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SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 216, Revision 1 (FGE.216Rev1). Consideration of genotoxic potential for $\alpha,\beta$-unsaturated 2-Phenyl -2-Alkenals from Subgroup 3.3 of FGE.19 ¹

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

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 five flavouring substances from subgroup 3.3 of FGE.19. In the Flavouring Group Evaluation 216 (FGE.216) additional genotoxicity data were requested. Additional genotoxicity studies have now been provided for the representative substance 2-phenylcrotonaldehyde [FL-no: 05.062]. Based on these new data the Panel concluded that the concern for genotoxicity could not be ruled out and requests a proof of sufficient systemic exposure of animals treated with 2-phenylcrotonaldehyde. Moreover, since the substance was genotoxic only without metabolic activation, it appears necessary to prove the absence of genotoxic effect locally in the gastrointestinal system using the Comet assay.

KEY WORDS

FGE.216, alpha,beta-unsaturated ketones, 3(2H)-furanones, flavouring substances, safety evaluation, Subgroup 3.3, FGE.19

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SUMMARY

Following a request from the European Commission the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF Panel) 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 5 flavouring substances in Flavouring Group Evaluation 216 (FGE.216) using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000.

The FGE.216 concerned five α,β-unsaturated 2-phenyl substituted aldehydes, 2-phenylcrotonaldehyde [FL-no: 05.062], 5-methyl-2-phenylhex-2-enal [FL-no: 05.099], 4-methyl-2-phenylpent-2-enal [FL-no: 05.100], 2-phenylpent-2-enal [FL-no: 05.175] and 2-phenyl-4-methyl-2-hexenal [FL-no: 05.222], corresponding to subgroup 3.3 of FGE.19. The conclusion of the Panel in FGE.216 was that the available data on genotoxicity were too limited to evaluate the five substances through the Procedure and additional genotoxicity studies were requested.

The Flavouring Industry has now submitted new data in reply to the above requested data for FGE.19 subgroup 3.3 (FGE.216) for the representative flavouring substance, 2-phenylcrotonaldehyde [FL-no: 05.062], covering the remaining four substances [FL-no: 05.099, 05.100, 05.175 and 05.222].

Based on these new data, the Panel concluded that the concern for genotoxicity could not be ruled out and requests a proof of sufficient systemic exposure of animals treated with 2-phenylcrotonaldehyde. Moreover, since the substance was genotoxic only without metabolic activation, it appears necessary to prove the absence of genotoxic effect locally in the gastrointestinal system using the Comet assay.
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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

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

EFSA has evaluated five flavouring substances, which correspond to subgroup 3.3 of FGE.19, in its evaluation of the flavouring group 216 (FGE.216). The opinion was adopted on 27 November 2008. The Panel concluded that a genotoxic potential of the five 2-phenyl-substituted aldehydes (i.e. 2-phenyl-2-alkenals) in the present FGE.216 could not be ruled out.

Information on one representative material, 2-phenylcrotonaldehyde [FL-no: 05.062], has now been submitted by the European Flavour Association. This information is intended to cover the re-evaluation of this substance and of the following four substances from FGE.19 subgroup 3.3:

- 5-Methyl-2-phenylhex-2-enal [FL-no: 05.099]
- 4-Methyl-2-phenylpent-2-enal [FL-no: 05.100]
- 2-Phenylpent-2-enal [FL-no: 05.175]
- 2-Phenyl-4-methyl-2-hexenal [FL-no: 05.222]

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 five substances: 2-phenylcrotonaldehyde [FL-no: 05.062], 5-methyl-2-phenylhex-2-enal [FL-no: 05.099], 4-methyl-2-phenylpent-2-enal [FL-no: 05.100], 2-phenylpent-2-enal [FL-no: 05.175] and 2-phenyl-4-methyl-2-hexenal [FL-no: 05.222] in accordance with Commission Regulation (EC) No 1565/2000.
**Flavouring Group Evaluation 216Rev1**

**HISTORY**

Regulation (EC) No 2232/96 of the European Parliament and the Council (EC, 1996) lays down a Procedure for the establishment of a list of flavouring substances, the use of which will be authorised to the exclusion of all other substances in the EU. In application of that Regulation, a Register of flavouring substances used in or on foodstuffs in the Member States was adopted by Commission Decision 1999/217/EC (EC, 1999), as last amended by Commission Decision 2009/163/EC (EC, 2009). Each flavouring substance is attributed a FLAVIS-number (FL-number) and all substances are divided into 34 chemical groups. Substances within a group should have some metabolic and biological behaviour in common.

Substances which are listed in the Register are to be evaluated according to the evaluation programme laid down in Commission Regulation (EC) No 1565/2000 (EC, 2000), which is broadly based on the opinion of the Scientific Committee on Food (SCF, 1999). For the submission of data by the manufacturer, deadlines have been established by Commission Regulation (EC) No 622/2002 (EC, 2002).

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

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. 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 (QSAR) 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, 2007) and four DTU-NFI MultiCASE Models (Gry et al., 2007; Nikolov et al., 2007) and the fact that there are available data on genotoxicity, *in vitro* and *in vivo*, as well as data on carcinogenicity for several substances. The Panel decided that 11 subgroups (1.1.2, 1.1.3, 1.1.4, 2.4, 2.6, 2.7, 3.1, 3.3, 4.1, 4.2 and 4.4) of FGE.19 (EFSA, 2008a) should be further examined to determine whether evaluation through the Procedure is feasible. Corresponding to these 11 subgroups, 11 Flavouring Group Evaluations (FGEs) were established (FGE.201, 202, 203, 210, 212, 213, 214, 216, 217, 218 and 220). If the Panel concludes for any substances in these 11 FGEs that they cannot be evaluated using the Procedure, then it has to be decided if there is a safety concern for certain substances or if additional data are required in order to finalise the evaluation. If the Panel concludes that a genotoxic potential can be ruled out for the substances, they will be merged with structurally related substances in other FGEs and evaluated using the Procedure.

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

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

The Flavouring Industry has now submitted additional data and the present revision of FGE.216 concerns the evaluation of these data requested on genotoxicity.

PRESENTATION OF THE SUBSTANCES BELONGING TO THE FLAVOURING GROUP EVALUATION 216 CORRESPONDING TO FGE.19 SUBGROUP 3.3

The Flavouring Group Evaluation 216 (FGE.216) concerns five \( \alpha,\beta \)-unsaturated 2-phenyl substituted aldehydes, 2-phenylcrotonaldehyde [FL-no: 05.062], 5-methyl-2-phenylhex-2-enal [FL-no: 05.099], 4-methyl-2-phenylpent-2-enal [FL-no: 05.100], 2-phenylpent-2-enal [FL-no: 05.175] and 2-phenyl-4-methyl-2-hexenal [FL-no: 05.222], which are presented in Table 1. The five substances correspond to subgroup 3.3 of FGE.19.

The \( \alpha,\beta \)-unsaturated aldehyde and ketone structures are structural alerts for genotoxicity (EFSA, 2008a). Accordingly, the available data on genotoxic or carcinogenic activity for the five aldehydes [FL-no: 05.062, 05.099, 05.100, 05.175 and 05.222] will be considered in this FGE.
### Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 216Rev1

<table>
<thead>
<tr>
<th>FL-no</th>
<th>EU Register name</th>
<th>Structural formula</th>
<th>FEMA no</th>
<th>Mol.formula</th>
<th>Solubility 1) Solubility in ethanol 2)</th>
<th>Boiling point, °C 3)</th>
<th>Melting point, °C</th>
<th>Refrac. Index 4)</th>
<th>Spec.gravity 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>05.062</td>
<td>2-Phenylcrotonaldehyde</td>
<td><img src="image" alt="Structural formula" /></td>
<td>3224</td>
<td>C₁₀H₁₀O</td>
<td>Practically insoluble or insoluble</td>
<td>177 (20 hPa)</td>
<td>NMR 97 %</td>
<td>1.558-1.564</td>
<td>1.031-1.037</td>
</tr>
<tr>
<td>05.099</td>
<td>5-Methyl-2-phenylhex-2-enal</td>
<td><img src="image" alt="Structural formula" /></td>
<td>3199</td>
<td>C₁₃H₁₆O</td>
<td>Freely soluble</td>
<td>96-100 (0.9 hPa)</td>
<td>NMR 96 %</td>
<td>1.531-1.536</td>
<td>0.970-0.976</td>
</tr>
<tr>
<td>05.100</td>
<td>4-Methyl-2-phenylpent-2-enal</td>
<td><img src="image" alt="Structural formula" /></td>
<td>3200</td>
<td>C₁₂H₁₄O</td>
<td>Practically insoluble or insoluble Freely soluble</td>
<td>96 (0.9 hPa)</td>
<td>NMR 95 %</td>
<td>1.533-1.539</td>
<td>0.980-0.986</td>
</tr>
<tr>
<td>05.175</td>
<td>2-Phenylpent-2-enal 6)</td>
<td><img src="image" alt="Structural formula" /></td>
<td>3491-63-2</td>
<td>C₁₁H₁₂O</td>
<td></td>
<td>126 (15 hPa)</td>
<td>MS 95 %</td>
<td>1.545-1.553</td>
<td>1.005-1.015</td>
</tr>
<tr>
<td>05.222</td>
<td>2-Phenyl-4-methyl-2-hexenal</td>
<td><img src="image" alt="Structural formula" /></td>
<td>4194</td>
<td>C₁₃H₁₆O</td>
<td>Insoluble</td>
<td>97 (0.6 hPa)</td>
<td>95 %</td>
<td>1.522-1.530</td>
<td>0.965-0.975</td>
</tr>
</tbody>
</table>

1) Solubility in water, if not otherwise stated.
2) Solubility in 95 % ethanol, if not otherwise stated.
3) At 1013.25 hPa, if not otherwise stated.
4) At 20°C, if not otherwise stated.
5) At 25°C, if not otherwise stated.
ASSESSMENT

1. History of the FGE.216 Evaluation

In the first scientific opinion on FGE.216 (EFSA, 2009), the Panel concluded that a genotoxic potential of the five 2-phenyl-substituted aldehydes (i.e. 2-phenyl-2-alkenals) could not be ruled out and therefore the five substances could not be evaluated through the Procedure. Additional data on genotoxicity for representative substances of this subgroup should be provided, according to the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19 (EFSA, 2008b).

In the EFSA Opinion “List of α,β-unsaturated aldehydes and ketones representative of FGE.19 substances for genotoxicity testing” (EFSA, 2008c), a representative flavouring substance has been selected for FGE.19, subgroup 3.3, corresponding to FGE.216. The representative substance is 2-phenylcrotonaldehyde [FL-no: 05.062] (Table 2).

The Panel viewed the previous JEFCA evaluation (JECFA, 2006) and reached the conclusions based on the data available at that time. These included a (Q)SAR Prediction analysis (Table 4). No data from genotoxicity or carcinogenicity studies with any of the substances in FGE.216 were available.

In Table 4, the outcome of the (Q)SAR predictions for possible genotoxic activity in the five in vitro (Q)SAR models (ISS Local Model-Ames test, DTU-NFI MultiCASE-Ames test, -Chromosomal aberration test in Chinese hamster ovary cells (CHO), Chromosomal aberration test in Chinese hamster lung cells (CHL), and Mouse lymphoma test) are presented.

The data available were insufficient to rule out the concern for genotoxicity.

2. Additional Genotoxicity Data Submitted for FGE.19, subgroup 3.3

The present revision of FGE.216, Revision 1 (FGE.216Rev1) concerns the evaluation of new genotoxicity data submitted by European Flavour and Fragrance Association (EFFA), in response to the request by EFSA in FGE.216, for the representative substance 2-phenylcrotonaldehyde [FL-no: 05.062], which is supposed to cover the genotoxicity evaluation of the four other substances in FGE.19, subgroup 3.3, 5-methyl-2-phenylhex-2-enal [FL-no: 05.099], 4-methyl-2-phenylpent-2-enal [FL-no: 05.100], 2-phenylpent-2-enal [FL-no: 05.175] and 2-phenyl-4-methyl-2-hexenal [FL-no: 05.222].
The new data submitted covers *in vitro* assays in bacteria and mammalian cell systems and *in vivo* data in the rat.

### 2.1. *In vitro* data

#### 2.1.1. Bacterial Reverse Mutation Assay

Ames assays were conducted in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 to assess the mutagenicity of 2-phenylcrotonaldehyde [FL-no: 05.062] (98.1% sum of isomers), 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 assays using both standard plate incorporation and modified pre-incubation treatments (Kilford, 2010). The protocol followed OECD Test Guideline 471 (OECD, 1997a) and the study was performed according to GLP.

In assay 1, no increases in revertant numbers were observed when *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 were incubated with 2-phenylcrotonaldehyde [FL-no: 05.062] up to 5000 μg/plate in the absence and presence of S9-mix using the standard plate incorporation method. A weak to moderate bacteriostatic activity was noted at concentrations of 1000 μg/plate and above in strains TA98 and TA102 in the absence of S9-mix and in strains TA1537 and TA102 in the presence of S9-mix.

In assay 2, no increases in revertant numbers were observed when *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 were treated with 2-phenylcrotonaldehyde [FL-no: 05.062] up to 5000 μg/plate in the absence of S9-mix. In the presence of S9-mix, the same concentrations were tested on strains TA98, TA100 and TA1535, whereas TA1537 and TA102 were treated up to 2000 μg/plate due to an excessive level of cytotoxicity in the first assay. A marked reduction in revertant numbers and/or slight thinning of the bacterial lawn was noted in all the high doses tested. No increase of revertants was observed except in the treatments of the TA100 strain in the absence of S9-mix at a concentration of 2000 μg/plate and in the presence of S9-mix at a concentration of 320 μg/plate. The increase in revertant mutations was statistically significant (p < 0.01), but these results were isolated and not reproducible in further assays.

To further explore the increase in mutations seen only in *S. typhimurium* strain TA100, assay 3 was performed in all tester strains in the presence of S9-mix and in the absence of S9-mix in strain TA100. No mutagenic effect was demonstrated.

Under these conditions, 2-phenylcrotonaldehyde [FL-no: 05.062] demonstrated no mutagenic activity in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 both in the absence and in the presence of metabolic activation.

#### 2.1.2. Micronucleus assays

2-Phenylcrotonaldehyde [FL-no: 05.062] was tested to determine its clastogenic or aneugenic potential in mammalian cells *in vitro* using the micronucleus test in cultured human peripheral blood lymphocytes with and without metabolic activation (Lloyd, 2012). The test was performed according the OECD Test Guideline 487 (OECD, 2010) (except that the assay with metabolic activation was not repeated) and performed according to GLP Guidelines.

The range of doses was determined in a preliminary range finding study.

In assay 1, 2-phenylcrotonaldehyde [FL-no: 05.062] was added for 3 hours with a 21 hours recovery period in the absence of S9-mix and examined at concentrations of 40, 60, 100 and 120 μg/mL. The frequency of MNBN cells was statistically higher (p < 0.001) than vehicle controls at 100 and 120 μg/mL with 26 and 66% of cytotoxicity, respectively. The frequencies of MNBN cells exceeded the 95th percentile observed range only at 120 μg/mL (in both cultures), indicating a weak but significant induction of chromosomal damage. It was also added to cultures for 3 hours with 21 hours recovery in
the presence of S9-mix at concentrations of 100, 130 and 140 μg/mL. The frequency of MNBN cells were significantly higher (p < 0.05) at the two highest concentrations analysed, 130 and 140 μg/mL, but fell clearly within normal ranges based on historical control data. Cultures were also treated for 24 + 0 hours in the absence of S9-mix at concentrations of 20, 23 and 26 μg/mL in the absence of S9-mix. The frequencies of MNBN cells were significantly higher (p < 0.05) than those observed in concurrent vehicle controls at all three concentrations (20, 23 and 26 μg/mL), but also fell within normal ranges based on historical control data. These data were considered difficult to interpret due to the steep concentration related cytotoxicity that was observed under all three treatment conditions as indicated by decreases in the replication index values of 13, 25 and 43 %, respectively.

In assay 2, cultures were treated with 2-phenylcrotonaldehyde [FL-no: 05.062] at concentrations of 20, 60, 70 and 80 μg/mL for 3 hours with 21 hours recovery in the absence of S9-mix. The frequency of MNBN cells was significantly higher (p < 0.01) compared to those observed in concurrent vehicle controls at 20, 70 and 80 μg/mL, but not at 60 μg/mL. The MNBN cell frequencies in both cultures at 20 and 70 μg/mL and in one culture at 80 μg/mL exceeded the 95th percentile of the historical control range. These observations indicate the induction of micronuclei at concentrations at or below the limit of cytotoxicity.

In conclusion, 2-phenylcrotonaldehyde [FL-no: 05.062] induced a significant increase of micronuclei in cultured human peripheral blood lymphocytes when tested for 3 + 21 hours in the absence of rat liver metabolic activation (S9-mix). In the same test system, 2-phenylcrotonaldehyde did not induce micronuclei when tested up to toxic concentrations for 3 + 21 hours in the presence of S9-mix and for 24 + 0 hours in the absence of S9-mix.

A summary of the in vitro data are presented in Table 5.

2.2. In vivo data

2.2.1. Bone Marrow Micronucleus Induction Assay in the rat

An in vivo micronucleus assay in rats was performed in compliance with OECD Test Guideline 474 (OECD, 1997b) (Henderson, 2012) to determine whether the results obtained in the initial in vitro micronucleus assay reflect the situation in vivo.

An initial Range-Finding study was conducted in Han-Wistar rats to estimate the Maximum Tolerated Dose (MTD) of 2-phenylcrotonaldehyde [FL-no: 05.062], administered by oral gavage. The dose of 700 mg/kg body weight (bw)/day was selected as the MTD based on displayed toxicity at the higher dose levels.

Groups of six male Han-Wistar rats were treated via gavage with 2-phenylcrotonaldehyde [FL-no: 05.062] at doses of 0 (vehicle control), 70, 350 and 700 mg/kg bw/day. Animals were dosed at 0 and 24 hours, followed by sacrifice and harvest of the femoral bone marrow at 24 hours after the last treatment.

Rats treated with 2-phenylcrotonaldehyde [FL-no: 05.062] at all doses exhibited group mean % of PCE that were similar to the vehicle control group. This parameter cannot be used to demonstrate systemic exposure of animals.

In rats treated with 2-phenylcrotonaldehyde [FL-no: 05.062] there were no statistically significant increases in micronucleus frequency for any of the groups receiving the test article, compared to the concurrent vehicle control, with the exception of the intermediate dose group, which was nonetheless well within the historic control range and the difference was due to the very low concurrent control frequencies.
The authors of the report concluded that 2-phenylcrotonaldehyde [FL-no: 05.062] did not induce micronuclei in the polychromatic erythrocytes of the bone marrow of male rats treated at 70 and 700 mg/kg bw/day but that it induced a small statistically significant increase in MN PCE observed at the intermediate dose (350 mg/kg bw/day), and they concluded that the small increase observed at 350 mg/kg bw/day is of questionable biological relevance. The intermediate dose produced a mean MN frequency that was two fold greater than and statistically (p < 0.05) higher than the vehicle control group. The mean value (2.83 MN/2000 PCE) was well within the laboratory’s historical range (0.74 - 4.46 MN/2000 PCE). However, individual results of the first reading demonstrated that MN frequency of 4 out of the 6 treated animals exceed the 95 % confidence interval for mean of historical controls and all the individual values of the control animals were within the limit of historical controls.

The data generated from a second set of 2000 PCE gave a similar response across all test article groups with all individual values falling ‘normally’ within the historical distribution. However, the concurrent vehicle control frequencies were distributed at the low end of the historical data producing a low background level for comparison.

The values obtained in this second reading were:

<table>
<thead>
<tr>
<th>% MNPCE</th>
<th>Reading 1</th>
<th>Reading 2</th>
<th>Reading mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.10</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td>75 mg/kg</td>
<td>0.09</td>
<td>0.12</td>
<td>0.10</td>
</tr>
<tr>
<td>350 mg/kg</td>
<td>0.18*</td>
<td>0.10</td>
<td>0.14*</td>
</tr>
<tr>
<td>700 mg/kg</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
</tr>
</tbody>
</table>

* P < 0.01

The results demonstrate that after a second reading the increase is still significant when the second reading are pooled.

Plasma of animals of a satellite group were taken but not analysed for 2-phenylcrotonaldehyde [FL-no: 05.062] content. Under these conditions no clear proof of exposure was given.

Moreover, the product 2-phenylcrotonaldehyde [FL-no: 05.062] was found to be positive without activation in the in vitro micronucleus test, i.e. after oral absorption, the gastrointestinal tract is the organ most exposed to high concentrations, which will not be found after systemic passage at the medullary level. Under these conditions, it appears necessary to have information of genotoxic potential of the product 2-phenylcrotonaldehyde [FL-no: 05.062] in the gastrointestinal mucosa by a Comet assay in the stomach or duodenum.

A summary of the in vivo data are presented in Table 6.

3. Conclusion

The FGE.216 concerned five α,β-unsaturated 2-phenyl substituted aldehydes, 2-phenylcrotonaldehyde [FL-no: 05.062], 5-methyl-2-phenylhex-2-enal [FL-no: 05.099], 4-methyl-2-phenylpent-2-enal [FL-no: 05.100], 2-phenylpent-2-enal [FL-no: 05.175] and 2-phenyl-4-methyl-2-hexenal [FL-no: 05.222], corresponding to subgroup 3.3 of FGE.19. The conclusion of the Panel in FGE.216 was that the available data on genotoxicity were too limited to evaluate the five substances through the Procedure and additional genotoxicity data were requested.

The Flavouring Industry has now submitted new data in reply to the above requested data for FGE.19 subgroup 3.3 (FGE.216) for the representative flavouring substance, 2-phenylcrotonaldehyde [FL-no: 05.062], covering the remaining four substances [FL-no: 05.099, 05.100, 05.175 and 05.222].
The product 2-phenylcrotonaldehyde [FL-no: 05.062] did not demonstrate any mutagenic effect in a bacterial test with and without metabolic activation. However, it showed a genotoxic effect in the *in vitro* micronucleus test in cultured human lymphocytes in the absence of metabolic activation.

In order to verify that this genotoxic potential demonstrated *in vitro* was confirmed *in vivo*, a micronucleus test was conducted in the rat bone marrow by oral route which led to an ambiguous result, because only the intermediate dose induced a statistically significant increase of MNPCE, even after rereading the slides. No evidence of systemic exposure of animals was provided in this study, in particular, no change in the percentage of PCE in the bone marrow was noted and plasma of animals sampled in a satellite group have not been analysed.

Under these conditions it appears necessary to provide proof of sufficient systemic exposure of animals treated with 2-phenylcrotonaldehyde.

Moreover, since the substance was genotoxic only without metabolic activation, it appears necessary to prove the absence of genotoxic effect locally in the gastrointestinal system using the Comet assay.
SUMMARY OF SAFETY EVALUATION APPLYING THE PROCEDURE (BASED ON THE MSDI APPROACH)

Table 3:  Summary of Safety Evaluation Applying the Procedure (based on intakes calculated by the MSDI approach) (JECFA, 2006)

<table>
<thead>
<tr>
<th>FL-no</th>
<th>EU Register name</th>
<th>Structural formula</th>
<th>MSDI 1) (µg/capita/day)</th>
<th>Class 2) Evaluation procedure path 3) (JECFA)</th>
<th>Outcome on the named compound [4 or 5]</th>
<th>EFSA comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>05.062</td>
<td>2-Phenylcrotonaldehyde</td>
<td></td>
<td>1.7</td>
<td>Class I A3: Intake below threshold</td>
<td>4)</td>
<td>Evaluated in FGE.216Rev1, additional genotoxicity data required.</td>
</tr>
<tr>
<td>05.099</td>
<td>5-Methyl-2-phenylhex-2-enal</td>
<td></td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>05.100</td>
<td>4-Methyl-2-phenylpent-2-enal</td>
<td></td>
<td>0.34</td>
<td>Class II A3: Intake below threshold</td>
<td>4)</td>
<td>Evaluated in FGE.216Rev1, additional genotoxicity data required.</td>
</tr>
<tr>
<td>05.175</td>
<td>2-Phenylpent-2-enal</td>
<td></td>
<td>0.011</td>
<td>Class II No evaluation</td>
<td></td>
<td>Not evaluated by JECFA</td>
</tr>
<tr>
<td>05.222</td>
<td>2-Phenyl-4-methyl-2-hexenal</td>
<td></td>
<td>3.0</td>
<td>No evaluation</td>
<td></td>
<td>Evaluated in FGE.216Rev1, additional genotoxicity data required.</td>
</tr>
</tbody>
</table>

1) EU MSDI: Amount added to food as flavour in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita/day.
2) Thresholds of concern: Class I = 1800 µg/person/day, Class II = 540 µg/person/day, Class III = 90 µg/person/day.
3) Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.
4) No safety concern based on intake calculated by the MSDI approach of the named compound.
5) Data must be available on the substance or closely related substances to perform a safety evaluation.
### Table 4: QSAR Predictions on Mutagenicity for Five Aldehydes from Subgroup 3.3

<table>
<thead>
<tr>
<th>FL-no</th>
<th>JEFCFA-no</th>
<th>Sub-group</th>
<th>EU Register name</th>
<th>Structural formula</th>
<th>FEMA no</th>
<th>CAS no</th>
<th>ISS Local Model Ames Test TA100</th>
<th>MultiCASE Ames test</th>
<th>MultiCASE Mouse lymphoma test</th>
<th>MultiCASE Chromosomal aberration test in CHO</th>
<th>MultiCASE Chromosomal aberration test in CHL</th>
</tr>
</thead>
<tbody>
<tr>
<td>05.062</td>
<td>1474</td>
<td>3.3</td>
<td>2-Phenylcrotonaldehyde</td>
<td><img src="image" alt="Structural formula" /></td>
<td>3224</td>
<td>670</td>
<td>4411-89-6</td>
<td>NEG</td>
<td>OD</td>
<td>OD</td>
<td>OD</td>
</tr>
<tr>
<td>05.099</td>
<td>1472</td>
<td>3.3</td>
<td>5-Methyl-2-phenylhex-2-enal</td>
<td><img src="image" alt="Structural formula" /></td>
<td>3199</td>
<td>10365</td>
<td>21834-92-4</td>
<td>NEG</td>
<td>OD</td>
<td>OD</td>
<td>OD</td>
</tr>
<tr>
<td>05.100</td>
<td>1473</td>
<td>3.3</td>
<td>4-Methyl-2-phenylpent-2-enal</td>
<td><img src="image" alt="Structural formula" /></td>
<td>3200</td>
<td>10366</td>
<td>26643-91-4</td>
<td>NEG</td>
<td>OD</td>
<td>OD</td>
<td>OD</td>
</tr>
<tr>
<td>05.175</td>
<td></td>
<td>3.3</td>
<td>2-Phenylpent-2-enal 6)</td>
<td><img src="image" alt="Structural formula" /></td>
<td>-</td>
<td>3491-63-2</td>
<td></td>
<td>NEG</td>
<td>OD</td>
<td>OD</td>
<td>OD</td>
</tr>
<tr>
<td>05.222</td>
<td></td>
<td>3.3</td>
<td>2-Phenyl-4-methyl-2-hexenal</td>
<td><img src="image" alt="Structural formula" /></td>
<td>-</td>
<td>26643-92-5</td>
<td></td>
<td>NEG</td>
<td>OD</td>
<td>OD</td>
<td>OD</td>
</tr>
</tbody>
</table>

Column 2: Structure group 4.4: α,β-unsaturated ketones.
Column 6: Local model on aldehydes and ketones, Ames TA100. (NEG: Negative; POS: Positive; OD*: out of domain).
Column 7: MultiCase Ames test (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
Column 8: MultiCase Mouse lymphoma test (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
Column 9: MultiCase Chromosomal aberration in CHO (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
Column 10: MultiCase Chromosomal aberration in CHL (OD*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

* OD, out of applicability domain: not matching the range of conditions where a reliable prediction can be obtained in this model. These conditions may be physicochemical, structural, biological etc.
### SUMMARY OF ADDITIONAL GENOTOXICITY DATA ON 2-PHENYLCROTONALDEHYDE [FL-NO: 05.062] SUBMITTED BY INDUSTRY

#### Table 5: Summary of Additionally submitted genotoxicity data on [FL-no: 05.062] of subgroup 3.3 (*in vitro*)

<table>
<thead>
<tr>
<th>FL-no</th>
<th>Chemical Name</th>
<th>Test System <em>in vitro</em></th>
<th>Test Object</th>
<th>Concentrations of Substance and Test Conditions</th>
<th>Result</th>
<th>Reference</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>05.062</td>
<td>2-Phenylcrotonaldehyde</td>
<td>Reverse Mutation</td>
<td>S. typhimurium TA98, TA100, TA1535, TA1537 and TA102</td>
<td>1.6, 8, 40, 200, 1000 and 5000 μg/plate [1,2]</td>
<td>Negative</td>
<td>(Kilford, 2010)</td>
<td>Toxicity was observed in all strains at 5000 μg/plate in the absence and presence of S9, and at 1000 μg/plate and above in strains TA98 and TA102 in the absence of S9 and in strains TA1537 and TA102 in the presence of S9. All strains were negative. Study design complied with current recommendations. Acceptable top concentration was achieved.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S. typhimurium TA98, TA100, TA1535, TA1537 and TA102</td>
<td>20.48, 51.2, 128, 320, 800, 2000 and 5000 μg/plate [2,4]</td>
<td>Negative</td>
<td>(Kilford, 2010)</td>
<td>Toxicity was observed in strains TA1537 and TA102 at 2000 μg/plate and above in the absence of S9 and at 320 μg/plate in the presence of S9. Similar toxicity was also observed in strains TA98, TA100 and TA1535 at 5000 μg/plate in the absence of S9 and at 800 μg/plate and above in the presence of S9. Statistically significant differences in mutation frequency were observed only in strain TA100 and only at levels of toxicity (in the absence of S9-mix at a concentration of 2000 μg/plate and in the presence of S9-mix at 320 μg/plate). Study design complied with current recommendations. Acceptable top concentration was achieved.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S. typhimurium TA98, TA100, TA1535, TA1537 and TA102</td>
<td>51.2, 128, 320, 800, 2000 and 5000 μg/plate [3,5]</td>
<td>Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S. typhimurium TA1537 and TA102</td>
<td>31.25 - 1000 μg/plate [3,5]</td>
<td>Negative</td>
<td>(Kilford, 2010)</td>
<td>Toxicity was observed at 3500 μg/plate and above in strain TA100 in the absence of S9. In the presence of S9, toxicity was observed at 250 μg/plate and above in strains TA1537 and TA102 and at 1000 μg/plate and above in strains TA100, TA98 and TA1535.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S. typhimurium TA1537, TA102</td>
<td>15.625 - 500 μg/plate [3,5]</td>
<td>Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>S. typhimurium TA100</td>
<td>320 - 5000 μg/plate [2,4]</td>
<td>Negative</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 5: Summary of Additionally submitted genotoxicity data on [FL-no: 05.062] of subgroup 3.3 (*in vitro*)

<table>
<thead>
<tr>
<th>FL-no</th>
<th>Chemical Name</th>
<th>Test System <em>in vitro</em></th>
<th>Test Object</th>
<th>Concentrations of Substance and Test Conditions</th>
<th>Result</th>
<th>Reference</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Micronucleus induction</td>
<td>Human peripheral blood lymphocytes</td>
<td>40, 60, 100, 120 μg/mL [4,6]</td>
<td>Positive</td>
<td>(Lloyd, 2012)</td>
<td>The MNBN cell frequencies increases were statistically significant at the top two concentrations but only slightly exceeded the 95% range of historic controls at the highest dose. All other treated cultures fell within the normal range. The study complies with OECD Test Guideline 487 (OECD, 2010).</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>100, 130, 140 μg/mL [5,6]</td>
<td>Negative</td>
<td>(Lloyd, 2012)</td>
<td>The MNBN cell frequencies increases were statistically significant at the top two concentrations but all treated cultures fell within the normal range. The study complies with OECD Test Guideline 487 (OECD, 2010).</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20, 23, 26 μg/mL [4,7]</td>
<td>Negative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20, 60, 70 and 80 μg/mL [4,6]</td>
<td>Positive</td>
<td>(Lloyd, 2012)</td>
<td>The MNBN cell frequencies in both cultures at 20 and 70 μg/mL and in one culture at 80 μg/mL exceeded the 95th percentile of the historical control range. The study complies with OECD Test Guideline 487 (OECD, 2010).</td>
<td></td>
</tr>
</tbody>
</table>

[1] With and without S9 metabolic activation.  
Table 6: Summary of Additionally Genotoxicity Data [FL-no: 05.062] of Subgroup 3.3 (in vivo)

<table>
<thead>
<tr>
<th>FL-no</th>
<th>Chemical Name</th>
<th>Test System in vivo</th>
<th>Test Object Route</th>
<th>Concentrations of Substance and Test Conditions</th>
<th>Result</th>
<th>Reference</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>[05.062]</td>
<td>2-Phenyldienealdehyd</td>
<td>Micronucleus induction</td>
<td>Rat Gavage</td>
<td>70, 350, and 700 mg/kg bw/day</td>
<td>Negative</td>
<td>(Henderson, 2012)</td>
<td>The study complies with OECD Test Guideline 474 (OECD, 1997b). Acceptable levels of cytotoxicity achieved at the top concentrations used.</td>
</tr>
</tbody>
</table>
REFERENCES


Flavouring Group Evaluation 216Rev1


ABBREVIATIONS

BW       Body Weight
CAS      Chemical Abstract Service
CEF      Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
         Chemical Abstract Service
CHO      Chinese hamster ovary (cells)
CHL      Chinese hamster lung (cells)
CoE      Council of Europe
EC       European Commission
EFFA     European Flavour and Fragrance Association
EFSA     The European Food Safety Authority
EU       European Union
FAO      Food and Agriculture Organization of the United Nations
FEMA     Flavor and Extract Manufacturers Association
FGE      Flavouring Group Evaluation
FLAVIS (FL) Flavour Information System (database)
GLP      Good Laboratory Practice
ID       Identity
JECFA    The Joint FAO/WHO Expert Committee on Food Additives
MNBN     MicroNucleated BiNucleate cells
MS       Mass spectrometry
MTD      Maximum Tolerated Dose
NMR      Nuclear Magnetic Resonance
No       Number
OECD     Organisation for Economic Co-operation and Development
PCE      PolyChromatic Erythrocytes
(Q)SAR   (Quantitative) Structure-Activity Relationship
SCF      Scientific Committee on Food
WHO      World Health Organisation