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SCIENTIFIC OPINION

Flavouring Group Evaluation 216 Revision 3 (FGE.216Rev3): Consideration of the genotoxic potential of α,β-unsaturated 2-phenyl-2-alkenals from subgroup 3.3 of FGE.19

EFSA Panel on Food Additives and Flavourings (FAF) | Laurence Castle | Monica Andreassen | Gabriele Aquilina | Maria Bastos | Polly Boon | Biagio Fallico | Rex FitzGerald | María José Frutos Fernández | Bettina Grasl-Kraupp | Ursula Gundert-Remy | Rainer Gürtler | Eric Houdeau | Marcin Kurek | Henriqueta Louro | Patricia Morales | Sabina Passamonti | Claudia Bolognesi | Eugenia Cordelli | Gisela Degen | Karl-Heinz Engel | Maria Carfí | Alessandra Tosato | Carla Martino

Correspondence: Ask a Question

The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts.

Abstract

The EFSA Panel on Food Additives and Flavourings (FAF) evaluated the genotoxic potential of two flavouring substances, 2-phenylcrotonaldehyde [FL-no: 05.062] and 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] from subgroup 3.3 of FGE.19, in the Flavouring Group Evaluation 216 revision 3 (FGE.216Rev3). In FGE.216Rev2, the Panel concluded that the use of the flavouring [FL-no: 05.062] at the reported use levels in several food categories would raise a concern for aneugenicity and requested substance-specific data for the other related compounds [FL-no: 05.099, 05.100, 05.175 and 05.222]. New data were provided only for [FL-no: 05.062 and 05.099]. For [FL-no: 05.062], the results of a new plasma analysis of the exposed animals were considered to be sufficient to rule out a concern for systemic aneugenicity of the substance. However, the data available do not overrule the concern for aneugenicity of 2-phenylcrotonaldehyde at the site of contact, where the concentrations will be maximal, taking into account that the positive findings in the in vitro micronucleus (MN) assay were seen only in the absence of metabolic activation. Therefore, in line with the principles described in the EFSA Scientific Committee guidance on aneugenicity, the Panel compared the lowest concentration resulting in aneugenicity in the in vitro MN assay (20 μg/mL) with the reported use levels of 2-phenylcrotonaldehyde [FL-no: 05.062] in food (up to 2 mg/kg) and noted that they are one order of magnitude below the concentration for which an aneugenic effect was observed in the in vitro MN assay. Based on this comparison, the Panel concluded that the use of the flavouring substance [FL-no: 05.062] in foods, including beverages, would not raise a concern for aneugenicity if the use levels were not greater than 2 mg/kg or mg/L. For [FL-no: 05.099], based on the new data available, the Panel concluded that there is no concern for genotoxicity.

KEYWORDS

α,β-unsaturated 2-phenyl-2-alkenals, FGE.19, FGE.216, flavouring substances, subgroup 3.3

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1 | INTRODUCTION

The present revision of FGE.216 (FGE.216Rev3) addresses newly submitted data on plasma analysis from animals administered with 2-phenylcrotonaldehyde [FL-no: 05.062] and new genotoxicity data for 5-methyl-2-phenylhex-2-enal [FL-no: 05.099]. In addition, updated information on use levels for both substances has been provided.

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

1.1.1 | Background

The use of flavourings is regulated under Regulation (EC) No 1334/2008[1] of the European Parliament and Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods. On the basis of Article 9(a) of this Regulation an evaluation and approval are required for flavouring substances.

The Union List of flavourings and source materials was established by Commission Implementing Regulation (EC) No 872/2012.[2] The list contains flavouring substances for which the safety evaluation should be completed in accordance with Commission Regulation (EC) No 1565/2000.[3]

In the scientific opinion on Flavouring group evaluation 216 revision 2 (FGE.216Rev2) of 29 June 2022, EFSA identified an aneugenic potential for 5 substances (FL No. 05.062, FL No. 05.099, FL No. 05.100, FL No. 05.175 and FL No. 05.222).

Following this evaluation there was an indication that the applicants were no longer interested to support the evaluation of the substances FL No. 05.100, FL No. 05.175 and FL No. 05.222. Therefore, these substances were flagged for deletion from the Union List. However, in May 2024 the applicants provided the relevant data for the substances FL No. 05.062 and FL No. 05.099.

1.1.2 | Terms of Reference

The European Commission requests the European Food Safety Authority (EFSA) to evaluate this new information submitted and, depending on the outcome, proceed to the full evaluation of the substances FL No. 05.062 and FL No. 05.099 in accordance with Commission Regulation (EC) No 1565/2000.

In case the genotoxic potential cannot be ruled out, EFSA is asked to estimate the exposure.

1.2 | History of the evaluation of FGE.19 substances

Flavouring Group Evaluation 19 (FGE.19) contains 360 flavouring substances from the EU Register[4] being α,β-unsaturated aldehydes or ketones and precursors thereof 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 on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) 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 AFC Panel noted that, for most of these models, internal and external validation had 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 AFC Panel considered that it was not appropriate, at this stage, to rely entirely on (Q)SAR predictions and decided not to take substances through the Procedure based on negative (Q)SAR predictions only.

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

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

[3]Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. OJ L 180, 19.7.2000, pp. 8–16.

[4]Commission Decision of 23 February 1999 adopting a register of flavouring substances used in or on foodstuffs drawn up in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council of 28 October 1996. OJ L 84, 27.3.1999, pp. 1–215.

The AFC 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.

Based on these data, the AFC Panel decided that substances from 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, 2008b) could not be evaluated through the Procedure due to concern with respect to genotoxicity. Corresponding to these subgroups, 15 Flavouring Group Evaluations (FGEs) were established: FGE.200, 204, 205, 206, 207, 208, 209, 211, 215, 219, 221, 222, 223, 224 and 225.

For 11 subgroups, the AFC 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 on genotoxicity from the Flavouring industry. 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 have been 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 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.

1.3 | Presentation of the substances in flavouring group evaluation 216

The Flavouring Group Evaluation 216 (FGE.216), corresponding to FGE.19 subgroup 3.3, 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 Appendix B, Table B1. In the EFSA Opinion 'List of α,β-unsaturated aldehydes and ketones representative of FGE.19 substances for genotoxicity testing' (EFSA, 2008c), 2-phenylcrotonaldehyde [FL-no: 05.062] (Table 1) was selected as representative flavouring substance for FGE.19, subgroup 3.3, corresponding to FGE.216.

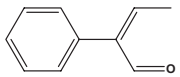
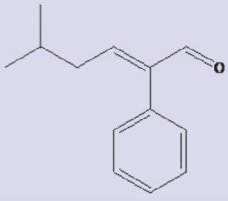
The α,β-unsaturated aldehyde and ketone functional groups are considered structural alerts for genotoxicity (EFSA, 2008a). Accordingly, the available data on genotoxic or carcinogenic activity for the five aldehydes [FL-no: 05.062, 05.099, 05.100, 05.175 and 05.222] were considered in FGE.216, FGE.216Rev1 and FGE.216Rev2.

After the publication of FGE.216Rev2, authorisation for the use of [FL-no: 05.100,[5] 05.175[6] and 05.222[6]] as flavouring substances in the EU has been withdrawn. These substances are not considered any further in FGE.216Rev3.

For the sake of completeness, the information on the identity of all substances is presented in Appendix B (summary of safety evaluation by JECFA). Information on specifications is only presented for the substances which are currently in the Union List[1] (see Appendix A, Table A1). For substances that are no longer in the Union List, FGE.216Rev2 should be consulted.

The substances [FL-no: 05.062 and 05.099] assessed in FGE.216Rev3 are presented in Table 1.

TABLE 1 Flavouring substances from subgroup 3.3 of FGE.19 assessed in FGE.216Rev3.

FL-no JECFA-no	Subgroup	Chemical name	Structural formula	Comments
05.062 1474	3.3	2-Phenylcrotonaldehyde		Data evaluated in FGE.216, FGE.216Rev1, FGE.216Rev2 and FGE.216Rev3
05.099 1472	3.3	5-Methyl-2-phenylhex-2-enal		Data evaluated in FGE.216Rev3

[5]Commission Regulation (EU) 2025/147 of 29 January 2025 amending Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council as regards the removal of the flavouring substance 4-Methyl-2-phenylpent-2-enal (FL No 05.100) from the Union list. OJ L, 2025/147, 30.1.2025.

[6]Commission Regulation (EU) 2024/234 of 15 January 2024 amending Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council as regards the removal of certain flavouring substances from the Union list. OJ L, 2024/234, 16.1.2024.

1.4 | History of the evaluation of the substances belonging to FGE.216

FGE.216

2-Phenylcrotonaldehyde [FL-no: 05.062], 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] and 4-methyl-2-phenylpent-2-enal [FL-no: 05.100] were evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and considered as of no safety concern (JECFA, 2006) (see Appendix B, Table B1).

As reported in Section 1.3, 2-phenylcrotonaldehyde [FL-no: 05.062] (Table 1) was selected as representative flavouring substance for the other four substances in FGE.19, subgroup 3.3, considered in FGE.216: 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 the first scientific opinion on FGE.216 (EFSA, 2009a), no data from genotoxicity or carcinogenicity studies with any of the substances in FGE.216 were available. The (Q)SAR predictions of these substances were limited to one endpoint (gene mutations in one strain of *Salmonella*) (see Appendix C, Table C1). Since the data available were insufficient to rule out the concern for genotoxicity, the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF Panel) requested data on genotoxicity for the representative substance of this subgroup (2-phenylcrotonaldehyde [FL-no: 05.062]), according to the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19 (EFSA, 2008b).

FGE.216Rev1

The CEF Panel continued the assessment of 2-phenylcrotonaldehyde [FL-no: 05.062] (representative flavouring substance for [FL-no: 05.099, 05.100, 05.175 and 05.222]) in FGE.216Rev1 (EFSA CEF Panel, 2013), based on the submission of additional data: a bacterial reverse mutation assay (Kilford, 2010), an in vitro micronucleus (MN) assay in human peripheral blood lymphocytes (Lloyd, 2012) and an in vivo MN assay in rat bone marrow (Henderson, 2013) (see Table 2; Appendix D, Tables D1 and D2).

In FGE.216Rev1 (EFSA CEF Panel, 2013), the CEF Panel considered that 2-phenylcrotonaldehyde [FL-no: 05.062] did not exhibit any mutagenic effect in a bacterial test with and without metabolic activation. However, it showed a genotoxic effect in the in vitro MN test in cultured human lymphocytes only in the absence of metabolic activation.

The genotoxic potential observed in the in vitro MN test was further investigated in an in vivo MN test (Henderson, 2013) conducted in the rat bone marrow after oral administration of 2-phenylcrotonaldehyde at the following doses: 0, 70, 350 and 700 mg/kg bw per day. Animals were dosed at 0 and 24 h, followed by bone marrow sampling at 24 h after the last treatment. In this in vivo study, an equivocal result was observed because only the intermediate dose (350 mg/kg bw per day) induced a statistically significant increase in the frequency of micronucleated polychromatic erythrocytes (MNPCE), even after re-scoring the slides. No evidence of systemic exposure of the animals was provided. In particular, no change in the percentage of polychromatic erythrocytes (PCE) in bone marrow was noted, and no analytical data on plasma was presented.

Under these conditions, the CEF Panel considered it necessary to provide proof of sufficient systemic exposure of animals orally treated with 2-phenylcrotonaldehyde.

Moreover, since the substance was genotoxic in vitro only without metabolic activation, the CEF Panel requested to prove the absence of genotoxic effects locally in the gastrointestinal tract (GIT) tissues using the in vivo comet assay.

JECFA evaluated the same studies in 2014 (JECFA, 2015) together with a second in vivo MN assay (Covance, 2013), which was evaluated also in FGE.216Rev2 (EFSA FAF Panel, 2022, see below). JECFA considered that additional data are needed to address the potential genotoxicity of 2-phenylcrotonaldehyde [FL-no: 05.062] and of the other α,β-unsaturated 2-phenyl compounds [FL-no: 05.099, 05.100, 05.222]. JECFA recommended that the evaluations of [FL-no: 05.062, 05.099, 05.100] (which were already assessed by JECFA in 2006) should be reconsidered, given the potential genotoxicity of 2-phenylcrotonaldehyde [FL-no: 05.062]. JECFA also concluded that the Procedure cannot be applied to [FL-no: 05.222] until the concerns regarding genotoxicity are resolved.

The studies evaluated in FGE.216Rev1 are listed in Table 2 and summarised in Appendix D.

FGE.216Rev2

Following the request for additional data expressed by the CEF Panel in FGE.216Rev1 (EFSA CEF Panel, 2013), an additional in vivo MN assay (Covance, 2013) was conducted using the same experimental conditions in rats as described in the study by Henderson (2013), and at the same dose levels (i.e. 0, 70, 350 and 700 mg/kg bw per day). Animals were dosed at 0 and 24 h, followed by bone marrow sampling at 24 h after the last treatment. In addition, in order to demonstrate bone marrow exposure in the in vivo MN assays (Henderson, 2013; Covance, 2013), industry investigated the presence of 2-phenylcrotonaldehyde [FL-no: 05.062] in the plasma of satellite groups of animals from these two in vivo MN assays (Covance, 2014). These studies were assessed by the Panel in FGE.216Rev2 (EFSA FAF Panel, 2022), which includes the representative substance [FL-no: 05.062] and the flavouring substances [FL-no: 05.099, 05.100, 05.175 and 05.222].

In the in vivo MN assay (Covance, 2013), 2-phenylcrotonaldehyde [FL-no: 05.062] did not induce micronuclei in PCE of male rats treated up to 700 mg/kg bw per day [an estimate of the maximum tolerated dose (MTD) for this study]. The ratio between polychromatic and normochromatic erythrocytes (PCE/NCE ratio) was not affected by the treatment with

2-phenylcrotonaldehyde [FL-no: 05.062], indicating that the substance did not induce bone marrow toxicity. No signs of toxicity were observed up to the highest dose tested (700 mg/kg bw per day). In the plasma analysis (Covance, 2014), the Panel noted that the linearity of spiked plasma extracts was in the range of 10–400 µg/mL, but the highest concentration reported for 2-phenylcrotonaldehyde in rat plasma samples was below 10 µg/mL. Moreover, the recovery and accuracy of the method were only determined from 50 µg/mL and above. No animals in the vehicle control group showed any detectable levels of 2-phenylcrotonaldehyde in plasma, while less than 10 µg/mL of 2-phenylcrotonaldehyde was detected in the plasma of animals treated with 700 mg/kg bw per day, half an hour after oral dosage. The Panel, however, noted that there was some variability in plasma levels for each time point among treated animals. Although these results indicated that a very low amount of 2-phenylcrotonaldehyde might be present in plasma shortly after dosing, the Panel concluded that the validation package provided by the study is not robust enough on its own to show that bone marrow was exposed in the two in vivo MN assays (Henderson, 2013; Covance, 2013).

Therefore, the Panel evaluated the results of these in vivo MN assays still as inconclusive.

In reply to the request from the CEF Panel in FGE.216Rev1, 2-phenylcrotonaldehyde was also tested for its potential to induce DNA damage in the duodenum of treated rats tested in an in vivo comet assay (Covance, 2016). Male rats were dosed orally at 0, 175, 300 or 700 mg/kg bw per day on two consecutive days. There were no clinical chemistry or histopathological findings associated with the administration of 2-phenylcrotonaldehyde. No statistically significant increases in tail intensity at any dose levels of 2-phenylcrotonaldehyde were observed compared to the vehicle control. All individual animal data at all dose levels were consistent with the data from vehicle control animals and fell within the laboratory's historical control data. The Panel concluded that 2-phenylcrotonaldehyde did not induce DNA damage in the duodenum of rats treated up to 700 mg/kg bw per day.

Since the available data did not allow to evaluate the aneugenic potential of 2-phenylcrotonaldehyde [FL-no: 05.062], the Panel requested to test this substance in an in vitro MN assay with centromere staining. Industry provided the study requested (BioReliance, 2018a) and additional studies: a bacterial reverse mutation assay (BioReliance, 2016) and an in vivo gene mutation assay in transgenic mice (BioReliance, 2017, 2018b) that were evaluated in FGE.216Rev2 (see Appendix E, Tables E1 and E2). In addition, new data on uses and use levels were provided to estimate the exposure (Documentation provided to EFSA No. 9).

2-Phenylcrotonaldehyde [FL-no: 05.062] was negative in a bacterial gene mutation assay (Kilford, 2010) with and without metabolic activation (assessed in FGE.216Rev1). In a second study (assessed in FGE.216Rev2), a statistically significant increase in revertants was observed in *S*. Typhimurium strain TA1535, without S9-mix, and in *E. coli* WP2 uvrA in the presence and absence of S9-mix (BioReliance, 2016).

In an in vivo follow-up study (BioReliance, 2017, 2018b), 2-phenylcrotonaldehyde was tested for gene mutations in Big Blue® transgenic mice in which duodenum, liver and bone marrow were analysed. No statistically significant increase in mutation frequency was observed in the tissues analysed. The Panel considered that 2-phenylcrotonaldehyde did not induce gene mutations in this in vivo assay.

Positive results were observed in two in vitro MN assays, in human peripheral blood lymphocytes (Lloyd, 2012) and in TK6 cells (BioReliance, 2018a) in the absence of metabolic activation.

In the in vitro MN assay in TK6 cells, a statistically significant increase in micronucleated cell frequency was observed in the treatment for 27 h, at a concentration of 20 µg/mL (the highest concentration evaluated for MN induction), in the absence of metabolic activation. The results of the CREST analysis indicate that 77% of MN induced by 2-phenylcrotonaldehyde were positive for kinetochore staining. The clastogen positive control mitomycin C (MMC) showed 30% of MN positive for kinetochore staining. The negative control showed 60% of MN positive for kinetochore staining. The aneugen positive control vinblastine (VB) had 87% of MN positive for kinetochore staining. The CREST analysis showed that 2-phenylcrotonaldehyde [FL-no: 05.062] induced MN mainly through an aneugenic mechanism.

Since 2-phenylcrotonaldehyde was found to be positive without metabolic activation in the in vitro MN test, the Panel considered that, after oral exposure, the GIT would be the most exposed and relevant target for investigating potential genotoxic effects at the first site of contact.

The Panel noted that the negative results observed in the in vivo comet assay (Covance, 2016) in duodenum can overrule the potential clastogenicity at the site of contact, but not a possible aneugenic effect.

The Panel also noted that there are no validated in vivo tests for the investigation of aneugenicity in the GIT. Thus, the Panel followed the recommendations of the EFSA Scientific Committee (EFSA Scientific Committee, 2021) on this endpoint and compared the concentration resulting in aneugenicity in vitro (20 µg/mL, the highest concentration tested in the treatment for 27 h) with the estimated concentration of 2-phenylcrotonaldehyde in the GIT following ingestion of food or beverage. Since the dilution in the upper parts of the GIT is expected to be small, the estimated concentration of a substance in this part of the GIT would be in the same order of magnitude as its concentration in food and beverages.

In FGE.216Rev2, the Panel noted that the normal use levels and/or the maximum use levels (up to 5.13 mg/kg) of 2-phenylcrotonaldehyde [FL-no: 05.062], for some food categories, are less than one order of magnitude below the concentration for which an aneugenic effect of this flavouring substance was observed in the in vitro MN assay (i.e. 20 µg/mL). Therefore, the use of [FL-no: 05.062] at the reported use levels in these food categories would raise a concern for aneugenicity (EFSA FAF Panel, 2022).

Based on structural similarity, for the remaining four substances in this FGE [FL-no: 05.099, 05.100, 05.175 and 05.222], an aneugenic potential may also be anticipated. To rule out this concern for the other four substances falling under FGE.216, generation of new relevant data on aneugenicity (an in vitro MN test with centromeres analysis) was requested by the

Panel. In case of an increase in micronucleated cells frequency, an appropriate follow-up test should be done, according to the EFSA guidance on genotoxicity testing strategy (EFSA Scientific Committee, 2011) and the EFSA guidance on aneugenicity (EFSA Scientific Committee, 2021).

The studies evaluated in FGE.216Rev2 are listed in Table 2 and summarised in Appendix E.

TABLE 2 Data evaluated in FGE.216Rev1 (EFSA CEF Panel, 2013) and in FGE.216Rev2 (EFSA FAF Panel, 2022).

Chemical name [FL-no]	Data assessed	References
Data assessed in FGE.216Rev1		
2-Phenylcrotonaldehyde [05.062]	Bacterial reverse mutation assay	Kilford (2010)
	In vitro MN test in human peripheral blood lymphocytes	Lloyd (2012)
	In vivo MN assay in rat bone marrow	Henderson (2013)
Data assessed in FGE.216Rev2		
2-Phenylcrotonaldehyde [05.062]	Bacterial reverse mutation assay	BioReliance (2016)
	In vitro MN assay with CREST staining in TK6 cells	BioReliance (2018a)
	Development and limited validation of a method for the analysis of plasma samples which may contain 2-phenylcrotonaldehyde	Covance (2014)
	In vivo MN assay in rat bone marrow	Covance (2013)
	In vivo comet assay in duodenum of rats	Covance (2016)
	In vivo oral dose range finding assay in C57BL/6 mice	BioReliance (2017)
	In vivo mutation assay at the cII locus in Big Blue® transgenic C57BL/6 mice	BioReliance (2018b)
	Use levels	Documentation provided to EFSA No. 9
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]	Use levels	Documentation provided to EFSA No. 9

Table 3 provides a compilation of the three opinions on FGE.216.

TABLE 3 References to the opinions on FGE.216.

FGE	References	Substances[a]
FGE.216	EFSA (2009a)	5
FGE.216Rev1	EFSA CEF Panel (2013)	5
FGE.216Rev2	EFSA FAF Panel (2022)	5
FGE.216Rev3	Current opinion	2

[a]Substances considered in: FGE.216, FGE.216Rev1 and FGE.216Rev2: [FL-no: 05.062, 05.099, 05.100, 05.175 and 05.222]; FGE.216Rev3: [FL-no: 05.062, 05.099].

2 | DATA AND METHODOLOGIES

2.1 | Data

In the present revision (FGE.216Rev3), the following data were considered:

- Analysis of the presence of 2-phenylcrotonaldehyde [FL-no: 05.062] in the plasma of rats after oral administration of the flavouring substance (Product Safety Labs, 2024a).
- New genotoxicity studies on 5-methyl-2-phenylhex-2-enal [FL-no: 05.099]: in vitro MN assay (Labcorp, 2024), in vivo MN assay (Product Safety Labs, 2024b) and, upon EFSA request (EFSA letter sent on 25 February 2025), an in vivo comet assay (Documentation provided to EFSA No. 13).
- Updated information on uses and use levels for 2-phenylcrotonaldehyde [FL-no: 05.062] and 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] (Documentation provided to EFSA No. 12).

In the dossier provided to EFSA (Documentation provided to EFSA No. 10), industry informed about a publication reporting positive results for 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] tested in an in vitro chromosomal aberration

(CA) assay, but the reference to the publication was not provided. Upon EFSA request (EFSA letter sent on 28 November 2024), industry clarified the correct reference to this publication (Documentation provided to EFSA No. 11) that is Honma et al. (2022). Taking into account the results presented by Honma et al. (2022) and the results of the studies submitted for 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] (see description in Sections from 3.2 to 3.7), EFSA requested to investigate potential effects at the first site of contact by the in vivo comet assay, performed at least in duodenum (EFSA letter sent on 25 February 2025). Following this request, an in vivo comet assay (Documentation provided to EFSA No. 13) was provided and considered in the assessment (see Section 3.8). The publication by Honma et al. (2022) reports also a bacterial reverse mutation assay and an in vivo MN assay with 5-methyl-2-phenylhex-2-enal [FL-no: 05.099], which are considered in the assessment. Since Honma et al. (2022) indicated that the Ames test data (including raw data) were already reported in a previous study by Kasamatsu et al. (2021); also, this publication was considered.

These studies evaluated in the present opinion, FGE.216Rev3, are listed in Table 4, summarised in Appendix F, and discussed in the following sections. For the two substances [FL-no: 05.062 and 05.099] assessed in the current opinion, specifications from JECFA evaluation (JECFA, 2004) are reported in Appendix A.

TABLE 4 Data evaluated in FGE.216Rev3.

Chemical name [FL-no]	**Additional data submitted**	**References**
2-Phenylcrotonaldehyde [05.062]	Plasma analysis in rats	Product Safety Labs (2024a)
	Use levels	Documentation provided to EFSA No. 12
5-Methyl-2-phenylhex-2-enal [05.099]	In vitro MN test in human peripheral blood lymphocytes	Labcorp (2024)
	In vitro CA test in Chinese hamster lung (CHL) cells	Honma et al. (2022)
	Bacterial reverse mutation assay	Kasamatsu et al. (2021); Honma et al. (2022) (same study reported in these publications)
	In vivo MN assay in mouse bone marrow	Honma et al. (2022)
	In vivo MN assay in rat peripheral blood, including analysis of blood samples for measuring the concentration of 5-methyl-2-phenylhex-2-enal	Product Safety Labs (2024b)
	In vivo comet assay in rat duodenum	Documentation provided to EFSA No. 13
	Use levels	Documentation provided to EFSA No. 12

2.2 | Methodologies

This opinion was prepared following the principles described in the EFSA Guidance on transparency with regard to scientific aspects of risk assessment (EFSA, 2009b) and following the relevant existing Guidelines from the EFSA Scientific Committee. As requested in the European Commission mandate, the assessment strategy applied is in line with the evaluation programme of flavouring substances, as laid down in Commission Regulation (EC) No. 1565/2000,[3] which is based on the Opinion on a Programme for the Evaluation of Flavouring substances of the Scientific Committee on Food (SCF, 1999).

3 | ASSESSMENT

The present revision 3 of FGE.216 (FGE.216Rev3) deals with the genotoxicity assessment of 2-phenylcrotonaldehyde [FL-no: 05.062] and 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] for which new data have been provided as described in Section 2.

3.1 | Plasma analysis for 2-phenylcrotonaldehyde [FL-no: 05.062]

For 2-phenylcrotonaldehyde [FL-no: 05.062], two in vivo MN assays in Han-Wistar rats had been assessed in FGE.216Rev1 (study by Henderson, 2013) and in FGE.216Rev2 (study by Covance, 2013). In both studies, no evidence of bone marrow exposure to the tested substance could be demonstrated (see Section 1.4).

To support a possible reinterpretation of the results of the two in vivo MN studies, a new kinetic study involving plasma analysis (Product Safety Labs, 2024a) has been provided to demonstrate systemic exposure of rats administered with 2-phenylcrotonaldehyde.

For the new plasma analysis (Product Safety Labs, 2024a), male Han-Wistar rats were grouped in three groups (6 animals/group) and received a single dose of 700 mg/kg bw of 2-phenylcrotonaldehyde (purity > 98%) via oral gavage. This dose is the highest of the three doses tested in the in vivo MN studies (70, 350 and 700 mg/kg bw per day, for 2 days) that

were assessed in FGE.216Rev1 and FGE.216Rev2. Blood samples were collected at different time points from 0.5 min to 24 h. No test substance-related mortalities were observed, nor any clinical findings.

The analysis of plasma samples was performed via liquid chromatography–tandem mass spectrometry (LC-MS/MS) after derivatisation of 2-phenylcrotonaldehyde to the respective hydrazone; 3-(trifluoromethoxy)benzaldehyde was used as internal standard. Validation data demonstrated linearity of the employed assay in the range of 10–2000 ng/mL.

The substance was detected in plasma at low concentrations: the highest mean concentration was about 1 µg/mL, with variability among individual animals. The presence of [FL-no: 05.062] in plasma was observed for a short period (up to 90 min) after administration; thereafter, the concentration declined rapidly.

The Panel noted also that the electrophilic nature of the α, β-unsaturated aldehydes suggests their rapid conjugation with glutathione and/or other endogenous nucleophilic thiol compounds (JECFA, 2006), resulting in very low plasma levels of these substances as free compounds. The glutathione conjugate can be further enzymatically transformed, resulting in the mercapturic acid which is excreted in urine.

The plasma analysis shows that the substance is systemically available following a dose of 700 mg/kg bw. However, the concentrations in plasma are very low, maximum concentrations being around 1 µg/mL.

Thus, the Panel considered that the low circulating levels of 2-phenylcrotonaldehyde as a free compound are unlikely to elicit a detectable response in the bone marrow MN assay. This is supported by the observation that, as reported in FGE.216Rev2 (EFSA FAF Panel, 2022), 2-phenylcrotonaldehyde at concentrations of 5 and 15 µg/mL (concentrations higher than those measured in vivo, up to 1 µg/mL in plasma) did not increase the frequency of micronucleated cells in the in vitro MN assay (BioReliance, 2018a), following an exposure period of 27 h.

Altogether, the results of the plasma analysis are considered by the Panel to be sufficient to rule out a concern for systemic aneugenicity of the substance. However, the data available do not overrule the concern for aneugenicity of 2-phenylcrotonaldehyde at the site of contact, where the concentrations will be maximal, taking into account that the positive findings in the in vitro MN assay were seen only in the absence of metabolic activation.

3.2 | Bacterial reverse mutation assay with 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] – Kasamatsu et al. (2021)

Results from a bacterial reverse mutation assay were reported by Honma et al. (2022), but more detailed information was reported by Kasamatsu et al. (2021). 5-Methyl-2-phenylhex-2-enal [FL-no: 05.099] (purity 96.5%) was tested in a bacterial reverse mutation test (preincubation method) in *Salmonella* Typhimurium TA100, TA98, TA1535, TA1537, and *Escherichia coli* WP2 uvrA in the presence and absence of metabolic activation. The test was conducted according to OECD Test Guideline (TG) 471 (OECD, 1997) and in compliance with the principles of good laboratory practice (GLP). Positive control chemicals and vehicle control (dimethyl sulfoxide, DMSO) were evaluated concurrently.

Six concentrations of 5-methyl-2-phenylhex-2-enal from 4.88 to 5000 µg/plate were tested in a first experiment. Growth inhibition was observed in all bacterial strains starting from the concentration of 313 µg/plate in the presence and absence of S9-mix. A second experiment was conducted with six concentrations of 5-methyl-2-phenylhex-2-enal from 9.77 to 313 µg/plate in the presence and absence of S9-mix. Two confirmatory experiments were conducted; in the first one, six concentrations of 5-methyl-2-phenylhex-2-enal from 9.77 to 313 µg/plate in the presence and absence of S9-mix were used; in the second one, only TA100 and TA98 strains were exposed in the absence of metabolic activation to five concentrations from 81.3 to 313 µg/plate.

All the positive control chemicals induced significant increases in revertant colony numbers. No increase in the mean number of revertant colonies was observed at any tested concentration of 5-methyl-2-phenylhex-2-enal in any tested strains in the absence or presence of metabolic activation. The Panel considered the study reliable without restrictions and the negative results of high relevance.

3.3 | In vitro micronucleus assay with 5-methyl-2-phenylhex-2-enal [FL-no: 05.099]

5-Methyl-2-phenylhex-2-enal [FL-no: 05.099] (purity 98.6%) was tested to determine its clastogenic or aneugenic potential in an in vitro MN test in human peripheral blood lymphocytes with and without metabolic activation (S9-mix). This study (Labcorp, 2024) was performed according to OECD TG 487 (OECD, 2016a) and in compliance with the principles of GLP.

The range of concentrations was selected on the basis of the results of a preliminary range finding study carried out up to the maximum concentration of 1890 µg/mL (equivalent of approximately 10 mM). The highest concentration selected for the MN test following all treatment conditions was one at which 50%–60% cytotoxicity was achieved based on the replication index (RI).

In the main experiment, duplicate cultures of whole blood cells were treated for 3 h followed by a 21-h recovery period (3 h + 21 h), in the absence and presence of S9-mix (from β-naphthoflavone/phenobarbital induced Sprague Dawley rats) or for 24 h in the absence of S9-mix with a 24-h recovery period. Positive controls were MMC, cyclophosphamide (CP) and VB.

In the short-term treatment (3 h + 21 h), the cell cultures were treated at 34, 48, 56 and 68 µg/mL in the absence of S9-mix and at 50, 72, 90 and 100 µg/mL in the presence of S9-mix. In the 24-h treatment, cell cultures were exposed to 24, 36 and 40 µg/mL in the absence of S9-mix.

Cytotoxicity of 52%, 58% and 51% was reported at the highest concentration tested in short-term treatment with S9-mix, without S9-mix and in the 24-h treatment, respectively.

The frequencies of binucleated micronucleated cells (MNBNs) reported in cell cultures treated with 5-methyl-2-phenylhex-2-enal were not significantly higher than those observed in concurrent vehicle controls and no indication of concentration-related effect was observed (non-significant linear trend tests). In addition, the frequencies of MNBNs in 5-methyl-2-phenylhex-2-enal treated cultures fell within the ranges of negative historical controls.

The Panel considered that 5-methyl-2-phenylhex-2-enal did not induce an increase in the frequency of MNBNs under the test conditions applied in this study. The study was considered reliable without restrictions and the results of high relevance.

3.4 | In vitro chromosomal aberration assay with 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] – Honma et al. (2022)

In the data set provided to EFSA (Documentation provided to EFSA No. 10, 11), industry informed about a publication (Honma et al., 2022) reporting positive results for 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] (purity 96.5%) tested in an in vitro CA assay and negative results in an in vivo MN assay, for which it was unclear if bone marrow was exposed (for the considerations of this in vivo MN assay, see Section 3.5).

In the CA test (reported by Honma et al., 2022 as conducted according to OECD TG 473 and in compliance with GLP), performed in Chinese hamster lung cells, 5-methyl-2-phenylhex-2-enal was tested in a short-term treatment of 6 h in the presence of metabolic activation (S9-mix) (concentrations tested: 140, 160 and 180 µg/mL) and in the absence of S9-mix (concentrations tested: 60, 70 and 80 µg/mL). An additional long-term treatment of 24 h in the absence of S9-mix was conducted at 40, 50 and 60 µg/mL. In this study, an increase in structural aberrations was observed at 24-h treatment in the absence of S9-mix (Honma et al., 2022).

The study was considered reliable with restrictions and the results of limited relevance, since the number of metaphases scored is 200 instead of 300 (as recommended in OECD TG 473, 2016b).

Based on the positive results observed in this in vitro study, the study authors tested the flavouring substance [FL-no: 05.099] in an in vivo MN assay (Honma et al., 2022).

3.5 | In vivo micronucleus assay with 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] – Honma et al. (2022)

Results from an in vivo MN assay in bone marrow were reported by Honma et al. (2022).

Four groups of five male CD1 mice each were treated via gavage with 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] (purity 96.5%) at doses of 0, 250, 500 and 1000 mg/kg bw per day, respectively. Animals were dosed at 0 and 24 h. An additional group of five animals was administered with mytomycin C (MMC), 1 mg/kg, via intraperitoneal route. Mice were sacrificed at 23–24 h after the last administration.

The authors reported that the study was performed according to OECD TG 474 and in compliance with the principles of GLP. However, the Panel noted that it is unclear whether the MTD was reached. No increase in MNPCE was observed at any dose of 5-methyl-2-phenylhex-2-enal. Given that no changes in %PCE were observed in the treated groups compared to the negative control, and that no signs of toxicity were reported in the publication (Honma et al., 2022), the exposure of bone marrow was not demonstrated. Therefore, the Panel considered the results of this in vivo MN assay as inconclusive. The study was considered not reliable and the results of low relevance.

3.6 | In vivo micronucleus assay with 5-methyl-2-phenylhex-2-enal [FL-no: 05.099]

5-Methyl-2-phenylhex-2-enal [FL-no: 05.099] (purity 99.2%) was tested in a mammalian erythrocytes micronucleus test in Sprague Dawley rats (Product Safety Labs, 2024b) according to OECD TG 474 (OECD, 2016c) and in compliance with the principles of GLP.

A dose range finding study was carried out in rats at 0, 1000 and 2000 mg/kg bw per day administered by gavage for 3 days in groups of three males and three females. At 2000 mg/kg bw per day, two females and one male died. At 1000 mg/kg bw per day, there were no signs of cytotoxicity as measured by the reticulocyte counts in peripheral blood relative to the control. Based on these results, the dose of 1000 mg/kg bw per day was considered by the study authors as the MTD.

In the main study, four groups of 10 animals each (five male and five female rats) were treated with 5-methyl-2-phenylhexy-2-enal at doses of 250, 500 and 1000 mg/kg bw per day, respectively, by gavage once per day for 3 days, with 24-h interval. An additional group of five male and five female rats was administered with the positive control (cyclophosphamide 10 mg/kg bw). Corn oil was used as vehicle control. Blood samples were collected for the analysis of micronucleated cells 20–24 h after the last treatment.

For the blood analysis of the compound, six male and six female rats were dosed at 1000 mg/kg bw. Blood samples were collected at different time points from 3 min to 24 h following administration.

No clinical signs were reported, other than slight hypersalivation in three of five animals, and slight moist rales in one animal at 1000 mg/kg bw per day. No changes in body weight were observed.

There were no statistically significant changes in the percent of reticulocytes (%RET) and micronucleated reticulocytes (%MN-RETs) at any dose tested compared with the negative controls.

The levels of 5-methyl-2-phenylhexy-2-enal measured in whole blood were generally below the limit of quantification of the method (< 5 μg/mL) and no further toxicokinetic assessment was performed.

The Panel considered the results of this in vivo MN assay as inconclusive because the bone marrow exposure was not demonstrated. In addition, the Panel considered that the MTD was not reached, since no signs of toxicity were observed at 1000 mg/kg bw per day. An intermediate dose between 1000 and 2000 mg/kg bw per day should have been tested. The study was considered not reliable and the results of low relevance.

3.7 | Background information for the request of the in vivo comet assay

The Panel considered the new studies (in vitro and in vivo MN assays) on 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] and the results reported in the publication by Honma et al. (2022). The Panel noted that the positive result, in the absence of metabolic activation, in the in vitro CA test reported by Honma et al. (2022) is not consistent with the negative result of the in vitro MN assay (Labcorp, 2024). The negative result of the in vivo follow-up study (in vivo MN test) reported in the same publication is in line with the in vivo MN study provided in the dossier (Product Safety Labs, 2024b).

However, the Panel considered that the information reported in the in vivo MN study (Product Safety Labs, 2024b) and the one reported in the publication by Honma et al. (2022) is not sufficient to demonstrate that animals were systemically exposed to 5-methyl-2-phenylhex-2-enal [FL-no: 05.099].

Based on the data available and described in previous sections, it was noted:

- positive results were observed in the in vitro CA in the absence of metabolic activation (Honma et al., 2022);
- absence of bone marrow toxicity (PCE/NCE) detected in both in vivo MN studies (Honma et al., 2022; Product Safety Labs, 2024b);
- lack of demonstration of systemic exposure to 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] in the in vivo MN study (Product Safety Labs, 2024b), since the plasma concentrations of the compound were below the limit of quantification (< 5 μg/mL). The local concentration of [FL-no: 05.099] at the site of contact is expected to be higher than that in bone marrow.

Therefore, the Panel requested to investigate potential effects at the first site of contact by the in vivo comet assay, performed at least in the duodenum (EFSA letter sent on 25 February 2025). Such a study has been provided (Documentation provided to EFSA No. 13) and is described below.

3.8 | In vivo comet assay with 5-methyl-2-phenylhex-2-enal [FL-no: 05.099]

In a dose range-finding assay, a total of 20 Han Wistar rats (males and females) were administered by gavage twice with 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] (purity 97.8%) at 0 (day 1) and 24 h (day 2) at doses of 250 (two rats), 500 (six rats), 750 (10 rats) and 1000 (two rats) mg/kg bw per day (Documentation provided to EFSA No. 13).

The stability of test article formulations (10, 25 and 50 mg/mL) was confirmed through a validated analytical method (HPLC-UV) for the determination of 5-methyl-2-phenylhex-2-enal in corn oil (5–80 mg/mL).

At 1000 mg/kg bw per day (tested on one male and one female rat), the male rat showed slight to moderate signs of toxicity (including reduced spontaneous activity and hunched posture), while the female showed severe signs of toxicity (including hypothermia and ataxia).

At 500 and 750 mg/kg bw per day on four animals (one male and one female for each dose group), slight signs of toxicity were observed in females and moderate signs of toxicity in males.

At 250 mg/kg bw per day, slight signs of toxicity (piloerection in both genders and reduced spontaneous activity in male rats) were observed.

Based on these results, two additional groups of animals were tested at 500 (two males and two females) and 750 mg/kg bw per day (four males and four females).

At 750 mg/kg bw per day, male rats and one female showed severe signs of toxicity (including abnormal breathing, dehydration, hunched posture). In the remaining females of this group, slight signs of toxicity were observed (including piloerection and reduced spontaneous activity).

At 500 mg/kg bw per day, no signs of toxicity were observed. Based on this study, the dose of 500 mg/kg bw per day was considered as the MTD and selected for the main study. As no gender differences were observed in the range-finder experiment, the main experiment was performed only in male rats.

In the main study, 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] (purity 97.8%) was tested for its potential to induce DNA damage in the duodenum of treated rats (Documentation provided to EFSA No. 13). The study was performed according to OECD TG 489 (OECD, 2016d) and in compliance with the principles of GLP.

Five male Han Wistar rats (seven rats for the high dose group) were dosed twice with 5-methyl-2-phenylhex-2-enal at 0 (day 1) and 24 h (day 2) by gavage. Dose levels were 0, 100, 250 or 500 mg/kg bw per day. The vehicle was corn oil and the positive control was ethyl methanesulfonate (250 mg/kg bw per day, single oral administration 4 h before sacrifice).

Clinical signs of toxicity were observed in animals of the highest dose group (e.g. reduced spontaneous activity, piloerection, hunched posture). Similar signs of toxicity (except hunched posture) were observed in animals of the mid-dose group. Four hours after the final administration not all animals of the high-dose group were fully recovered. No specific clinical findings were observed in animals of the low-dose group or animals of the vehicle and positive control groups.

A slight weight loss was observed in one animal of each dose group. The remaining animals showed a slight weight gain or no weight change.

Duodenum was sampled on day 2, 4 h after the last administration. Histopathology was not performed.

The tail intensity value for vehicle control (4.91%) was at the upper limit of the laboratory's historical data (1.43%–4.88%, 95% confidence limits). The positive control resulted in a statistically significant increase in tail intensity (14.42%) that was comparable with the laboratory's historical positive control data (6.21%–27.63%, 95% confidence limits).

There was no dose-related increase in % hedgehogs in duodenum following treatment with 5-methyl-2-phenylhex-2-enal, thus suggesting that the treatment did not cause excessive DNA damage, which could interfere with comet analysis.

A statistically significant decrease in tail intensity compared to the vehicle control was observed at all dose levels. The tail intensity values (%) of one animal of the mid-dose group and two animals of the high-dose group were below the lower limit of the historical vehicle control range.

Taking into account (a) the chemical structure of the substance, (b) the negative control value being at the upper level of the historical negative control range and (c) the negative results of the in vitro MN assay (Labcorp, 2024), the Panel did not consider the lower tail intensity values observed at all doses compared to the concurrent negative control as an indication for potential crosslinking activity.

The Panel concluded that 5-methyl-2-phenylhex-2-enal did not induce DNA damage in the duodenum of rats treated up to 500 mg/kg bw per day (estimated MTD). The study was considered reliable without restrictions and the results are of high relevance.

3.9 | Data on uses and use levels and natural occurrence

New data on uses and use levels were provided for 2-phenylcrotonaldehyde [FL-no: 05.062] and 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] (Documentation provided to EFSA No. 12), which are reported in Appendix G. This latest submission (Documentation provided to EFSA No. 12) replaces data on uses and use levels that were submitted in 2022 (Documentation provided to EFSA No. 9) and that were considered in FGE.216Rev2 (EFSA FAF Panel, 2022).

The Panel noted that the highest maximum use level for 2-phenylcrotonaldehyde [FL-no: 05.062], among all food categories, was 2 mg/kg food (see Section 4; Appendix G, Table G1).

In addition, based on information retrieved by EFSA from the Volatile Compounds in Food (VCF) database (VCF online database, 2026), 2-phenylcrotonaldehyde [FL-no: 05.062] is reported to be present in natural food sources and processed food (Appendix G, Table G4).

4 | DISCUSSION

The present revision of FGE.216 (FGE.216Rev3) addresses newly submitted data on plasma analysis from animals administered with 2-phenylcrotonaldehyde [FL-no: 05.062] and new genotoxicity data for 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] (Appendix F, Tables F1 and F2). In addition, for both substances, updated information on use levels has been considered.

Only these two substances [FL-no: 05.062 and 05.099] are considered in the present FGE.216Rev3, because the other three substances [FL-no: 05.100,[5] 05.175[6] and 05.222[6]], previously allocated to FGE.19 subgroup 3.3, are no longer authorised as flavouring substances in the EU.

In FGE.216Rev2 (EFSA FAF Panel, 2022), the Panel concluded that 2-phenylcrotonaldehyde [FL-no: 05.062] does not raise concerns for gene mutation or clastogenicity on the basis of negative in vivo findings, whereas the concern for aneugenicity could not be resolved. In fact, the positive in vitro micronucleus results, including evidence of an aneugenic mechanism, were not adequately clarified by the available in vivo follow-up studies (i.e. MN studies in bone marrow) which were considered inconclusive due to the lack of clear evidence of bone marrow exposure.

To support a possible reinterpretation of the results of the two in vivo MN studies (Henderson, 2013; Covance, 2013), a new plasma analysis (Product Safety Labs, 2024a) has been provided to demonstrate systemic exposure of rats to 2-phenylcrotonaldehyde in these in vivo MN studies. These new data have been evaluated in the current opinion (FGE.216Rev3).

When administered as single exposure at the highest dose tested in the in vivo MN studies (700 mg/kg bw per day), 2-phenylcrotonaldehyde was detected in plasma at low concentrations (the highest mean concentration was about 1 μg/mL, with variability among individual animals) and for a short period after administration.

The results of the plasma analysis are considered by the Panel to be sufficient to rule out a concern for systemic aneugenicity of the substance. However, the data available do not overrule the concern for aneugenicity of 2-phenylcrotonaldehyde

at the site of contact, where the concentrations will be maximal, taking into account that the positive findings in the in vitro MN assay were seen only in the absence of metabolic activation.

Therefore, the Panel concluded that the results of these two in vivo MN studies remain inconclusive with respect to the aneugenicity of 2-phenylcrotonaldehyde at the site of contact after oral exposure.

As already reported in FGE.216Rev2 (EFSA FAF Panel, 2022), the Panel noted that, at present, there are no validated in vivo tests for the investigation of aneugenicity in the GIT. Thus, also in the present opinion (FGE.216Rev3), the Panel followed the recommendations of the EFSA Scientific Committee guidance on aneugenicity (EFSA Scientific Committee, 2021) and compared the concentration resulting in aneugenicity in vitro (20 µg/mL, the highest concentration tested in the treatment for 27 h) with the estimated concentration of the substance in the upper part of the GIT following ingestion of food or beverage.

The Panel noted that, for 2-phenylcrotonaldehyde [FL-no: 05.062], a concern for aneugenicity was raised in the previous opinion (FGE.216Rev2; EFSA FAF Panel, 2022) because for some food categories, the previously reported use levels (up to 5.13 mg/kg) were less than one order of magnitude below the concentration of 20 µg/mL for which an aneugenic effect of this flavouring substance was observed in the in vitro MN assay. However, based on newly reported data on normal and maximum use levels (now up to 2 mg/kg, Appendix G, Table G1), the Panel considered that the new maximum use level of 2 mg/kg is one order of magnitude lower than the concentration resulting in an aneugenic effect in vitro. Therefore, use levels not greater than 2 mg/kg or mg/L would not give rise to concern with respect to aneugenicity.

In addition, the Panel noted that the values for the natural occurrence of 2-phenylcrotonaldehyde in foods and food ingredients are generally below 2 mg/kg, with the exception of cocoa beans reported to be up to 3 mg/kg (Table G4, Appendix G). Taking into account the generally low concentrations along with the dilution effect of food formulation and food preparation using these foods and food ingredients, the Panel concluded that, based on the information available, the concentrations of 2-phenylcrotonaldehyde present naturally in food sources do not give rise to a concern for aneugenicity.

Based on new in vitro genotoxicity data provided for 5-methyl-2-phenylhex-2-enal [FL-no: 05.099], the Panel noted that the substance did not induce an increase in the frequency of micronucleated cells and it did not induce gene mutations in studies assessed as of high relevance. However, positive results were reported in an in vitro CA assay (Honma et al., 2022) in the absence of metabolic activation, considered by the Panel as of limited relevance. In the same publication (Honma et al., 2022), a negative in vivo MN assay was also reported, but it was unclear if bone marrow was exposed to the substance and if the MTD was reached.

5-Methyl-2-phenylhex-2-enal [FL-no: 05.099] was tested in a new in vivo MN study in rats (Product Safety Labs, 2024b), where also the presence of the substance in blood was analysed. The levels of 5-methyl-2-phenylhex-2-enal measured in whole blood were generally below the levels of quantification of the method (< 5 µg/mL). The Panel considered the results of this in vivo MN assay as inconclusive because the bone marrow exposure was not demonstrated.

An in vivo comet assay in duodenum was provided (Documentation provided to EFSA No. 13), based on which the Panel concluded that 5-methyl-2-phenylhex-2-enal did not induce DNA damage in the duodenum of rats treated up to 500 mg/kg bw per day (estimated MTD).

5 | CONCLUSIONS

Information on the analysis of 2-phenylcrotonaldehyde [FL-no: 05.062] in rat plasma, intended to demonstrate systemic exposure at the highest dose tested in the previously evaluated in vivo MN studies, has been assessed.

The results of the plasma analysis are considered by the Panel to be sufficient to rule out a concern for systemic aneugenicity of the substance. However, the data available do not overrule the concern for aneugenicity of 2-phenylcrotonaldehyde at the site of contact on oral exposure, where the concentrations will be maximal, taking into account that the positive findings in the in vitro MN assay were seen only in the absence of metabolic activation.

Therefore, in line with the principles described in the EFSA Scientific Committee guidance on aneugenicity (EFSA Scientific Committee, 2021), the Panel compared the lowest concentration resulting in aneugenicity in vitro with the reported use levels of 2-phenylcrotonaldehyde [FL-no: 05.062] in food, and noted that they are one order of magnitude below the concentration for which an aneugenic effect of this flavouring substance was observed in the in vitro MN assay (i.e. 20 µg/mL). Therefore, based on this comparison, the Panel concluded that the use of the flavouring substance [FL-no: 05.062] in foods, including beverages, would not raise a concern for aneugenicity if the use levels were not greater than 2 mg/kg or mg/L.

Concerning 5-methyl-2-phenylhex-2-enal [FL-no: 05.099], based on the new genotoxicity data evaluated in this opinion, the Panel concluded that there is no concern for genotoxicity.

Accordingly, 2-phenylcrotonaldehyde [FL-no: 05.062] and 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] can proceed to the full evaluation in accordance with Commission Regulation (EC) No 1565/2000, which in this case will be conducted within FGE.55 Revision 1.

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ABBREVIATIONS

AFC	Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food
BW	body weight
CAS	Chemical Abstract Service
CEF	Panel on Scientific Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CHL	Chinese hamster lung (cells)

CHO	Chinese hamster ovary (cells)
CoE	Council of Europe
CP	cyclophosphamide
CREST	calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly and telangiectasia
FAF	Panel on Food Additives and Flavourings
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS	Flavour Information System database
FL-no	Flavis number
GIT	gastrointestinal tract
GLP	Good Laboratory Practice
ID	identity
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LC–MS/MS	liquid chromatography–tandem mass spectrometry
MMC	mitomycin C
MN	micronucleus
MNBN	micronucleated binucleate cells
MNPCE	micronucleated polychromatic erythrocytes
MS	mass spectrometry
MSDI	maximised survey-derived daily intake
mTAMDI	modified theoretical added maximum daily intake
MTD	maximum tolerated dose
NCE	normochromatic erythrocytes
NEG	negative
NMR	nuclear magnetic resonance
No	number
OD	out of applicability domain
OECD	Organisation for Economic Co-operation and Development
PCE	polychromatic erythrocytes
(Q)SAR	(quantitative) structure–activity relationship
RET	reticulocytes
SCF	Scientific Committee on Food
VB	vinblastine
VCF	volatile compounds in food
WHO	World Health Organization

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PANEL MEMBERS

Laurence Castle, Monica Andreassen, Gabriele Aquilina, Maria Bastos, Polly Boon, Biagio Fallico, Rex FitzGerald, María José Frutos Fernández, Bettina Grasl-Kraupp, Ursula Gundert-Remy, Rainer Gürtler, Eric Houdeau, Marcin Kurek, Henriqueta Louro, Patricia Morales and Sabina Passamonti.

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APPENDIX A

Specification summary of the substances in the Flavouring Group Evaluation 216Rev3

TABLE A1 Specification summary of the substances in the Flavouring Group Evaluation 216Rev3 (JECFA, 2004).

FL-no / JECFA-no	Chemical name	Structural formula	FEMA no / CoE no / CAS no	Phys. form / Mol. formula / Mol. weight	Solubility in water[a] / Solubility in ethanol[b]	Boiling point, °C[c] / Melting point, °C / ID test / Assay minimum	Refrac. Index[d] / Spec. gravity[e]
05.062	2-Phenylcrotonaldehyde		3224 / 670 / 4411-89-6	Liquid / $C_{10}H_{10}O$ / 146.19	Insoluble / Soluble	177 (20 hPa) / n.a. / NMR / 97%	1.558–1.564 / 1.031–1.037
1474							
05.099	5-Methyl-2-phenylhex-2-enal		3199 / 10365 / 21834-92-4	Liquid / $C_{13}H_{16}O$ / 188.27	Insoluble / Soluble	96–100 (0.9 hPa) / n.a. / NMR / 96%	1.531–1.536 / 0.970–0.976
1472							

Abbreviations: CAS, Chemical Abstract Service; CoE, Council of Europe; FEMA, Flavor and Extract Manufacturers Association; FLAVIS, Flavour Information System (database); FL-no, FLAVIS number; ID, identity; JECFA, The Joint FAO/WHO Expert Committee on Food Additives; n.a., not applicable; NMR, nuclear magnetic resonance.

[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.

APPENDIX B

Summary of safety evaluation by JECFA, applying the Procedure

TABLE B1 Summary of safety evaluation by JECFA of the substances in FGE.19 subgroup 3.3 (assessed for genotoxicity in FGE.216), applying the Procedure (based on intakes calculated by the MSDI approach) (JECFA, 2006, 2015).

FL-no JECFA-no CAS no	Chemical name	Structural formula	MSDI[a] (μg/capita per day)	Class[b] Evaluation procedure path[c] (JECFA)	Outcome on the named compound (JECFA, 2006)	Outcome on the named compound (JECFA, 2015)	EFSA conclusion on the named compound (genotoxicity)
05.062 1474 4411-89-6	2-Phenylcrotonaldehyde		1.7	Class I A3: Intake below threshold	[d]	[e]	Evaluated in FGE.216Rev3. No concern for aneugenicity at use levels ≤ 2 mg/kg or mg/L.
05.099 1472 21834-92-4	5-Methyl-2-phenylhex-2-enal		15	Class II A3: Intake below threshold	[d]	[e]	Evaluated in FGE.216Rev3, no concern for genotoxicity.
05.100[7] 1473 26643-91-4	4-Methyl-2-phenylpent-2-enal		0.34	Class II A3: Intake below threshold	[d]	[e]	Additional genotoxicity data requested in FGE.216Rev2. Authorisation for use as flavouring substance in EU has been withdrawn.[7]
05.175[8] – 3491-63-2	2-Phenylpent-2-enal		0.011	No evaluation	Not evaluated by JECFA	Not evaluated by JECFA	Additional genotoxicity data requested in FGE.216Rev2. Authorisation for use as flavouring substance in EU has been withdrawn.[8]
05.222[8] 2069 26643-92-5	2-Phenyl-4-methyl-2-hexenal		3.0	No evaluation	Not evaluated by JECFA	[f]	Additional genotoxicity data requested in FGE.216Rev2. Authorisation for use as flavouring substance in EU has been withdrawn.[8]

[a]EU MSDI: Amount added to food as flavour in (kg/year) × 10E^9/(0.1 × population in Europe (=375 × 10E^6) × 0.6 × 365) = μg/capita per day, calculated by EFSA based on the most recent data submitted.

[b]Thresholds of toxicological concern: Class I = 1800 μg/person per day, Class II = 540 μg/person per day, Class III = 90 μg/person per day.

[c]Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B, substances cannot be predicted to be metabolised to innocuous products.

[d]No safety concern based on intake calculated by the MSDI approach of the named compound.

[e]Given the potential genotoxicity of 2-phenylcrotonaldehyde (JECFA No. 1474), JECFA recommended that the evaluation of [FL-no: 05.062, 05.099, 05.100] should be reconsidered (JECFA, 2015).

[f]The Procedure cannot be applied until concerns regarding genotoxicity of 2-phenylcrotonaldehyde (JECFA No. 1474) are resolved.

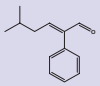
[7]Commission Regulation (EU) 2025/147 of 29 January 2025 amending Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council as regards the removal of the flavouring substance 4-Methyl-2-phenylpent-2-enal (FL No 05.100) from the Union list. OJ L, 2025/147, 30.1.2025.

[8]Commission Regulation (EU) 2024/234 of 15 January 2024 amending Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council as regards the removal of certain flavouring substances from the Union list. OJ L, 2024/234, 16.1.2024.

APPENDIX C

(Q)SAR predictions on mutagenicity in five models for five aldehydes from Subgroup 3.3 (as reported in EFSA, 2009a)

TABLE C1 (Q)SAR predictions on mutagenicity for five aldehydes from Subgroup 3.3 considered in FGE.216 (EFSA, 2009a).

FL-no JECFA-no	Chemical name	Structural formula[a]	FEMA no CoE no CAS no	ISS local model Ames test TA100[b]	MultiCASE Ames test[c]	MultiCASE mouse lymphoma test[d]	MultiCASE chromosomal aberration test in CHO[e]	MultiCASE chromosomal aberration test in CHL[f]
05.062 1474	2-Phenylcrotonaldehyde		3224 670 4411-89-6	NEG	OD	OD	OD	OD
05.099 1472	5-Methyl-2-phenylhex-2-enal		3199 10365 21834-92-4	NEG	OD	OD	OD	OD
05.100 1473	4-Methyl-2-phenylpent-2-enal		3200 10366 26643-91-4	NEG	OD	OD	OD	OD
05.175	2-Phenylpent-2-enal		– – 3491-63-2	NEG	OD	OD	OD	OD
05.222	2-Phenyl-4-methyl-2-hexenal		– – 26643-92-5	NEG	OD	OD	OD	OD

Abbreviations: (Q)SAR, (quantitative) structure–activity relationship; CAS, Chemical Abstract Service; CHL, Chinese hamster lung (cells); CHO, Chinese hamster ovary (cells); CoE, Council of Europe; FEMA, Flavor and Extract Manufacturers Association; FLAVIS, Flavour Information System (database); FL-no, FLAVIS number; JECFA, The Joint FAO/WHO Expert Committee on Food Additives; NEG, negative; 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.).

[a]Structure group 3.3: α,β-unsaturated 2-phenyl substituted aldehydes.

[b]Local model on aldehydes and ketones, Ames TA100.

[c]MultiCase Ames test.

[d]MultiCase mouse lymphoma test.

[e]MultiCase chromosomal aberration in CHO.

[f]MultiCase chromosomal aberration in CHL.

APPENDIX D

Genotoxicity data on 2-phenylcrotonaldehyde [FL-no: 05.062] evaluated in FGE.216Rev1 (as reported in EFSA CEF Panel, 2013)

TABLE D1 Summary of in vitro genotoxicity data on 2-phenylcrotonaldehyde [FL-no: 05.062] of subgroup 3.3.

Chemical name [FL-no]	Test system in vitro	Test object	Concentrations of substance and test conditions	Result	References	Comments
2-Phenylcrotonaldehyde [FL-no: 05.062]	Reverse mutation	*S.* Typhimurium TA98, TA100, TA1535, TA1537 and TA102	1.6, 8, 40, 200, 1000 and 5000 μg/plate[a,b]	Negative	Kilford (2010)	Toxicity was observed in all strains at 5000 μg/plate in the absence and presence of S9-mix, and at 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. All strains were negative. Study design complied with current recommendations. Acceptable top concentration was achieved.
		S. Typhimurium TA98, TA100, TA1535 TA1537 and TA102	51.2, 128, 320, 800, 2000 and 5000 μg/plate[b,d]	Negative	Kilford (2010)	Toxicity was observed in strains TA1537 and TA102 at 2000 μg/plate and above in the absence of S9-mix and at 320 μg/plate in the presence of S9-mix. 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-mix. 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[c,e]	Negative		
		S. Typhimurium TA1537 and TA102	20.48, 51.2, 128, 320, 800, 2000 μg/plate[c,e]	Negative		
		S. Typhimurium TA98, TA100, TA1535	31.25–1000 μg/plate[c,e]	Negative	Kilford (2010)	Toxicity was observed at 3500 μg/plate and above in strain TA100 in the absence of S9-mix. In the presence of S9-mix, 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[c,e]	Negative		
		S. Typhimurium TA100	320–5000 μg/plate[b,d]	Negative		

(Continues)

TABLE D1 (Continued)

Chemical name [FL-no]	Test system in vitro	Test object	Concentrations of substance and test conditions	Result	References	Comments
	Micronucleus induction	Human peripheral blood lymphocytes	40, 60, 100, 120 µg/mL[d,f]	Positive	Lloyd (2012)	The MNBN cell frequencies increases were statistically significant at the top two concentrations, but only slightly exceeded the 95% range of historical controls at the highest dose. All other treated cultures fell within the normal range. The study complies with OECD TG 487.
			100, 130, 140 µg/mL[e,f]	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 TG 487.
			20, 23, 26 µg/mL[d,g]	Negative		
			20, 60, 70 and 80 µg/mL[d,f]	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 TG 487.

[a]With and without S9 metabolic activation.
[b]Plate incorporation method.
[c]Pre-incubation method.
[d]Without S9 metabolic activation.
[e]With S9 metabolic activation.
[f]3-h incubation with 21-h recovery period.
[g]24-h incubation with no recovery period.

TABLE D2 Summary of in vivo genotoxicity data on 2-phenylcrotonaldehyde [FL-no: 05.062] of subgroup 3.3.

Chemical name		Test object				
[FL-no]	Test system in vivo	Route	Doses	Result	References	Comments
2-Phenylcrotonaldehyde [FL-no: 05.062]	Micronucleus assay in bone marrow	Rat Gavage	70, 350, and 700 mg/kg bw per day	Negative	Henderson (2013)	The study complies with OECD TG 474. No clear evidence of bone marrow exposure, therefore the test is inconclusive.

APPENDIX E

Genotoxicity data on 2-phenylcrotonaldehyde [FL-no: 05.062] evaluated in FGE.216Rev2 (as reported in EFSA FAF Panel, 2022)

TABLE E1 Summary of additional in vitro genotoxicity data on 2-phenylcrotonaldehyde [FL-no: 05.062] of subgroup 3.3.

Chemical name [FL-no]	Test system in vitro	Test object	Concentrations of substance and test conditions	Result	References	Comments
2-Phenylcrotonaldehyde [FL-no: 05.062]	Reverse mutation test	*S.* Typhimurium TA98, TA100, TA1535, TA1537 *E. coli* WP2 uvrA	1.5, 5.0, 15, 50, 150, 500, 1500 and 5000 µg/plate[a,b,c]	Positive	BioReliance (2016)	Reliable without restrictions. Study performed according to OECD TG 471 and in compliance with GLP.
			15, 50, 150, 500, 1000, 1500 and 5000 µg/plate[a,b]			
	Micronucleus assay with CREST staining	Human TK6 cells	20, 40, 45 µg/mL[d,f]	Negative	BioReliance (2018a)	Reliable without restrictions. Study performed according to OECD TG 487 and in compliance with GLP.
			40, 60, 80 µg/mL[e,f]	Negative		The given concentrations are those for the cultures that were scored for micronuclei.
			5, 15, 20 µg/mL (assay 1) 16, 18, 20 µg/mL (assay 2)[d,g]	Positive		CREST analysis indicates that 2-phenylcrotonaldehyde induced MN by an aneugenic mechanism.

[a]With and without S9 metabolic activation.
[b]Plate incorporation method.
[c]Preliminary toxicity and mutagenicity test.
[d]Without S9 metabolic activation.
[e]With S9 metabolic activation.
[f]4-h incubation with 23-h recovery period.
[g]27-h incubation with no recovery period.

TABLE E2 Summary of additional in vivo genotoxicity data on 2-phenylcrotonaldehyde [FL-no: 05.062] of subgroup 3.3.

Chemical name		Test object				
[FL-no]	**Test system in vivo**	**Route**	**Doses**	**Result**	**References**	**Comments**
2-Phenylcrotonaldehyde [FL-no: 05.062]	Micronucleus assay in bone marrow	Rat Gavage	70, 350 and 700 mg/kg bw per day	Inconclusive	Covance (2013)	Reliable with restrictions (no clear evidence of bone marrow exposure). Study performed in compliance with GLP and according to OECD TG 474. The highest dose tested (700 mg/kg bw per day) was an estimate of the MTD according to the micronucleus study by Henderson (2013).
	Plasma bioanalysis (Micronucleus assay)[a]	Rat Gavage	700 mg/kg bw per day	Inconclusive	Covance (2014)	GC-MSD method validated (recovery, accuracy and precision). Linearity and working range were assessed. The concentration of 2-phenylcrotonaldehyde detected was below the linearity range. Not a GLP study.
	Comet assay in duodenum	Rat Gavage	175, 350 and 700 mg/kg bw per day	Negative	Covance (2016)	Reliable without restrictions. Study performed in compliance with GLP and according to OECD TG 489. The dose of 700 mg/kg bw per day was an estimate of the MTD according to the micronucleus study by Henderson (2013).
	Gene mutation assay in duodenum, liver and bone marrow	Big Blue® C57BL/6 transgenic mice Gavage	75, 250 and 500 mg/kg bw per day	Negative	BioReliance (2018b)	Reliable without restrictions. Study performed in compliance with GLP and according to OECD TG 488.

[a]Plasma obtained from satellite group of animals in the study by Henderson (2013) and from satellite group of animals in the study by Covance (2013).

APPENDIX F

Genotoxicity data evaluated in FGE.216Rev3

TABLE F1 Summary of in vitro genotoxicity data on 5-methyl-2-phenylhex-2-enal [FL-no: 05.099].

Chemical name [FL-no]	Test system in vitro	Test object	Concentrations of substance and test conditions	Result	Reliability/comments	Relevance of test system/relevance of the result	References
5-Methyl-2-phenylhex-2-enal [FL-no: 05.099]	Reverse mutation test	*S.* Typhimurium TA98, TA100, TA1535, TA1537 *E. coli* WP2 uvrA	Experiment 1: 4.88–5000 µg/plate (with and without S9-mix) Experiment 2: 9.77–313 µg/plate (with and without S9-mix) Confirmatory experiment 1: 9.77–313 µg/plate (with and without S9-mix) Confirmatory experiment 2: 81.3–313 µg/plate (only without S9-mix, only in strains TA98 and TA100)	Negative	Reliable without restrictions. The study was performed according to OECD TG 471 and in compliance with GLP.	High/High	Kasamatsu et al. (2021); Honma et al. (2022)
	Micronucleus assay	Human peripheral blood lymphocytes	34, 48, 56, 68 µg/mL (3 h + 21 h, without S9-mix) 50, 72, 90, 100 µg/mL (3 h + 21 h, with S9-mix) 24, 36, 40 µg/mL (24 h + 24 h, without S9-mix)	Negative	Reliable without restrictions. Study performed according to OECD TG 487 and in compliance with GLP. The given concentrations are those for the cultures that were scored for micronuclei.	High/High	Labcorp (2024)
	Chromosomal aberration	Chinese hamster lung cells	60, 70, 80 µg/mL (6 h, without S9-mix) 140, 160, 180 µg/mL (6 h, with S9-mix) 40, 50, 60 µg/mL (24 h, without S9-mix)	Negative Negative Positive	Reliable with restrictions (200 metaphases scored instead of 300). The study was performed according to OECD TG 473 and in compliance with GLP.	High/Limited	Honma et al. (2022)

TABLE F2 Summary of in vivo studies on 2-phenylcrotonaldehyde [FL-no: 05.062] and 5-methyl-2-phenylhex-2-enal [FL-no: 05.099].

Chemical name [FL-no]	Test system in vivo	Test object Route	Doses (mg/kg bw per day) and exposure conditions	Result	Reliability/comments	Relevance of test system/ relevance of the result	References
2-Phenylcrotonaldehyde [FL-no: 05.062]	Plasma analysis	Rats Gavage	700 mg/kg bw [highest dose tested in the in vivo MN assays, Henderson (2013) and Covance (2013)], single administration	Very low levels (highest mean concentration was about 1 μg/mL, with variability among individual animals)	Validated LC-MS/MS method. The concentration of 2-phenylcrotonaldehyde detected was inside the linearity range (10–2000 ng/mL).	n.a.	Product Safety Labs (2024a)
5-Methyl-2-phenylhex-2-enal [FL-no: 05.099]	Micronucleus assay in peripheral blood	Rats Gavage	250, 500 and 1000, once per day for 3 days, with 24 h interval	Inconclusive (negative, but without demonstration of bone marrow exposure)	Not reliable (no clear evidence of bone marrow exposure, the MTD was not reached). Study performed according to OECD TG 474 and in compliance with GLP.	High/Low	Product Safety Labs (2024b)
	Blood bioanalysis		1000 mg/kg bw (highest dose tested in the in vivo MN assay), single administration	Inconclusive	Validated LC–MS/MS method. The concentration of 5-methyl-2-phenylhex-2-enal detected was below the linearity range.	n.a.	
	Micronucleus assay in bone marrow	Mice Gavage	250, 500 and 1000, at 0 and 24 h	Inconclusive (negative, but without demonstration of bone marrow exposure)	Not reliable (no clear evidence of bone marrow exposure, unclear whether the MTD was reached). The study was performed according to OECD TG 474 and in compliance with GLP.	High/Low	Honma et al. (2022)
	Comet assay in duodenum	Rat Gavage	100, 250 and 500, at 0 and 24 h	Negative	Reliable without restrictions. Study performed according to OECD TG 489 and in compliance with GLP.	High/High	Documentation provided to EFSA No. 13

Abbreviation: n.a., not applicable.

APPENDIX G

Exposure

G.1 | Uses and use levels as provided by the Flavour Industry

For each substance, normal and maximum use levels have been provided by industry (documentation provided to EFSA No. 12) according to the EFSA Guidance on the data required for the risk assessment of flavourings to be used in or on foods (EFSA CEF Panel, 2010). Use level data (Table G1) have been used to calculate the 'modified Theoretical Added Maximum Daily Intake' (mTAMDI).[9] Since the calculation of mTAMDI is based on food categories as reported in Annex III of Regulation (EC) 1565/2000 (Table G2), for the substances [FL-no: 05.062, 05.099], the highest values of the normal use levels among the subcategories have been selected.

TABLE G1 Use levels reported by industry for the flavouring substances in FGE.19 subgroup 3.3.

CODEX code	Food categories[a,b]	Standard portions[c] (g)	Use level as added flavouring substance (mg/kg)			
			Normal	Max	Normal	Max
Flavouring substances FL-no			**05.062**		**05.099**	
01.0	Dairy products and analogues, excluding products of category 02.0					
01.1	Milk and dairy-based drinks	200	0.25	0.50	1.50	5.00
01.6	Cheese and analogues	40	0.30	1.40	1.60	3.20
01.7	Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	125	0.40	1.30	2.10	5.00
02.0	Fats and oils and fat and oil emulsions (type water-in-oil)					
02.1	Fats and oils essentially free from water	15	0.20	1.6	2.00	5.00
02.2	Fat emulsions mainly of type water-in-oil	15	0.20	1.6	2.00	5.00
02.3	Fat emulsions mainly of type water-in-oil, including mixed and/or flavoured products based on fat emulsions	15	0.20	1.6	2.00	5.00
02.4	Fat-based desserts excluding dairy-based dessert products of category 1.7	50	0.50	1.30	0.80	5.00
03.0	Edible ices, including sherbet and sorbet	50	0.40	1.15	3.50	5.00
04.1.2	Processed fruit	125	0.03	0.05	0.50	1.10
04.1.2.5	Jams, jellies, marmalades	30	0.30	0.30	0.80	2.70
04.2.2	Processed vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes, and aloe vera), seaweed, and nut and seed purees and spreads (e.g. peanut butter) and nuts and seeds	200	0.03	0.20	0.05	0.50
05.0	Confectionery					
05.1	Cocoa products and chocolate products, including imitations and chocolate substitutes	40	1.00	2.00	3.50	5.00

(Continues)

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

TABLE G1 (Continued)

CODEX code	Food categories[a,b]	Standard portions[c] (g)	Use level as added flavouring substance (mg/kg)			
			Normal	Max	Normal	Max
Flavouring substances FL-no			**05.062**		**05.099**	
05.2	Confectionery, including hard and soft candy, nougats, etc., other than 05.1, 05.3 and 05.4	30	0.50	2.00	3.50	5.00
05.3	Chewing gum	3	1.60	2.00	3.50	5.00
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	35	0.10	1.10	3.50	5.00
06.0	Cereals and cereal products, incl. flours & starches from roots & tubers, pulses & legumes, excluding bakery					
06.3	Breakfast cereals, including rolled oats	30	0.40	2.00	0.80	2.10
06.4	Pastas and noodles and like products (e.g. rice paper, rice vermicelli, soya bean pastas and noodles)	200	0.01	0.04	0.03	0.30
06.5	Cereal and starch based desserts (e.g. rice pudding, tapioca pudding)	200	0.20	1.30	1.00	1.60
06.6	Batters (e.g. for breading or batters for fish or poultry)	30	0.04	0.30	1.00	3.00
06.7	Pre-cooked or processed rice products, including rice cakes (Oriental type only)	200	0.30	0.60	0.02	0.06
06.8	Soya bean products (excluding soya bean products of food category 12.9 and fermented soya bean products of food category 12.10)	100	0.05	0.30	0.40	1.60
07.0	Bakery wares					
07.1	Bread and ordinary bakery wares	50	0.50	2.00	1.70	5.00
07.2	Fine bakery wares (sweet, salty, savoury) and mixes	80	1.20	2.00	5.00	5.00
08.0	Meat and meat products, including poultry and game					
08.2	Processed meat, poultry and game products in whole pieces or cuts	100	0.70	0.80	0.08	0.20
08.3	Processed comminute meat, poultry and game products	100	0.70	0.80	0.08	0.20
09.0	Fish and fish products, including molluscs, crustaceans and echinoderms					
09.2	Processed fish and fish products, including molluscs, crustaceans and echinoderms	100	0.40	0.50	4.10	5.00
09.3	Semi-preserved fish and fish products, including molluscs, crustaceans and echinoderms	100	0.40	0.50	4.10	5.00
09.4	Fully preserved, including canned or fermented, fish and fish products, including molluscs, crustaceans and echinoderms	100	0.40	0.50	4.10	5.00
10.0	Eggs and egg products					
10.2	Egg products	100	0.02	0.20	0.05	0.50
10.3	Preserved eggs, including alkaline. salted and canned eggs	100	0.02	0.20	0.05	0.50
10.4	Egg-based desserts (e.g. custard)	125	0.80	2.00	5.00	5.00
11.0	Sweeteners, including honey					
11.1	Refined and raw sugar	10	–	–	1.00	2.40

TABLE G1 (Continued)

CODEX code	Food categories[a,b]	Standard portions[c] (g)	Use level as added flavouring substance (mg/kg)			
			Normal	Max	Normal	Max
Flavouring substances FL-no			**05.062**		**05.099**	
11.2	Brown sugar excluding products of food category 11.1	10	–	1.50	0.70	5.00
11.3	Sugar solutions and syrups, and (partially) inverted sugars, including molasses and treacle, excluding products of food category 11.1	30	0.01	1.50	0.20	5.00
11.4	Other sugars and syrups (e.g. xylose, maple syrup, sugar toppings)	30	0.40	1.50	1.30	5.00
11.6	Table-top sweeteners, including those containing high-intensity sweeteners	1	–	–	0.004	0.02
12.0	Salts, spices, soups, sauces, salads, protein products, etc.					
12.2	Herbs, spices, seasonings and condiments (e.g. seasoning for instant noodles)	1	0.40	0.60	0.20	5.00
12.3	Vinegars	15	0.30	1.80	1.00	3.00
12.4	Mustards	15	0.10	0.20	2.00	5.00
12.5	Soups and broths	200	0.20	0.40	0.07	0.20
12.6	Sauces and like products	30	0.30	1.90	0.40	1.60
12.7.1	Salads 120 g (e.g. macaroni salad, potato salad) excluding cocoa- and nut-based spreads of food categories	120	0.02	0.20	0.05	0.60
12.7.2	Sandwich spreads (20 g), excluding cocoa- and nut-based spreads of food categories	20	0.02	0.20	2.30	5.00
12.9	Protein products	15	2.00	2.00	5.00	5.00
12.10	Fermented soya bean products	40	1.00	1.00	5.00	5.00
13.0	Foodstuffs intended for particular nutritional uses					
13.3	Dietetic foods intended for special medical purposes (excluding food products of category 13.1)	200	0.30	2.00	0.50	1.10
13.4	Dietetic formulae for slimming purposes and weight reduction	200	0.30	2.00	0.50	1.10
13.5	Dietetic foods (e.g. supplementary foods for dietary use), excluding products of food categories 13.1–13.4 and 13.6	200	0.30	2.00	0.50	1.10
13.6	Food supplements	5	0.00004	0.00004	1.10	5.00
14.1	Non-alcoholic ('soft') beverages	300	0.50	2.00	1.50	5.00
14.2	Alcoholic beverages, incl. alcohol-free and low-alcoholic counterparts		–	–	0.11	0.11
14.2.1	Beer and malt beverages	300	0.10	0.20	0.50	1.25
14.2.2	Grape wines	150	0.20	0.30	2.50	5.00
14.2.3	Mead	150	0.50	0.50	4.00	5.00
14.2.4	Spirituous beverages	30	0.40	1.50	0.50	5.00
15.0	Ready-to-eat savouries	30	1.20	2.00	3.50	5.00

(Continues)

TABLE G1 (Continued)

CODEX code	Food categories[a,b]	Standard portions[c] (g)	Use level as added flavouring substance (mg/kg)			
			Normal	Max	Normal	Max
Flavouring substances FL-no			**05.062**		**05.099**	
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) – foods that could not be placed in categories 01.0–15.0	300	0.10	0.40	0.50	2.30

[a]Most of the categories reported are the subcategories of Codex GSFA (General Standard for Food Additives, available at http://www.codexalimentarius.net/gsfaonline/CXS_192e.pdf) used by JECFA in the SPET technique (FAO/WHO, 2008).

[b]Only food categories for which use has been reported are included in the table.

[c]For adults. In case of foods marketed as powder or as concentrates, use levels must be reported for the reconstituted product, considering the instructions reported on the product label or one of the standard dilution factors established by JECFA (FAO/WHO, 2008):
– 1/25 for powder used to prepare water-based drinks such as coffee, containing no additional ingredients;
– 1/10 for powder used to prepare water-based drinks containing additional ingredients such as sugars (iced tea, squashes, etc.);
– 1/7 for powder used to prepare milk, soups and puddings;
– 1/3 for condensed milk.

TABLE G2 Distribution of the 18 food categories listed in Commission Regulation (EC) No 1565/2000[3] into the seven SCF food categories used for TAMDI calculation (SCF, 1995).

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

G.2 | Intake data from intended use

The Panel noted that exposure estimates were requested according to the Terms of Reference (Section 1.1.2) only for those substances for which a concern for genotoxicity could not be ruled out. However, since EU MSDI value were already available and new use levels have been provided for the substances under assessment in FGE.216Rev3, i.e. [FL-no: 05.062 and 05.099], their exposure estimates have been included for information in Table G3.

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

Use levels for 2-phenylcrotonaldehyde [FL-no: 05.062] and 5-methyl-2-phenylhex-2-enal [FL-no: 05.099] provided by industry (Documentation provided to EFSA No. 12) and listed in Table G1 have been used to calculate the mTAMDI. Since use levels for subcategories are reported according to the EFSA Guidance on the data required for the risk assessment of flavourings (EFSA CEF Panel, 2010), the highest value of the normal use levels among the subcategories was selected as the value for each main category for the calculation of mTAMDI.

The EU MSDI and mTAMDI exposure estimates are given in Table G3.

TABLE G3 Estimated exposure to 2-phenylcrotonaldehyde [FL-no: 05.062] and 5-methyl-2-phenylhex-2-enal [FL-no: 05.099].

		EU MSDI	mTAMDI
FL-no	**Chemical name**	**μg/capita per day**	**μg/person per day**
05.062	2-phenylcrotonaldehyde	1.7	442
05.099	5-methyl-2-phenylhex-2-enal	15	1500

G.3 | Presence in food

The Panel noted that estimates of exposure were requested according to the Terms of Reference (Section 1.1.2), only for those substances for which a concern for genotoxicity could not be ruled out. These estimates should take into account also the natural occurrence of these substances in food.

Since, in the present opinion, the Panel concluded that for 2-phenylcrotonaldehyde [FL-no: 05.062] use levels not greater than 2 mg/kg would not give rise to concern with respect to aneugenicity, the Panel considered that information on the natural occurrence of the substance can also be relevant to address the Terms of Reference. For this purpose, EFSA used the Volatile Compounds in Food (VCF) database (VCF online database, 2026).

According to the VCF database,[10] 2-phenylcrotonaldehyde [FL-no: 05.062] is reported to be present in natural food sources and processed food as listed in Table G4. This table reports a list of foodstuffs containing [FL-no: 05.062], updated as of April 2026.

TABLE G4 Examples of 2-phenylcrotonaldehyde [FL-no: 05.062] occurrence in foodstuff.

Food item	Process	Qualitative	Quantitative (ppm)	References
Asparagus (*Asparagus officinalis* L.)	Cooked		0.01	Tressl et al. (1977)
	Raw	Yes		
Barley	Malting		0.01–0.045	Kossa et al. (1979)
	Raw	Yes		Farley and Nursten (1980)
Black garlic	Thermally processed (55°C–85°C, 1–12 days)		0.2453–1.7449	Yu et al. (2024)
Black tea	Raw	Yes		Sato et al. (1970)
	Information not available	Yes		Bricout et al. (1967)
	Fermentation and drying (with and without initial shaking and standing process)		0.02	Wang et al. (2024)
	Microbial fermentation	Yes		Kawakami et al. (1987)
	Fermentation/microbial fermentation	Yes		Kobayashi and Kawakami (1991)
	Brewing		0.1–0.15	Schreier and Mick (1984)

(Continues)

[10] https://www.vcf-online.nl/VcfCompoundDetails.cfm?volatkey=0309050300. Database accessed on 22nd April 2026.

TABLE G4 (Continued)

Food item	Process	Qualitative	Quantitative (ppm)	References
Buckwheat flour	Boiled	Yes		Yajima et al. (1983)
Chinese rice wine (Guyelongshan Huadiao)	From fermentation of cooked glutinous rice		0.199	Chen et al. (2013)
Cocoa beans	Fermentation and drying (with and without inoculum addition)	Yes		Chagas Junior et al. (2021)
	Roasting		2.5–3	Silwar (1988)
	Roasting	Yes		Van Praag et al. (1968)
	Raw/roasting	Yes		Ziegleder (1991)
Cocoa butter	From roasted and unroasted beans	Yes		Carlin et al. (1982)
Cocoa liquor	Beans are roasted, shelled and finely ground into liquor	Yes		Counet et al. (2004) Baigrie and Rumbelow (1987) Müggler-Chavan and Reymond (1967)
Coffee	Roasting		0.1–0.2	Silwar et al. (1987)
	Information not available		0.6	Silwar (1982)
Dark chocolate	Produced *in house*	Yes		Afoakwa et al. (2009)
	Produced by roasted beans and conching			Owusu et al. (2012)
Filbert hazelnut (*Corylus avellano*)	Roasting	Yes		Kinlin et al. (1972)
Honey (*Rhus chinesis*)	Natural capping (10–25 days)		0.1984–0.2652	Li et al. (2024)
Katsuobushi (Dried bonito – variety of fish)	Drying – soup stock	Yes		Washino et al. (1989)
Macadamia nut (*Macadamia integrifolia*)	Roasting and grinding	Yes		Crain Jr and Tang (1975)
Miso (soybean, rice or fish meat)	Fermentation	Yes		Giri et al. (2010)
Milk chocolate	Storage	Yes		Ziegleder and Stojacic (1988)
Mushrooms	Raw		0.1	Pyysalo (1975)
	Raw and pressed into juices	Yes		Pyysalo (1976)
	Raw	Yes		Pyysalo and Honkanen (1976)
Nampla (fish sauce)	Premium		0.00002	Giri et al. (2010)
	Standard		0.00001	
Okra (*Hibiscus esculentus* L.)	Raw	Yes		Ames and Macleod (1990)
Peanut oil	Cold-pressed from raw and roasted peanuts		0.1	Brown et al. (1973)
	Roasted peanuts	Yes		Johnson et al. (1971)
Pork (liver)	Pressure cooked	Yes		Mussinan and Walradt (1974)
Potato chips (American)	Frying	Yes		Buttery (1973)
Rice bran	Raw	Yes		Tsugita et al. (1978)
Sake	Sokujo-brewed (addition of pure lactic acid to start fermentation)		0.00050–0.0033	Sasaki et al. (2023)
	Yamahai-brewed (naturally growing lactic acid to start fermentation)		0.00056–0.0041	
Sesame seed	Roasting	Yes		Manley et al. (1974)
Sesame seed oil (white)	From roaster white sesame seeds	Yes		Yin et al. (2021)
Soy sauce	Dark		0.00018	Giri et al. (2010)
	Light		0.00010	Giri et al. (2010)
Soy sauce moromi (pre-fermented soy sauce)	From heat-treated soya beans and wheat inoculated with koji fungus and mixed with high concentrated salt water	Yes		Mizuno et al. (2024)
Soya bean	Extrusion from defatted flour	Yes		Ames and Macleod (1984)

TABLE G4 (Continued)

Food item	Process	Qualitative	Quantitative (ppm)	References
Yeast extract (*Saccharomyces cerevisiae*)	Processed in different ways and supplied as powder	Yes		Ames and Elmore (1992)
	Processed in different ways and supplied as paste	Yes		Mahadevan and Farmer (2006)