	4)	Evaluated in FGE.204, additional genotoxicity data required
07.101 1131	4-Methylpent-3-en-2-one		0.34 ND	Class II A3: Intake below threshold	4)	No safety concern with respect to genotoxicity. To be evaluated through the Procedure.
07.105 1127	Hept-3-en-2-one		0.16 0.07	Class II A3: Intake below threshold	4)	Evaluated in FGE.204, additional genotoxicity data required
07.106 1132	5-Methylhex-3-en-2-one		ND 0.1	Class II A3: Intake below threshold	4)	Evaluated in FGE.204, additional genotoxicity data required
07.107 1128	Oct-3-en-2-one		0.63 1	Class II A3: Intake below threshold	4)	Evaluated in FGE.204, additional genotoxicity data required
07.121 1130	Dec-3-en-2-one		0.012 ND	Class II A3: Intake below threshold	4)	Evaluated in FGE.204, additional genotoxicity data required
07.177 1135	7-Methyl-3-octenone-2		ND 2	Class II A3: Intake below threshold	4)	Evaluated in FGE.204, additional genotoxicity data required
07.188 1136	Non-3-en-2-one		13 13	Class II A3: Intake below threshold	4)	Evaluated in FGE.204, additional genotoxicity data required

Table 2: Summary of Safety Evaluation of the JECFA substances in the present group (JECFA, 2002c)

FL-no JECFA-no	EU Register name	Structural formula	EU MSDI 1) US MSDI (µg/*capita*/day)	Class 2) Evaluation procedure path 3)	JECFA Outcome on the named compound [4) or 5)]	EFSA conclusion on the named compound (genotoxicity)
07.244 1138	trans-6-Methyl-3-hepten-2-one		3.4 3	Class II A3: Intake below threshold	4)	Evaluated in FGE.204, additional genotoxicity data required
07.187	Non-2-en-4-one		0.0012	Class II No evaluation	Not evaluated by the JECFA	Evaluated in FGE.204, additional genotoxicity data required
07.258	6-Methyl-3-hepten-2-one		0.061	Class II No evaluation	Not evaluated by the JECFA	Evaluated in FGE.204, additional genotoxicity data required
07.261	4-Methyl-3-hepten-5-one			No evaluation	Not evaluated by the JECFA	Evaluated in FGE.204, 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.
ND Not determined.

TABLE 3: GENOTOXICITY (*IN VITRO*)

Table 3: Summary of Additionally Submitted Genotoxicity Data on the Representative Substance of Subgroup 1.2.1

FL-no	Chemical Name	Test System *in vitro*	Test Object	Concentrations of Substance and Test Conditions	Result	Reference	Comments
[07.101]	4-Methylpent-3-en-2-one	Reverse Mutation	*S.typhimurium* TA98, TA100, TA102, TA1535 and TA1537	1.6 - 5000 µg/plate [1]	Negative	(Williams, 2009b)	Valid. Study design complies with current recommendations.
				156.25 - 5000 µg/plate [1,2]	Negative		
		Micronucleus Assay	Human peripheral blood lymphocytes	600 - 981.4 µg/ml [3]	Negative	(Stone, 2011)	Valid. Complies with OECD Guideline 487.
				200 - 981.4 µg/ml [4]	Negative		
				100 - 500 µg/ml [4]	Negative		
				100 - 300 µg/ml [5]	Negative		
[07.177]	7-methyl-3-octenone-2	Reverse Mutation	*S.typhimurium* TA102	1.6 - 5000 µg/plate [1]	Negative	(Ballantyne, 2011b)	Valid. Studies combined comply with current recommendations.
				51.2 - 5000 µg/plate [1,2]	Negative		
			S.typhimurium TA98, TA100, TA1535, TA1537, and TA1538	15 - 5000 µg/plate [1]	Negative	(Thompson, 1996a)	
		Micronucleus Assay	Human peripheral Blood lymphocytes	5 - 15 µg/ml [3]	Equivocal	(Lloyd, 2009d)	Valid. Testing strategies including FISH analyses for determination of potential clastogenicity or aneugenicity Comply with OECD Guideline 487.
				30 - 60 µg/ml [4]	Equivocal		
				2 - 6 µg/ml [5]	Positive		
				5.5 - 8 µg/ml [5]	Positive	(Lloyd, 2010c)	

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 inappropriate / not validated test system).

Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided, text not in a Community language).

[1] With and without S9-mix metabolic activation
[2] Assay modified with pre-incubation in the presence of S9-mix.
[3] Without metabolic activation, 3 hours treatment + 21 hours recovery
[4] With metabolic activation, 3 hours treatment + 21 hours recovery
[5] Without metabolic activation, 24 hours + 0 hours recovery

TABLE 4: GENOTOXICITY (*IN VIVO*)

Table 4: Summary of Additionally Submitted *In Vivo* Genotoxicity Data on 7-Methyl-3-octenone-2

FL-no	Chemical Name	Test System *in vivo*	Test Object / Sex No per group / groups	Route	Concentrations of Substance	Result	Reference	Comments
[07.177]	7-methyl-3-octenone-2	Micronucleus Assay	Male Han Wister rats / 6 animals/group	Gavage on 2 occasions 24 hours apart	0, 500, 1000 and 2000 mg/kg bw/day	Negative	(Henderson, 2011)	Valid. Complies with OECD Guideline 474. Althoug the study was performed at the maximum recommended highest dose-level (2000 mg/kg) no clear indication of toxicity was observed indicating that test substance might not have been sistemically available. This is also supported by positive findings observed in the *in vitro* micronucleus assay in the absence of S9 metabolism which indicates a direct reactivity of test compound.

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 inappropriate / not validated test system).

Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided, text not in a Community language).

TABLE 5: COMPARISON OF TEST GROUPS PCE (%) AND HISTORICAL CONTROL GROUP PCE (%)

Table 5: Comparison of Reported Group Mean Values of PCE (%) for 7-Methyl-3-octenone-2 Treatment Groups and Historical Group Mean Values of PCE (%) for vehicle control (Henderson, 2012)

Chemical Name	Test system (*in vivo*)	Test object	No. of animals	Route of administration	Dose-levels mg/kg bw	Group mean PCE (%)	Historical group mean range values of (%) PCE for the vehicle control group
7-methyl-3-octenone-2	Bone marrow micronucleus test	Male rats	6	Oral gavage 2 occasions 24 hours apart	0	42.60	32.41 - 56.26
					500	39.15	
					1000	38.38	
					2000	36.85	
Cyclophosphamide (positive control)				Oral gavage Once 24 hour before sacrifice	20	46.38	

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ABBREVIATIONS

CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CoE	Council of Europe
DNA	Deoxyribonucleic acid
EFSA	The European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FGE	Flavouring Group Evaluation
FISH	Fluorescence In Situ Hybridization
FLAVIS (FL)	Flavour Information System (database)
GLP	Good Laboratory Practice
ID	Identity
IR	Infrared Spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
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
PHA	PhytoHaemAgglutinin
(Q)SAR	(Quantitative) Structure Activity Relationship
RI	Replication Index
S9-mix	Rat Liver Metabolic Activation System
SCF	Scientific Committee on Food
WHO	World Health Organisation