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SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 215 (FGE.215): Seven α,β-Unsaturated Cinnamyl Ketones from subgroup 3.2 of FGE.19[1]

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

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids of the European Food Safety Authority was requested to evaluate the genotoxic potential of flavouring substances from subgroup 3.2 of FGE.19 in the Flavouring Group Evaluation 215 (FGE.215). The Flavour Industry has provided *in vitro* genotoxicity studies for the representative substances in FGE.215, namely 4-phenylbut-3-en-2-one [FL-no: 07.024] and 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030]. Based on these genotoxicity data, the Panel concluded that the genotoxicity concern could not be ruled out and *in vivo* genotoxicity data are requested.

KEY WORDS

α,β-unsaturated aldehydes, straight chain, FGE.215, α,β-unsaturated cinnamyl ketones, subgroup 3.2, FGE.19

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SUMMARY

Following a request from the European Commission, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) was asked to deliver a scientific opinion on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Panel was asked to evaluate seven α,β-unsaturated substances in the Flavouring Group Evaluation 215 (FGE.215) using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000.

The present FGE.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 [FL-no: 02.066] for such ketones. The seven substances under consideration in FGE.215 are listed in Table 3.

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

The Flavour Industry has now 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 are going to cover the evaluation of the other five substances in this subgroup 3.2 of FGE.19.

The 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 (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 liver and duodenum are requested.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The use of flavouring is regulated under Regulation (EC) No 1334/2008[4] 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[5]. The list contains flavouring substances for which the scientific evaluation should be completed in accordance with Commission Regulation (EC) No 1565/2000[6].

At the 26th Plenary meeting of the AFC Panel on 27 - 29 November 2007, EFSA discussed the flavouring group evaluation 19 (FGE.l9). FGE.19 contains those flavouring substances which are α,β-unsaturated aldehydes or ketones and their precursors which could give rise to such carbonyl substances via hydrolysis and/or oxidation. The α,β-unsaturated aldehyde and ketone structure is considered to be a structural alert for genotoxicity. FGE.l9 was divided into subgroups. For subgroup 3.2 EFSA concluded that there is a need for additional information before conclusions on the substances in this subgroup can be reached.

Genotoxicity data on two representative materials, namely 4-phenylbut-3-en-2-one [FL-no: 07.024] and 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] from subgroup 3.2, have now been submitted by the European Flavour Association (EFFA). This information is intended to cover the re-evaluation of the above mentioned substances and of the following five substances from FGE.19 subgroup 3.2:

- 4-Phenylbut-3-en-2-ol [FL-no: 02.066]
- 3-Methyl-4-phenylbut-3-en-2-one [FL-no: 07.027]
- 1-(4-Methoxyphenyl)-4-methylpent-1-en-3-one [FL-no: 07.049]
- 4-(2,3,6-Trimethylphenyl)but-3-en-2-one [FL-no: 07.206]
- Vanillylidene acetone [FL-no: 07.046]

The Commission asks EFSA to evaluate this new information and depending on the outcome proceed to the full evaluation of the flavouring substances.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The European Commission requests the European Food Safety Authority to carry out a safety assessment on the following seven substances included in subgroup 3.2 of FGE.19: 4-phenylbut-3-en-2-ol [FL-no: 02.066], 4-phenylbut-3-en-2-one [FL-no: 07.024], 3-methyl-4-phenylbut-3-en-2-one [FL-no: 07.027], 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] and 4-(2,3,6-trimethylphenyl)but-3-en-2-one [FL-no: 07.206] in accordance with Commission Regulation (EC) No 1565/2000.

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

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

[6] Commission Regulation (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.

HISTORY OF FGE.19

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

The α,β-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity (EFSA, 2008a). The Panel noted that there were limited genotoxicity data on these flavouring substances but that positive genotoxicity studies were identified for some substances in the group.

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

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

The Panel took note of the (Q)SAR predictions by using two ISS Local Models (Benigni and Netzeva, 2007a; Benigni and Netzeva, 2007b) and four DTU-NFI MultiCASE Models (Gry et al., 2007; Nikolov et al., 2007) and the fact that there are available data on genotoxicity, *in vitro* and *in vivo*, as well as data on carcinogenicity for several substances. Based on these data the Panel decided that 15 subgroups (1.1.1, 1.2.1, 1.2.2, 1.2.3, 2.1, 2.2, 2.3, 2.5, 3.2, 4.3, 4.5, 4.6, 5.1, 5.2 and 5.3) (EFSA, 2008a) could not be evaluated through the Procedure due to concern with respect to genotoxicity. Corresponding to these subgroups, 15 Flavouring Group Evaluations (FGEs) were established: FGE.200, 204, 205, 206, 207, 208, 209, 211, 215, 219, 221, 222, 223, 224 and 225.

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

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

The Flavouring industry has now submitted additional genotoxicity data according to the list of representative substances and test strategy for each subgroup and the present FGE concerns the evaluation of these data.

ASSESSMENT

1. Presentation of the Substances in the Flavouring Group

1.1. Description

The present Flavouring Group Evaluation 215 (FGE.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 Table 3.

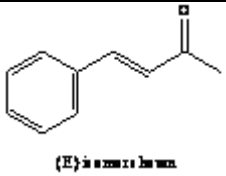
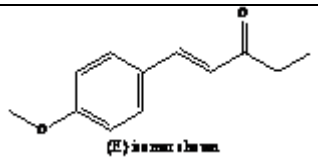
Six of the flavouring substances were previously evaluated by the JECFA (JECFA, 2002). A summary of their current evaluation status by the JECFA and the outcome of this consideration is presented in Table 4.

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.

1.2. Representative Substance for Subgroup 3.2

The Panel has identified two substances in subgroup 3.2 which will 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 representative substances are shown in Table 1.

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

FL-no JECFA-no	EU Register name	Structural formula
07.024 820	4- Phenylbut-3-en-2-one	
07.030 826	1-(4-Methoxyphenyl)pent-1-en-3-one	

2. Genotoxicity Data on the Representative Substances of Subgroup 3.2

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, *S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102, ± S9-mix (Lillford, 2009)
		in vitro Micronucleus assay in human peripheral blood lymphocytes, 3+21 hours with recovery ± S9-mix and 24+0 hours without recovery – S9-mix (Stone, 2011; Watters, 2013)
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 hours with recovery ± S9-mix and 24+0 hours without recovery – S9-mix (Stone, 2012)

2.1. *In vitro* Data

2.1.1. 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 1254-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, 1997). 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, 1000 and 5000 µg/plate, plus negative vehicle and positive controls. Evidence of toxicity in the form of complete killing of the background lawn was observed at 5000 µg/plate in the absence and presence of S9-mix. 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, 1000 and 3000 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 5000 µg/plate in the presence of S9-mix. The standard plate incorporation method was employed. Evidence of toxicity was observed at 3000 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 1000 µg/plate in strain TA1535 in the absence of S9-mix. Complete killing was observed at 5000 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, 1500 and 3000 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 minutes). Evidence of toxicity ranging from a marked reduction in revertant numbers and/or slight thinning of the bacterial lawn to a complete killing of the test bacteria was observed at 750 and/or 1500 and/or 3000 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 - 1500 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 1500 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 5000 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 pre-incubation treatments (Bowen, 2011). Study design complies with OECD Guideline 471 (OECD, 1997).

In experiment 1, all *S. typhimurium* strains were incubated with 0.32, 1.6, 8, 40, 200, 1000 and 5000 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 1000 µg/plate in strains TA1537 and TA102 and complete killing of bacteria was observed at 5000 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, 1250, 2500 and 5000 µg/plate and strains TA1537 and TA102 were also treated at a lower concentration of 78.13. The maximum test concentration of 5000 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 1250 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 2500 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 pre-incubation methodology, due to the toxicity reported in experiment 2. The maximum test concentration was reduced to 2500 µg/plate based on toxicity observed previously. Narrowed concentration intervals were employed including 19.53, 39.06, 78.13, 156.3, 312.5, 625, 1250 and 2500 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 5000 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.

2.1.2. 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 in cultured human peripheral blood lymphocytes (whole blood cultures pooled from 2 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 1462 µg/ml at 48 hours following culture initiation (stimulation by phytohaemagglutinin) either for 3 hours treatment plus 21 hours recovery in the absence or presence of S9-mix, or for 24 hours treatment without recovery in the absence of S9-mix. In the assay with 3 hours treatment and 21 hours 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 hours 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 hours recovery in the absence and presence of S9-mix and 24 hours 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 hours 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 hours 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 - 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 hours 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 hours 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 - 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 micronuclei 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 hours 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 micronuclei in cultured human peripheral blood lymphocytes when tested up to the limits of cytotoxicity for 3 + 21 hours in the absence and presence of rat liver metabolic activation (S9-mix), or for 24 + 0 hours 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 micronuclei in cultured human peripheral blood lymphocytes (pooled blood of 2 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 hours following culture initiation (stimulation by phytohaemagglutinin) either for 3 hours treatment plus 21 hours recovery in the absence or presence of S9-mix, or for 24 hours treatment without recovery in the absence of S9-mix. In the assay with 3 hours treatment and 21 hours 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-hour 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 hours 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 was above the

historical control range. For treatment with 4-phenylbut-3-en-2-one for 3 + 21 hours 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 hours 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 hours 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 hours 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 hours 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 hours 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 hours in the presence of S9-mix were also scored by 2 Analysts. The data from both Analysts showed that the treatment of cells for 3 + 21 hours 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 datasets 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 hours 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 hours 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 micronuclei in cultured human peripheral blood lymphocytes when tested up to the limits of cytotoxicity for 3 + 21 hours 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 micronuclei following 24 + 0 hours 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 micronuclei 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, 1141 and 1902 µg/ml at 48 hours following culture initiation (stimulation by phytohaemagglutinin) either for 3 hours treatment plus 21 hours recovery in the absence or presence of S9-mix, or for 24 hours treatment without recovery in the absence of S9-mix. In the assay with 3 hours treatment and 21 hours 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 hours 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 in the 24-hour 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 hours 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 hours 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 micronuclei in cultured human peripheral blood lymphocytes when tested up to the limits of cytotoxicity for 3 + 21 hours in the presence of rat liver metabolic activation (S9-mix) and weakly in the absence of metabolic activation. It did not induce micronuclei in cells treated for 24 + 0 hours in the absence of S9-mix up to toxic concentrations.

2.2. *In vivo* Genotoxicity Tests

There are no new relevant studies available.

CONCLUSION

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 micronuclei in cultured human peripheral blood lymphocytes when tested up to the limits of cytotoxicity for 3 + 21 hours in the absence and presence of rat liver metabolic activation (S9-mix). Similarly, 1-(4-methoxyphenyl)pent-1-en-3-one [FL-no: 07.030] did not induce gene mutation in bacteria. It induced micronuclei in cultured human peripheral blood lymphocytes when tested up to the limits of cytotoxicity for 3 + 21 hours 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 liver and duodenum are requested.

SPECIFICATION SUMMARY OF THE SUBSTANCES IN THE FLAVOURING GROUP EVALUATION 215

Table 3: Specification Summary of the Substances in the Present Group Evaluation

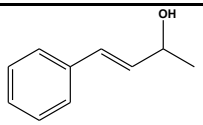
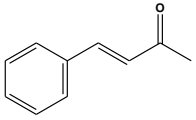
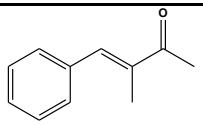
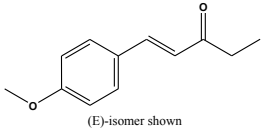
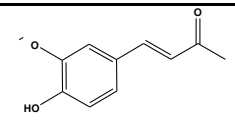
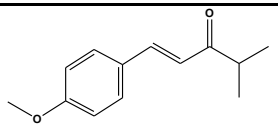
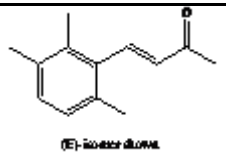
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C [c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity[e]
02.066 819	4-Phenylbut-3-en-2-ol	 (E)-isomer shown	2880 2032 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	 (E)-isomer shown	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	 (E)-isomer shown	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	 (E)-isomer shown	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	 (E)-isomer shown	3738 691 1080-12-2	Solid $C_{11}H_{12}O_3$ 192.21	Slightly soluble Moderately soluble	 129-130 IR 97 %	n.a. n.a.
07.049 829	1-(4-Methoxyphenyl)-4-methylpent-1-en-3-one	 (E)-isomer shown	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

Table 3: Specification Summary of the Substances in the Present Group Evaluation

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)]
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.

(a): Solubility in water, if not otherwise stated.
(b): Solubility in 95 % ethanol, if not otherwise stated.
(c): At 1013.25 hPa, if not otherwise stated.
(d): At 20°C, if not otherwise stated. (e): At 25°C, if not otherwise stated.

SUMMARY OF SAFETY EVALUATION APPLYING THE PROCEDURE

Table 4: Summary of Safety Evaluation of the JECFA Substances in the Present Group

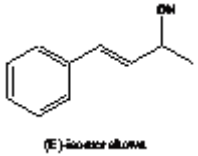
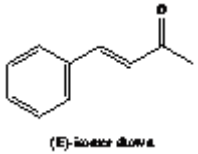
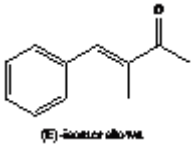
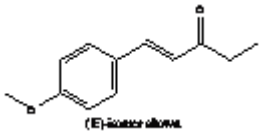
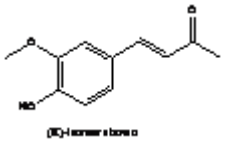
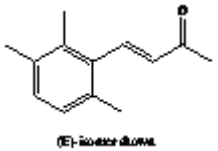
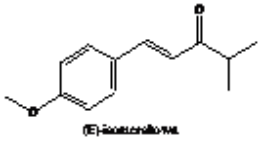
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [(a)] US MSDI (μg/*capita*/day)	Class [(b)] Evaluation procedure path [(c)]	JECFA Outcome on the named compound [(d) or (e)]	EFSA conclusion on the named compound:
02.066 819	4-Phenylbut-3-en-2-ol		2.3 0.1	Class I A3: Intake below threshold	(d)	Evaluated in FGE.215, additional genotoxicity data required
07.024 820	4-Phenylbut-3-en-2-one		2.4 7	Class I A3: Intake below threshold	(d)	Evaluated in FGE.215, additional genotoxicity data required
07.027 821	3-Methyl-4-phenylbut-3-en-2-one		0.12 0.1	Class I A3: Intake below threshold	(d)	Evaluated in FGE.215, additional genotoxicity data required
07.030 826	1-(4-Methoxyphenyl)pent-1-en-3-one		0.37 110	Class I A3: Intake below threshold	(d)	Evaluated in FGE.215, additional genotoxicity data required
07.046 732	Vanillylidene acetone		0.11 0.1	Class I A3: Intake below threshold	(d)	Evaluated in FGE.215, additional genotoxicity data required

Table 4: Summary of Safety Evaluation of the JECFA Substances in the Present Group

FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [(a)] US MSDI (µg/*capita*/day)	Class [(b)] Evaluation procedure path [(c)]	JECFA Outcome on the named compound [(d) or (e)]	EFSA conclusion on the named compound:
07.206	4-(2,3,6-Trimethylphenyl)but-3-en-2-one		0.0012	Class I No evaluation	Not evaluated by the JECFA	Evaluated in FGE.215, additional genotoxicity data required
07.049 829	1-(4-Methoxyphenyl)-4-methylpent-1-en-3-one		28 0.3	Class II A3: Intake below threshold	(d)	Evaluated in FGE.215, additional genotoxicity data required

(a): EU MSDI: Amount added to food as flavour in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita/day.
(b): Thresholds of concern: Class I = 1800 µg/person/day, Class II = 540 µg/person/day, Class III = 90 µg/person/day.
(c): Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.
(d): No safety concern based on intake calculated by the MSDI approach of the named compound.
(e): Data must be available on the substance or closely related substances to perform a safety evaluation.

GENOTOXICITY DATA

Table 5: 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	*S. typhimurium* TA98, TA1535, TA1537 and TA102	1.6, 8, 40, 200, 1000 and 3000 µg/plate [1,2]	Negative	Lillford, 2009	Evidence of toxicity was observed at 3000 µg/plate in TA100 in the absence of S9-mix and in all other strains in the absence and presence of S9-mix; at 1000 µg/plate in strain TA1535 in the absence of S9-mix; complete killing was observed at 5000 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, 1000 and 3000 µg/plate [4,2]	Negative		
			1.6, 8, 40, 200, 1000 and 5000 µg/plate [5,2]	Positive		
		S. typhimurium TA98, TA1535, TA1537, TA102	93.75, 187.5, 375, 750, 1500 and 3000 µg/plate [4, 2] or [5,3]	Negative		Evidence of toxicity was observed at 750 and/or 1500 and/or 3000 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, 1500 and 3000 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 1500 µg/plate [5,3]	Negative		Evidence of cytotoxicity was observed at 1500 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 1500 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
			40, 70, 80 and 85 µg/ml [5,6]	Negative		

Register name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
			12, 14, 17.5 and 20 µg/ml [4,7]	Negative		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.
			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 hours 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		
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, 1000 and 5000 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 1000 µg/plate in strains TA1537 and TA102 and complete killing of bacteria was observed at 5000 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, 1250, 2500 and 5000 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 1250 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 2500 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, 1250, 2500 and 5000 µg/plate [4,2] or [5,3]	Negative		
		S. typhimurium TA1535 and TA1537	19.53, 39.06, 78.13, 156.3, 312.5, 625, 1250 and 2500 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
			90, 150, 160 and 180 µg/ml [5,6]	Positive		

Register name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
			10, 15, 16 and 18 µg/ml [4,7]	Negative		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 hours 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.

[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-hour incubation with 21-hour recovery period.
[7] 24-hour incubation with no recovery period.

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ABBREVIATIONS

CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CoE	Council of Europe
EFFA	European Flavour Association
EFSA	The European Food Safety Authority
EU	European Union
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
GLP	Good Laboratory Practice
ID	Identity
IOFI	International Organization of the Flavor Industry
IR	Infrared spectroscopy
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MN	Micronuclei
MNBN	MicroNucleated BiNucleate cells
MS	Mass spectra
MSDI	Maximised Survey-derived Daily Intake
NMR	Nuclear Magnetic Resonance
No	Number
OECD	Organisation for Economic Co-operation and Development
(Q)SAR	(Quantitative) Structure Activity Relationship
WHO	World Health Organisation