EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2013. Scientific Opinion on Flavouring Group Evaluation 216, Revision 1 (FGE.216Rev1). Consideration of genotoxic potential for ,-unsaturated 2-Phenyl -2-Alkenals from Subgroup 3.3 of FGE.19

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SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 216, Revision 1 (FGE.216Rev1). Consideration of genotoxic potential for α,β-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, 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 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 gastro intestinal 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

[1] On request from the European Commission, Question No EFSA-Q-2013-00170, EFSA-Q-2013-00171, EFSA-Q-2013-00172, EFSA-Q-2013-00173, EFSA-Q-2013-00174, adopted on 4 July 2013

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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.

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 (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, 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.

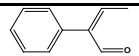
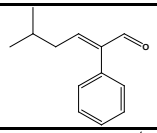
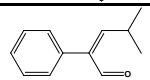
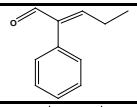
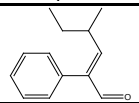
PRESENTATION OF THE SUBSTANCES BELONGING TO THE FLAVOURING GROUP EVALUATION 216 CORRESPONDING TO FGE.19 SUBGROUP 3.3

The Flavouring Group Evaluation 216 (FGE.216) concerns five 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], which are presented in Table 1. The five substances correspond to subgroup 3.3 of FGE.19.

The α,β-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.

SPECIFICATION SUMMARY OF THE SUBSTANCES IN THE FLAVOURING GROUP EVALUATION 216REV1

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 216Rev1

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
05.062 1474	2-Phenylcrotonaldehyde		3224 670 4411-89-6	 $C_{10}H_{10}O$ 146.19		177 (20 hPa) NMR 97 %	1.558-1.564 1.031-1.037
05.099 1472	5-Methyl-2-phenylhex-2-enal		3199 10365 21834-92-4	Liquid $C_{13}H_{16}O$ 188.27		96-100 (0.9hPa) NMR 96 %	1.531-1.536 0.970-0.976
05.100 1473	4-Methyl-2-phenylpent-2-enal		3200 10366 26643-91-4	Liquid $C_{12}H_{14}O$ 174.24		96 (0.9 hPa) NMR 95 %	1.533-1.539 0.980-0.986
05.175	2-Phenylpent-2-enal 6)		 3491-63-2	Liquid $C_{11}H_{12}O$ 160.22	Practically insoluble or insoluble Freely soluble	126 (15 hPa) MS 95 %	1.545-1.553 1.005-1.015
05.222	2-Phenyl-4-methyl-2-hexenal		4194 26643-92-5	Liquid $C_{13}H_{16}O$ 188	Insoluble Soluble	97 (0.6 hPa) 95 %	1.522-1.530 0.965-0.975

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.

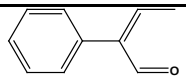
ASSESSMENT

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).

Table 2: Representative substance for subgroup 3.3 of FGE.19 (EFSA, 2008c)

FL-no JECFA-no	Subgroup	EU Register name	Structural formula	Comments
05.062	3.3	2-Phenylcrotonaldehyde		Data submitted in accordance to request in FGE.216

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.

GE	Adopted by EFSA	Link	No. of Substances
FGE.216	27 November 2008	http://www.efsa.europa.eu/en/efsajournal/doc/881.pdf	5
FGE.216Rev1	4 July 2013		5

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.

No second assay was performed with S9-mix.

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 group 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:

% MNPCE			
	Reading 1	Reading 2	Reading mean
Vehicle	0.10	0.04	0.07
75 mg/kg	0.09	0.12	0.10
350 mg/kg	0.18*	0.10	0.14*
700 mg/kg	0.12	0.12	0.12

* $P < 0.01$

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.

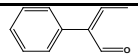
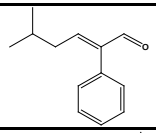
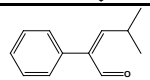
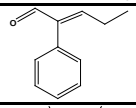
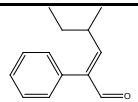
In order to verify that this genotoxic potential demonstrated *in vitro* was confirmed *in vivo*, a micronucleus test was conducted in the rat bone marrow by oral route which led to an ambiguous result, because only the intermediate dose induced a statistically significant increase of MNPCE, even after 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)

FL-no JECFA-no	EU Register name	Structural formula	MSDI 1) (µg/capita/day)	Class 2) Evaluation procedure path 3) (JECFA)	Outcome on the named compound [4) or 5]	EFSA comments
05.062 1474	2-Phenylcrotonaldehyde		1.7 0.07	Class I A3: Intake below threshold	4)	Evaluated in FGE.216Rev1, additional genotoxicity data required.
05.099 1472	5-Methyl-2-phenylhex-2-enal		15 6	Class II A3: Intake below threshold	4)	Evaluated in FGE.216Rev1, additional genotoxicity data required.
05.100 1473	4-Methyl-2-phenylpent-2-enal		0.34 5	Class II A3: Intake below threshold	4)	Evaluated in FGE.216Rev1, additional genotoxicity data required.
05.175	2-Phenylpent-2-enal		0.011	Class II No evaluation	Not evaluated by JECFA	Evaluated in FGE.216Rev1, additional genotoxicity data required.
05.222	2-Phenyl-4-methyl-2-hexenal		3.0	No evaluation	Not evaluated by JECFA	Evaluated in FGE.216Rev1, additional genotoxicity data required.

1) EU MSDI: Amount added to food as flavour in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita/day.

2) Thresholds of concern: Class I = 1800 µg/person/day, Class II = 540 µg/person/day, Class III = 90 µg/person/day.

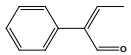
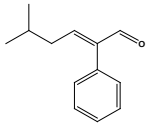
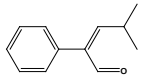
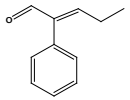
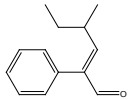
3) Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.

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

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

QSAR PREDICTIONS ON MUTAGENICITY IN FIVE MODELS FOR FIVE ALDEHYDES FROM SUBGROUP 3.3

Table 4: QSAR Predictions on Mutagenicity for Five Aldehydes from Subgroup 3.3

FL-no JECFA-no	Sub-group	EU Register name	Structural formula	FEMA no CoE no CAS no	ISS Local Model Ames Test TA100	MultiCASE Ames test	MultiCASE Mouse lymphoma test	MultiCASE Chromosomal aberration test in CHO	MultiCASE Chromosomal aberration test in CHL
05.062 1474	3.3	2-Phenylcrotonaldehyde		3224 670 4411-89-6	NEG	OD	OD	OD	OD
05.099 1472	3.3	5-Methyl-2-phenylhex-2-enal		3199 10365 21834-92-4	NEG	OD	OD	OD	OD
05.100 1473	3.3	4-Methyl-2-phenylpent-2-enal		3200 10366 26643-91-4	NEG	OD	OD	OD	OD
05.175	3.3	2-Phenylpent-2-enal 6)		- - 3491-63-2	NEG	OD	OD	OD	OD
05.222	3.3	2-Phenyl-4-methyl-2-hexenal		- - 26643-92-5	NEG	OD	OD	OD	OD

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 Lymphona 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*)

FL-no	Chemical Name	Test System *in vitro*	Test Object	Concentrations of Substance and Test Conditions	Result	Reference	Comments
[05.062]	2-Phenylcrotonaldehyde	Reverse Mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537 and TA102	1.6, 8, 40, 200, 1000 and 5000 µg/plate [1,2]	Negative	(Kilford, 2010)	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.
			S. typhimurium TA98, TA100, TA1535 TA1537 and TA102	20.48, 51.2, 128, 320, 800, 2000 and 5000 µg/plate [2,4]	Negative	(Kilford, 2010)	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.
			S. typhimurium TA98, TA100*, TA1535	51.2, 128, 320, 800, 2000 and 5000 µg/plate [3,5]	Negative		
			*S. typhimurium*TA1537 and TA102	51.2, 128, 320, 800, 2000 µg/plate [3,5]	Negative		
			S. typhimurium TA98, TA100, TA1535	31.25 - 1000 µg/plate [3,5]	Negative	(Kilford, 2010)	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.
			S. typhimurium TA1537, TA102	15.625 - 500 µg/plate [3,5]	Negative		
			S. typhimurium TA100	320 - 5000 µg/plate [2,4]	Negative		

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

FL-no	Chemical Name	Test System *in vitro*	Test Object	Concentrations of Substance and Test Conditions	Result	Reference	Comments
		Micronucleus induction	Human peripheral blood lymphocytes	40, 60, 100, 120 µg/mL [4,6]	Positive	(Lloyd, 2012)	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).
				100, 130, 140 µg/mL [5.6]	Negative	(Lloyd, 2012)	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).
				20, 23, 26 µg/mL [4,7]	Negative		
				20, 60, 70 and 80 µg/mL [4,6]	Positive	(Lloyd, 2012)	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).

[1] With and without S9 metabolic activation.
[2] Plate incorporation method.
[3] Pre-incubation method.
[4] Without S9 metabolic activation.
[5] With S9 metabolic activation.
[6] 3-hour incubation with 21-hour recovery period.
[7] 24-hour incubation with no recovery period.

Table 6: Summary of Additionally Genotoxicity Data [FL-no: 05.062] of Subgroup 3.3 (*in vivo*)

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

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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