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Scientific Opinion on Flavouring Group Evaluation 215 Revision 1 (FGE.215Rev1): seven α,β-unsaturated cinnamyl ketones from subgroup 3.2 of FGE.19

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Abstract

The Panel on Food Additives and Flavourings of the European Food Safety Authority was requested to evaluate the genotoxic potential of flavouring substances from subgroup 3.2 of FGE.19 in the Flavouring Group Evaluation 215, Revision 1 (FGE.215Rev1). In FGE.215, the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids concluded that the concern for genotoxicity could not be ruled out and requested *in vivo* data for the two representative substances 4-phenylbut-3-en-2-one [FL-no: 07.024] and 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030]. The Flavour Industry has provided additional genotoxicity studies for both representative substances [FL-no: 07.024] and [FL-no: 07.030]. Based on these new data, the Panel concluded that the concern for genotoxicity is ruled out for the representative substance [FL-no: 07.024] and for the structurally related substances 4-phenylbut-3-en-2-ol [FL-no: 02.066] and 3-methyl-4-phenylbut-3-en-2-one [FL-no: 07.027] which can accordingly be evaluated through the Procedure in FGE.69. For the representative substance 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030], the Panel concluded that [FL-no: 07.030] is aneugenic *in vitro*. For such substances, there is currently no agreed follow-up strategy to finalise their safety assessment. The Panel is aware that the EFSA Scientific Committee is going to address this issue and a statement clarifying the assessment of *in vitro* aneugenic substances is under preparation. The Panel concluded therefore that, for the time being, the representative substance 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] and the structurally related substances vanillylidene acetone [FL-no: 07.046] and 1-(4-methoxyphenyl)-4-methylpent-1-en-3-one [FL-no: 07.049] cannot be evaluated through the Procedure. The Panel further concluded that 4-(2,3,6-trimethylphenyl)but-3-en-2-one [FL-no: 07.206] is to be considered as a stand-alone substance due to the presence of the methyl groups, therefore, *in vitro* genotoxicity data were requested for [FL-no: 07.206]. Industry communicated that the evaluation of [FL-no: 07.206] is not supported any longer, therefore additional data were not submitted.

Keywords: FGE.215, α,β-unsaturated cinnamyl ketones, flavouring substances, safety evaluation, FGE.19, subgroup 3.2

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

The use of flavourings is regulated under Regulation (EC) No 1334/2008[1] 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 872/2012[2]. The list contains flavouring substances for which the scientific evaluation should be completed in accordance with Commission Regulation (EC) No 1565/2000[3].

On 26 March 2014, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (EFSA CEF Panel) adopted an opinion on Flavouring Group Evaluation 215 (FGE.215): Consideration of genotoxic potential for FGE.215 α,β-unsaturated aldehydes, straight chain, α,β-unsaturated cinnamyl ketones, subgroup 3.2, FGE.19.

The Panel concluded that for (4-phenylbut-3-en-2-one [FL-no: 07.024] and 1-(4-methoxyphenyl) pent-1-en-3-one [FL-no: 07.030]) of subgroup 3.2 of FGE.19 the Panel's concern with respect to genotoxicity could not be ruled out and subsequently additional data are requested.

On 5 November 2014 the applicant submitted additional studies on the representative substances [FL-no: 07.024] and [FL-no: 07.030] in response to this EFSA evaluation (Ares (2015) 786221).

1.1.1. Terms of Reference

The European Commission requests the European Food Safety Authority (EFSA) to evaluate the new information and, depending on the outcome, proceed to the full evaluation on the flavouring substance in accordance with Commission Regulation (EC) No 1565/2000.[3]

1.1.2. Interpretation of the Terms of Reference

In the most recent requests from the European Commission related to the evaluation of genotoxicity data submitted for the substances belonging to FGE.19, if the genotoxicity concern could not be ruled out, the Panel is requested to include information on the natural occurrence in food and on the exposure of the substance of concern. In line with these most recent European Commission requests the Panel has reported information on occurrence in food and on exposure (Appendix E).

2. Data and methodologies

2.1. 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 that were available at that time (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

[1] 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.

[2] 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.

[3] Commission Regulation (EC) 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.

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,b) 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 were 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 were requested, EFSA worked out a list of representative substances for each subgroup (EFSA, 2008c). In selecting the representative substances expert judgement was applied. In each subgroup, the representative substances were selected taking into account chain length, chain branching, lipophilicity and additional functional groups. 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.

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

2.2. Presentation of the substances belonging to FGE.215

The Flavouring Group Evaluation 215, corresponding to subgroup 3.2 of FGE.19, concerns six cinnamyl ketones with the α,β-unsaturation in the side-chain [FL-no: 07.024, 07.027, 07.030, 07.046, 07.049 and 07.206] and one precursor for such ketones [FL-no: 02.066]. The seven substances under consideration in FGE.215 are listed in Appendix A, Table A.1.

Six of the flavouring substances were previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2001a,b, 2002). A summary of their current evaluation status by the JECFA and the outcome of this consideration are presented in Appendix B, Table B.1.

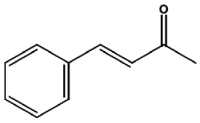
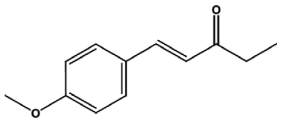
The α, β-unsaturated ketone structure is a structural alert for genotoxicity (EFSA, 2008a) and the data on genotoxicity previously available did not rule out the concern for genotoxicity for these seven flavouring substances.

2.3. History of the evaluation of the substances in subgroup 3.2

The CEF Panel has identified two substances in FGE.19 subgroup 3.2 (see Table 1) which represent the other five substances in this subgroup (EFSA, 2008c). For these substances, genotoxicity data according to the test strategy (EFSA, 2008b), have been requested.

The Flavour Industry submitted *in vitro* data for two representative substances (4-phenylbut-3-en-2-one [FL-no: 07.024] and 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030]) which have been evaluated in FGE.215 (EFSA CEF Panel, 2014). The CEF Panel has evaluated these data and concluded that the genotoxicity concern could not be ruled out. To further assess the genotoxic potential of both representative substances [FL-no: 07.024] and [FL-no: 07.030], combined *in vivo* micronucleus and comet assays in the liver and duodenum were requested (EFSA CEF Panel, 2014).

Table 1: Representative substances for subgroup 3.2 of FGE.19 (EFSA, 2008c)

FL-no	EU Register name	Structural formula
07.024	4- Phenylbut-3-en-2-one	
07.030	1-(4-Methoxyphenyl)pent-1-en-3-one	

FGE: Flavouring Group Evaluation; FLAVIS (FL): Flavour Information System (database); FL-no: FLAVIS number.

Following the request for additional data for the representative substances [FL-no: 07.024] and [FL-no: 07.030] indicated by the CEF Panel in FGE.215 (EFSA CEF Panel, 2014), industry has submitted an *in vivo* combined micronucleus and comet assay for each substance. For [FL-no: 07.030] an *in vitro* micronucleus assay in human peripheral blood lymphocytes and an *in vitro* micronucleus assay in TK6 cells with CREST staining were submitted. For [FL-no: 07.024] an *in vitro* micronucleus assay in TK6 cells and an *in vitro* micronucleus assay in human peripheral blood lymphocytes with fluorescence *in situ* hybridisation (FISH) analysis were submitted. These data are evaluated in the present revision of FGE.215 (FGE.215Rev1).

During the evaluation process, the Panel noted that 4-(2,3,6-trimethylphenyl)but-3-en-2-one [FL-no: 07.206] would be expected to follow a different metabolic pathway compared with the other substances in this FGE due to the presence of the methyl groups. Therefore, the Panel requested to test [FL-no: 07.206] *in vitro* in a bacterial reverse mutation test (OECD TG 471) and in a micronucleus test (OECD TG 487), in accordance with the EFSA Scientific Committee opinion on genotoxicity testing strategy (EFSA Scientific Committee, 2011). Industry communicated that the evaluation of 4-(2,3,6-trimethylphenyl)but-3-en-2-one [FL-no: 07.206] is not supported any longer, therefore additional data were not submitted.

FGE	Adopted by EFSA	Link	No. of substances
FGE.215	26 March 2014	https://www.efsa.europa.eu/en/efsajournal/pub/3623	7
FGE.215Rev1	26 September 2019	https://www.efsa.europa.eu/en/efsajournal/pub/5875	7

FGE: Flavouring Group Evaluation.

Section 2.4 of this opinion reports the same information that was presented in FGE.215 (EFSA CEF Panel, 2014). Section 3 reports the evaluation of the new data submitted by industry.

2.4. Data evaluated by the Panel in FGE.215[4]

The data on genotoxicity, submitted by the Industry (EFFA, 2013), for the representative substances of this subgroup are listed in Table 2.

Table 2: Data Submitted for Representative substances of FGE.215

FL-no JECFA-no	EU Register name	Data submitted
07.024 820	4-Phenylbut-3-en-2-one	Ames test, *Salmonella* Typhimurium TA98, TA100, TA1535, TA1537 and TA102, ± S9-mix (Lillford, 2009)
		In vitro micronucleus assay in human peripheral blood lymphocytes, 3 + 21 h with recovery ± S9-mix and 24 + 0 h without recovery – S9-mix (Stone, 2011; Watters, 2013)

[4] The data presented in Section 2.4 are cited from FGE.215. These data are the basis for the conclusions in FGE.215 requesting additional genotoxicity data.

FL-no JECFA-no	EU Register name	Data submitted
07.030 826	1-(4-Methoxyphenyl)pent-1-en-3-one	Ames test, *S.* Typhimurium TA98, TA100, TA1535, TA1537 and TA102, ± S9-mix (Bowen, 2011)
		In vitro micronucleus assay in human peripheral blood lymphocytes, 3 + 21 h with recovery ± S9-mix and 24 + 0 h without recovery – S9-mix (Stone, 2012)

FGE: Flavouring Group Evaluation; FLAVIS (FL): Flavour Information System (database); FL-no: FLAVIS number; JECFA: The Joint FAO/WHO Expert Committee on Food Additives.

2.4.1. *In vitro* data

2.4.1.1. Bacterial reverse mutation assay

4-phenylbut-3-en-2-one [FL-no: 07.024]

Ames assays were conducted in *Salmonella* Typhimurium strains TA98, TA100, TA1535, TA1537 and TA102 to assess the mutagenicity of 4-phenylbut-3-en-2-one [FL-no: 07.024] (purity 99.6%), both in the absence and in the presence of metabolic activation by an Aroclor 1,254-induced rat liver postmitochondrial fraction (S9-mix) in three separate experiments using both standard plate incorporation and modified pre-incubation treatments (Lillford, 2009). Study design complies with OECD Guideline 471 (OECD, 1997a). An initial toxicity range-finding experiment was carried out in triplicate 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, 1,000 and 5,000 μg/plate, plus negative vehicle and positive controls. Evidence of toxicity in the form of complete killing of the background lawn was observed at 5,000 μg/plate in the absence and presence of S9-mix. Since mutagenicity was observed at 40 μg/plate and above in the presence of S9-mix, the strain was included in experiment 1 for further assessment.

In experiment 1, *S.* Typhimurium strains TA98, TA1535, TA1537 and TA102 were incubated with 1.6, 8, 40, 200, 1,000 and 3,000 μg/plate of 4-phenylbut-3-en-2-one in the absence and presence of S9-mix. Strain TA100 was incubated with the same concentrations in the absence of S9-mix, but with a highest concentration of 5,000 μg/plate in the presence of S9-mix. The standard plate incorporation method was employed. Evidence of toxicity was observed at 3,000 μg/plate in TA100 in the absence of S9-mix and in all other strains in the absence and presence of S9-mix, in the form of a slight thinning of the background bacterial lawn or complete killing of the bacteria. A reduction in the numbers of revertants and/or a slight thinning of the background bacterial lawn was also observed at 1,000 μg/plate in strain TA1535 in the absence of S9-mix. Complete killing was observed at 5,000 μg/plate in strain TA100 in the presence of S9-mix. A statistically significant concentration-related increase in mutation rate was observed in strain TA100 at 40 μg/plate and above in the presence of S9-mix.

In experiment 2, *S.* Typhimurium strains TA98, TA100, TA1535, TA1537 and TA102 were treated with 93.75, 187.5, 375, 750, 1,500 and 3,000 μg/plate of 4-phenylbut-3-en-2-one in the absence and in the presence of S9-mix, to assess mutagenicity close to the limits of toxicity. The assays run in the presence of S9-mix were further modified by the inclusion of a pre-incubation step (60 min).

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 750 and/or 1,500 and/or 3,000 μg/plate in the absence and presence of S9-mix. Treatments of strain TA100 in the presence of S9-mix at concentrations of 375 μg/plate and above showed a statistically significant concentration-related increase in revertant mutations ($p \leq 0.01$).

To further explore the increase in mutations reported in *S.* Typhimurium strain TA100, experiment 3 was performed in all tester strains only in the presence of S9-mix, using the pre-incubation method, at concentrations of 46.88–1,500 μg/plate of 4-phenylbut-3-en-2-one. Evidence of cytotoxicity in the form of complete killing of the test bacteria was observed at 1,500 μg/plate in all strains. Treatments of the TA100 strain in the presence of S9-mix at concentrations equal to or higher than 187.5 μg/plate showed a statistically significant increase in revertant mutations ($p \leq 0.01$).

Although there was evidence of toxicity towards all the bacterial strains tested, it was concluded that 4-phenylbut-3-en-2-one induced reproducible, concentration-related and statistically significant mutations in strain TA100 of *S.* Typhimurium in the presence of metabolic activation. It did not induce mutations in any other strain when tested under the conditions of this study. These conditions included

treatments at concentrations up to either the limit of toxicity or 5,000 μg/plate (the maximum recommended concentration according to current regulatory guidelines), in the absence and in the presence of a rat liver metabolic activation system (S9-mix) (Lillford, 2009).

1-(4-Methoxyphenyl)pent-1-en-3-one [FL-no: 07.030]

The mutagenicity of 1-(4-methoxyphenyl)pent-1-en-3-one (purity 98%) was assessed in *S.* Typhimurium strains TA98, TA100, TA1535, TA1537 and TA102, 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 separate experiments using both standard plate incorporation and modified preincubation treatments (Bowen, 2011). Study design complies with OECD Guideline 471 (OECD, 1997a).

In experiment 1, all *S.* Typhimurium strains were incubated with 0.32, 1.6, 8, 40, 200, 1,000 and 5,000 μg/plate of 1-(4-methoxyphenyl)pent-1-en-3-one, in the absence and presence of S9-mix. Toxicity was observed in the form of slight thinning of background bacteria lawn and reduced numbers of revertants at 1,000 μg/plate in strains TA1537 and TA102 and complete killing of bacteria was observed at 5,000 μg/plate in all strains, in the absence and presence of S9-mix. No increases in revertants were observed in any strain under any treatment condition.

In experiment 2, all tester strains were treated with 1-(4-methoxyphenyl)pent-1-en-3-one in the absence and in the presence of S9-mix at more narrow concentration intervals. All strains were treated with concentrations of 156.3, 312.5, 625, 1,250, 2,500 and 5,000 μg/plate and strains TA1537 and TA102 were also treated at a lower concentration of 78.13. The maximum test concentration of 5,000 μg/plate was retained for all strains, in order to examine more closely the ability of 1-(4-methoxyphenyl)pent-1-en-3-one to induce mutations at concentrations approaching the maximum test concentration and/or toxicity limit. In addition, all treatments in the presence of S9-mix were further modified by the inclusion of a pre-incubation step. Evidence of toxicity in the form of thinning of the background lawn and/or a reduction in revertant numbers to a complete killing of the test bacteria was observed at 625 μg/plate and above in strains TA1535, TA1537 and TA102 in the presence of S9-mix, at 1,250 μg/plate and above in strains TA1537 and TA102 in the absence of S9-mix and TA100 in the presence of S9-mix, and at 2,500 μg/plate and above in strains TA98 in the absence and presence of S9-mix, and TA100 and TA1535 in the absence of S9-mix. No increases in revertants were observed in any strain under any treatment condition.

Experiment 3 was performed in strains TA1535 and TA1537 in the presence of S9-mix, using a preincubation methodology, due to the toxicity reported in experiment 2. The maximum test concentration was reduced to 2,500 μg/plate based on toxicity observed previously. Narrowed concentration intervals were employed including 19.53, 39.06, 78.13, 156.3, 312.5, 625, 1,250 and 2,500 μg/plate. Under these conditions, evidence of toxicity was observed at 625 μg/plate and above in both of these strains in the presence of S9-mix, ranging from thinning of the background bacteria lawn and/or a reduction in revertant numbers to a complete killing of the test bacteria. Consistent with previous experiments, no increases in revertants were observed in either strain under these treatment conditions.

It was concluded that 1-(4-methoxyphenyl)pent-1-en-3-one did not induce mutation in five histidine requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *S.* Typhimurium when tested under the conditions of this study. These conditions included treatments at concentrations up to 5,000 μg/plate (the maximum recommended concentration according to current regulatory guidelines), in the absence and in the presence of a rat liver metabolic activation system.

2.4.1.2. Micronucleus induction assay

4-phenylbut-3-en-2-one [FL-no: 07.024]

4-Phenylbut-3-en-2-one [FL-no: 07.024] (purity 99%) was assayed for the induction of chromosome damage and potential clastogenic and/or aneugenic events 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 two healthy male donors) in three separate trials, treated in the absence and presence of S9-mix (Stone, 2011). Study design complies with OECD Guideline 487 (OECD, 2010).

In a range-finding experiment, 4-phenylbut-3-en-2-one was added at concentrations of 5.304, 8.840, 14.73, 24.56, 40.93, 68.21, 113.7, 189.5, 315.8, 526.3, 877.2 and 1,462 μg/mL at 48 h following culture initiation (stimulation by phytohaemagglutinin (PHA)) either for 3 h treatment plus 21 h recovery in the absence or presence of S9-mix, or for 24 h treatment without recovery in the absence of S9-mix.

In the assay with 3 h treatment and 21 h of recovery, a steep increase in cytotoxicity was observed between the concentrations of 24.56 and 40.93 μg/mL (32 and 72%, respectively) in the absence of S9-mix, and between the concentrations of 40.93, 68.21 and 113.7 μg/mL (18, 35 and 87%, respectively) in the presence of S9-mix. In the assay of 24 h treatment with no recovery period, a steep increase in cytotoxicity was observed between the concentrations of 8.840, 14.73 and 24.56 μg/mL (13, 54 and 92%, respectively). According to the study report, in the cytotoxicity range-finder experiment, the pH and osmolality were measured in the medium after treatment of cells. Their values remained within physiological limits, however, the details of the data were not reported. Positive and negative control substances produced the expected responses.

In the main micronucleus experiment, three separate trials were conducted for each set of treatment conditions (3 + 21 h recovery in the absence and presence of S9-mix and 24 h without recovery in the absence of S9-mix) in order to identify an appropriate range of concentrations that would include adequate level of cytotoxicity.

In the first trial, treatment was conducted for 3 + 21 h recovery in the absence of S9-mix at concentrations of 5, 10, 15, 20, 25, 27.5, 30, 32.5, 35, 37.5, 40 and 50 μg/mL 4-phenylbut-3-en-2-one were selected for micronucleus analysis based on the cytotoxicity observations of the range-finding experiment. No suitable maximum concentrations could be selected due to insufficient cytotoxicity (< 30% at the highest concentration). A second trial with treatment for 3 + 21 h recovery in the absence of S9-mix was conducted using 4-phenylbut-3-en-2-one concentrations of 10, 15, 20, 25, 30, 35, 40, 50, 55, 60, 65, 70, 75, 80 and 100 μg/mL. No suitable range could be selected due to a steep increase in cytotoxicity between the concentrations of 35 and 40 μg/mL (33 and 65%, respectively). In a third trial using a range between 10 and 100 μg/mL, with more narrowly spaced concentration intervals of 2.5 μg/mL between 25 and 40 μg/mL, the observed cytotoxicity range was considered adequate. Concentrations of 30, 40 and 50 μg/mL were selected from the third trial for analysis of micronucleus induction by 4-phenylbut-3-en-2-one treatment for 3 + 21 h recovery in the absence of S9-mix. The frequency of micronucleated binucleate (MNBN) cells was similar to vehicle controls at all concentrations tested (with cytotoxicity of 14, 32 and 56%, respectively).

For treatment with 4-phenylbut-3-en-2-one for 3 + 21 h recovery in the presence of S9-mix, a suitable concentration range could not be identified in the first of three trials that included a range of 20–120 μg/mL, spaced at 10 μg/mL intervals (and 5 μg/mL intervals between 70 and 90 μg/mL), due to a steep increase in cytotoxicity (38, 47 and 67% at concentrations of 85, 90 and 100 μg/mL). Using the same concentration range, 20–120 μg/mL, a second trial was performed with more narrowly spaced intervals of 2.5 μg/mL between 85 and 100 μg/mL. Due to steep concentration-related toxicity, the trial was repeated a third time. The third trial conducted at the same concentration range (20–120 μg/mL) resulted in insufficient cytotoxicity levels (48% at the highest concentration), unlike those observed in the second trial. Therefore, concentrations from trial two were selected for MN analysis. Although cytotoxicity observed in the second trial was not within the desired target range (50–60%), the reduction of 65% in replicative index achieved at 85 μg/mL was considered acceptable. The following concentrations were selected for micronucleus analysis: 40, 70, 80 and 85 μg/mL. The frequency of MNBN cells was similar to vehicle controls at all concentrations scored (cytotoxicity of 15, 27, 42 and 65%, respectively).

Cultures were also treated for 24 + 0 h in the absence of S9-mix at concentrations of 12, 14, 17.5 and 20 μg/mL (with cytotoxicity of 11, 20, 46 and 60%, respectively), selected from a single trial conducted at concentration range of 2–20 μg/mL of 4-phenylbut-3-en-2-one. The frequencies of MNBN cells were similar to those observed in concurrent vehicle controls at all concentrations analysed. All frequencies of MNBN fell within normal ranges based on historical control data.

In this study, 4-phenylbut-3-en-2-one did not induce MN in cultured human peripheral blood lymphocytes when tested up to the limits of cytotoxicity for 3 + 21 h in the absence and presence of rat liver metabolic activation (S9-mix), or for 24 + 0 h in the absence of S9-mix (Stone, 2011).

The inconsistent cytotoxicity curves generated in the first micronucleus study for 4-phenylbut-3-en-2-one could not be easily explained, and on that basis a second study was initiated.

4-Phenylbut-3-en-2-one (purity 99.9%) was reassessed *in vitro* for the induction of chromosome damage and potential clastogenic and/or aneugenic events in mammalian cells by examining its effect on the frequency of MN in cultured human peripheral blood lymphocytes (pooled blood of two healthy female donors in three separate experiments and of two male donors in a single experiment) treated in the absence and presence of S9-mix (Watters, 2013).

In a range-finding experiment, 4-phenylbut-3-en-2-one was added at concentrations of 2.5, 5.0, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, 80.0, 100.0, 120.0 and 150.0 μg/mL at 48 h following culture

initiation (stimulation by PHA) either for 3 h treatment plus 21 h recovery in the absence or presence of S9-mix, or for 24 h treatment without recovery in the absence of S9-mix. In the assay with 3 h treatment and 21 h of recovery, a steep increase in cytotoxicity was observed between the concentrations of 40.0 and 50.0 μg/mL (16 and 65% toxicity, respectively) in the absence of S9-mix, and between the concentrations of 80.0 and 100.0 μg/mL (6 and 55% toxicity, respectively) in the presence of S9-mix. In the assay of 24-h treatment with no recovery period, a steep increase in cytotoxicity was observed between the concentrations of 10.0 and 20.0 μg/mL (11 and 89%, respectively). According to the study report, in the cytotoxicity range-finder experiment, the pH and osmolality were measured in the medium after treatment of cells. Their values remained within physiological limits. Positive and negative control substances produced the expected responses.

In the first trial, treatment was conducted for 3 + 21 h recovery in the absence of S9-mix and concentrations of 30, 40, 44 and 46 μg/mL were selected for analysis of micronucleus induction. The frequency of MNBN cells was similar to vehicle controls at all concentrations tested (with cytotoxicity of 5, 32, 44 and 50%, respectively). The frequencies of MNBN in the vehicle control were above the historical control range. For treatment with 4-phenylbut-3-en-2-one for 3 + 21 h recovery in the presence of S9-mix, concentrations of 85, 95, 100 and 105 μg/mL were chosen for micronucleus analysis, and the frequency of MNBN cells was statistically significant and higher than vehicle controls at all concentrations scored (cytotoxicity of 8, 28, 41 and 51%, respectively). Cultures were also treated for 24 + 0 h in the absence of S9-mix and concentrations of 8, 13, 14 and 15 μg/mL (with cytotoxicity of 0, 29, 45 and 59%, respectively) were chosen for micronucleus analysis. The frequencies of MNBN cells were similar to those observed in concurrent vehicle controls at all concentrations analysed. All frequencies of MNBN fell within normal ranges based on historical control data.

In the first trial, the micronucleus data for both of the 3 + 21 h treatments showed inconsistent MNBN cell frequencies between replicate cultures in all four 4-phenyl-3-buten-2-one concentrations analysed and the concurrent vehicle control when scored by Analyst 1 (statistically significant heterogeneity was apparent; $p \leq 0.05$). Therefore, the slides were sent to a peer review Analyst (Analyst 2) for re-analysis. A comparison of the analysts' results is as follows:

Analyst 1: Treatment of cells with 4-phenyl-3-buten-2-one for 3 + 21 h in the absence of S9-mix resulted in frequencies of MNBN cells that were inconsistent between replicate cultures for all concentrations analysed. The MNBN cell frequency of a single replicate culture from the concurrent vehicle control and in each of the four test article concentrations analysed exceeded the normal range (0.1–1.1% MNBN cells). The mean MNBN cell frequency for the vehicle control and each test article marginally exceeded the normal range.

Analyst 2: Treatment of cells for 3 + 21 h in the absence of S9-mix resulted in frequencies of MNBN cells that were significantly higher ($p \leq 0.01$) than those observed in concurrent vehicle controls for an intermediate and the highest concentration analysed (40 and 46 μg/mL, respectively).

The MNBN cell frequency of both cultures at 40 and 46 μg/mL exceeded the normal range. Single replicate cultures also exceeded the normal range in the concurrent vehicle control and the other two concentrations analysed (30 and 44 μg/mL).

As the MNBN cell frequency of one replicate culture of the vehicle control exceeded the normal range when scored by both analysts, the validity of the dataset was considered questionable. Therefore, a confirmatory experiment was performed for the 3 + 21 h treatment without S9-mix using the same concentrations (trial 2). These data would also aid biological relevance interpretation of the test article-related increases in MNBN cell frequency determined by Analyst 2.

Treatment of cells with 4-phenyl-3-buten-2-one in the absence of S9-mix in trial 2 resulted in frequencies of MNBN cells that were significantly higher ($p \leq 0.05$) than those observed in concurrent vehicle controls at the lowest and two highest concentrations analysed (30, 46 and 48 μg/mL, respectively). The MNBN cell frequency of both treated cultures at 46 and 48 μg/mL exceeded the normal range.

Data obtained from the treatment of cells (female donors) for 3 + 21 h in the presence of S9-mix were also scored by two analysts. The data from both analysts showed that the treatment of cells for 3 + 21 h in the presence of S9-mix resulted in frequencies of MNBN cells that were significantly higher ($p \leq 0.05$) than those observed in concurrent vehicle controls for all concentrations analysed.

The MNBN cell frequency of all but a single replicate culture at the lowest concentration analysed (by Analyst 1) exceeded the normal range. Although it was noted that Analyst 2 scored higher MNBN

cell frequencies compared to Analyst 1, both data sets demonstrated increases in MNBN cell frequencies above the normal range for all concentrations analysed, indicative of a positive result.

These data confirmed that the increases in MNBN cell frequency observed in trial 1 were test article related despite some inconsistencies in micronucleus data. These results are, however, entirely inconsistent with those previously collected (Stone, 2011).

The authors tried to attribute these inconsistencies to the use of pooled blood collected from male donors in the study by Stone (Stone, 2011), compared to the study by Watters (Watters, 2013) where pooled blood collected from female donors was used. On this basis, they investigated possible differences in outcomes due to sex differences in donors, performing a third trial for 3 + 21 h in the presence of S9-mix using different replicate cultures prepared from pooled blood obtained by either two female donors or two male donors treated at the same concentrations for scoring as in trials 1 and 2. The MNBN cell frequencies of treated cultures at 95, 105 and 110 μg/mL exceeded the normal range and were significantly higher ($p \leq 0.001$) than those observed in concurrent vehicle controls for both replicate cultures obtained from male and female donors. Results obtained indicate that sex differences did not play a role on the study outcome. Inconsistencies in the cytotoxicity and MNBN cell frequencies of treated cultures between the two studies could be ascribed, plausibly, to methodological differences since in the study by Stone (Stone, 2011) gentamicin at 50 μg/mL in culture medium was used instead of 0.52% penicillin/streptomycin, as foreseen by standard protocol, which is considered a shortcoming.

4-Phenylbut-3-en-2-one was tested on pooled blood lymphocytes cultures from female donors for 24 h with no recovery period, in the absence of S9-mix. Concentrations of 4-phenylbut-3-en-2-one at 8, 13, 14 and 15 μg/mL were selected for micronucleus analysis corresponding to a cytotoxicity of 0, 29, 45 and 59%, respectively. Frequencies of MNBN cells were not statistically significant higher than those observed in concurrent vehicle controls for all concentrations analysed (Watters, 2013).

In conclusion, in this study, 4-phenylbut-3-en-2-one did induce MN in cultured human peripheral blood lymphocytes when tested up to the limits of cytotoxicity for 3 + 21 h in the absence and presence of rat liver metabolic activation (S9-mix). In the same test system, 4-phenylbut-3-en-2-one did not induce MN following 24 + 0 h treatment in the absence of S9-mix, when analysed up to cytotoxic concentrations.

1-(4-Methoxyphenyl)pent-1-en-3-one [FL-no: 07.030]

1-(4-Methoxyphenyl)pent-1-en-3-one (purity 98%) was assayed for the induction of chromosome damage and potential clastogenic and/or aneugenic events in mammalian cells *in vitro* by examining its effect on the frequency of MN in cultured human peripheral blood lymphocytes (whole blood cultures pooled from 2 healthy female volunteers) in a single experiment, treated in the absence and presence of S9-mix (Stone, 2012). Study design complies with OECD Guideline 487 (OECD, 2010).

In a range-finding experiment, 1-(4-methoxyphenyl)pent-1-en-3-one was added at concentrations of 6.9, 11.5, 19.17, 31.95, 53.24, 88.74, 147.9, 246.5, 410.8, 684.7, 1,141 and 1,902 μg/mL at 48 h following culture initiation (stimulation by PHA) either for 3 h treatment plus 21 h recovery in the absence or presence of S9-mix, or for 24 h treatment without recovery in the absence of S9-mix. In the assay with 3 h treatment and 21 h of recovery, cytotoxicity was observed at concentrations of 88.74 μg/mL (32%) and above in the absence of S9-mix, and 53.24 μg/mL (24%) and above in the presence of S9-mix. In the assay of 24 h treatment with no recovery period, a steep increase in cytotoxicity was observed already at the lowest concentration (27%). Precipitation was observed at 147.9 μg/mL and above. The replication index could not be measured at 410 μg/mL and above in the pulse assays and at 246.5 μg/mL in the 24-h treatment assay. According to the study report, in the cytotoxicity range-finder experiment, the pH and osmolality were measured in the medium after treatment of cells. Their values remained within physiological limits. Positive and negative control substances produced the expected responses.

Treatment of cells with 1-(4-methoxyphenyl)pent-1-en-3-one was conducted for 3 + 21 h at concentrations of 60, 100, 110, 120 and 130 μg/mL in the absence of S9-mix (cytotoxicity of 12, 30, 51, 60 and 46%, respectively) and 90, 150, 160 and 180 μg/mL in the presence of S9-mix (cytotoxicity of 2, 28, 49 and 61%, respectively). The concentrations were selected from a single trial covering the range 15–175 μg/mL in the absence of S9-mix and 15–225 μg/mL in the presence of S9-mix. Treatment resulted in concentration-dependent increases in frequencies of MNBN cells. In the absence of S9-mix, micronucleus frequency increases were statistically significant ($p \leq 0.05$) at the highest two selected concentrations compared to the concurrent control. At the concentration of 120 μg/mL, only a single replicate exceeded the historical control range. In the presence of S9-mix, statistically significant

($p \leq 0.001$) increases in MNBN frequencies were observed at 150 μg/mL and above compared to concurrent vehicle controls, but exceeded the historical range at the top two concentrations and in a single replicate at 150 μg/mL.

Cultures were also treated for 24 + 0 h in the absence of S9-mix at concentrations of 10, 15, 16 and 18 μg/mL (with cytotoxicity of 8, 32, 45 and 52%, respectively), selected from the second of two trials. The first trial conducted at a concentration range of 1–17.5 μg/mL of 1-(4-methoxyphenyl)pent-1-en-3-one resulted in insufficient toxicity (up to 40% at the top concentration) and the second trial covered the range 2–25 μg/mL and provided adequate cytotoxicity range. Although increases in MNBN cells were statistically significant at the lowest and one intermediate concentration (10 and 16 μg/mL) compared to concurrent control cultures, frequencies of MNBN cells at all concentrations fell within normal ranges based on historical control data. Therefore, these data indicate absence of micronucleus induction.

In conclusion, 1-(4-methoxyphenyl)pent-1-en-3-one induced MN in cultured human peripheral blood lymphocytes when tested up to the limits of cytotoxicity for 3 + 21 h in the presence of rat liver metabolic activation (S9-mix) and weakly in the absence of metabolic activation. It did not induce MN in cells treated for 24 + 0 h in the absence of S9-mix up to toxic concentrations.

2.4.2. *In vivo* genotoxicity tests

There are no new relevant studies available.

2.4.3. Conclusion on genotoxicity in FGE.215

The two representative substances for subgroup 3.2 of FGE.19, 4-phenylbut-3-en-2-one [FL-no: 07.024] and 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] showed an *in vitro* genotoxic potential. 4-Phenylbut-3-en-2-one [FL-no: 07.024] induced reproducible, concentration-related and statistically significant increases in the mutation frequencies, in strain TA100 of *S.* Typhimurium in the presence of metabolic activation. It also induced MN in cultured human peripheral blood lymphocytes when tested up to the limits of cytotoxicity for 3 + 21 h in the absence and presence of rat liver metabolic activation (S9-mix). 1-(4-Methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] did not induce gene mutation in bacteria. It induced MN in cultured human peripheral blood lymphocytes when tested up to the limits of cytotoxicity for 3 + 21 h in the presence of rat liver metabolic activation (S9-mix) and a weak increase in its absence. On this basis, to further assess the genotoxic potential of both representative substances (4-phenylbut-3-en-2-one [FL-no: 07.024] and 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030]) of subgroup 3.2 of FGE.19, a combined *in vivo* micronucleus and Comet assays in the liver and duodenum are requested.

3. Assessment

3.1. Additional data evaluated by the Panel in FGE.215Rev1

In response to the CEF Panel request for further genotoxicity data for the two representative substances of subgroup 3.2 (4-phenylbut-3-en-2-one [FL-no: 07.024] and 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030]), as described in FGE.215 (EFSA CEF Panel, 2014), industry has submitted two *in vivo* combined bone marrow micronucleus and comet assays with analysis of the liver and duodenum (Table 3 and Appendix D).

Additional information was provided by the applicant during the assessment process in response to requests from EFSA sent on 19/5/2015, 4/7/2018 (see Documentation provided to EFSA n. 3, 4, 5, 13). Information requested is summarised below.

The *in vivo* micronucleus studies in bone marrow were negative, but the exposure of the bone marrow was not demonstrated, therefore the Working Group on Genotoxicity (WG) of the CEF Panel requested to provide evidence of bone marrow exposure to 4-phenylbut-3-en-2-one and to 1-(4-methoxyphenyl)pent-1-en-3-one by plasma analysis as recommended in the OECD TG 474 (EFSA letter dated 19/5/2015).

Following this request, a technical hearing was held with the applicant on 19 January 2016 (EFSA, 2016) to clarify the challenges observed by the applicant in performing the plasma analysis and demonstrating bone marrow exposure.

As follow-up of the technical hearing, additional information on the plasma analysis already performed (EFSA letter dated 2/2/2016) was requested. After reviewing these data, the WG suggested

to suspend the activities related to plasma analysis and requested to test both representative substances in an *in vitro* micronucleus assay with centromere analysis, in order to investigate the mechanism inducing MN *in vitro* (clastogenicity or aneugenicity) (EFSA letter dated 29/4/2016).

Following this request (EFSA letter dated 29/4/2016), a second technical hearing was held with the applicant on 24 January 2017 (EFSA, 2017) to clarify the applicant's proposal to test the representative substances with a new method instead of the *in vitro* micronucleus with centromere analysis.

Following the second technical hearing, the WG reiterated the request for an *in vitro* micronucleus assay with centromere analysis for investigating the mode of action of the representative substances (EFSA letter dated 10/4/2017). The applicant provided the requested data on 10 April 2018 (EFFA, 2018, see Documentation provided to EFSA n. 13) which are listed in Table 3 and evaluated in the present revision of FGE.215 (FGE.215Rev1).

In the new *in vitro* micronucleus test on 4-phenylbut-3-en-2-one [FL-no: 07.024] (BioReliance, 2018a), an increase in MN frequency was not observed. These results are in contrast with the previous submitted *in vitro* micronucleus study on human peripheral blood lymphocytes (Watters, 2013). Therefore, a new *in vitro* micronucleus assay in cultured peripheral blood lymphocytes with FISH staining, complying with OECD TG 487 (OECD, 2016) was requested (EFSA letter dated 4/7/2018). This study was submitted on 17/04/2019 (BioReliance, 2019).

The applicant also submitted for the substance [FL-no: 07.030] an *in vitro* micronucleus study in human peripheral blood lymphocytes (Covance, 2013).

The substance 4-(2,3,6-trimethylphenyl)but-3-en-2-one [FL-no: 07.206] is considered as a stand-alone because it would be expected to follow a different metabolic pathway compared with the other substances in this FGE due to the presence of the methyl groups. Therefore, the Panel requested to test [FL-no: 07.206] *in vitro* in a bacterial reverse mutation test and in a micronucleus test (EFSA letter dated 4/7/2018), but industry communicated that the evaluation of 4-(2,3,6-trimethylphenyl)but-3-en-2-one [FL-no: 07.206] is not supported any longer. Therefore, the additional data requested were not submitted.

The new studies evaluated in the present revision of FGE.215 (FGE.215Rev1) are listed in Table 3. A summary of results is reported in Appendix D. All these studies were performed in accordance with respective OECD guidelines and in compliance with Good Laboratory Practice (GLP).

Table 3: List of genotoxicity studies evaluated in FGE.215Rev1

Test substance	Additional data submitted	Reference
4-phenylbut-3-en-2-one [FL-no: 07.024]	*In vivo* combined bone marrow micronucleus test and comet assay in the liver and duodenum	Covance (2014a)
	In vitro micronucleus assay without centromere analysis in TK6 cells	BioReliance (2018a)
	In vitro micronucleus test in human peripheral blood lymphocytes with FISH analysis	BioReliance (2019)
1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030]	*In vivo* combined bone marrow micronucleus test and comet assay in the liver and duodenum	Covance (2014b)
	In vitro micronucleus assay with CREST staining in TK6 cells	BioReliance (2018b)
	In vitro micronucleus test in human peripheral blood lymphocytes	Covance (2013)

FGE: Flavouring Group Evaluation; FLAVIS (FL): Flavour Information System (database); FL-no: FLAVIS number.

3.2. Data on 4-phenylbut-3-en-2-one [FL-no: 07.024]

3.2.1. 4-Phenylbut-3-en-2-one [FL-no: 07.024] – *in vivo* combined bone marrow micronucleus test and comet assay

The genotoxic potential of 4-phenyl-3-buten-2-one [FL-no: 07.024] (purity 99.9%) was assessed *in vivo* using the bone marrow micronucleus assay combined with the comet assay in the duodenum and liver of rats (Covance, 2014a). The study was conducted following GLP. The micronucleus study was conducted in accordance with OECD TG 474 (OECD, 1997b). The comet study was conducted before the publication of the first relevant OECD test guideline (OECD TG 489, 2014b), but it was based on the guidance provided by International Workshops on Genotoxicity Testing (Burlinson et al.,

2007), the international validation of the *in vivo* comet assay by the Japanese Center for the Validation of Alternative Methods (JaCVAM) and literature available at that time (Tice et al., 2000; Hartmann et al., 2003, 2004; Smith et al., 2008).

In a dose range-finder assay, groups of three male and three female Han Wistar rats were given three administrations by gavage (at 0, 24 and 45 h) of 4-phenyl-3-buten-2-one at 1,000, 1,400 or 2,000 mg/kg body weight (bw) per day.

Doses of 1,400 and 2,000 mg/kg bw per day resulted in severe signs of toxicity (including prostration, ataxia, decreased breathing) which led to the early termination of all male and female animals on day 2. Dosing at 1,000 mg/kg bw per day resulted in moderate, but tolerated post dose observations (including ataxia, decreased activity and/or piloerection) in all male and female animals.

Based on this study, a maximum tolerated dose (MTD) of 1,000 mg/kg bw per day was established. As no gender specific effects were seen, only male rats were used in the main study.

In the main experiment, groups of six male Han Wistar rats per dose group were administered doses by gavage of 0 (corn oil), 250, 500 or 1,000 mg/kg bw per day on three consecutive days (0, 24 and 45 h). A positive control group of six male rats were given doses of 150 mg ethyl methanesulfonate (EMS)/kg bw at the same time intervals as the dosed groups.

After 48 h (i.e. 3 h after the final administration), bone marrow, liver and duodenum were sampled from the same animals for micronucleus assay and comet assay. Four out of six animals in the highest dose group (1,000 mg/kg bw per day) showed ataxia, reduced activity and/or a cold body approximately 0.5 h post dose on day 3. These signs were transient and all animals appeared normal approximately 1 h after dosing on day 3. In the same dose group, a body weight loss of 4.5% was observed.

No post-dose signs of toxicity were observed in animals of the other dose groups.

Clinical chemistry analysis showed no treatment-related changes. A high number of blood samples were lipaemic, some of them were not suitable for the analysis of clinical chemistry.

Upon dissection, no macroscopic findings were observed in any tissues.

Histopathological evaluation revealed in the liver increased hepatocyte mitosis and decreased incidence and/or severity of glycogen vacuolation in animals from all treated groups, related to administration of the test article. There were no findings related to the test article treatment in duodenum.

Micronucleus assay

Bone marrow from the femurs was prepared for MN scoring. A total of at least 500 polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) were scored to calculate the degree of bone marrow toxicity by the relative decrease in PCE. For MN analysis, 2,000 PCE per animal were scored for the presence of MN.

Rats treated with 4-phenyl-3-buten-2-one exhibited group mean frequencies of micronucleated polychromatic erythrocytes (MNPCE) that were similar to and not statistically different from those observed in the concurrent vehicle control for all dose groups. The treatment with 4-phenyl-3-buten-2-one did not reduce the percentage of PCE, therefore no indication of bone marrow toxicity was observed. The positive control (EMS) induced a statistically significant increase in the incidence of MNPCE.

Under the conditions of this test, 4-phenyl-3-buten-2-one did not induce MN in bone marrow of rats tested up to 1,000 mg/kg bw per day (MTD), by gavage.

Considerations on the micronucleus assay

In the *in vivo* micronucleus assay, 4-phenyl-3-buten-2-one [FL-no: 07.024] did not induce MN in bone marrow PCE of male rats following gavage administration up to 1,000 mg/kg bw per day.

Considering that the percentage of PCE in the bone marrow of treated animals was not changed compared to the concurrent vehicle control group, it was unclear whether the bone marrow was exposed and therefore additional evidence of systemic exposure through plasma analysis was requested to the applicant in order to conclude on the *in vivo* micronucleus assay.

However, in view of technical problems reported by the applicant with the plasma analysis, the Panel decided to waive its initial request for additional evidence of systemic exposure and requested the applicant to investigate the mechanism of MN induction (clastogenicity or aneugenicity) through an *in vitro* micronucleus assay with centromere analysis. The clarification of the mechanism of genotoxicity would allow to interpret the already available *in vivo* data or to identify an appropriate *in vivo* follow-up study.

Comet assay

Liver and duodenum cells were prepared for comet analysis. Tail moment and tail intensity (%) of a total of 150 cells per animal were recorded. Both in the liver and in the duodenum, no statistically significant increase in group mean tail intensity and tail moment values were observed in any test substance treatment group compared to the vehicle control group. Liver exposure was indicated by an increase in hepatocyte mitosis in all treatment samples.

In most of liver samples, the % of clouds exceeded the historical vehicle control. Since the observed increase of clouds was similar in all treated groups, including the vehicle controls, and it was not associated with a higher tail intensity and tail moment values, the Panel agreed with the study authors who considered this effect not related to the treatment, but to mechanical damage during cell isolation. The Panel considered the negative outcome of this study as reliable.

Therefore, the Panel concluded that 4-phenyl-3-buten-2-one [FL-no: 07.024] did not induce primary DNA damage in the liver and duodenum of rats after oral administration.

Study results are summarised in Appendix D, Table D.2.

3.2.2. 4-Phenylbut-3-en-2-one [FL-no: 07.024] – *in vitro* micronucleus test in TK6 cells without centromere analysis

4-Phenylbut-3-en-2-one [FL-no: 07.024] (purity 99.1%) was tested in an *in vitro* micronucleus assay in the human lymphoblastoid cell line TK6 cells (BioReliance, 2018a), with the purpose of evaluating the aneugenic and clastogenic potential of the tested substance. The study was performed according to GLP and OECD TG 487 (OECD, 2014a).

TK6 cells were treated for 4 h with 23 h of recovery period (4 + 23 h) in the absence and presence of S9-mix (from Aroclor 1254-induced rats), or for 27 h in the absence of S9-mix.

Positive controls were: cyclophosphamide (CPA) and vinblastine (VB). The vehicle control was dimethyl sulfoxide (DMSO).

In the preliminary cytotoxicity assay, 4-phenylbut-3-en-2-one was tested at concentrations from 0.146 to 1,460 μg/mL. Precipitate was observed at the highest concentration tested (1,460 μg/mL) in all treatment conditions. Cytotoxicity, indicated by a decrease in relative population doubling (RPD) ≥ 50% compared to the vehicle control, was observed at concentrations ≥ 43.8 μg/mL in the 4 + 23 h treatment in the absence of S9-mix; at concentrations ≥ 146 μg/mL in the 4 + 23 h treatment in presence of S9-mix; and at ≥ 14.6 μg/mL in the 27-h treatment in the absence of S9-mix.

In the main experiment, MN frequencies were evaluated at 5, 20 and 25 μg/mL in the non-activated 4 + 23 h treatment, with cytotoxicities determined to be 15, 46 and 59%, respectively; 5, 20 and 40 μg/mL in the 4 + 23 h treatment with metabolic activation, with cytotoxicities determined to be 4, 16 and 53%, respectively; and 2.5, 7, 10 and 12 μg/mL in the 27-h treatment without metabolic activation, with cytotoxicities determined to be 4, 27, 48 and 57%, respectively. At least 2,000 mononucleated cells were scored per each concentration. The positive controls induced statistically significant increases in MN frequency. Negative controls were within the historical vehicle control ranges. In all three treatment groups, no statistically significant or concentration-related increases in MN induction were observed.

The Panel noted that the negative results observed in this study in TK6 cells for all treatment conditions are in contrast with results obtained in the *in vitro* micronucleus study in human peripheral blood lymphocytes (Watters, 2013). In this study, 4-phenylbut-3-en-2-one [FL-no: 07.024] increased the frequency of MN in the short-term treatment in the absence and in the presence of S9-mix (Watters, 2013). Therefore, the Panel requested to test 4-phenylbut-3-en-2-one [FL-no: 07.024] in a new *in vitro* micronucleus assay in cultured human peripheral blood lymphocytes with FISH analysis.

Study results are summarised in Appendix D, Table D.1.

3.2.3. 4-Phenylbut-3-en-2-one [FL-no: 07.024] – *in vitro* micronucleus test in human lymphocytes with FISH analysis

4-Phenylbut-3-en-2-one [FL-no: 07.024] (purity 99.06%) was tested in an *in vitro* micronucleus assay with FISH analysis in human peripheral blood lymphocytes both in the presence and in the absence of metabolic activation (Aroclor 1254-induced rat liver, S9-mix). The study was carried out according to OECD TG 487 (OECD, 2016) and GLP (BioReliance, 2019).

Vehicle control (DMSO) and appropriate positive controls were included: mitomycin C (MMC), CPA and VB. Cytochalasin B (cytoB) was included in all testing conditions: 4 h treatment followed by 20 h

of recovery period (4 + 20 h) in the presence or absence of S9-mix and 24 h treatment in the absence of S9-mix followed by 0 h of recovery period (24 h).

In a concentration range-finder experiment, 4-phenylbut-3-en-2-one was tested at concentrations ranging from 0.146 to 1,460 μg/mL. Precipitate was observed at the highest concentrations tested. Cytotoxicity was observed at concentrations above 146 μg/mL in all the three testing conditions.

In the main experiment, MN frequencies were evaluated at 15, 40, 70 μg/mL in the non-activated 4 + 20 h treatment, with cytotoxicities determined to be 9, 13, 51%, respectively; at 15, 40, 80 μg/mL in the 4 + 20 h treatment with metabolic activation, with cytotoxicities determined to be 18, 16, 51%, respectively; and at 5, 30, 50 μg/mL in the 24 h treatment without metabolic activation, with cytotoxicities determined to be 14, 32, 57%, respectively. At least 2,000 binucleated cells per concentration were analysed from duplicate cultures.

In the absence of metabolic activation, 4-phenylbut-3-en-2-one [FL-no: 07.024] did not induce any statistically significant increase of MN frequency both at 4 + 20 h and at 24 h treatment.

At 4 + 20 h in the presence of S9-mix, 4-phenylbut-3-en-2-one induced a statistically significant increase of MN frequency at 80 μg/mL (cytotoxicity 51%), the highest concentration analysed for MN. The increase of MN frequency observed (0.85%) was outside the range of historical controls (95% reference range from 0.06% to 0.54%).

Slides from 80 μg/mL cultures were stained for FISH. Slides from cultures treated with CPA or VB were scored as positive controls for clastogenic or aneugenic mode of action, respectively.

This analysis showed that, in the sample treated with 4-phenylbut-3-en-2-one, the percentage of MN positive for the DNA probe (C + MN) was 28%. Comparing this result with the percentage of (C + MN) observed in the concurrent positive controls (CPA induced 32% of C + MN and VB induced 77% of C + MN) and the data available in literature (Darroudi et al., 1996; Young et al., 2014) this result indicates that 4-phenylbut-3-en-2-one induced MN via a clastogenic mechanism.

The Panel noted that the analysis was based on a limited number of cells (43 MNBN cells), but this was sufficient to clarify the mode of action.

Study results are summarised in Appendix D, Table D.1.

3.2.4. Literature data on 4-phenylbut-3-en-2-one [FL-no: 07.024]

3.2.4.1. *In vitro* data

4-Phenylbut-3-en-2-one was reported to be positive for mutagenicity in *S.* Typhimurium strain TA100 (Seifried et al., 2006). In the same study, when tested in the *in vitro* mouse lymphoma assay (L5178Y/tk+/-), 4-phenylbut-3-en2-one was negative for mutagenicity in the presence of metabolic activation and positive for mutagenicity in the absence of metabolic activation (Seifried et al., 2006).

4-Phenylbut-3-en-2-one was also tested in another study in *S.* Typhimurium strains TA100 and TA98 in the absence of metabolic activation or in the presence of rat or hamster S9-mix. 4-Phenylbut-3-en-2-one induced gene mutations in strain TA100 in the presence of rat S9-mix (NTP, 1994).[5]

In another NTP report (NTP, 2012), 4-phenylbut-3-en-2-one (methyl *trans*-styryl ketone CAS n 1896-62-4) was positive in *S.* Typhimurium strain TA100 in the presence of metabolic activation (S9-mix). No mutagenicity was observed in *S.* Typhimurium strain TA98 with or without S9-mix. No mutagenicity was observed in *Escherichia coli* strain WP2uvrA/pKM101 in the absence of S9-mix while inconsistent responses were reported in two trials in the presence of S9-mix (NTP, 2012).

Overall, the Panel considered that although most of these data are poorly reported (NTP, 1994; Seifried et al., 2006) they are consistent with the results reported by industry on the mutagenic activity of 4-phenylbut-3-en-2-one observed *in vitro* in S. Typhimurium strain TA100 in the presence of S9-mix.

3.2.4.2. *In vivo* data

In an *in vivo* micronucleus assay in bone marrow cells from B6C3F1 mice treated three times intraperitoneal (i.p.) with doses of 4-phenylbut-3-en-2-one from 37.812 to 605 mg/kg and sacrificed 24 h after the final treatment, no induction of MN PCE was observed (NTP, 1994)[5].

A comprehensive report was published by the National Toxicology Program (NTP, 2012) for methyl *trans*-styryl ketone (CAS n 1896-62-4) which is one possible isomer of the flavouring substance 4-phenylbut-3-en-2-one. The NTP report indicates that methyl *trans*-styryl ketone is used as a synthetic flavouring agent and a fragrance additive in food and personal care products. This NTP report includes

[5] https://manticore.niehs.nih.gov/cebssearch/test_article/122-57-6

both *in vitro* and *in vivo* genotoxicity tests, 3-month oral and dermal toxicity studies, and 2-year dermal carcinogenicity studies in male and female F344/N rats and B6C3F1 mice (NTP, 2012).

The *in vivo* MN test was conducted on peripheral blood samples from male or female mice administered 4-phenylbut-3-en-2-one for 3 months via feed (up to 0.4% in the feed) or via dermal application (up to 350 mg/kg bw). No increases in the frequencies of micronucleated NCE was observed in any testing conditions. However, no treatment-related changes in the percentage of PCE among the total erythrocyte population were observed, so there is no indication of bone marrow exposure.

Data from absorption, distribution, metabolism, and excretion (ADME) studies in mice and rats indicate that tissue exposure to methyl *trans*-styryl ketone is expected to be very low by both oral and dermal routes (NTP, 2012).

3.3. Data on 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030]

3.3.1. 1-(4-Methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] – *in vivo* combined bone marrow micronucleus test and comet assay

The genotoxic potential of 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] (purity 99.5%) was assessed *in vivo* using the bone marrow micronucleus assay combined with the comet assay in the duodenum and liver of rats (Covance, 2014b). The study was conducted in compliance with GLP. The micronucleus study was conducted in accordance with OECD TG 474 (OECD, 1997b). The comet study was conducted before the publication of the first relevant OECD test guideline (OECD TG 489, 2014b), but it was based on the guidance provided by International Workshops on Genotoxicity Testing (Burlinson et al., 2007), the international validation of the *in vivo* comet assay by the JaCVAM and literature available at that time (Tice et al., 2000; Hartmann et al., 2003, 2004; Smith et al., 2008).

In a dose range-finder assay, groups of three male and three female Han Wistar rats were given three administrations by gavage (at 0, 24 and 45 h) of 1-(4-methoxyphenyl)pent-1-en-3-one, at 1,000, 1,400 and 2,000 mg/kg bw per day. Post-dose effects were reported on day 2 and day 3 at 1,400 and 2,000 mg/kg bw per day, including prostration, decreased activity and ataxia. At the same doses, body weight reductions were observed (from 2% to 8%). Based on this study, the dose of 1,000 mg/kg bw per day was selected for follow-up research.

As no gender differences were observed in the range-finder experiment, the main experiment was performed only in male rats.

Groups of six male Han Wistar rats per dose group were administered doses by gavage of 0 (corn oil), 250, 500 or 1,000 mg/kg bw of 1-(4-methoxyphenyl)pent-1-en-3-one on three consecutive days (0, 24 and 45 h). A positive control group of six male rats were given doses of 150 mg EMS/kg bw at the same time intervals as the dosed groups.

Animals were killed and sampled at 48 h post the initial dose.

No clinical signs of toxicity were observed in any animal following treatments with 1-(4-methoxyphenyl)pent-1-en-3-one, with the vehicle or with EMS.

A small decrease of body weight was observed at the two highest doses.

Histopathological evaluation revealed increased hepatocyte mitosis in animals treated with 500 and 1,000 mg/kg bw per day, which indicates liver exposure. No findings were observed in the duodenum.

Micronucleus assay

Bone marrow from the femurs was prepared for micronucleus scoring. A total of at least 500 PCE and NCE were scored to calculate the degree of bone marrow toxicity by the relative decrease in PCE. For MN analysis 2,000 PCE per animal were scored for the presence of MN.

Rats treated with 1-(4-methoxyphenyl)pent-1-en-3-one exhibited group mean frequencies of MNPCE that were similar to and not statistically different from those observed in the concurrent vehicle control for all dose groups. The treatment with 1-(4-methoxyphenyl)pent-1-en-3-one did not reduce the percentage of PCE, therefore no indication of bone marrow toxicity was observed.

Under the conditions of this study 1-(4-methoxyphenyl)pent-1-en-3-one did not induce MN in bone marrow of rats tested up to 1,000 mg/kg bw per day, by gavage.

Considerations on the micronucleus assay

In the *in vivo* micronucleus assay, 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] did not induce MN in bone marrow PCE of male rats following gavage administration up to 1,000 mg/kg bw per day.

Considering that the percentage of PCE in the bone marrow of treated animals was not changed compared to the concurrent vehicle control group, it was unclear whether the bone marrow was exposed and therefore additional evidence of systemic exposure through plasma analysis was requested to the applicant in order to conclude on the *in vivo* micronucleus assay.

However, in view of technical problems reported by the applicant with the plasma analysis, the Panel decided to waive its initial request for additional evidence of systemic exposure and requested the applicant to investigate the mechanism of MN induction (clastogenicity or aneugenicity) through an *in vitro* micronucleus assay with centromere analysis. The clarification of the mechanism of genotoxicity would allow to interpret the already available *in vivo* data or to identify an appropriate *in vivo* follow-up study.

Comet assay

Liver and duodenum cells were prepared for comet analysis. Tail moment and tail intensity (%) of a total of 100 cells per animal were recorded. No dose-related increase in % clouds was observed following treatment with 1-(4-methoxyphenyl)pent-1-en-3-one demonstrating that treatment did not cause excessive DNA damage that could have interfered with comet analysis.

Both in the liver and the duodenum, no statistically significant increase in group mean tail intensity and tail moment values were observed in any test substance treatment group compared to the vehicle control group. Liver exposure was indicated by an increase in hepatocyte mitosis at the highest doses tested (500 and 1,000 mg/kg bw per day).

Therefore, the Panel concluded that 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] did not induce primary DNA damage in the liver and duodenum of rats after oral administration.

Study results are summarised in Appendix D, Table D.2.

3.3.2. 1-(4-Methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] – *in vitro* micronucleus test in human lymphocytes

1-(4-Methoxyphenyl)pent-1-en-3-one (purity 99.5%) was tested in an *in vitro* micronucleus assay using duplicate human lymphocytes cultures (Covance, 2013). The cells were treated with a broad range of concentrations at narrow intervals, both in the absence and presence of metabolic activation (S9-mix) from Aroclor 1,254-induced rats. Treatments were conducted for 3 h in the presence or in the absence of S9-mix, followed by 21 h recovery period (3 + 21 h) or for 24 h in the absence of S9-mix without recovery (24 h). A preliminary cytotoxicity Range Finder Experiment was conducted to assess the effect of 1-(4-methoxyphenyl)pent-1-en-3-one on the replication index (RI) and determine the highest concentrations to be used in the main micronucleus experiment (limited by cytotoxicity). The treatments were initiated 48 h following mitogen stimulation by PHA. MN were analysed at concentrations of 40, 70 and 90 μg/mL for 3 + 21 h without S9-mix, with cytotoxicities determined to be 22, 34, 55%, respectively, and at 90, 120, 130 and 150 μg/mL for 3 + 21 h with S9-mix, with cytotoxicities determined to be 1, 20, 39, 54%, respectively. MN were also analysed at 14, 18, 22 and 25 μg/mL for 24-h without S9-mix, with cytotoxicities determined to be 3, 21, 43, 50%, respectively.

MMC and VB were used as clastogenic and aneugenic positive controls, respectively, in the absence of S9-mix and CPA was used as a clastogenic positive control in the presence of S9-mix. The frequency of MNBN cells in the vehicle control and positive control cultures fell within 95th percentiles of the respective historical control ranges and the test system was considered sensitive and valid.

The frequencies of MNBN cells following treatment with 1-(4-methoxyphenyl)pent-1-en-3-one for 3 + 21 h and 24 h in the absence of S9-mix were similar to those observed in concurrent vehicle controls for all concentrations analysed and they all fell within the normal range.

At 3 + 21 h in the presence of S9-mix, the MNBN cells frequencies were significantly higher ($p \leq 0.05$) compared to the concurrent vehicle control at the highest two concentrations analysed (130 and 150 μg/mL). The mean MNBN cell frequencies exceeded the historical control range of negative controls (95% reference range 0.0–1.0) at 130 μg/mL (1.08%) and 150 μg/mL (1.55%) (Covance, 2013).

The Panel concluded that 1-(4-methoxyphenyl)pent-1-en-3-one induced MN in cultured human peripheral blood lymphocytes, when tested up to cytotoxic concentrations for 3 + 21 h, in the presence of metabolic activation, similarly to results obtained in a previous study (Stone, 2012).

Study results are summarised in Appendix D, Table D.1.

3.3.3. 1-(4-Methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] – *in vitro* micronucleus test in TK6 cells with CREST staining

1-(4-Methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] (purity 100%) was tested in an *in vitro* micronucleus assay with kinetochores staining in the human lymphoblastoid cell line TK6 cells (BioReliance, 2018b), with the purpose of evaluating the aneugenic and clastogenic potential of the tested substance. DMSO was used as the vehicle. The study was performed according to GLP and OECD TG 487 (OECD, 2014a).

TK6 cells were treated for 4 h with 23 h of recovery period (4 + 23 h) in the absence and presence of S9-mix (from Aroclor 1254-induced rats), or for 27 h in the absence of S9-mix.

Positive controls were CPA and VB. The vehicle control was DMSO.

In the preliminary cytotoxicity assay, 1-(4-methoxyphenyl)pent-1-en-3-one was tested at concentrations from 0.19 to 1,900 μg/mL. Precipitate was observed at concentrations ≥ 570 μg/mL in all treatment conditions. Cytotoxicity, indicated by a decrease in RPD of ≥ 50% compared to the vehicle control (DMSO), was observed at concentrations ≥ 190 μg/mL in the 4 + 23 h treatment with and without S9-mix, and at ≥ 57 μg/mL in the 27-h treatment without S9-mix.

Based on these results, concentrations used in the micronucleus assay were 5–150 μg/mL for the 4 + 23 h treatment without metabolic activation, 5–250 μg/mL for the 4 + 23 h treatment with metabolic activation and 5–75 μg/mL for the 27-h treatment without metabolic activation.

At least 2,000 mononucleated cells were scored per each concentration. MN frequency was evaluated at 5, 25 and 55 μg/mL in the non-activated 4 + 23 h treatment with cytotoxicities determined to be 3, 12, 55%, respectively, and at 5, 25 and 50 μg/mL in the 4 + 23 h treatment group with metabolic activation with cytotoxicities determined to be 3, 22 and 60%, respectively. MN were also analysed at 5, 15 and 25 μg/mL in the 27-h treatment without metabolic activation with cytotoxicities determined to be 0, 24 and 59%, respectively. In all treatment conditions, statistically significant and concentration-related increases in MN induction were observed: in the 4 + 23 h treatment without S9-mix at 55 μg/mL (2.55%, $p \leq 0.01$); in the 4 + 23 h treatment with S9-mix at 25 and 50 μg/mL (1.65 and 1.90%, $p \leq 0.01$); and in the 27-h treatment without S9-mix at 15 and 25 μg/mL (1.40% and 2.70%, $p \leq 0.05$ and $p \leq 0.01$, respectively).

To confirm these results, the micronucleus assay was repeated at concentrations ranging from 5 to 70 μg/mL in the 4 + 23 h in the absence and presence of S9-mix, and from 5 to 40 μg/mL in 27-h treatment without S9-mix.

Concentrations selected for MN frequency evaluation were 5, 40, 55 and 60 μg/mL in the 4 + 23 h treatment without S9-mix with cytotoxicities determined to be 4, 22, 55 and 60%, respectively, and 5, 20 and 50 μg/mL in the 4 + 23 h treatment with S9-mix with cytotoxicities determined to be 2, 9 and 53%, respectively. MN were also analysed at 5, 15, 18, 25 and 28 μg/mL in the 27-h treatment without S9-mix with cytotoxicities determined to be 5, 18, 17, 50, 56%, respectively.

In this repeated experiment, statistically significant and concentration-related increases were observed in the 4 + 23 h treatment without S9-mix at the concentration of 60 μg/mL (1.65%, which is outside the historical vehicle control at 95% reference range 0.19–0.92). In the 4 + 23 h treatment with S9-mix, the MN frequency increase seen in the first experiment was not reproduced. In the 27-h treatment without S9-mix, a statistically significant increase in MN induction was observed at 28 μg/mL (1.25%, which is outside the historical vehicle control at 95% reference range 0.18–1.07).

The positive controls induced statistically significant increases in MN frequency and the system was considered sensitive and valid. Negative controls were within the historical vehicle control ranges.

Since positive responses were observed, kinetochores staining (CREST staining) was applied in order to determine the mode of action (aneugenicity or clastogenicity).

One concentration, 60 μg/mL, from the 4 + 23 h treatment without S9-mix and the positive controls were analyzed. A total of 100 MN, for each sample, were evaluated for the presence (K+) or absence (K-) of kinetochore staining.

This analysis showed that, in the sample treated with 1-(4-methoxyphenyl)pent-1-en-3-one, the percentage of MN positive for the CREST antibody (K + MN) was 70%. Comparing this result with the percentage of (K + MN) observed in the concurrent positive controls (CPA induced 38% of K + MN and VB induced 94% of K + MN) and the data available in literature (Eastmond and Tucker, 1989; Young et al., 2014) this result indicates that 1-(4-methoxyphenyl)pent-1-en-3-one induced MN via an aneugenic mechanism.

The Panel concluded that 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] induced MN in TK6 cells via an aneugenic mechanism.

3.4. Discussion

3.4.1. 4-Phenylbut-3-en-2-one [FL-no: 07.024]

4-Phenylbut-3-en-2-one [FL-no: 07.024] induced reproducible, concentration-related and statistically significant increases in mutation frequencies, in strain TA100 of *S.* Typhimurium in the presence of metabolic activation. It also induced MN in cultured human peripheral blood lymphocytes when tested up to the limits of cytotoxicity for 3 + 21 h in the absence and presence of rat liver metabolic activation (S9-mix).

In FGE.215, the Panel requested to test the representative substance 4-phenylbut-3-en-2-one [FL-no: 07.024] in an *in vivo* combined micronucleus and comet assay in the liver and duodenum, to further assess the genotoxic potential of [FL-no: 07.024].

In response to this request for further genotoxicity data, industry submitted an *in vivo* combined bone marrow micronucleus test and comet assay in the liver and duodenum for [FL-no: 07.024].

The results of *in vivo* comet assay were negative both in the liver and in the duodenum with evidence of liver exposure.

The results of the *in vivo* micronucleus assay in bone marrow were negative, but with limited evidence of bone marrow exposure, therefore these results are considered as inconclusive.

The Panel requested to provide evidence of bone marrow exposure to substance 4-phenylbut-3-en-2-one [FL-no: 07.024] by plasma analysis as recommended in the OECD TG 474. Due to challenges in the plasma analysis, the Panel decided to waive this request, but requested to test the flavouring substance in an *in vitro* micronucleus assay with centromere analysis, in order to investigate the mechanism inducing MN *in vitro* (clastogenicity or aneugenicity). The clarification of the mechanism of action would allow to identify the most appropriate follow-up study.

Industry submitted an *in vitro* micronucleus assay in TK6 cells for [FL-no: 07.024], where no induction of MN was observed, therefore the kinetochores staining was not applied.

The Panel noted that the negative results observed in this study in TK6 cells are in contrast with results obtained in the *in vitro* micronucleus study in human peripheral blood lymphocytes (Watters, 2013).

The Panel noted that the negative results reported in the assay conducted in TK6 cells could be related to a reduced sensitivity of the test due to the experimental conditions used. In particular, the protocol without application of cytoB in the TK6 cells study, may have limited the detection of DNA damage. Since no cytokinesis block with cytoB was applied (as it was performed in the studies on human lymphocytes), it was not clear if the analysis was limited to cells that had divided only once after the treatment (binucleated cells).[6] Therefore, the Panel considered the results of the *in vitro* micronucleus assay performed in human peripheral blood lymphocytes, in which 4-phenylbut-3-en-2-one [FL-no: 07.024] clearly increased the frequency of MN both in the presence and absence of metabolic activation, as more reliable.

Therefore, the Panel requested to test 4-phenylbut-3-en-2-one [FL-no: 07.024] in a new *in vitro* micronucleus assay in cultured peripheral blood lymphocytes with FISH analysis.

In this new *in vitro* micronucleus assay, 4-phenylbut-3-en-2-one induced a statistically significant increase of MN frequency at 4 + 20 h in the presence of metabolic activation. The FISH analysis on MNBN cells showed that 4-phenylbut-3-en-2-one induced MN mainly via a clastogenic mechanism.

In the *in vivo* comet assay, 4-phenylbut-3-en-2-one [FL-no: 07.024] did not induce primary DNA damage in the liver and duodenum of rats after oral administration, therefore the potential clastogenicity in the presence and absence of metabolic activation is ruled out. Data from literature (NTP, 1994, 2012) which were provided by the applicant in the course of the evaluation, were consistent with this conclusion.

3.4.2. 1-(4-Methoxyphenyl)pent-1-en-3-one [FL-no: 07.030]

1-(4-Methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] did not induce gene mutation in bacteria. It induced MN in cultured human peripheral blood lymphocytes when tested up to the limits of cytotoxicity for 3 + 21 h in the presence of rat liver metabolic activation (S9-mix) and a weak increase in its absence.

In FGE.215, the Panel requested to test the representative substance 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] in an *in vivo* combined micronucleus and comet assay in the liver and duodenum, to further assess the genotoxic potential of [FL-no: 07.030].

[6] Further division of cells would result in a lower frequency of MN. This would be prevented by the use of cytoB.

In response to this request for further genotoxicity data, industry submitted an *in vivo* combined bone marrow micronucleus test and comet assay in the liver and duodenum for [FL-no: 07.030].

The results of *in vivo* comet assay were negative both in the liver and in the duodenum with evidence of liver exposure.

The results of the *in vivo* micronucleus assay in bone marrow were negative, but with no evidence of bone marrow exposure, therefore these results are considered as inconclusive.

The Panel requested to provide evidence of bone marrow exposure to substance 4-phenylbut-3-en-2-one [FL-no: 07.030] by plasma analysis as recommended in the OECD TG 474. Due to challenges in the plasma analysis, the Panel decided to waive this request, but requested to test the flavouring substance in an *in vitro* micronucleus assay with centromere analysis, in order to investigate the mechanism inducing MN *in vitro* (clastogenicity or aneugenicity). The clarification of the mode of action would allow to identify the most appropriate follow-up study.

Industry submitted an *in vitro* micronucleus assay in human peripheral blood lymphocytes and an *in vitro* micronucleus assay in TK6 cells with kinetochores staining for [FL-no: 07.030].

The *in vitro* micronucleus assay in human peripheral blood lymphocytes confirmed the increase of MN frequency in the presence of metabolic activation as observed in the previously available study.

In a first experiment in TK6 cells, 1-(4-methoxyphenyl)pent-1-en-3-one induced an increase of MN frequency, both in the presence and in the absence of metabolic activation. However, the repetition of the tests confirmed the increase of MN frequency only in the absence of metabolic activation. Therefore, the kinetochores staining was applied only to the cells from the short-term treatment in the absence of S9-mix. CREST analysis indicated that 1-(4-methoxyphenyl)pent-1-en-3-one induced MN mainly by an aneugenic mechanism.

In the *in vivo* comet assay, 1-(4-methoxyphenyl)pent-1-en-3-one did not induce primary DNA damage in the liver and duodenum of rats after oral administration, therefore the potential clastogenicity is ruled out.

However, the available data are not sufficient to rule out the potential aneugenicity of [FL-no: 07.030] *in vivo*.

Since the genotoxicity concern cannot be ruled out for the representative substance [FL-no: 07.030] and the structurally related substances [FL-no: 07.046 and 07.049], data on exposure are reported in Appendix E.

The available data on production volume and use levels for the representative substance 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] and the structurally related substances vanillylidene acetone [FL-no: 07.046] and 1-(4-methoxyphenyl)-4-methylpent-1-en-3-one [FL-no: 07.049], as chemically defined flavouring substance, are reported with the respective calculation of Maximised Survey-derived Daily Intake (MSDI) and modified Theoretical Added Maximum Daily Intake (mTAMDI) (Appendix E).

The Triskelion database of Volatile Compounds in Food version 16.6.1 has been searched for the three substances [FL-no: 07.030, 07.046 and 07.049]. None of them have been reported to occur in any natural or processed food products according to the Triskelion database (Triskelion, 2019).

3.5. Conclusions

3.5.1. 4-Phenylbut-3-en-2-one [FL-no: 07.024]

The Panel concluded that the concern for genotoxicity is ruled out for the representative substance 4-phenylbut-3-en-2-one [FL-no: 07.024] and for the structurally related substances 4-phenylbut-3-en-2-ol [FL-no: 02.066] and 3-methyl-4-phenylbut-3-en-2-one [FL-no: 07.027] which can accordingly be evaluated through the Procedure in FGE.69.

3.5.2. 1-(4-Methoxyphenyl)pent-1-en-3-one [FL-no: 07.030]

Based on the available data, the Panel concluded that the representative substance 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] is aneugenic *in vitro*. For such substances, there is currently no agreed follow-up strategy to finalise their safety assessment. The Panel is aware that the EFSA Scientific Committee is going to address this issue and a statement clarifying the assessment of *in vitro* aneugenic substances is under preparation.[7]

[7] http://registerofquestions.efsa.europa.eu/raw-war/wicket/page?3

The Panel concluded therefore that, for the time being, the representative substance 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] and the structurally related substances vanillylidene acetone [FL-no: 07.046] and 1-(4-methoxyphenyl)-4-methylpent-1-en-3-one [FL-no: 07.049] cannot be evaluated through the Procedure.

3.5.3. 4-(2,3,6-trimethylphenyl)but-3-en-2-one [FL-no: 07.206]

During the evaluation process, the Panel concluded that 4-(2,3,6-trimethylphenyl)but-3-en-2-one [FL-no: 07.206] is to be considered as a stand-alone substance due to the presence of the methyl groups. Therefore, the Panel requested to test [FL-no: 07.206] *in vitro* in a bacterial reverse mutation test and in a micronucleus test. Industry communicated that the evaluation of 4-(2,3,6-trimethylphenyl)but-3-en-2-one [FL-no: 07.206] is not supported any longer, therefore additional data were not submitted.

Documentation provided to EFSA

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2) Benigni R and Netzeva T, 2007b. Report on a QSAR model for prediction of genotoxicity of α,ß-unsaturated ketones in *S. typhimurium* TA100 and its application for predictions on α,ß-unsaturated aldehydes in Flavouring Group Evaluation 19 (FGE.19). Unpublished report submitted by FLAVIS Secretariat to EFSA.
3) BioReliance, 2018a. *In Vitro* mammalian cell micronucleus assay in TK6 cells: 4-Phenylbut-3-en-2-one. BioReliance Corporation. Study Number AE84HH.361.BTL. February 2018. Unpublished report submitted by EFFA.
4) BioReliance, 2018b. *In Vitro* mammalian cell micronucleus assay in TK6 Cells: 1-(4-Methoxyphenyl)pent-1-en-3-one. BioReliance Corporation. Study Number AE84HM.361.BTL. April 2018. Unpublished report submitted by EFFA.
5) BioReliance, 2019. *In Vitro* mammalian cell micronucleus assay in human peripheral blood lymphocytes: 4-Phenylbut-3-en-2-one. BioReliance Corporation. Study Number AE84HH.348FISH.BTL. March 2019. Unpublished report submitted by EFFA.
6) Bowen R, 2011. Reverse mutation in five histidine-requiring strains of *Salmonella typhimurium*. 1-(4-Methoxyphenyl)pent-1-en-3-one. Covance Laboratories Ltd. Study no. 8241436. August 2011. Unpublished report submitted by EFFA to FLAVIS Secretariat.
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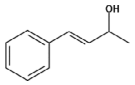
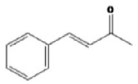
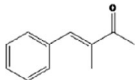
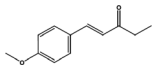
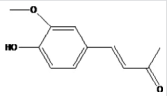
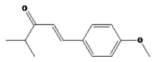
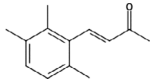
Abbreviations

ADME	absorption, distribution, metabolism, and excretion
bw	body weight
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CoE	Council of Europe
CPA	cyclophosphamide

CREST staining	anti-kinetochore antibody staining
cytoB	cytochalasin B
DMSO	dimethylsulfoxide
EFFA	European Flavour Association
EMS	ethyl methanesulfonate
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FISH	fluorescence *in situ* hybridisation
FLAVIS (FL)	Flavour Information System (database)
GLP	Good Laboratory Practice
ID	Identity
i.p.	intraperitoneal
IR	infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MMC	mitomycin C
MN	micronuclei
MNBN	micronucleated binucleate cells
MNPCE	micronucleated polychromatic erythrocytes
MS	Mass spectra
MSDI	Maximised Survey-derived Daily Intake
MTD	maximum tolerated dose
mTAMDI	modified Theoretical Added Maximum Daily Intake
NCE	normochromatic erythrocytes
No	Number
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
PCE	polychromatic erythrocytes
PHA	phytohaemagglutinin
(Q)SAR	(quantitative) structure–activity relationship
RI	Replication index
RPD	relative population doubling
VIN	vinblastine

Appendix A – Specification Summary of the Substances in the Flavouring Group Evaluation 215Rev1

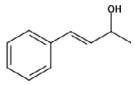
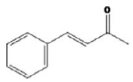
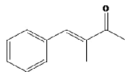
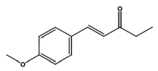
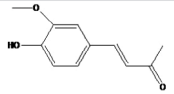
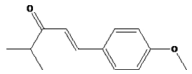
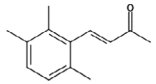
Table A.1: Specification Summary of the Substances in the Flavouring Group Evaluation 215 Revision 1 (JECFA 2001a)

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]	EFSA comments
02.066 819	4-Phenylbut-3-en-2-ol		2880 2032 17488-65-2	Liquid $C_{10}H_{12}O$ 148.21	Insoluble Miscible	140 (16 hPa) IR 96%	1.558-1.567 1.006-1.012	
07.024 820	4-Phenylbut-3-en-2-one		2881 158 122-57-6	Solid $C_{10}H_{10}O$ 146.19	Insoluble Very soluble	260 39-42 IR 97%	n.a. n.a.	
07.027 821	3-Methyl-4-phenylbut-3-en-2-one		2734 161 1901-26-4	Solid $C_{11}H_{12}O$ 160.22	Insoluble Very soluble	124-125(13 hPa) 38-40 NMR 97%	n.a. n.a.	
07.030 826	1-(4-Methoxyphenyl) pent-1-en-3-one		2673 164 104-27-8	Solid $C_{12}H_{14}O_2$ 190.24	Insoluble Very soluble	278 60 IR 98%	n.a. n.a.	
07.046 732	Vanillylidene acetone		3738 691 1080-12-2	Solid $C_{11}H_{12}O_3$ 192.21	Slightly soluble Moderately soluble	Decomposes 129-130 IR 97%	n.a. n.a.	
07.049 829	1-(4-Methoxyphenyl)-4-methylpent-1-en-3-one		3760 719 103-13-9	Liquid $C_{13}H_{16}O_2$ 204.27	Insoluble Miscible	201 (13 hPa) NMR 97%	1.510-1.515 1.016-1.026	
07.206	4-(2,3,6-Trimethylphenyl)but-3-en-2-one		56681-06-2	Solid $C_{13}H_{16}O$ 188.27	Practically insoluble or insoluble Freely soluble	97 (0.1 hPa) 75 MS 95%	n.a. n.a.	Substance no longer supported by Industry

CAS: Chemical Abstract Service; CoE: Council of Europe; FEMA: Flavor and Extract Manufacturers Association; FLAVIS (FL): Flavour Information System (database); FL-no: FLAVIS number; ID: Identity; IR: Infrared spectroscopy; JECFA: The Joint FAO/WHO Expert Committee on Food Additives; MS: mass spectra; n.a.: not analysed; NMR: nuclear magnetic resonance.
(a): Solubility in water, if not otherwise stated.
(b): Solubility in 95% ethanol, if not otherwise stated.
(c): At 1,013.25 hPa, if not otherwise stated.
(d): At 20°C, if not otherwise stated.
(e): At 25°C, if not otherwise stated.

Appendix B – Summary of Safety Evaluation Applying the Procedure

Table B.1: Summary of Safety Evaluation Applying the Procedure (JECFA, 2001b, 2002)

FL-no JECFA-no	EU Union List chemical name	Structural formula	EU MSDI[(a)] US MSDI (μg/capita per day)	Class[(b)] Evaluation procedure path[(c)]	JECFA Outcome on the named compound[(d)] or [(e)]	EFSA conclusion on the named compound (genotoxicity)
02.066 819	4-Phenylbut-3-en-2-ol		0[(f),(g)] 0.1	Class I A3: Intake below threshold	d	Evaluated in FGE.215Rev1, genotoxicity concern can be ruled out. The substance can be evaluated through the Procedure
07.024 820	4-Phenylbut-3-en-2-one		1.83[(f)]; 1.22[(g)] 7	Class I A3: Intake below threshold	d	Evaluated in FGE.215Rev1, genotoxicity concern can be ruled out. The substance can be evaluated through the Procedure
07.027 821	3-Methyl-4-phenylbut-3-en-2-one		0[(f),(g)] 0.1	Class I A3: Intake below threshold	d	Evaluated in FGE.215Rev1, genotoxicity concern can be ruled out. The substance can be evaluated through the Procedure
07.030 826	1-(4-Methoxyphenyl) pent-1-en-3-one		0.24[(f)]; 0.04[(g)] 110	Class I A3: Intake below threshold	d	Evaluated in FGE.215Rev1, as aneugenic *in vitro*. There is currently no agreed follow-up strategy to finalise the genotoxicity assessment
07.046 732	Vanillylidene acetone		0[(f),(g)] 0.1	Class I A3: Intake below threshold	d	Evaluated in FGE.215Rev1, as potential aneugenic *in vitro*. There is currently no agreed follow-up strategy to finalise the genotoxicity assessment
07.049 829	1-(4-Methoxyphenyl)-4-methylpent-1-en-3-one		0[(f),(g)] 0.3	Class II A3: Intake below threshold	d	Evaluated in FGE.215Rev1, as potential aneugenic *in vitro*. There is currently no agreed follow-up strategy to finalise the genotoxicity assessment
07.206 –	4-(2,3,6-Trimethylphenyl)but-3-en-2-one		0[(f),(g)]	Class I No evaluation	Not evaluated by JECFA	Substance no longer supported by Industry

FLAVIS (FL): Flavour Information System (database); FL-no: FLAVIS number; JECFA: The Joint FAO/WHO Expert Committee on Food Additives; JECFA no: JECFA number; MSDI: Maximised Survey-derived Daily Intake.
(a): EU MSDI: Amount to food as flavour in (kg/year) × 10E9/(0.1 × population in Europe (= 375 × 10E6) × 0.6 × 365) = μg/capita per day.
(b): Thresholds of concern: Class I = 1,800 μg/person per day, Class II = 540 μg/person per day, Class III = 90 μg/person per day.
(c): Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.
(d): No safety concern based on intake calculated by the MSDI approach of the named compound.
(e): Data must be available on the substance or closely related substances to perform a safety evaluation.
(f): MSDI value calculated based on EFFA poundage survey covering 2010, submitted by EFFA to European Commission (EFFA, 2017).
(g): MSDI value calculated based on EFFA poundage survey covering 2015, submitted by EFFA to European Commission (EFFA, 2017).

Appendix C – Genotoxicity studies evaluated in FGE.215

Table C.1: Genotoxicity data (*in vitro*) considered by the Panel in FGE.215

Register name [FL-no]	Test system	Test object	Concentration	Result	Reference	Comments
4-Phenylbut-3-en-2-one [07.024]	Reverse mutation	*Salmonella* Typhimurium TA98, TA1535, TA1537 and TA102	1.6, 8, 40, 200, 1,000 and 3,000 μg/plate [1, 2]	Negative	Lillford (2009)	Evidence of toxicity was observed at 3,000 μg/plate in TA100 in the absence of S9-mix and in all other strains in the absence and presence of S9-mix; at 1,000 μg/plate in strain TA1535 in the absence of S9-mix; complete killing was observed at 5,000 μg/plate in strain TA100 in the presence of S9-mix. Statistically significant concentration-related increase in mutations was observed in strain TA100 at 40 μg/plate and above in the presence of S9-mix. Study design complies with current recommendations (OECD Guideline 471). Acceptable top concentration was achieved
		S. Typhimurium TA100	1.6, 8, 40, 200, 1,000 and 3,000 μg/plate [4, 2]	Negative		
			1.6, 8, 40, 200, 1,000 and 5,000 μg/plate [5, 2]	Positive		
		S. Typhimurium TA98, TA1535, TA1537, TA102	93.75, 187.5, 375, 750, 1,500 and 3,000 μg/plate [4, 2] or [5, 3]	Negative		Evidence of toxicity was observed at 750 and/or 1,500 and/or 3,000 μg/plate in the absence and presence of S9-mix. Statistically significant concentration-related increase in mutations was observed only in strain TA100 at 375 μg/plate and above in the presence of S9-mix. Study design complies with current recommendations (OECD Guideline 471). Acceptable top concentration was achieved
		S. Typhimurium TA100	93.75, 187.5, 375, 750, 1,500 and 3,000 μg/plate [4, 2] or [5, 3]	Positive [5, 3]		
		S. Typhimurium TA98, TA1535, TA1537, TA102	46.88, 93.75, 187.5, 375, 750 and 1,500 μg/plate [5, 3]	Negative		Evidence of cytotoxicity was observed at 1,500 μg/plate in all strains. Statistically significant concentration-related increase in mutations was observed only in strain TA100 at 187.5 μg/plate and above. Study design complies with current recommendations (OECD Guideline 471). Acceptable top concentration was achieved
		S. Typhimurium TA100	46.88, 93.75, 187.5, 375, 750 and 1,500 μg/plate [5, 3]	Positive		
	Micronucleus induction	Human peripheral blood lymphocytes	30, 40 and 50 μg/mL [4, 6]	Negative	Stone (2011)	Although acceptable levels of cytotoxicity were achieved at the top concentrations used in all parts of the study and the MNBN cell frequencies were similar to vehicle controls in all treated cultures, the inconsistent cytotoxicity curves generated among the three trials in this study could not be easily explained. On this basis a second study was performed (Watters, 2013). The study complies with OECD Guideline 487
			40, 70, 80 and 85 μg/mL [5, 6]	Negative		
			12, 14, 17.5 and 20 μg/mL [4, 7]	Negative		
			30, 40, 44, and 46 μg/mL [4, 6]	Positive	Watters (2013)	Acceptable levels of cytotoxicity were achieved at the top concentrations used in all parts of the study. Test compound induced micronuclei in cultured human peripheral blood lymphocytes from both male and female donors, following 3+21 h treatment in the absence and presence of S9-mix. The study complies with OECD Guideline 487
			85, 95, 100, and 105 μg/mL [5, 6]	Positive		
			8, 13, 14 and 15 μg/mL [4, 7]	Negative		

Register name [FL-no]	Test system	Test object	Concentration	Result	Reference	Comments
1-(4-Methoxyphenyl) pent-1-en-3-one [07.030]	Reverse mutation	*S.* Typhimurium TA98, TA100, TA1535, TA1537 and TA102	0,32, 1.6, 8, 40, 200, 1,000 and 5,000 μg/plate [1, 2]	Negative	Bowen (2011)	Toxicity was observed in the form of slight thinning of background bacteria lawn and reduced numbers of revertants at 1,000 μg/plate in strains TA1537 and TA102 and complete killing of bacteria was observed at 5,000 μg/plate in all strains, in the absence and presence of S9-mix. Study design complies with current recommendations (OECD Guideline 471). Acceptable top concentration was achieved
		S. Typhimurium TA98, TA100, TA1535	156.3, 312.5, 625, 1,250, 2,500 and 5,000 μg/plate [4, 2] or [5, 3]	Negative		Evidence of toxicity was observed at 625 μg/plate and above in strains TA1535, TA1537 and TA102 in the presence of S9-mix, at 1,250 μg/plate and above in strains TA1537 and TA102 in the absence of S9-mix and TA100 in the presence of S9-mix, and at 2,500 μg/plate and above in strains TA98 in the absence and presence of S9-mix, and TA100 and TA1535 in the absence of S9-mix. Study design complies with current recommendations (OECD Guideline 471). Acceptable top concentration was achieved
		S. Typhimurium TA1537 and TA102	78.13, 156.3, 312.5, 625, 1,250, 2,500 and 5,000 μg/plate [4, 2] or [5, 3]	Negative		
		S. Typhimurium TA1535 and TA1537	19.53, 39.06, 78.13, 156.3, 312.5, 625, 1,250 and 2,500 μg/plate [5, 3]	Negative		Evidence of toxicity was observed at 625 μg/plate and above in both strains
	Micronucleus induction	Human peripheral blood lymphocytes	60, 100, 110, 120 and 130 μg/mL [4, 6]	Positive (weak)	Stone (2012)	In pulse treatment with recovery in the absence of S9-mix, MNBN cell frequency was higher than the historical range only at the highest concentration. In the presence of S9-mix, statistically significant increases in MNBN were observed from 150 μg/mL and above and exceeded the historical range at the top two concentrations and a single replicate of 150 μg/mL. After 24 h treatment without S9-mix, all frequencies were within historical control range although statistically significant increases in MNBN cells were observed at 10 and 16 μg/mL. Study design complies with OECD Guideline 487. Acceptable levels of cytotoxicity were achieved at the top concentrations in all parts of the study
			90, 150, 160 and 180 μg/mL [5, 6]	Positive		
			10, 15, 16 and 18 μg/mL [4, 7]	Negative		

FLAVIS (FL): Flavour Information System (database); FL-no: FLAVIS number; MNBN: Micronucleated Binucleate cells; OECD: Organisation for Economic Co-operation and Development; S9-mix: metabolic activation system.
[1]: With and without S9-mix metabolic activation.
[2]: Plate incorporation method.
[3]: Pre-incubation method.
[4]: Without S9-mix metabolic activation.
[5]: With S9-mix metabolic activation.
[6]: 3-h incubation with 21-h recovery period.
[7]: 24-h incubation with no recovery period.

Appendix D – Genotoxicity studies evaluated in FGE.215Rev1

Table D.1: Summary of *in vitro* genotoxicity data for [FL-no: 07.024 and 07.030] evaluated in FGE.215Rev1

Chemical name [FL-no]	Test system	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
4-Phenylbut-3-en2-one [07.024]	Micronucleus assay	Human TK6 cells	5, 20 and 25 μg/mL[(a)]	Negative	BioReliance (2018a)	Reliable with restrictions. Study performed in compliance with GLP and according to OECD TG 487
			5, 20 and 40 μg/mL[(b)]	Negative		
			2.5, 7, 10 and 12 μg/mL[(c)]	Negative		
	Micronucleus assay with FISH analysis	Human peripheral blood lymphocytes	15, 40, 70 μg/mL[(d)]	Negative	BioReliance (2019)	Reliable without restrictions. Study performed in compliance with GLP and according to OECD TG 487 FISH analysis indicates that 4-phenylbut-3-en2-one induced MN by a clastogenic mechanism
			15, 40, 80 μg/mL[(e)]	Positive		
			5, 30, 50 μg/mL[(f)]	Negative		
1-(4-Methoxyphenyl) pent-1-en-3-one [07.030]	Micronucleus assay	Human peripheral blood lymphocytes	40, 70, and 90 μg/mL[(g)]	Negative	Covance (2013)	Reliable without restrictions. Study performed in compliance with GLP and according to OECD TG 487
			90, 120, 130 and 150 μg/mL[(h)]	Positive		
			14, 18, 22 and 25 μg/mL[(f)]	Negative		
	Micronucleus assay with CREST staining	Human TK6 cells	5, 25 and 55 μg/mL[(a)]	Positive	BioReliance (2018b)	First experiment. Reliable without restrictions. Study performed in compliance with GLP and according to OECD TG 487. Not clear why the inconsistent result between the first and the second experiment in the presence of metabolic activation
			5, 25 and 50 μg/mL[(b)]	Positive		
			5, 15 and 25 μg/mL[(c)]	Positive		
			5, 40, 55 and 60 μg/mL[(a)]	Positive		Repeated assay conducted due to the positive results observed in the first experiment Reliable without restrictions. Study performed in compliance with GLP and according to OECD TG 487 CREST analysis indicates that 1-(4-methoxyphenyl)pent-1-en-3-one induced MN by an aneugenic mechanism
			5, 20 and 50 μg/mL[(b)]	Negative		
			5, 15, 18, 25 and 28 μg/mL[(c)]	Positive		

FLAVIS (FL): Flavour Information System (database); FL-no: FLAVIS number; TK6: a lymphoblastoid cell line of human origin; MNBN: micronucleated binucleate cells; S9-mix: Metabolic activation system; GLP: Good Laboratory Practice; FISH: fluorescence *in situ* hybridisation.
(a): Without S9-mix metabolic activation, 4 + 23 h treatment.
(b): With S9-mix metabolic activation, 4 + 23 h treatment.
(c): Without S9-mix metabolic activation, 27 h treatment.
(d): Without S9-mix metabolic activation, 4 + 20 h treatment.
(e): With S9-mix metabolic activation, 4 + 20 h treatment.
(f): Without S9-mix metabolic activation, 24 h treatment.
(g): Without S9-mix metabolic activation, 3 +21 h treatment.
(h): With S9-mix metabolic activation, 3 +21 h treatment.

Table D.2: Summary of *in vivo* Genotoxicity Data for [FL-no:07.024 and 07.030] evaluated in FGE.215Rev1

Chemical name [FL-no]	Test system	Test object	Route	Dose	Result	Reference	Comments
4-Phenylbut-3-en-2-one [07.024]	Micronucleus assay (bone marrow)	Han Wistar rats (M)	Gavage	250, 500, and 1,000 mg/kg bw per day[(a)]	Negative	Covance (2014a)	Reliable with restrictions (no clear evidence of bone marrow exposure). Study performed in compliance with GLP and according to OECD TG 474
	Comet assay (liver and duodenum)				Negative		Reliable without restrictions. The study was performed in compliance with recommendations of the Comet and IWGT workshop, Japanese Center for the Validation of Alternative Methods (JaCVAM) and current literature
1-(4-Methoxyphenyl) pent-1-en-3-one [07.030]	Micronucleus assay (bone marrow)	Han Wistar rats (M)	Gavage	250, 500, and 1,000 mg/kg bw per day [(a)]	Negative	Covance (2014b)	Reliable with restrictions (no clear evidence of bone marrow exposure). Study performed in compliance with GLP and according to OECD TG 474
	Comet assay (liver and duodenum)				Negative		Reliable without restrictions. The study was performed in compliance with recommendations of the Comet and IWGT workshop, Japanese Center for the Validation of Alternative Methods (JaCVAM) and current literature

bw: body weight; FLAVIS (FL): Flavour Information System (database); FL-no: FLAVIS number; M: male; OECD: Organisation for Economic Co-operation and Development; GLP: Good Laboratory Practice.
(a): Administered via gavage in 3 doses at times 0, 24 and 45 h with sacrifice and harvest at 48 h.

Appendix E – Exposure estimates

The Triskelion database of Volatile Compounds in Food (version 16.6.1) has been searched for the three substances [FL-no: 07.030, 07.046 and 07.049]. None of them have been reported to occur in any natural or processed food products according to the Triskelion database (Triskelion, 2019).

Intended use and use levels as provided by the Flavour Industry

Use levels in the different food categories reported in Annex III of Reg. (EC) 1565/2000[3] have been submitted by the flavour industry and are reported in Table E.1 for the substances [FL-no: 07.030, 07.046 and 07.049] (EFFA, 2019).

Table E.1: Normal and Maximum use levels (mg/kg) for the substances in FGE.215Rev1 (EFFA, 2019)

FL-no	Food categories Normal use levels[(a)] (mg/kg) Maximum use levels (mg/kg)																		
	01.0	**02.0**	**03.0**	**04.1**	**04.2**	**05.0**	**05.3[(b)]**	**06.0**	**07.0**	**08.0**	**09.0**	**10.0**	**11.0**	**12.0**	**13.0**	**14.1**	**14.2**	**15.0**	**16.0**
07.030	0.36 0.41	– –	– –	– –	– –	33.22 74.19	– –	0.27 0.31	1.02 1.88	– –	– –	– –	– –	25.5 51	– –	1.05 2.06	0.1 0.1	– –	– –
07.046[(c)]	3.76 –	0.1 –	0.55 –	– –	– –	6.065 –	30 –	4.125 –	6.885 –	10 –	– –	– –	– –	0.4 –	– –	1.485 –	1 –	– –	– –
07.049[(c)]	3.76 –	0.1 –	0.55 –	– –	– –	6.065 –	30 –	4.125 –	6.885 –	10 –	– –	– –	– –	0.4 –	– –	1.485 –	1 –	– –	– –

(a): 'Normal use' is defined as the average of reported usages and 'maximum use' is defined as the 95th percentile of reported usages (EFFA, 2002).
(b): Additional food category 05.3 (chewing gum as per Annex II part D of Reg. (EC) 1333/2008) for which EFFA submitted use levels (EFFA, 2019). These have been considered in the calculation of mTAMDI.
(c): According to the information reported by industry (EFFA, 2019), no 'surveyed use levels' were available, but the data from the iterated median use levels for chemical group are representing the use of this substance. These data are representative of normal use levels.

Table E.2: Distribution of the 18 food categories listed in Commission Regulation (EC) No 1565/2000[3] into the seven SCF food categories used for TAMDI calculation (SCF, 1995)

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

TAMDI: Theoretical Added Maximum Daily Intake.
(a): For explanation of exceptions see SCF (1995).

Intake data from intended use

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

Use levels for 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030], vanillylidene acetone [FL-no: 07.046], 1-(4-methoxyphenyl)-4-methylpent-1-en-3-one [FL-no: 07.049] provided by industry (EFFA, 2019) are listed in Table 1. These data have been used to calculate the 'modified Theoretical Added Maximum Daily Intake' (mTAMDI).[8]

The MSDI and mTAMDI exposure estimates are given in Table E.3.

Table E.3: Estimated intakes based on the MSDI approach and the mTAMDI approach

FL-no	EU Register name	MSDI EU (μg/capita per day)	mTAMDI (μg/person per day)
07.030	1-(4-Methoxyphenyl)pent-1-en-3-one	0.24[(a)] 0.04[(b)]	1,900[(c)]
07.046	Vanillylidene acetone	0[(a),(b)]	2,070[(d)]
07.049	1-(4-Methoxyphenyl)-4-methylpent-1-en-3-one	0[(a),(b)]	2,070[(d)]

MSDI: Maximised Survey-derived Daily Intake; mTAMDI: modified Theoretical Added Maximum Daily Intake.
(a): MSDI value calculated based on EFFA poundage survey covering 2010, submitted by EFFA to European Commission (EFFA, 2017).
(b): MSDI value calculated based on EFFA poundage survey covering 2015, submitted by EFFA to European Commission (EFFA, 2017).
(c): mTAMDI calculated based on survey data (EFFA, 2019).
(d): mTAMDI calculated based on iterated use levels data (EFFA, 2019).

[8] mTAMDI estimation is based on an approach used by the SCF up to 1995 (SCF, 1995) and is calculated on the basis of standard portions and normal use levels for flavoured beverages and foods in general, with exceptional levels for particular foods.