SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 4 May 2017

doi: 10.2903/j.efsa.2017.4847

Scientific Opinion on Flavouring Group Evaluation 226 Revision 1 (FGE.226Rev1): consideration of genotoxicity data on one α,β-unsaturated aldehyde from chemical subgroup 1.1.1(b) of FGE.19

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF), Vittorio Silano, Claudia Bolognesi, Laurence Castle, Jean-Pierre Cravedi, Karl-Heinz Engel, Paul Fowler, Roland Franz, Konrad Grob, Trine Husøy, Sirpa Kärenlampi, Wim Mennes, Maria Rosaria Milana, André Penninks, Andrew Smith, Maria de Fátima Tavares Poças, Christina Tlustos, Detlef Wölfle, Holger Zorn, Corina-Aurelia Zugravu, Mona-Lise Binderup, Riccardo Crebelli, Francesca Marcon, Daniel Marzin, Pasquale Mosesso, Maria Anastassiadou, Maria Carfì, Siiri Saarma and Rainer Gürtler

Abstract

The EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids was requested to evaluate the genotoxic potential of one flavouring substance from subgroup 1.1.1(b) of FGE.19 in the Flavouring Group Evaluation 226 (FGE.226). The flavour industry provided genotoxicity studies for the substance 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071]. Based on these data, the Panel concluded in FGE.226 that 4,5-epoxydec-2(*trans*)-enal did not induce gene mutations in bacterial cells but was positive in an *in vitro* micronucleus assay, so, 4,5-epoxydec-2(*trans*)-enal is considered an *in vitro* genotoxic agent. The negative results obtained in an *in vivo* micronucleus assay cannot overrule the positive results of the *in vitro* micronucleus assay with and without S9-mix due to the lack of demonstration of bone marrow exposure. Following this, the flavour industry has provided plasma analysis of a satellite group of rats treated with 4,5-epoxydec-2(*trans*)-enal in order to investigate the systemic exposure of animals in the *in vivo* micronucleus assay. However, the plasma analysis did not provide enough evidence of target tissue exposure. An *in vivo* Comet assay in rodents was recommended in FGE.226, in order to investigate possible genotoxic effects at the first site of contact (e.g. stomach/duodenum cells) and in the liver. An *in vivo* Comet assay in liver and duodenum was provided that suggests that 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] did not induce DNA damage in the duodenum of rats. However, the genotoxic effect observed *in vitro* was confirmed in the *in vivo* Comet assay in the liver of rats. The Panel concluded that 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] does raise a safety concern with respect to genotoxicity and, therefore, it cannot be evaluated according to the Procedure.

Keywords: 4,5-epoxydec-2(*trans*)-enal, subgroup 1.1.1(b), FGE.19, FGE.226

Requestor: European Commission

Question number: EFSA-Q-2015-00205

Correspondence: fip@efsa.europa.eu

Panel members: Claudia Bolognesi, Laurence Castle, Jean-Pierre Cravedi, Karl-Heinz Engel, Paul Fowler, Roland Franz, Konrad Grob, Rainer Gürtler, Trine Husøy, Sirpa Kärenlampi, Wim Mennes, Maria Rosaria Milana, André Penninks, Vittorio Silano, Andrew Smith, Maria de Fátima Tavares Poças, Christina Tlustos, Detlef Wölfle, Holger Zorn and Corina-Aurelia Zugravu.

Acknowledgements: The Panel wishes to thank the hearing experts: Vibe Beltoft and Karin Nørby and EFSA staff members: Annamaria Rossi for the support provided to this scientific output.

Suggested citation: EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), Silano V, Bolognesi C, Castle L, Cravedi J-P, Engel K-H, Fowler P, Franz R, Grob K, Husøy T, Kärenlampi S, Mennes W, Milana MR, Penninks A, Smith A, Tavares Poças MF, Tlustos C, Wölfle D, Zorn H, Zugravu C-A, Binderup M-L, Crebelli R, Marcon F, Marzin D, Mosesso P, Anastassiadou M, Carfì M, Saarma S and Gürtler R, 2017. Scientific Opinion on Flavouring Group Evaluation 226 Revision 1 (FGE.226Rev1): consideration of genotoxicity data on one α,β-unsaturated aldehyde from chemical subgroup 1.1.1(b) of FGE.19. EFSA Journal 2017;15(5):4847, 24 pp. https://doi.org/10.2903/j.efsa.2017.4847

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Summary

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

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

The Flavouring Group Evaluation 226 (FGE.226), corresponding to subgroup 1.1.1(b) of FGE.19, concerns one α,β-unsaturated aldehyde which is also an epoxide, 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071]. These structural elements are considered to be structural alerts for genotoxicity and the data on genotoxicity previously available did not rule out the concern for genotoxicity.

To evaluate the genotoxic potential of 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071], the Panel has therefore requested additional genotoxicity data according to the test strategy worked out by the Panel.

According to the above requirements, the Industry has submitted genotoxicity studies for 4,5-epoxydec-2(*trans*)-enal. Based on these data the Panel noted, in FGE.226, that 4,5-epoxydec-2(*trans*)-enal did not induce gene mutations in bacterial cells, but was positive in an *in vitro* micronucleus assay, so, 4,5-epoxydec-2(*trans*)-enal is considered an *in vitro* genotoxic agent. The negative results obtained in an *in vivo* micronucleus assay cannot overrule the positive results of the *in vitro* micronucleus assay with and without S9-mix due to the lack of cytotoxicity in the bone marrow. On this basis, an *in vivo* Comet assay in rodents was recommended in order to investigate possible genotoxic effects at the first site of contact (e.g. stomach/duodenum cells) and in liver.

Subsequently, the flavour industry has provided plasma analysis of a satellite group of rats treated with 4,5-epoxydec-2(*trans*)-enal in order to investigate the systemic exposure of animals in the *in vivo* micronucleus assay and an *in vivo* Comet assay in liver and duodenum.

The Panel concluded that the plasma analysis did not provide enough evidence of target tissue exposure.

Comet assay data provided suggest that 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] did not induce DNA damage in the duodenum of rats treated up to 300 mg/kg body weight (bw) per day by oral route (an estimate of the maximum tolerated dose in male rats). However, the genotoxic effect observed *in vitro* was confirmed in an *in vivo* comet assay in the liver of rats.

Overall, the Panel concluded that 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] does raise a safety concern with respect to genotoxicity, and therefore, it cannot be evaluated according to the Procedure.

Table of contents

1. Introduction

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

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

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

On 5 July 2014, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) adopted an opinion on Flavouring Group Evaluation 226 (FGE.226): consideration of genotoxicity data on one α,β–unsaturated aldehyde from chemical subgroup 1.1.1(b) of FGE.19.

The Panel concluded that for 4,5-epoxydec-2(*E*)-enal [FL-no: 16.071] of subgroup 1.1.1 b of FGE.19, the Panel's concern with respect to genotoxicity could not be ruled out and subsequently additional data are requested.

On 9 January 2015, the applicant has submitted the final study on plasma analysis on the above mentioned substance [FL-no: 16.071] in relation to this EFSA evaluation.

1.1.1. Terms of Reference

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

2. Data and methodologies

2.1. History of the evaluation of FGE.19 substances

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

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

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

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

The Panel took note of the (Q)SAR predictions by using two ISS Local Models (Benigni and Netzeva, 2007) and four DTU-NFI MultiCASE Models (Gry et al., 2007; Nikolov et al., 2007) and the fact that there are available data on genotoxicity, *in vitro* and *in vivo*, as well as data on carcinogenicity for several substances. Based on these data the Panel decided that 15 subgroups (1.1.1, 1.2.1, 1.2.2, 1.2.3, 2.1, 2.2, 2.3, 2.5, 3.2, 4.3, 4.5, 4.6, 5.1, 5.2 and 5.3) (EFSA, 2008a) could not be evaluated through the

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

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

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

Procedure due to concern with respect to genotoxicity. Corresponding to these subgroups, 15 FGEs were established: FGE.200, 204, 205, 206, 207, 208, 209, 211, 215, 219, 221, 222, 223, 224 and 225.

For 11 subgroups, the Panel decided, based on the available genotoxicity data and (Q)SAR predictions that a further scrutiny of the data should take place before requesting additional data 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.

The flavouring industry has now submitted additional data and the present revision of FGE.226 concerns the evaluation of these data requested on genotoxicity.

2.2. History of the evaluation of FGE.226

The Flavouring Group Evaluation 226 (FGE.226) concerns the evaluation of the genotoxic properties of one aliphatic aldehyde with the α,β-unsaturation in conjugation with an epoxide moiety. This substance was originally allocated to FGE.200 (FGE.19 subgroup 1.1.1).

Subgroup 1.1.1 of FGE.19 originally covered 71 α,β-unsaturated aliphatic aldehydes. Seventy of these are simple, aliphatic, α,β-unsaturated aldehydes, or precursors for such, with or without additional double bonds, which is not in conjugation with the α,β-unsaturated structure. These 70 substances were allocated to subgroup 1.1.1(a) in FGE.200. The one remaining aliphatic, α,β-unsaturated aldehyde contains an epoxide moiety which is not present within the other 70 members of FGE.19 subgroup 1.1.1. On this basis, it would be anticipated to have different chemical reactivity potential, and would have metabolic options that are not available to the other members of this subgroup. For these reasons, the Panel decided that this substance should be allocated to a separate subgroup, subgroup 1.1.1(b) and evaluated in a separate FGE, FGE.226.

The present revision of FGE.226 (FGE.226Rev1) deals with the evaluation of the genotoxicity data submitted by the flavouring industry for substance 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] from subgroup 1.1.1(b).

FGE	Adopted by EFSA	Link	No. of substances
FGE.226	5 July 2012	http://www.efsa.europa.eu/en/efsajournal/pub/2838	1
FGE.226Rev1	4 May 2017	http://www.efsa.europa.eu/en/efsajournal/pub/4847	1

FGE: Flavouring Group Evaluation.

2.3. Presentation of the substances in flavouring group evaluation 226

The Flavouring Group Evaluation 226 (FGE.226), corresponding to subgroup 1.1.1(b) of FGE.19, concerns one aliphatic aldehyde with the α,β-unsaturation in conjugation with an epoxide moiety, 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071]. These structural elements are considered to be structural alerts for genotoxicity. The substance is shown in Table 1.

4,5-Epoxydec-2(*trans*)-enal [FL-no: 16.071] has previously been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2006, 2009b). A summary of the current evaluation status by JECFA and the outcome of the present consideration is presented in Appendix A, Table A.1.

As the α,β-unsaturated aldehyde and ketone structures are considered alerts for genotoxicity (EFSA, 2008b) and the data on genotoxicity previously available did not rule out the concern for genotoxicity, the Panel has requested additional genotoxicity data for 4,5-epoxydec-2(*trans*)-enal according to the test strategy (EFSA, 2008b).

The flavouring industry has submitted data requested by the Panel in FGE.226 that are evaluated in the present revision of FGE.226 (FGE.226Rev1).

Section 2.4 of this opinion reports the same information that was presented in FGE.226 (EFSA CEF Panel, 2012). Section 3 reports the evaluation of the new data submitted by industry.

Table 1: Specification summary of the substance in the present group (JECFA, 2006, 2009a)

FL-no JECFA-no	EU register name	Structural formula	FEMA no CoE no CAS no	Phys. form Mol. formula Mol. weight	Solubility[(a)] Solubility in ethanol[(b)]	Boiling point, °C Melting point, °C ID test Assay minimum	Refrac. index[(c)] Spec. gravity[(d)]
16.071 1570	4,5-Epoxydec-2(*trans*)-enal		4037 - 188590-62-7	Liquid $C_{10}H_{16}O_2$ 168.23	Soluble Soluble	80–83 (0.8 hPa) IR NMR MS 87% (*trans* isomer) and 8–10% (*cis* isomer)	1.472–1.478 0.943–0.949

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

2.4. Genotoxicity data on 4,5-epoxydec-2(*trans*)-enal evaluated in FGE.226[4]

The Industry has submitted *in vitro* and *in vivo* genotoxicity data for the representative and only substance for this subgroup 1.1.1(b), 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] (EFFA, 2011).

2.4.1. *In vitro* Data

In vitro genotoxicity assays have been performed in bacteria and mammalian cells with the α,β-unsaturated aldehyde 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071].

2.4.1.1. Bacterial Reverse Mutation Assay

An Ames assay was conducted in *Salmonella* Typhimurium strains TA98, TA100, TA1535, TA1537 and TA102 to assess the mutagenicity of 4,5-epoxydec-2(*trans*)-enal, both in the absence and in the presence of metabolic activation by phenobarbital and β-naphthoflavone induced rat liver S9-mix, in two experiments (Sokolowski, 2001). It is a Good Laboratory Practice (GLP) study conducted in accordance with OECD Test Guideline 471. An initial toxicity range-finding experiment was carried out in the absence and presence of S9-mix in strains TA98 and TA100 only, using final concentrations of 4,5-epoxydec-2(*trans*)-enal at 3, 10, 33, 100, 333 and 1,000 μg/plate, plus negative (solvent) and positive controls. Evidence of toxicity, in terms of a decrease in revertant count, was apparent on all plates treated at 333 μg/plate and above in the absence and at 1,000 μg/plate in the presence of S9-mix. In the first experiment, 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] was tested in all five strains in the absence and presence of S9-mix using plate incorporation methodology and final concentrations of either 1, 3, 10, 33, 100, and 333 μg/plate (TA1535, TA1537 and TA102 without S9-mix) or 3, 10, 33, 100, 333 and 1,000 μg/plate (TA1535, TA1537 and TA102 with S9-mix; TA100 and TA98 with and without S9-mix). Following these treatments, evidence of toxicity was observed in all strains at concentrations of 333 and/or 1,000 μg/plate, both in the absence and in the presence of S9-mix. No strains produced a biologically significant increase in the number of revertants.

In the second experiment, treatments of all the tester strains were performed in the absence and presence of S9-mix using the same concentrations as in the first experiment using the pre-incubation methodology. Following these treatments, evidence of toxicity was observed in all strains at concentrations of 333 and/or 1,000 μg/plate. No biologically significant increases in the number of revertants were seen in any strain (Appendix B, Table B.1).

[4] Data presented in Section 2.4 are cited from the first scientific opinion on FGE.226. These data are the basis for the conclusions in FGE.226 requesting additional genotoxicity data. Only minor changes were made when the data were reconsidered in FGE.226Rev1. However, this does not affect the conclusion that was drawn in FGE.226.

It was concluded that 4,5-epoxydec-2(*trans*)-enal did not induce mutations in five strains of *S.* Typhimurium when tested up to toxic concentrations in the absence and in the presence of a rat liver metabolic activation system (Sokolowski, 2001).

2.4.1.2. Micronucleus Assays

4,5-Epoxydec-2(*trans*)-enal was assayed for the induction of chromosome damage, and potential aneugenic effects, in mammalian cells *in vitro* by examining the effect on the frequency of micronuclei in cultured human peripheral blood lymphocytes (whole blood cultures pooled from two healthy male volunteers) treated in the absence and presence of rat liver metabolising system (S9-mix) (Lloyd, 2009). This GLP study complies with OECD Test Guideline 487. 4,5-Epoxydec-2(*trans*)-enal was added at 48 h following culture initiation (stimulation by phytohaemagglutinin) either for 3 h in the absence or presence of S9-mix, or for 24 h in the absence of S9-mix. Cytochalasin B (6 μg/mL) was added either at the start of treatment (24-h treatments) or at the start of recovery (after 3-h treatments) in order to block cytokinesis and generate binucleate cells for analysis. It remained in the cultures until they were harvested 24 h after the start of treatment. A preliminary range-finding experiment had been conducted with and without S9-mix treatment in order to determine the effect of treatment upon replication index (RI), which was used as a basis for choosing a range of concentrations to be evaluated in the main study.

In the main assay, micronuclei were analysed at multiple concentrations for each treatment group. For 3-h treatment without S9-mix the concentrations were 1, 2, 4 and 5 μg/mL, for 3-h treatment with S9-mix the concentrations were 9, 10.5 and 12 μg/mL, and for 24-h treatment without S9-mix the concentrations were 2.5, 3, 3.5 and 4 μg/mL. The levels of cytotoxicity (reduction in RI) at the top concentrations reached 61%, 52% and 55% in the 3-h treatment in the absence of S9-mix, the 3-h treatment in the presence of S9-mix and the 24-h treatment in the absence of S9-mix, respectively. These are within or very close to the target (50–60%) range. One thousand binucleate cells per culture from two replicate cultures per concentration were scored for micronuclei. The study is therefore considered to comply with OECD Test Guideline 487.

Following the 3-h treatment without S9-mix, there was an increase in the frequency of micronucleated binucleate cells (MNBN) from 0.1% in the solvent control to 0.65% ($p < 0.01$) and 0.45% ($p < 0.05$) at the two highest concentrations. However, the increases observed at 4 and 5 μg/mL were small and were amplified because the MNBN cell frequencies in both vehicle control cultures (0.1% in both cases) were at the lower end of the normal range (0–1.0%). Furthermore, the MNBN cell frequencies in all treated cultures under this treatment condition fell within the 95th percentile of the normal range. Therefore, these observations were not considered by the authors of this study to represent clear evidence of a biologically relevant response, although the results cannot be considered clearly negative.

Following the 3-h treatment in the presence of S9-mix at the highest concentration analysed (12 μg/mL), the frequency of MNBN cells (2.25%) was significantly higher ($p < 0.001$) than those observed in concurrent vehicle controls (0.2%). The MNBN cell frequencies in both cultures at 12 μg/mL exceeded the normal ranges, and therefore, this was considered to be a positive result. Similarly, for the 24-h treatment at the lowest (2.5 μg/mL) and two highest concentrations (3.5 and 4.0 μg/mL), the frequencies of MNBN cells were significantly higher (1.25% $p < 0.05$, 3.19% $p < 0.001$ and 3.80% $p < 0.001$, respectively) than those observed in the concurrent vehicle control (0.65%). The MNBN cell frequencies in both cultures at each of these concentrations exceeded the normal ranges, and therefore, this was considered to be a positive result (Lloyd, 2009).

On the basis of these results, a new GLP study to determine whether 4,5-epoxydec-2(trans)-enal was acting as a clastogen or an aneugen using fluorescence *in situ* hybridisation (FISH) analysis was attempted (Lloyd, 2011). Micronuclei were analysed at multiple concentrations for each treatment group, and the maximum concentrations were based on the toxicity displayed in the previous study. For 3-h treatment with S9-mix the concentrations were 0, 12, 15 and 17.5 μg/mL, with MNBN cell frequencies of 0.30%, 0.20%, 0.50% and 0.45%, respectively, with historical control range of 0.0–0.7%. For 24-h treatment without S9-mix the concentrations were 0, 4, 5 and 7.5 μg/mL, with MNBN cell frequencies of 0.35%, 0.25%, 0.55% and 0.20%, respectively, with a historical control range of 0.1–0.9%. The levels of cytotoxicity (reduction in RI) were 16%, 36% and 48% for the three concentrations in the 3-h treatment in the presence of S9-mix and 3%, 10% and 56% for the three concentrations in the 24-h treatment in the absence of S9-mix, respectively. 48% and 56% at the top concentrations are within or very close to the target (50–60%) range. One thousand

binucleate cells per culture from two replicate cultures per concentration were scored for micronuclei. The study is therefore considered to comply with OECD Test Guideline 487.

The MNBN cell frequencies in all cultures under both treatment conditions fell within the normal range, thereby giving negative results (Appendix B, Table B.1). These data are in marked contrast to the previously described study (Lloyd, 2009). However, the Panel noted that this study has a shortcoming, i.e. in the absence of S9-mix, one replicate culture in the positive control vinblastine resulted in an effect very close to the range of historical negative controls, which limits the reliability of the outcome. Because no induction of micronuclei was observed following 3 + 21 h with S9-mix and 24 + 0 h without S9-mix treatments, further analysis (FISH) was not conducted. Different blood donors were used in the first and second studies on 4,5-epoxydec-2(*trans*)-enal. A subsequent study in which peripheral blood from the donors used in both experiments were compared in a single experiment confirmed the existence of a donor effect for this compound (data not provided). It is not known why this difference occurred, but the positive responses observed in the previous study (Lloyd, 2009) cannot be dismissed. The Panel considered the result of the negative study less reliable than the positive one.

2.4.2. *In vivo* Data

2.4.2.1. *In vivo* Micronucleus Assays

On the basis of the *in vitro* micronucleus studies reported above, it was concluded that 4,5-epoxydec-2(*trans*)-enal induced micronuclei in human lymphocytes and it was considered that the most appropriate *in vivo* follow up was an *in vivo* micronucleus assay, in order to determine whether the results obtained in the initial *in vitro* micronucleus assay could be confirmed *in vivo*. Therefore, groups of Han-Wistar rats were administered 4,5-epoxydec-2(*trans*)-enal via gavage and the induction of micronuclei in the polychromatic erythrocyte (PCE) of the bone marrow of treated rats was examined (Henderson, 2011).

In an initial range-finding experiment to identify a maximum tolerated dose (MTD), groups of male and female (up to 3 animals/sex per group) Han-Wistar rats were administered 4,5-epoxydec-2(*trans*)-enal by oral gavage at doses of 250, 350, 500, 700, 1,000, 1,400 and 2,000 mg/kg body weight (bw) per day until an estimate of the MTD was established. Animals were dosed once daily for two consecutive days with the test article and observations made over a 2-day period following the final administration. Clinical signs of toxicity and body weight were recorded. At doses of 500 mg/kg bw per day and above, clinical signs of toxicity such as decreased activity and piloerection were observed in all animals, and mortality was induced. At doses of 350 mg/kg bw per day and below no clinical signs of toxicity were observed, except in one female at 350 mg/kg bw per day, for which decreased activity, piloerection and hunched posture were observed. Both male and female groups at 350 mg/kg bw per day showed mean body weight loss. On the basis of these concentrations, the MTD was considered to be 350 mg/kg bw per day. Additionally, as there were no differences between sexes in apparent toxicity, only male animals were subsequently used in the micronucleus experiment.

In the micronucleus experiment, groups of male (6 animals/group) rats were administered 4,5-epoxydec-2(*trans*)-enal by oral gavage at 87.5, 175 and 350 mg/kg bw per day on two occasions 24 h apart. Animals were sampled 24 h after the final administration, thus enabling examination of cells exposed to the test article over a period of 24–48 h prior to sampling. At the highest dose on day 2, decreased activity was observed in all animals 1-h post-dose, and at 2-h post-dose, piloerection was also noted in all animals. For the highest dose group, one animal was found dead at end of day 2. However, there was no evidence of bone marrow exposure.

Rats treated with 4,5-epoxydec-2(*trans*)-enal at all doses exhibited group mean % PCE that were similar to the vehicle control group. These values were comparable with the historical control data for this experiment at the testing laboratory, thus confirming there was no evidence of test article related bone marrow toxicity. Additionally, rats treated with 4,5-epoxydec-2(*trans*)-enal at all doses exhibited micronucleus (MN) PCE frequencies that were similar to the vehicle control group and which were considered consistent with the laboratory's historical data. There were no statistically significant increases in micronucleus frequency for any of the groups receiving the test article, compared to the concurrent vehicle control (Appendix B, Table B.2). On this basis, it was concluded that 4,5-epoxydec-2(*trans*)-enal did not induce micronuclei in the PCEs of the bone marrow of male rats treated up to 350 mg/kg per day (a dose which exceeded the MTD).

2.4.3. Discussion of Genotoxicity Data

4,5-Epoxydec-2(*trans*)-enal did not induce gene mutations in a valid Ames test. In a valid *in vitro* micronucleus assay, 4,5-epoxydec-2(*trans*)-enal was clearly positive in both treatments for 3 + 21 h in the presence of S9-mix and for 24 + 0 h in the absence of S9-mix. In the same study, in the treatment for 3 + 21 h in the absence of S9-mix, statistically significant increases of MNBN cell frequencies were reported at the two highest concentrations. These increases were not considered biologically relevant because the MNBN cell frequencies in the vehicle control cultures (0.1%) were at the lower end of the historical control range (0.0–1.0%) and because all the MNBN cell frequencies fell within the 95th percentile of the normal range. On this basis, the results of this part of this study should be considered as equivocal. Overall, the results of this study indicate that 4,5-epoxydec-2(*trans*)-enal is an *in vitro* genotoxic agent both in the presence and in the absence of metabolic activation.

The positive results of the first study (Lloyd, 2009) could not be confirmed in a second study, in which different blood donors were used (Lloyd, 2011). According to the study authors, the existence of a donor effect for this substance was confirmed in a subsequent study in which peripheral blood from the donors used in both studies were compared in a single experiment. However, data related to this experiment were not provided and also an explanation for this difference was not given. Therefore, the concern for the genotoxic potential of 4,5-epoxydec-2(*trans*)-enal remains.

4,5-Epoxydec-2(*trans*)-enal was found negative in an *in vivo* micronucleus assay in rats treated by oral gavage up to 350 mg/kg bw, considered as the MTD, on two occasions 24 h apart. At this dose and below, no clinical signs of toxicity were observed, except one female; both male and female groups showed only mean body weight loss. Clinical signs, including some mortality, were observed at the dose of 500 mg/kg bw, used in the initial range-finding experiment. At 350 mg/kg bw, there was no evidence of any test article-induced toxicity to the bone marrow. There was no proof that the bone marrow was exposed. In addition, the negative results of this *in vivo* micronucleus assay do not allow to exclude site of contact effects. Therefore, an *in vivo* Comet assay should be performed.

The request for a Comet assay is in line with the recommendations of the AFC Panel (EFSA, 2008b) and Scientific Committee opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA Scientific Committee, 2011).

2.4.4. Conclusion drawn in FGE.226

4,5-Epoxydec-2(*trans*)-enal did not induce gene mutations in bacterial cells (Ames test). It was positive in an *in vitro* micronucleus assay in cultured human lymphocytes with and without metabolic activation. Although these results could not be confirmed in a second study in which different blood donors were used, 4,5-epoxydec-2(*trans*)-enal is considered an *in vitro* genotoxic agent in the presence and in the absence of S9-mix. The negative results obtained in an *in vivo* micronucleus assay do not allow to exclude possible first site of contact effects. In addition, there was no proof that the bone marrow was exposed. On this basis, an *in vivo* Comet assay in rodents is required, in order to verify possible genotoxic effects at the first site of contact (e.g. stomach/duodenum cells) and in liver.

The request for a Comet assay is in line with the recommendations of the AFC Panel (EFSA, 2008b) and Scientific Committee opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA Scientific Committee, 2011).

3. Assessment of new data

Following the request for additional data expressed by the Panel in FGE.226, the industry has investigated the presence of 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] in the plasma of a satellite group of animals from the *in vivo* micronucleus assay by Henderson, 2011 (Mallinson, 2014). The industry has submitted an *in vivo* comet assay with scoring of duodenum and liver cells (Beevers, 2016). These additional data are evaluated in the present revision of FGE.226 (FGE.226Rev1).

3.1. Plasma bioanalysis

In order to demonstrate the bone marrow exposure of animals treated with 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] in the micronucleus assay by Henderson (2011) (see Section 2.4.2), a plasma analysis of a satellite group of animals was provided. Six male Han-Wistar rats were dosed, by oral gavage, with 350 mg/kg bw per/day of 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071]. A method was developed for the analysis of 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] using gas chromatography with mass selective detection (GC-MSD) (Mallinson, 2014).

According to the applicant, satisfactory linearity, recovery and repeatability were found for 4,5-epoxydec-2(*trans*)-enal when the substance was spiked and analysed in rat plasma samples.

The Panel, however, noted that linearity in plasma extracts was in the range of 5–100 μg/mL, but the highest concentration reported for 4,5-epoxydec-2(*trans*)-enal in rat plasma samples (0.14 μg/mL) was far below this range. Moreover, the recovery and accuracy of the method were only determined from 50 μg/mL and above. Therefore, the data of plasma analysis obtained *in vivo* are unreliable and cannot be considered as demonstration of sufficient bone marrow exposure (Appendix C, Table C.1).

3.2. *In vivo* Comet assay

4,5-Epoxydec-2(*trans*)-enal [FL-no: 16.071] was tested in an *in vivo* Comet assay in Han-Wistar rats via oral gavage (Beevers, 2016). The dose-range finding test was not repeated because data from the *in vivo* micronucleus study (Henderson, 2011) were used. Based on this study, the following doses were selected: 75, 150 and 300 mg/kg bw per day. The study authors considered 300 mg/kg bw per day as an estimate of the MTD (Beevers, 2016) because in the *in vivo* micronucleus study one animal of the highest dose group (350 mg/kg bw per day) was found dead at the end of day 2 (Henderson, 2011). As no gender differences in toxicity, metabolism or bioavailability have been previously identified, the study was conducted solely in male animals (6 animals/group). The vehicle control group was dosed with 0.5% (w/v) aqueous methylcellulose (0.5% MC) and the positive control group (3 animals) with ethyl methanesulfonate (EMS) 150 mg/kg bw per day. 4,5-Epoxydec-2(*trans*)-enal and the vehicle control were given as two administrations, at 0 and 21 h; the positive control was administered once only at 21 h. All animals were sampled at 24 h (Appendix C, Table C.1).

No clinical signs of toxicity were observed in animals following any treatments. A small but dose related reduction in body weight gain, resulting in weight loss at the highest dose (300 mg/kg per day) was observed. There was a generally dose-related decrease in albumin, and concomitant decreases in total protein and albumin:globulin ratio in animals from all groups treated with [FL-no: 16.071]. A dose-related decrease in calcium and increase in phosphate in animals from all groups given [FL-no: 16.071] were observed. There were marked increases in urea and creatinine in one animal given 300 mg/kg per day. A small increase in urea was seen in several animals dosed with [FL-no: 16.071] 150 or 300 mg/kg bw per day.

Macroscopically, stomach was gelatinous, thick, red, distended and/or contained abnormal contents (clear fluid) and duodenum was distended, pale and/or thick in animals from all groups. Jejunum and ileum were distended and/or contained abnormal yellow gelatinous contents in animals given 300 mg/kg per day. Caecum contained abnormal gritty contents in animals given 150 or 300 mg/kg per day and was distended in one animal given 300 mg/kg per day.

Microscopically, in the duodenum, single cell necrosis and villous atrophy were present in animals from all groups. There was a decrease in hepatocyte glycogen in animals from all groups given 4,5-epoxydec-2(*trans*)-enal, with a dose-related effect.

In the Comet assay, treatment with 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] did not cause excessive tissue damage in the liver or duodenum which could interfere with the outcome of the Comet assay as indicated by the lack of significant increase in % hedgehogs that could have interfered with the assay.

3.2.1. Comet assay in liver

Statistically significant increases in tail intensity in the liver were observed following dose administration at 75 mg/kg bw per day ($p \leq 0.05$) and 300 mg/kg bw per day ($p \leq 0.01$), no statistically significant increase was observed in the intermediate dose group (150 mg/kg per day). However, a significant linear trend was also apparent across the data ($p \leq 0.05$).

The tail intensity values for all animals fell within the laboratory's historical control data range (95% reference ranges of 0.05–7.14%). However, this range is very wide. Additionally, considering tail intensity data for the individual animals, the tail intensities of all animals in the highest dose group, except one animal (tail intensity value of 0.38) are higher than the highest tail intensity value (tail intensity value of 0.56) in the concurrent control group.

The clinical chemistry and histopathology data show no clear evidence of hepatotoxicity associated with 4,5-epoxydec-2(*trans*)-enal treatment.

The Panel considered that the following two criteria for evaluation and interpretation of results as positive (OECD TG 489) were fulfilled:

a) at least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control,
b) the increase is dose-related when evaluated with an appropriate trend test.

The third criterion ('any of the results are outside the distribution of the historical negative control data for a given species, vehicle, route, tissue, and number of administrations') mentioned in the OECD TG 489 is not applicable in this case because the range for historical negative controls is very wide (95% reference range of 0.05–7.14%).

Therefore, the Panel concluded that 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] is genotoxic in this *in vivo* comet assay in the liver of rats.

3.2.2. Comet assay in duodenum

Group mean tail intensity and tail moment values in the duodenum for all groups of animals treated with 4,5-epoxydec-2(*trans*)-enal were comparable with the group mean vehicle control data. There were no statistically significant differences in tail intensity between treated and control groups. Therefore, the Panel concluded that 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] is not genotoxic in the duodenum of rats.

4. Conclusions

4,5-Epoxydec-2(*trans*)-enal [FL-no: 16.071] was negative in the Ames test both with and without metabolic activation. However, positive effects of 4,5-epoxydec-2(*trans*)-enal were demonstrated in an *in vitro* micronucleus assay both with and without metabolic activation. As an *in vivo* follow-up study, a rat bone marrow micronucleus study was performed by gavage. The negative outcome of this study is considered to be of limited relevance, because no clear indication of biological relevant exposure to the target tissue could be demonstrated. Since the substance was positive in the *in vitro* micronucleus assay both in the absence and in the presence of S9-mix, an *in vivo* Comet assay in the first site of contact (e.g. the duodenum) and in the liver was requested.

Comet assay data provided suggest that 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] did not induce DNA damage in the duodenum of rats treated up to 300 mg/kg per day by oral route (an estimate of the MTD in male rats). However, the genotoxic effect observed *in vitro* was confirmed in an *in vivo* comet assay in the liver of rats.

Overall, the Panel concluded that 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] does raise a safety concern with respect to genotoxicity, and therefore, it cannot be evaluated according to the Procedure.

Additional remarks

The Panel noted that the petitioner suggested to consider in the evaluation that the exposure to 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] is very low, based on the maximised survey-derived daily intake (MSDI) approach (see Appendix D). However, the Panel considered that even if the exposure would be below the threshold of concern for genotoxic carcinogens, such a comparison would only be applicable to substances not intentionally added to foods, like impurities or contaminants.

Documentation provided to EFSA

1) Beevers C, 2016. 4,5-Epoxydec-2(*trans*)-enal: Rat Alkaline Comet Assay. Covance Laboratories Ltd. Study number 8331879. September 2016. Unpublished report submitted by EFFA to EFSA.
2) Benigni R and Netzeva T, 2007a. Report on a QSAR model for prediction of genotoxicity of alpha,beta-unsaturated aldehydes in S. typhimurium TA100 and its application for predictions on alpha,beta-unsaturated aldehydes in Flavouring Group Evaluation 19 (FGE.19). Unpublished report submitted by FLAVIS Secretariat to EFSA.
3) Benigni R and Netzeva T, 2007b. Report on a QSAR model for prediction of genotoxicity of alpha,beta-unsaturated ketones in S. typhimurium TA100 and its application for predictions on alpha,beta-unsaturated aldehydes in Flavouring Group Evaluation 19 (FGE.19). Unpublished report submitted by FLAVIS Secretariat to EFSA.
4) EFFA (European Flavour & Fragrance Association), 2002. EFFA letter to the Commission and FLAVIS Secretariat on definitions and data clarifications. 31 October 2002.
5) EFFA (European Flavour & Fragrance Association), 2007. EFFA letter to the Commission on use levels of flavouring substances. 12 October 2007.

6) EFFA (European Flavour Association), 2011. Submission by the European Flavour Association to the European Food Safety Authority. Flavouring Group Evaluation 19 Subgroup 1.1.1 (corresponding to FGE.200): Submission of additional data related to FGE.19 subgroup 1.1.1 (Epoxydecenal). 29 December 2011. FLAVIS/8.137.
7) EFFA (European Flavour Association), 2016. EFFA letter to EFSA on additional information related to 4,5-epoxydec-2(trans)-enal. 9 September 2016.
8) Gry J, Beltoft V, Benigni R, Binderup M-L, Carere A, Engel K-H, Gürtler R, Jensen GE, Hulzebos E, Larsen JC, Mennes W, Netzeva T, Niemelä J, Nikolov N, Nørby KK and Wedebye EB, 2007. Description and validation of QSAR genotoxicity models for use in evaluation of flavouring substances in Flavouring Group Evaluation 19 (FGE.19) on 360 alpha,beta-unsaturated aldehydes and ketones and precursors for these. Unpublished report submitted by FLAVIS Secretariat to EFSA.
9) Henderson D, 2011. Induction of micronuclei in the bone marrow of treated rats. 4,5-Epoxydec-2(trans)-enal. Covance Laboratories Ltd. Study no. 8250466. December 2011. Unpublished report submitted by EFFA to FLAVIS Secretariat.
10) Lloyd M, 2009. Induction of micronuclei in cultured human peripheral blood lymphocytes. Epoxydecenal. Covance Laboratories Ltd. Study no. 8203181. September 4, 2009. Unpublished report submitted by EFFA to FLAVIS Secretariat.
11) Lloyd M, 2011. Induction of micronuclei in cultured human peripheral blood lymphocytes. 4,5-Epoxydec-2(trans)-enal. Unaudited draft report. Covance Laboratories LTD. Study no. 8233105. December 2011. Unpublished report submitted by EFFA to FLAVIS Secretariat.
12) Mallinson C, 2014. Development and limited validation of a method for the analysis of plasma samples which may contain trans-4,5-epoxy-2-decenal. Covance Laboratories Ltd. Study number 8298-913. Unpublished report submitted by EFFA to EFSA.
13) Nikolov N, Jensen GE, Wedebye EB and Niemelä J, 2007. Report on QSAR predictions of 222 alpha,beta-unsaturated aldehydes and ketones from Flavouring Group Evaluation 19 (FGE.19) on 360 alpha,beta-unsaturated aldehydes and ketones and precursors for these. Unpublished report submitted by FLAVIS Secretariat to EFSA.
14) Sokolowski A, 2001. Salmonella typhimurium reverse mutation assay with epoxydecenal (Giv 85-0703). Project no. 695302. RCC Cytotest Cell Research GMBH, Germany. August 14, 2001. Unpublished report submitted by EFFA to FLAVIS Secretariat.

References

Buettner A and Schieberle P, 1999. Characterization of the most odor-active volatiles in fresh, hand-squeezed juice of grapefruit (*Citrus paradisi* Macfayden). Journal of Agricultural and Food Chemistry, 47, 5189–5193.

Burdack-Freitag A and Schieberle P, 2010. Changes in the key odorants of Italian hazelnuts (*Coryllus avellana* L. Var. Tonda Romana) induced by roasting. Journal of Agricultural and Food Chemistry, 58, 6351–6359.

Cadwallader KR and Heo J, 2001. Aroma of roasted sesame oil: characterization by direct thermal desorption-gas chromatography-olfactometry and sample dilution analysis. In: Leland JV, Schieberle P, Buettner A and Acree TE (ed.). *Gas Chromatography-Olfactometry*. American Chemical Society, Washington, DC, USA. pp. 187–202.

Cemy C and Guntz R, 2004. Formation of aroma-active compounds during heating of egg yolk. State-of-the-art in flavour chemistry and biology. Proceedings of the Wartburg Symposium on Flavour Chemistry and Biology, 7th Eisenach, Germany, 182–189. Published by Deutsche Forschungsanstalt für lebensmittelchemie.

Derail C, Hofmann T and Schieberle P, 1999. Differences in key odorants of handmade juice of yellow-flesh peaches (*Prunus persica* L.) induced by the workup procedure. Journal of Agricultural and Food Chemistry, 47, 4742–4745.

EFSA (European Food Safety Authority), 2008a. Minutes of the 26th Plenary meeting of the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food, Held in Parma on 27–29 November 2007. Parma, 7 January 2008. Available online: http://www.efsa.europa.eu/EFSA/Event_Meeting/afc_minutes_26thplen_en.pdf

EFSA (European Food Safety Authority), 2008b. Genotoxicity test strategy for substances belonging to subgroups of FGE.19 - statement of the panel on food contact materials, enzymes, flavourings and processing aids (CEF). EFSA Journal 2008;6(12):854, 1–5, pp. https://doi.org/10.2903/j.efsa.2008.854

EFSA (European Food Safety Authority), 2008c. Scientific Opinion. List of α,β-unsaturated aldehydes and ketones representative of FGE.19 substances for genotoxicity testing - statement of the panel on food contact materials, enzymes, flavourings and processing aids (CEF). EFSA Journal 2008;6(12):910, 1–5, pp. https://doi.org/10.2903/j.efsa.2008.910

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2012. Scientific Opinion on Flavouring Group Evaluation 226 (FGE.226): consideration of genotoxicity data on one α, β-unsaturated aldehyde from chemical subgroup 1.1.1(b) of FGE.19 by EFSA. EFSA Journal 2012;10(7):2838, 18 pp. https://doi.org/10.2903/j.efsa.2012.2838

EFSA Scientific Committee, 2011. Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA Journal 2011;9(9):2379, 69 pp. https://doi.org/10.2903/j.efsa.2011.2379

Goicoechea E and Guillén MD, 2014. Volatile compounds generated in corn oil stored at room temperature. Presence of toxic compounds. European Journal of Lipid Science and Technology, 116, 395–406.

Guillén MD and Uriarte PS, 2012. Aldehydes contained in edible oils of a very different nature after prolonged heating at frying temperature: presence of toxic oxygenated α, β unsaturated aldehydes. Food Chemistry, 131, 915–926.

Guillén MD, Cabo N, Ibargoitia L and Ruiz A, 2005. Study of both sunflower oil and its headspace throughout the oxidation process. Occurrence in the headspace of toxic oxygenated aldehydes. Journal of Agricultural and Food Chemistry, 53, 1093–1101.

Helier C and Schieberle P, 1997. Quantitative instrumental and sensory studies on aroma compounds contributing to a metallic flavour defect in buttermilk. International Dairy Journal, 7, 659–666.

Hinterholzer A and Schieberle P, 1998. Identification of the most odour-active volatiles in fresh, hand-extracted juice of valencia late oranges by odour dilution techniques. Flavour and Fragrance Journal, 13, 49–55.

Hinterholzer A, Lemos T and Schieberle P, 1998. Identification of the key odorants in raw French beans and changes during cooking. Zeitschrift für Lebensmittel-Untersuchung und -Forschung, 207, 219–222.

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2006. Safety evaluation of certain food additives. Sixty-fifth meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). WHO Food Additives Series 56. Available online: http://apps.who.int/iris/bitstream/10665/43407/1/9241660562_eng.pdf

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2009a. JECFA Online Edition "Specification for Flavourings". Available online: http://www.fao.org/ag/agn/jecfa-flav/search.html

JECFA (Joint FAO/WHO Expert Committee on Food Additives), 2009b. Evaluation of certain food additives. Sixty-ninth report of the Joint FAO/WHO Expert Committee on Food Additives. WHO Technical Report Series, no. 952. Rome, 17–26 June 2008. Available online: http://whqlibdoc.who.int/trs/WHO_TRS_952_eng.pdf

Jezussek M, Juliano BO and Schieberle P, 2002. Comparison of key aroma compounds in cooked brown rice varieties based on aroma extract dilution analyses. Journal of Agricultural and Food Chemistry, 50, 1101–1105.

Kirchhoff E and Schieberle P, 2001. Determination of key aroma compounds in the crumb of a three-stage sourdough rye bread by stable isotope dilution assays and sensory studies. Journal of Agricultural and Food Chemistry, 49, 4304–4311.

Kirchhoff E and Schieberle P, 2002. Quantitation of odor-active compounds in rye flour and rye sourdough using stable isotope dilution assays. Journal of Agricultural and Food Chemistry, 50, 5378–5385.

Kobayashi N and Nishimura O, 2014. Availability of detection frequency method using three-port gas chromatography–olfactometry for rapid comparison of whole milk powders. Food Science and Technology Research, 20, 809–814.

Koutidou M, Grauwet T, Van Loey A and Acharya P, 2017. Impact of processing on odour-active compounds of a mixed tomato-onion puree. Food Chemistry, 228, 14–25.

Mahattanatawee K, Rouseff R, Valim FM and Naim M, 2005. Identification and aroma impact of norisoprenoids in orange juice. Journal of Agricultural and Food Chemistry, 53, 393–397.

Milo C and Grosch W, 1997. Potent odorants in boiled cod as affected by the storage of raw material. In: Shahidi F and Cadwallader KR (eds.). *Flavour and Lipid Chemistry of Seafoods*. American Chemical Society, Washington, DC, USA. pp. 110–119.

OECD, 2014. Genetic Toxicology: *In vivo* Mammalian Alkaline Comet Assay. In OECD Guidelines for the testing of chemicals. OECD Paris, Test Guideline 489.

Rengarajan R and Seitz LM, 2003. Analysis of flavor compounds from microwave popcorn using supercritical fluid Co_2 followed by dynamic/static headspace techniques. In: Deibler KD and Delwiche J (eds.). *Handbook of Flavor Characterization: Sensory Analysis, Chemistry and Physiology*. Marcel Dekker Inc, Ohio, U.S.A. pp. 311–334.

Ruiz Perez-Cacho P, Mahattanatawee K, Smoot JM and Rouseff R, 2007. Identification of sulfur volatiles in canned orange juices lacking orange flavor. Journal of Agricultural and Food Chemistry, 55, 5761–5767.

SCF (Scientific Committee for Food), 1999. Opinion on a programme for the evaluation of flavouring substances (expressed on 2 December 1999). Scientific Committee on Food. SCF/CS/FLAV/TASK/11 Final 6/12/1999. Annex I the minutes of the 119th Plenary meeting. European Commission, Health & Consumer Protection Directorate-General.

SCF (Scientific Committee for Food), 1995. First annual report on chemically defined flavouring substances. May 1995.

Schuh C and Schieberle P, 2006. Characterization of the key aroma compounds in the beverage prepared from Darjeeling black tea: quantitative differences between tea leaves and infusion. Journal of Agricultural and Food Chemistry, 54, 916–924.

Triqui R and Guth H, 1997. Determination of potent odorants in ripened anchovy (*Engraulis encrasicholus* L.) by aroma extract dilution analysis and by gas chromatography-olfactometry of headspace samples. In: Shahidi F and Cadwallader KR (eds.). *Flavour and Lipid Chemistry of Seafoods*. American Chemical Society, Washington, DC, USA. pp. 31–38.

Triskelion, 2017. VCF online. Volatile compounds in food. In: Nijssen B, van Ingen-Visscher K, Donders J (eds.). *Database Version 16.3*, Triskelion, Zeist, Netherlands. pp. 1992–2017.

Abbreviations

Bw	body weight
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CoE	Council of Europe
DNA	deoxyribonucleic acid
EFFA	European Flavour and Fragrance Association
EMS	ethyl methanesulfonate
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
GC-MSD	gas chromatography with mass selective detection
GLP	Good Laboratory Practice
ID	Identity
IR	infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MC	methylcellulose
MN	micronucleus
MNBN	micronucleated binucleated (cells)
MS	mass spectrometry
MSDI	maximised survey-derived daily intake
mTAMDI	modified Theoretical Added Maximum Daily Intake
MTD	maximum tolerated dose
NMR	nuclear magnetic resonance
OECD	Organisation for Economic Co-operation and Development
PCE	polychromatic erythrocytes
(Q)SAR	(quantitative) structure–activity relationship
RI	replication index
WHO	World Health Organization

Appendix A – Summary of Safety Evaluation

Table A.1: Summary of safety evaluation of the JECFA substance in the present group (JECFA, 2009b)

FL-no JECFA-no	EU register name	Structural formula	EU MSDI[(a)] US MSDI (μg/capita per day	Class[(b)] Evaluation procedure path[(c)]	JECFA outcome on the named compound[(d)] or [(e)]	EFSA conclusions on the named compound (genotoxicity)
16.071 1570	4,5-Epoxydec-2(trans)-enal		0.061[(f)] 0.2	Class III A3: Intake below threshold	[(d)]	Evaluated in FGE.200, additional genotoxicity data required
						Evaluated in FGE.226, additional genotoxicity data required
			0.04[(g)]			Evaluated in FGE.226Rev1 as of genotoxicity concern

FL-no: FLAVIS number; JECFA: The Joint FAO/WHO Expert Committee on Food Additives; MSDI: maximised survey-derived daily intake.
(a): EU MSDI: Amount added to food as flavour in (kg/year) × 10E9/(0.1 × population in Europe (= 375 × 10E6) × 0.6 × 365) = μg/capita per day.
(b): Thresholds of concern: Class I = 1,800 μg/person per day, Class II = 540 μg/person per day, Class III = 90 μg/person per day.
(c): Procedure path A, substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.
(d): No safety concern based on intake calculated by the MSDI approach of the named compound.
(e): Data must be available on the substance or closely related substances to perform a safety evaluation.
(f): EU MSDI calculated based on EU poundage of 0.5 kg/year.
(g): EU MSDI calculated based on EU poundage of 0.3 kg/year (EFFA, 2016).

Appendix B – Genotoxicity data evaluated in FGE.226

Table B.1: Summary of *in vitro* genotoxicity data on 4,5-epoxydec-2(*trans*)-enal

FL-no	Chemical name	Test system *in vitro*	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
16.071	4,5-Epoxydec-2 (*trans*)-enal	Reverse mutation	*Salmonella* Typhimurium TA98, TA100	3–1,000 μg/plate [1,2] 3–1,000 μg/plate [1,3]	Negative	Sokolowski (2001)	Reliable without restrictions. GLP study in compliance with OECD Test Guideline 471
			S. Typhimurium TA1535, TA1537 and TA102	1–333 μg/plate [2,4] 3–1,000 μg/plate [2,5] 1–333 μg/plate [3,4] 3–1,000 μg/plate [3,5]	Negative		
		Micronucleus assay	Human peripheral blood lymphocytes	1–5 μg/mL [4,6]	Equivocal	Lloyd (2009)	Reliable with minor restrictions (no data on historical positive controls were reported), otherwise in compliance with OECD Test Guideline 487. GLP study. Acceptable levels of cytotoxicity achieved at the top concentrations used in all parts of the study. Increases at 4.0 and 5.0 μg/mL observed after short treatment without metabolic activation, are of doubtful biological relevance due to low vehicle control and because are within the 95th percentile of the normal range
				9–12 μg/mL [5,6] 2.5–4 μg/mL [4,7]	Positive		
				12–17.5 μg/mL [5,6] 4–7.5 μg/mL [4,7]	Negative	Lloyd (2011)	Reliable with restrictions (no data on historical positive controls were reported; in the absence of S9-mix, one replicate culture in the positive control vinblastine resulted in an effect very close to the range of historical negative controls, which limits the reliability of the outcome). Otherwise, the study complies with OECD Test Guideline 487. Acceptable levels of cytotoxicity achieved at the top concentrations used in all parts of the study

GLP: Good Laboratory Practice; OECD: Organisation for Economic Co-operation and Development.
[1]: With and without S9 metabolic activation.
[2]: Plate incorporation method.
[3]: Pre-incubation method.
[4]: Without S9 metabolic activation.
[5]: With S9 metabolic activation.
[6]: 3 h incubation with 21 h recovery period.
[7]: 24 h incubation with no recovery period.

Table B.2: Summary of *in vivo* genotoxicity data on 4,5-epoxydec-2(trans)-enal

FL-no	Chemical name	Test system *in vivo*	Test object Sex/no per group	Route	Concentrations of substance and test conditions	Result	Reference	Comments
16.071	4,5-Epoxydec-2(*trans*)-enal	Micronucleus assay	Han-Wistar rats Male/6	Gavage	87.5, 175, 350 mg/kg bw per day	Negative	Henderson (2011)	Not reliable (no evidence of test article related bone marrow exposure), otherwise the study complies with OECD TG 474

bw: body weight; OECD: Organisation for Economic Co-operation and Development.

Appendix C – Genotoxicity data evaluated in FGE.226Rev1

Table C.1: Summary of additionally genotoxicity data (*in vivo*) on 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] of subgroup 1.1.1b

FL-no	Chemical name	Test system *in vivo*	Test object route	Concentrations of substance and test conditions	Result	Reference	Comments
16.071	4,5-Epoxydec-2(*trans*)-enal	Plasma concentrations (micronucleus assay)[(a)]	Rat gavage	350 mg/kg bw per day	Inconclusive	Mallinson (2014)	GC-MSD method validated (recovery, accuracy and precision). Linearity and working range were assessed. The concentration of 4,5-epoxydec-2(*trans*)-enal detected was below the linearity range. Not a GLP study
		Comet assay in duodenum	Rat gavage	75, 150, 300 mg/kg bw per day in 2 administrations	Negative	Beevers (2016)	Reliable without restrictions. The study complies with OECD Test Guideline 489 (OECD, 2014). The dose of 300 mg/kg per day was considered as the maximum tolerated dose based on the micronucleus study by Henderson (2011)
		Comet assay in liver			Positive		

bw: body weight; GLP: Good Laboratory Practice; OECD: Organisation for Economic Co-operation and Development.
(a): Plasma obtained from satellite group of animals in the study by Henderson (2011).

Appendix D – Exposure

D.1. Presence in food

According to the TNO database, the candidate substance [FL-no: 16.071] is not reported to be present in natural food sources (Triskelion, 2017). However, there are authors reporting the presence of the substance in processed and non-processed foods. Table D.1 reports a non-exhaustive list of foodstuff containing 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071].

Table D.1: Examples of 4,5-epoxydec-2(*trans*)-enal occurrence in foodstuff

Food item	Process	Qualitative	Quantitative	Reference
Orange juice	1) Hand-squeezed oranges 2) Juiced oranges with a commercial juice extractor model	Yes		1) Hinterholzer and Schieberle (1998); 2) Mahattanatawee et al. (2005)
Canned orange juice	Canned orange juice purchased in tin-coated steel cans	Yes		Ruiz Perez-Cacho et al. (2007)
Peach	a) Full ripe yellow-flesh peaches homogenised b) Full ripe yellow-flesh peaches cut and suspended in boiled water	Yes		Derail et al. (1999)
Grapefruit juice	Hand-squeezed grapefruit	Yes		Buettner and Schieberle (1999)
Whole milk powder	Whole milk powder purchased at domestic markets; process not specified	Yes		Kobayashi and Nishimura (2014)
Fresh rye bread crumb	Fermentation and baking		4.5 μg/kg	Kirchhoff and Schieberle (2001)
Rye sourdough	Fermentation		16 μg/kg (dry weight)	Kirchhoff and Schieberle (2002)
Rye flour	Freshly ground rye flour		0.5 μg/kg (dry weight)	
French beans	Raw beans and cooked beans	Yes		Hinterholzer et al. (1998)
Mixed tomato-onion puree	Heating	Yes		Koutidou et al. (2017)
Darjeeling black tea extract	Tea leaves and tea infusion	Yes		Schuh and Schieberle (2006)
Italian hazelnuts	Raw hazelnuts and roasted hazelnuts	Yes		Burdack-Freitag and Schieberle (2010)
Sunflower oil	Oxidation	Yes		Guillén et al. (2005)
Extra virgin olive oil Sunflower oil Virgin linseed oil	Heating at 190°C for several hours		• Extra virgin olive oil up to 75.11 ± 5.58 (μmol/L) • Sunflower oil up to 186.21 ± 15.24 (μmol/L) • Virgin linseed oil up to 6.31 ± 0.52 (μmol/L)	Guillén and Uriarte (2012)
Corn oil	Long-term storage at room temperature with different air–oil volume ratios and/or air–oil contact surfaces	Yes		Goicoechea and Guillén (2014)
Cooked brown rice	Boiling	Yes		Jezussek et al. (2002)

Food item	Process	Qualitative	Quantitative	Reference
Buttermilk	Fresh fermented sweet-cream buttermilk and stored sour-cream buttermilk	Yes		Helier and Schieberle (1997)
Egg yolk	Heating	Yes		Cemy and Guntz (2004)
Roasted sesame oil	Roasting	Yes		Cadwallader and Heo (2001)
Boiled cod	Boiling	Yes		Milo and Grosch (1997)
popcorn	Freshly popped	Yes		Rengarajan and Seitz (2003)
Ripened anchovy	Ripening	Yes		Triqui and Guth (1997)

D.2. Intended use and use levels as provided by the Flavour Industry

Use levels in the different food categories reported in Annex I of Reg. (EC) 1565/2000[5] have been submitted by the flavour industry and are reported in Table D.2 (EFFA, 2007).

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

Table D.2: Use levels of 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] in food categories listed in Annex I of Reg. (EC) 1565/2000 (EFFA, 2007)

FL-no	Food categories																	
	Normal use levels (mg/kg)[a] Maximum use levels (mg/kg)																	
	01.0	**02.0**	**03.0**	**04.1**	**04.2**	**05.0**	**06.0**	**07.0**	**08.0**	**09.0**	**10.0**	**11.0**	**12.0**	**13.0**	**14.1**	**14.2**	**15.0**	**16.0**
16.071	0.0000077	–	0.00035	–	–	–	–	–	0.0049	–	–	–	0.0000077	–	0.00012	–	0.000077	–
	0.00023	–	0.0023	–	–	–	–	–	0.023	–	–	–	–	–	0.0027	–	–	–

FL-no: FLAVIS number.
(a): 'Normal use' is defined as the average of reported usages and 'maximum use' is defined as the 95th percentile of reported usages (EFFA, 2002).

Table D.3: Distribution of the 18 food categories listed in Commission Regulation (EC) No 1565/2000[a] 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

	Food categories according to Commission Regulation 1565/2000	Distribution of the seven SCF food categories		
Key	**Food category**	**Foods**	**Beverages**	**Exceptions**
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) – foods that could not be placed in categories 01.0–15.0	Foods		

(a): Commission Regulation 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. Official Journal of the European Communities 19.7.2000, L 180, p. 8–16.

D.3. Intake data from intended use

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

Use levels for 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] provided by industry (EFFA, 2007) and listed in Table D.3, have been used to calculate the 'modified Theoretical Added Maximum Daily Intake' (mTAMDI).[6]

The MSDI and mTAMDI exposure estimates are given in Table D.4.

Table D.4: Exposure to 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071]

FL-no	Name	EU MSDI μg/capita per day	mTAMDI μg/person per day
16.071	4,5-Epoxydec-2(trans)-enal	0.04(a)	0.69(b)

FL-no: FLAVIS number; MSDI: maximised survey-derived daily intake; mTAMDI: modified theoretical added maximum daily intake.
(a): Based on EU poundage of 0.3 kg (EFFA, 2016).
(b): Based on use levels data from 2007 (EFFA, 2007).

[6] mTAMDI estimation is based in 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.