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Scientific Opinion on Flavouring Group Evaluation 200, Revision 1 (FGE.200 Rev.1): 74 α,β-unsaturated aliphatic aldehydes and precursors from chemical subgroup 1.1.1 of FGE.19

EFSA Panel on Food Additives and Flavourings (FAF),
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Abstract

The Panel on Food Additives and Flavourings of the European Food Safety Authority was requested to evaluate the genotoxic potential of 74 flavouring substances from subgroup 1.1.1 of FGE.19 in the Flavouring Group Evaluation 200 Revision 1 (FGE.200 Rev1). In FGE.200, genotoxicity studies were provided for one representative substance, namely hex-2(*trans*)-enal [FL-no: 05.073], and for other two substances in the same subgroup, namely 2-dodecenal [FL-no: 05.037] and 2-nonenal [FL-no: 05.171]. The Panel concluded that the concern still remains with respect to genotoxicity for the substances of this subgroup and requested an *in vivo* Comet assay performed in duodenum and liver for hex-2(*trans*)-enal [FL-no: 05.073]. For the two other representative substances of subgroup 1.1.1 (nona-2(*trans*),6(*cis*)-dienal [FL-no: 05.058] and oct-2-enal [FL-no: 05.060]), the Panel requested a combined *in vivo* Comet assay and micronucleus assay. These data have been provided and are evaluated in the present opinion FGE.200 Rev1. Industry submitted genotoxicity studies on *trans*-2-octenal [FL-no: 05.190], instead of oct-2-enal [FL-no: 05.060]. Based on the available data, the Panel concluded that the concern for genotoxicity can be ruled out for hex-2(*trans*)-enal [FL-no: 05.073], *trans*-2-octenal [FL-no: 05.190] and nona-2(*trans*),6(*cis*)-dienal [FL-no: 05.058], therefore all the 74 substances [FL-no: 02.020, 02.049, 02.050, 02.090, 02.112, 02.137, 02.156, 02.192, 02.210, 02.231, 05.037, 05.058, 05.060, 05.070, 05.072, 05.073, 05.076, 05.078, 05.102, 05.109, 05.111, 05.114, 05.120, 05.144, 05.150, 05.171, 05.172, 05.179, 05.184, 05.189, 05.190, 05.191, 05.195, 06.025, 06.031, 06.072, 09.054, 09.097, 09.109, 09.119, 09.146, 09.233, 09.244, 09.247, 09.276, 09.277, 09.303, 09.312, 09.385, 09.394, 09.395, 09.396, 09.397, 09.398, 09.399, 09.400, 09.410, 09.411, 09.469, 09.482, 09.489, 09.492, 09.493, 09.498, 09.678, 09.701, 09.719, 09.741, 09.790, 09.841, 09.866, 09.947, 09.948, 13.004] can be evaluated through the Procedure for flavouring substances.

Keywords: α,β-unsaturated aldehydes, straight chain, FGE.200, flavouring substances, safety evaluation, subgroup 1.1.1, FGE.19

Requestor: European Commission

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EFSA-Q-2018-00161, EFSA-Q-2018-00162, EFSA-Q-2018-00163, EFSA-Q-2018-00164, EFSA-Q-2018-00165, EFSA-Q-2018-00166, EFSA-Q-2018-00167, EFSA-Q-2018-00168, EFSA-Q-2018-00169, EFSA-Q-2018-00170, EFSA-Q-2018-00171, EFSA-Q-2018-00172, EFSA-Q-2018-00173, EFSA-Q-2018-00174, EFSA-Q-2018-00175, EFSA-Q-2018-00176, EFSA-Q-2018-00177, EFSA-Q-2018-00178, EFSA-Q-2018-00179, EFSA-Q-2018-00180, EFSA-Q-2018-00181, EFSA-Q-2018-00182, EFSA-Q-2018-00183, EFSA-Q-2018-00184, EFSA-Q-2018-00185, EFSA-Q-2018-00186, EFSA-Q-2018-00187, EFSA-Q-2018-00188, EFSA-Q-2018-00189, EFSA-Q-2018-00190, EFSA-Q-2018-00191, EFSA-Q-2018-00192, EFSA-Q-2018-00193, EFSA-Q-2018-00194, EFSA-Q-2018-00195, EFSA-Q-2018-00196, EFSA-Q-2018-00197, EFSA-Q-2018-00198, EFSA-Q-2018-00199, EFSA-Q-2018-00200, EFSA-Q-2018-00201, EFSA-Q-2018-00202, EFSA-Q-2018-00203, EFSA-Q-2018-00204, EFSA-Q-2018-00205, EFSA-Q-2018-00206, EFSA-Q-2018-00207, EFSA-Q-2018-00208, EFSA-Q-2018-00209, EFSA-Q-2018-00210, EFSA-Q-2018-00211, EFSA-Q-2018-00212, EFSA-Q-2018-00213, EFSA-Q-2018-00214, EFSA-Q-2018-00215, EFSA-Q-2018-00216, EFSA-Q-2018-00217, EFSA-Q-2018-00218, EFSA-Q-2018-00219, EFSA-Q-2018-00220 and EFSA-Q-2018-00626

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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 includes a number of flavouring substances for which the safety evaluation should be completed in accordance with Commission Regulation (EC) No 1565/2000[3].

In February 2011, the EFSA Panel had evaluated a first dossier submitted by Industry in response to the requested data for representative substances in FGE. 200. These data were not considered adequate to alleviate the genotoxicity concern for the substance in subgroup 1.1.1 and the Panel recommended at that time 'to perform in vivo dietary Comet assays (in drinking water or in feed, not by gavage) for the three linear representatives of subgroup 1.1.1 [FL-no: 05.073, 05.058 and 05.060]'.

Additional data was submitted in February and June 2013 by industry related to one representative substance of subgroup 1.1.1, hex-2(*trans*)-enal [FL-no: 05.073] and two other substances of the group.

On 21 May 2014 the EFSA CEF Panel adopted an opinion on this Flavouring Group Evaluation 200 (FGE.200). The Panel confirmed the need for an *in vivo* Comet assay performed in duodenum and liver for hex-2(*trans*)-enal [FL-no: 05.073]. For the two representative substances of subgroup 1.1.1 (nona-2(*trans*), 6(*cis*)-dienal [FL-no: 05.058] and oct-2-enal [FL-no: 05.060]), a combined *in vivo* Comet assay and micronucleus assay would be required and that evidence of bone marrow exposure should be provided.

New data concerning the three representative substances of this group addressing the EFSA opinion have been submitted during 2017. The data also included updated poundage and use levels concerning these substances.

The list of the substances referred to in this letter is included in Annex II.

1.1.1. Terms of Reference

The European Commission requests the European Food Safety Authority (EFSA) to evaluate the new information submitted and, depending on the outcome, proceed to full evaluation of the substances in this group in accordance with Commission Regulation (EC) No 1565/2000. In accordance with the usual practice by the CEF panel, the first step (assessment of the genotoxicity) should be completed within nine months. An additional 9 months if necessary is also established for the second step (evaluation through the CEF Procedure).

In case the genotoxic potential cannot be ruled out or the procedure cannot be applied in the first step, EFSA is asked to quantify the exposure.

Annex II: List of flavouring substances of FGE.200 included in this evaluation

FL-no:	Name of the substance
02.020	Hex-2-en-1-ol
02.049	Nona-2,6-dien-1-ol
02.050	Pent-2-en-1-ol
02.090	Non-2(*trans*)-en-1-ol
02.112	Non-2(*cis*)-en-1-ol
02.137	Dec-2-en-1-ol
02.156	Hex-2(*cis*)-en-1-ol

[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 (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.

FL-no:	Name of the substance
02.192	Oct-2-en-1-ol
02.210	Undec-2-en-1-ol
02.231	*trans*-2,*cis*-6-Nonadien-1-ol
05.037	2-Dodecenal
05.058	Nona-2(*trans*),6(*cis*)-dienal
05.060	Oct-2-enal
05.070	2-Heptenal
05.072	*trans*-2-Nonenal
05.073	Hex-2(*trans*)-enal
05.076	Dec-2-enal
05.078	Tridec-2-enal
05.102	Pent-2-enal
05.109	2-Undecenal
05.111	Octa-2(*trans*),6(*trans*)-dienal
05.114	4-Methylpent-2-enal
05.120	Dodeca-2,6-dienal
05.144	Dodec-2(*trans*)-enal
05.150	Hept-2(*trans*)-enal
05.171	Non-2-enal
05.172	Nona-2(*trans*),6(*trans*)-dienal
05.179	(*E*)-Tetradec-2-enal
05.184	Undec-2(*trans*)-enal
05.189	2-Hexenal
05.190	*trans*-2-Octenal
05.191	*trans*-2-Decenal
05.195	*trans*-2-Tridecenal
06.025	1,1-Diethoxynona-2,6-diene
06.031	1,1-Diethoxyhex-2-ene
06.072	1,1-Dimethoxyhex-2(*trans*)-ene
09.054	Allyl butyrate
09.097	Allyl heptanoate
09.109	Allyl nonanoate
09.119	Allyl octanoate
09.146	Allyl undec-10-enoate
09.233	Allyl propionate
09.244	Allyl hexanoate
09.247	Allyl crotonate
09.276	Oct-2-enyl acetate
09.277	Oct-2(*trans*)-enyl butyrate
09.303	Hept-2-enyl isovalerate
09.312	Allyl hexa-2,4-dienoate
09.385	Hept-2-enyl acetate
09.394	Hex-2(*E*)-enyl acetate
09.395	Hex-2(*E*)-enyl propionate
09.396	Hex-2-enyl butyrate
09.397	Hex-2-enyl formate
09.398	Hex-2(*E*)-enyl hexanoate
09.399	(2*E*)-Hexenyl isovalerate
09.400	Hex-2-enyl phenylacetate
09.410	Allyl 2-ethylbutyrate

FL-no:	Name of the substance
09.411	Allyl cyclohexanebutyrate
09.469	Allyl cyclohexanevalerate
09.482	Allyl cyclohexaneacetate
09.489	Allyl isovalerate
09.492	Allyl cyclohexanehexanoate
09.493	Allyl 2-methylcrotonate
09.498	Allyl cyclohexanepropionate
09.678	Pent-2-enyl hexanoate
09.701	Allyl phenoxyacetate
09.719	Allyl anthranilate
09.741	Allyl cinnamate
09.790	Allyl phenylacetate
09.841	2-Hexenyl octanoate
09.866	Allyl valerate
09.947	(*E*,*Z*)-2,6-Nonadienyl acetate
09.948	(2*E*)-2-Nonenyl acetate
13.004	Allyl 2-furoate

The following substance mentioned in the information submitted by the applicant was withdrawn from the Union List by Commission Regulation No 2017/1250[4] following the EFSA opinion on FGE.226 as regards its genotoxicity:

16.071 4,5-Epoxydec-2(*trans*)-enal

This substance is therefore not included in this mandate.

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 that were available at that time (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 alpha, beta-unsaturated carbonyls. Therefore, the Panel considered it inappropriate to totally rely on (Q)SAR predictions at this point in time and decided not to take substances through the procedure based on negative (Q)SAR predictions only.

The Panel took note of the (Q)SAR predictions by using two ISS Local Models (Benigni and Netzeva, 2007a,b) 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

[4] Commission Regulation (EU) 2017/1250 of 11 July 2017 amending Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council as regards removal from the Union list of the flavouring substance 4,5-epoxydec2(*trans*)-enal. OJ L 179, 12.7.2017, p. 3–5.

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 from the Flavouring Industry on genotoxicity. These subgroups were evaluated in FGE.201, 202, 203, 210, 212, 213, 214, 216, 217, 218 and 220. For the substances in FGE.202, 214 and 218, it was concluded that a genotoxic potential could be ruled out and accordingly these substances were 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 worked out a list of representative substances for each subgroup (EFSA, 2008c). In selecting the representative substances, expert judgement was applied. In each subgroup, the representative substances were selected taken into account chain length, branched chain, lipophilicity and possible additional functional groups. 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.

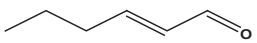
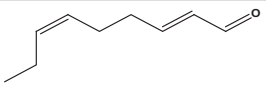
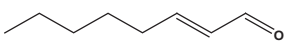
The Flavouring Industry has now submitted additional data and the present FGE concerns the evaluation of these data requested on genotoxicity.

2.2. History of the evaluation of the substances in FGE.19 subgroup 1.1.1

Subgroup 1.1.1 is one of the FGE.19 subgroups for which the Panel concluded that additional genotoxicity data are needed to perform the safety assessment of the genotoxic potential of the substances (EFSA, 2008a; EFSA CEF Panel, 2011). This conclusion was based on the *in vitro* and *in vivo* genotoxicity data available at that time (Appendix D, Tables D.1 and D.2) as well as on the outcome of the (Q)SAR predictions (Appendix C, Table C.1).

Hex-2(*trans*)-enal [FL-no: 05.073], nona-2(*trans*),6(*cis*)-dienal [FL-no: 05.058], oct-2-enal [FL-no: 05.060] and 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] were selected as representative substances to be tested for the subgroup 1.1.1 (EFSA, 2008c). The substance 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071] was subsequently considered structurally different from the other substances in subgroup 1.1.1 and was allocated to FGE.226 for evaluation on its own. The representative substances should be tested in accordance with the conditions set out in the 'Genotoxicity Test Strategy for Substances belonging to Subgroups of FGE.19' (EFSA, 2008b). The representative substances for subgroup 1.1.1 are shown in Table 1.

Table 1: Representative substances for Subgroup 1.1.1 of FGE.19 (EFSA, 2008c)

FL-no	EU register name	Structural formula	Comments
05.073	Hex-2(*trans*)-enal		Data from literature and new study reports (Beevers, 2013; Bhatia et al., 2010; Dittberner et al., 1995, 1997; Durward, 2009; Eder et al., 1992; Griffin and Segall, 1986; Honarvar, 2007a; Kato et al., 1989; Sokolowski, 2007a)
05.058	Nona-2(*trans*),6(*cis*)-dienal		Data from literature (Eder et al., 1992; Dittberner et al., 1995)
05.060	Oct-2-enal		Data from literature (Marnett et al.,1985; Canonero et al., 1990; Eder et al., 1993)

In October 2009, the Industry submitted the first dossier in response to the requested data (this dossier was replaced by an updated dossier in April 2010, (EFFA, 2010)).

The Panel considered these new data and its conclusion was given in an EFSA statement published in February 2011 (EFSA CEF Panel, 2011):

'*Supplementary information now provided includes both new data and arguments, which have been discussed by the Panel. Overall, the supplementary information provided by EFFA is not considered sufficient*.

- *Although some of arguments provided by EFFA (e.g. those on metabolism and GSH-depletion and those on the role of DNA damage) are plausible, they are not sufficient to alleviate concerns for the genotoxic and carcinogenic potential of the substances belonging to subgroup 1.1.1.*
- *The data provided are not compliant with the 'Genotoxicity Test Strategy for Substances in Subgroups of FGE.19'.*

Therefore, the need for additional genotoxicity data has not been alleviated and genotoxicity studies should be carried out for the representative substances of subgroup 1.1.1. In line with the Genotoxicity Test Strategy (EFSA 2008b), the Panel recommended to perform in vivo *dietary Comet assays (in drinking water or in feed, not by gavage) for the three linear representatives of subgroup 1.1.1 [FL-no: 05.073, 05.058 and 05.060]. The results may allow to identify whether there is a critical chain length for DNA damage'.*

The opinion on FGE.200 (EFSA CEF Panel, 2014a) dealt with the additional genotoxicity data submitted by the International Organization of the Flavor Industry (IOFI, 2013) in response to the EFSA statement on the first dossier submitted to EFSA on FGE.200 (EFSA CEF Panel, 2011). IOFI provided additional genotoxicity studies for one representative substance in FGE.200 (hex-2(*trans*)-enal [FL-no: 05.073]) and for other two substances in the same subgroup (2-dodecenal [FL-no: 05.037] and 2-nonenal [FL-no: 05.171] (Table 2). The CEF Panel evaluated these data and concluded that the concern still remains with respect to genotoxicity for the substances of this subgroup and their three representative substances. The Panel confirmed the need for an *in vivo* Comet assay performed in duodenum and liver for hex-2(*trans*)-enal [FL-no: 05.073]. For the two other representative substances of subgroup 1.1.1 (nona-2(*trans*),6(*cis*)-dienal [FL-no: 05.058] and oct-2-enal [FL-no: 05.060]), a combined *in vivo* Comet assay and micronucleus assay was required. For the latter, evidence of bone marrow exposure was asked.

Furthermore, four additional flavouring substances (*trans*-2,*cis*-6-nonadien-1-ol [FL-no: 02.231], undec-2(*trans*)-enal [FL-no: 05.184], *trans*-2-octenal [FL-no: 05.190] and *trans*-2-tridecenal [FL-no: 05.195]) were identified which are structurally related to the substances in subgroup 1.1.1 and were evaluated within this group.

In the present revision of FGE.200 (FGE.200 Revision 1), the new required genotoxicity studies submitted by Industry (Table 3) are evaluated.

FGE	Adopted by EFSA	Link	No. of substances
Statement on FGE.19 subgroup 1.1.1	21 February 2011	http://www.efsa.europa.eu/en/efsajournal/pub/2086	70
FGE.200	21 May 2014	http://www.efsa.europa.eu/en/efsajournal/pub/3709	74
FGE. 200Rev.1	13 September 2018	http://www.efsa.europa.eu/en/efsajournal/pub/5422	74

2.3. Presentation of the substances in flavouring group evaluation 200

FGE.200 concerns 74 straight chain, α,β-unsaturated aldehydes, with or without additional non-conjugated double bonds, or precursors for such structures. The 74 substances correspond to subgroup 1.1.1 of FGE.19. One former member of subgroup 1.1.1, 4,5-epoxydec-2(*trans*)-enal [FL-no: 16.071], was withdrawn from this subgroup and was evaluated in a new FGE (FGE.226Rev1, EFSA CEF Panel, 2017) as the Panel did not consider the substance to be sufficiently structurally related to the other 74 substances in subgroup 1.1.1. The flavouring substance [FL-no: 16.071] was withdrawn from the Union List by Commission Regulation No 2017/1250[5] following the EFSA Opinion on FGE.226 (EFSA CEF Panel, 2017) as regards its genotoxicity.

The chemical structures of the substances of subgroup 1.1.1 are shown in Appendix A (Table A.1) together with their specifications.

Section 2.4 of the present Opinion reports the same information that was presented in the FGE. 200. Section 3 reports the evaluation of the new data.

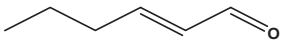
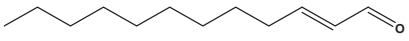
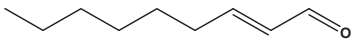
[5] Commission Regulation (EU) 2017/1250 of 11 July 2017 amending Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council as regards removal from the Union list of the flavouring substance 4,5-epoxydec-2(trans)-enal. OJ L 179, 12.07.2017, p. 3–5.

2.4. Data evaluated by Panel in FGE.200[6]

In February 2011, the Panel evaluated the first dossier submitted by the Industry in response to the requested data for representative substances in FGE.200. These data were not considered adequate to alleviate the genotoxicity concern for the substance in subgroup 1.1.1 and concluded: 'the Panel recommended to perform *in vivo* dietary Comet assays (in drinking water or in feed, not by gavage) for the three linear representatives of subgroup 1.1.1 [FL-no: 05.073, 05.058 and 05.060]'.

In February and June 2013, the Industry (IOFI, 2013) submitted the second dossier which included additional data on one [FL-no: 05.073] of the three representative substances originally selected by the Panel and supporting information to the data already submitted in the first dossier. In Table 2, the newly submitted data are listed.

Table 2: Overview of data submitted for subgroup 1.1.1 (IOFI, 2013)

Test substance	Test	Test conditions	Reference
Hex-2(*trans*)-enal [05.073] representative substance (purity: 98.2%) 	Bacterial reverse mutation assay	*Salmonella* Typhimurium strains TA98, TA100, TA102, TA1535 and TA1537 with and without metabolic activation up to 5,000 μg/plate	Sokolowski (2007a), Bhatia et al. (2010)
	In vivo micronucleus assay	Muta™mouse blood reticulocytes (days 1, 4 and 31) Treatment by oral gavage at doses of 120, 235 and 350 mg/kg bw per day for 28 days	Beevers (2013)
	Induction of *lacZ*-mutations in Muta™Mouse	Muta™Mouse treatment by oral gavage at doses of 120, 235 and 350 mg/kg bw per day for 28 days. Mutation frequencies (day 31) determined in the liver and the duodenum	Beevers (2013)
	In vivo micronucleus assay	Treatment by oral route at doses of 250, 500, 1,000 mg/kg bw per day. Sampling of bone marrow was done 24 and 48 h after treatment	Honarvar (2007a)
	In vivo rat liver unscheduled DNA synthesis (UDS) assay	Treatment by oral route at doses of 200 and 500 mg/kg bw per day. Liver was perfused at 16 and 3 h after dosing	Durward (2009)
2-Dodecenal [05.037] not representative (purity: 99.4%) 	Bacterial reverse mutation assay	*S.* Typhimurium strains TA98, TA100, TA102, TA1535 and TA1537 with and without metabolic activation up to 1,000 μg/plate	Sokolowski (2007b), Bhatia et al. (2010)
	In vivo micronucleus assay	Treatment by oral route at doses of 500, 1,000 and 2,000 mg/kg bw per day. Sampling of bone marrow was done 24 and 48 h after treatment. 2,000 PCE scored at 24 h (3 doses) and 48 h (top dose)	Honarvar (2007b), Bhatia et al. (2010)
2-Nonenal [05.171] not representative (purity: 96.2%)  (E)-isomer shown	*In vivo* Micronucleus assay	Treatment by oral route at doses of 500, 1,000 and 2,000 mg/kg bw per day. Sampling of bone marrow was done 24 and 48 h after treatment. 2,000 PCE scored at 24 h (3 doses) and 48 h (top dose)	Honarvar (2008), Bhatia et al. (2010)

bw: body weight; PCE: polychromatic erythrocytes.

[6] The data presented in Section 2.4 are cited from the Scientific Opinion FGE.200.

2.4.1. *In vitro* genotoxicity tests

Bacterial reverse mutation assays

*Hex-2(*trans*)-enal [FL-no: 05.073]*

Hex-2(*trans*)-enal (purity: 98.2%) was tested at concentrations up to 5,000 μg/plate (but concentrations higher than 200 μg/plate were bacteriostatic) in the *Salmonella* Typhimurium strains TA98, TA100, TA102, TA1535 and TA1537, in a Good Laboratory Practice (GLP) study performed according to OECD Guideline 471 (OECD, 1997a), with or without metabolic activation (Sokolowski, 2007a; Bhatia et al., 2010). A small but concentration-dependent increase in revertant colony numbers was observed using the pre-incubation method in strain TA100 without metabolic activation (concentrations tested 1–2,500 μg/plate). Toxic effects at higher concentrations reduced the number of revertants. Smaller increases (< 2-fold) were also seen in the presence of S9-mix. Therefore, a follow-up experiment, again using the pre-incubation method, was performed in strain TA100 over a narrow range of concentrations up to 200 μg/plate. In this follow-up experiment, a moderate concentration-dependent increase in revertant colony numbers was again observed without metabolic activation at 50 and 100 μg/plate. Based on the reproducibility of this effect, the author concluded a positive mutagenic outcome for this test. While the magnitude of the increase in revertant colony numbers is not substantial, these results do not exclude possible mutagenic potential in strain TA100 (Sokolowski, 2007a).

Kato et al. (1989) tested hex-2(*trans*)-enal (unknown purity) in the *S*. Typhimurium strains TA98, TA100 and TA104 and in *Escherichia coli* strain WP2uvrA/pKM101 with and without metabolic activation using the pre-incubation method (20 min at 37°C). According to the authors, hex-2(*trans*)-enal was 'suspected to be positive'; however, no further details were provided and the validity of this study is limited.

2-Dodecenal [FL-no: 05.037]

At concentrations up to 1,000 μg/plate with and without metabolic activation (but concentrations ≥ 100 μg/plate were bacteriostatic) 2-dodecenal (purity: 99.4%) was not mutagenic in the *S*. Typhimurium strains TA98, TA100, TA102, TA1535 and TA1537 in a GLP study performed according to OECD Guideline 471; the limiting factor was the bacteriostatic activity (Sokolowski, 2007b). Toxic effects (reduction in revertant numbers) were seen at the higher concentrations in all parts of the study. No genotoxic effect was noted with and without metabolic activation in the five strains.

The same data for the bacterial reverse mutation assay reported by Sokolowski (2007a, b) for hex-2(*trans*)-enal [FL-no: 05.073] and 2-dodecenal [FL-no: 05.037] were presented in a poster abstract (Bhatia et al., 2010).

Summary of the bacterial reverse mutation assays for both hex-2(*trans*)-enal [FL-no: 05.073] and 2-dodecenal [FL-no: 05.037] are reported in Appendix D, Table D.3.

2.4.2. *In vivo* genotoxicity tests

*Hex-2(*trans*)-enal [FL-no: 05.073]*

On the basis of the *in vitro* bacterial reverse mutation assay results reported above for hex-2(*trans*)-enal, it was considered most appropriate to probe its genotoxic potential using a Muta™Mouse (lacZ/GalE) assay with an *in vivo* micronucleus component included (Beevers, 2013). The assay was carried out in transgenic mice. This combined approach minimises the number of animals used in the experiments. Micronuclei were measured in peripheral blood, and in the mutation arm of the experiment, the liver and the duodenum were chosen as the most appropriate tissues, in order to address the potential for mutation at the site of most significant metabolism and at the site of first contact, respectively. Therefore, groups of Muta™Mouse CD2-lacZ80/HazfBR mice were administered hex-2(*trans*)-enal via gavage and the liver, duodenum and peripheral blood were analysed for the potential induction of DNA damage in a GLP study performed according to OECD Guidelines 474 (OECD, 1997b) and 488 (OECD, 2011). However, the Panel noted that there were some deviations from OECD guideline 474 (see below and Appendix D, Table D.4).

An initial range-finding study was conducted to estimate the maximum tolerated dose (MTD) of hex-2(*trans*)-enal (purity 99.5%) after administration by oral gavage to groups of three male and three female Muta™Mouse mice. Doses of 500 mg/kg body weight (bw)/day were clearly toxic to mice, with one animal being killed in extremis on day 4 and the rest of the animals exhibiting signs of toxicity (piloerection, hunched posture) but surviving to day 7. Further groups of animals were also dosed at

250 and 350 mg/kg bw per day. No clinical signs of toxicity were observed at 250 mg/kg bw per day, but at 350 mg/kg bw per day 1 animal showed signs of clinical toxicity (hunched posture, decreased activity and dyspnoea). As a result, 350 mg/kg bw per day was identified as the MTD. As no significant gender differences in clinical signs of toxicity were observed, it was concluded that male mice alone could be used in the main experiment. Two lower doses of 120 and 235 mg/kg bw per day were also selected for testing.

Groups of six male Muta™Mouse mice were treated daily by oral gavage with hex-2(*trans*)-enal at doses of 120, 235 and 350 mg/kg bw per day, including a vehicle control (corn oil) for 28 days with a 3-day recovery period prior to sacrifice. Concurrent positive control animals were not included in this study. Tissue-matched positive control DNA was included in all packaging reactions in order to confirm correct assay functioning. The positive control DNA originated from animals dosed with ethylnitrosurea. All individual packaging reaction resulted in at least 30,000 plaque-forming unit (PFU) and at least one mutant plaque. For all animals, data were generated for at least 200,000 PFU per tissue, from at least three independent packaging reactions. At least 1 million PFU were obtained per group, per tissue from a minimum of five animals. No significant increases in mutation frequency (MF) or significant dose-related trends were observed in the liver or the duodenum. Some of the hex-2(*trans*)-enal treatment groups showed duodenum MF that exceeded laboratory historical controls but were comparable to concurrent vehicle control values. The testing laboratory had a limited number of datasets that comprise the historical control data for the duodenum in this assay and considered its historical control for the duodenum in the Muta™Mouse assay to be narrow at the time of drafting this report.

Hex-2(*trans*)-enal was evaluated in a micronucleus assay in peripheral normochromatic erythrocytes (NCE) and reticulocytes for its ability to induce chromosomal damage (micronuclei (MN)) in mice on days 4 and 31 after 28 days of dosing, using a flow cytometry method. Where possible, 20,000 reticulocytes were analysed from each blood sample. No significant differences were observed in the frequency of peripheral blood reticulocytes (% RET) in all treatment groups on day 4 or 31 after 28 days of dosing. There were no significant increases in the frequency of micronuclei compared to concurrent controls on day 4 or 31 after 28 days of dosing. On day 31, it was noted that there was a significant linear trend in micronucleated reticulocyte (% MN-RET) frequency ($p \leq 0.05$); however, as the MN-RET frequencies for all treated animals (0.37 $\pm$ 0.04, 0.39 $\pm$ 0.05, 0.39 $\pm$ 0.06, 0.46 $\pm$ 0.09 at doses of 0, 120, 235, 350 mg/kg bw per day respectively) were highly consistent with the day 1 background levels of MN-RET (0.38 $\pm$ 0.04, 0.39 $\pm$ 0.05, 0.41 $\pm$ 0.05, 0.42 $\pm$ 0.05, at doses of 0, 120, 235, 350 mg/kg bw per day respectively), the significant linear response was considered to be an artefact and was not indicative of any accumulation of micronuclei over time (Beevers, 2013).

The Panel noted that in the micronucleus arm of the study, the peripheral blood was sampled 72 h after the treatment while the OECD Guideline 474 recommends: 'once between 36 and 48 h following the final treatment for the peripheral blood'. This point limited the reliability of the results obtained in the micronucleus part of the assay.

Hex-2(*trans*)-enal (purity: 98.2%) was evaluated in a micronucleus assay in bone marrow polychromatic erythrocytes (PCE) for its ability to induce chromosomal damage (MN) in mice in a GLP study performed according to OECD Guideline 474 (OECD, 1997b). Hex-2(*trans*)-enal dissolved in corn oil as a carrier was given orally to animals (5 males and 5 females) at doses of 250, 500 and 1,000 mg/kg bw. The high dose was determined in a preliminary toxicity study. Mice from all dose groups were sampled 24 h after dosing, and mice from the top-dose and control groups were also sampled 48 h after dosing (Honarvar, 2007a).

Cyclophosphamide (40 mg/kg bw) was given as the positive control and mice were sampled at 24 h. At least 2,000 PCE were scored for each animal for MN. At the highest dose given, two males and two females died, which indicates that higher doses could not have been used. Also, in the highest dose group the numbers of PCE were clearly decreased ($-$35% at 24 h) as compared to the mean value of PCE of the vehicle control. This indicates that hex-2(*trans*)-enal exerts cytotoxic effects in the bone marrow at this dose level and demonstrates, in the absence of toxicokinetic measures, that the target tissue was exposed. In comparison to the corresponding vehicle controls, there was no statistically significant increase in the frequency of the detected micronuclei at any preparation interval after administration of the test item with any dose level used (Honarvar, 2007a).

2-Hexenal (unspecified isomer and purity) was evaluated in an *in vivo* unscheduled DNA synthesis assay using oral administration in a GLP study performed according to OECD Guideline 486 (OECD, 1997c) (Durward, 2009). Male rats were given 200 or 500 mg/kg bw 2-hexenal. The top dose was proposed by the sponsor, and a preliminary test by the testing facility demonstrated no deaths at this dose. As no other dose levels were used, it is not clear that this was the MTD, and perhaps a higher dose

could have been used. In one experiment, livers were perfused approximately 16 h after dosing, and in a second experiment, 3 h after dosing. Following perfusion, hepatocytes were processed and areas of nucleus and cytoplasm scored for autoradiographic grains in 150 cells/animal at each sampling time using automated image analysis. A control group was given only corn oil, and the positive control groups were administered 2-acetylaminofluorene (16 h) or *N*,*N*'-dimethylhydrazine (3 h). Net nuclear grain counts were < 0 at the two harvest times and the percentage of cells in repair was low in all animals dosed with 2-hexenal at the 3-h harvest time. The percentage of cells in repair at the 16-h harvest time was weakly increased with 1.8 ± 1.7% and 2.2 ± 0.6% cells in repair at 200 and 500 mg/kg, respectively, vs 0.4 ± 0.6% in the concurrent control; however, these values are low and within those generally observed. In the absence of an increase in the number of net grain per cell, these variations have no meaning in term of genotoxic effect. There was therefore no evidence of induction of unscheduled DNA synthesis in animals dosed with the test material at either time point.

2-Dodecenal [FL-no: 05.037]

2-Dodecenal (purity: 99.4%) was evaluated in a micronucleus assay in bone marrow PCE for its ability to induce chromosomal damage in mice in a GLP study performed according to OECD Guideline 474 (OECD, 1997b). 2-Dodecenal, dissolved in corn oil as a carrier, was given orally to animals (5 males and 5 females) at doses of 500, 1,000 and 2,000 mg/kg bw. The top dose of 2,000 mg/kg bw is a limit dose for non-toxic substances. Mice from all dose groups were sampled 24 h after dosing, and mice from the top-dose and control groups were sampled also at 48 h after dosing. Cyclophosphamide (40 mg/kg bw) was given as the positive control and mice were sampled at 24 h. At least 2,000 PCE were scored for each animal for MN. No cytotoxic effects were observed at any dose, based on the ratio between PCE and NCE in each treated sample versus vehicle controls.

In comparison to the corresponding vehicle controls, there was no statistically significant increase in the frequency of the detected micronuclei at any preparation interval after administration of the test item with any dose level used (Honarvar, 2007b).

2-Nonenal [FL-no: 05.171]

2-Nonenal (purity: 96.2%) was evaluated in a micronucleus assay in bone marrow PCE for its ability to induce chromosomal damage in mice in a GLP study performed according to OECD Guideline 474 (OECD, 1997b). 2-Nonenal, dissolved in corn oil as a carrier, was given orally to animals (5 males and 5 females) at doses of 500, 1,000 and 2,000 mg/kg bw. The top dose of 2,000 mg/kg was estimated as suitable by a preliminary study on acute toxicity. Mice from all dose groups were sampled 24 h after dosing, and mice from the top-dose and control groups were sampled also at 48 h after dosing. Cyclophosphamide (40 mg/kg bw) was given as the positive control and mice were sampled at 24 h. At least 2,000 PCE were scored for each animal for MN. The numbers of PCE were slightly decreased, mainly in the top dose group at both sampling times, as compared to the mean value of PCE of the vehicle control (−13% at 24 and 48 h sampling times). However, the decrease in % PCE was small. In comparison to the corresponding vehicle controls, there was no statistically significant increase in the frequency of the detected micronuclei at any preparation interval after administration of the test item with any dose level used (Honarvar, 2008).

For both 2-dodecenal and 2-nonenal tested through micronucleus assays in mouse bone marrow PCE (Honarvar, 2007b, 2008), there was no direct confirmation that the bone marrow was exposed, as no toxicokinetic measures of the test substance in plasma were made.

Micronucleus data for hex-2(*trans*)-enal (Honarvar 2007a), 2-nonenal (Honarvar, 2008) and 2-dodecenal (Honarvar, 2007b) were reported also in a poster abstract (Bhatia et al., 2010).

The results of *in vivo* studies are summarised in Table D.4.

2.4.3. DNA adduct and related studies

DNA adduct studies in vitro

The ability of the α,β-unsaturated aldehydes to bind to isolated nucleosides and nucleotides *in vitro* has been reported (Eder et al., 1993; Eisenbrand et al., 1995; Golzer et al., 1996; Stout et al., 2008). 2-Hexenal and related α,β-unsaturated aldehydes are capable of forming 1,N^2-cyclic deoxyguanosine and 7,8-cyclic guanosine adducts.

DNA adduct studies in vivo *on hex-2(*trans*)-enal [FL-no: 05.073]*

Using a ^{32}P-post-labelling method based on nuclease P1 enrichment and thin-layer chromatography (TLC) separation of the labelled adducts,[7] *in vivo* studies on hex-2(*trans*)-enal report adducts formation. In a first study, Schuler et al. (1999) administered hex-2(*trans*)-enal at a single dose of 500 mg/kg bw by oral route to F344 male rats. No adducts were found in the control rats. In treated rats, an adduct (1,N^2-propanodeoxyguanosine (Hex-PdG)) was detected in the liver. Highest Hex-PdG adduct levels were found 2 days after gavage. Four days after gavage, the Hex-PdG adducts level was one-third of the maximum level but it was even higher than Hex-PdG adducts found after 1 day. No adducts were detected 8 h after gavage. This study demonstrates that after one single high dose of hex-2(*trans*)-enal, formation of DNA adducts were induced, that there was a delay before apparition of adducts in the liver and that these adducts were repaired only slowly.

Schuler and Eder (1999) detected Hex-PdG adducts in the forestomach, liver, oesophagus and kidneys of F344 rats at relatively high single doses, i.e. 200 and 500 mg/kg bw of hex-2(*trans*)-enal by gavage. At 50 mg/kg bw, Hex-PdG adducts were quantified only in the oesophagus. The covalent binding index was 0.06, 0.22 and 0.62 at 50, 200 and 500 mg/kg bw, respectively (Schuler and Eder, 1999).

In the study performed by Stout et al. (2008), using a liquid chromatography with tandem mass spectrometry (LC–MS/MS)[8] method, no adduct formation was reported at 50 mg/kg bw of hex-2 (*trans*)-enal except in forestomach DNA of one rat exposed to a single dose and sacrificed 2 days after (Stout et al., 2008). Quantifiable levels of Hex-PdG adducts were reported in the forestomach of animals exposed to 100 mg/kg bw per day of hex-2(*trans*)-enal for 1 or 4 weeks (once daily for 5 days per week) and at 200 mg/kg bw of hex-2(*trans*)-enal in single doses. However, Hex-PdG was not quantifiable in forestomach DNA of rats after exposure to 0, 10 or 30 mg/kg for 1 or 4 weeks (Stout et al., 2008). These data are indicative of a dose- and time dependence on DNA adducts formation with hex-2(*trans*)-enal. Hex-PdG was not quantifiable in liver DNA after exposure to 100 mg/kg for 1 or 4 weeks. These findings suggest that the genotoxicity of hex-2(*trans*)-enal was limited to the site of contact (forestomach) and DNA adduct formation occurred in the setting of severe tissue damage as demonstrated by histopathological observations. At these cytotoxic doses, cell proliferation was noted. The Panel noted that no DNA adducts were observed at 30 mg/kg per day and below.

2.4.4. Data on toxicokinetic

Analogous to other α,β-unsaturated aldehydes, *trans*-2-hexenal is readily oxidised *in vitro* to *trans*-2-hexenoic acid in the cytosolic fraction of mouse liver cells (Lamé and Segall, 1986) and by isoenzymes of rat aldehyde dehydrogenase (ALDH) present in mitochondrial, cytosolic and microsomal fractions (Mitchell and Petersen, 1987). In general, the members of the ALDH superfamily demonstrate higher catalytic activity *in vitro* for higher molecular weight and more lipophilic aldehydes (Nakayasu et al., 1978).

Prior to absorption, 15% of a 100 mg/kg bw dose of *trans*-2-nonenal given to rats was oxidised to *trans*-2-nonenoic acid (Grootveld et al., 1998).

Linear α,β-unsaturated aldehydes are rapidly absorbed, distributed, metabolised and excreted in the urine and, to a lesser extent, in the faeces. In *in vivo* experiments with *trans*-2-nonenal and *trans*-2-pentenal, male Wistar albino rats were administered a bolus dose of 100 mg/kg bw of one of the aldehydes by gavage in unheated olive oil. A control group of rats received only the unheated olive oil. Urine samples were collected prior to and after administration. Proton nuclear magnetic resonance (^{1}H-NMR) analysis indicated that both *trans*-2-nonenal and *trans*-2-pentenal entered systemic circulation from the gastrointestinal tract and were metabolised in the fatty acid pathway or were conjugated with glutathione (GSH) to yield the C-3 mercapturate conjugate that is excreted mainly in the urine within 24 h. Trace amounts of *trans*-2-nonenal and *trans*-2-pentenal were detected in the faeces (Grootveld et al., 1998).

PBK/D model

A recent physiologically based kinetic/dynamic (PBK/D) study supports a dose-dependent effect on hex-2(*trans*)-enal detoxification and development of DNA adducts (Kiwamoto et al., 2012, 2013). The detoxification of *trans*-2-hexenal proceeds via three pathways: oxidation to 2-hexenoic acid by ALDH, reduction to 2-hexen-1-ol by aldose reductase (AR), conjugation with reduced GSH either chemically or catalysed by glutathione *S*-transferase (GST) (Eisenbrand et al., 1995). Kiwamoto et al. (2012)

[7] Detection limit 0.03 adducts per 10^6 nucleotides.
[8] The limit of quantitation was 0.015 fmol Hex-PdG/μg DNA (200 μg DNA) or 0.006 fmol Hex-PdG/μg DNA (500 μg DNA).

developed a PBK/D model in rats determining *in vitro* kinetic parameters (e.g. K_m, V_{max} and catalytic efficiency) for each detoxification pathway. Performance of the model was evaluated against available *in vivo* data from literature on rats exposed to high doses of *trans*-2-hexenal (Schuler and Eder, 1999; Stout et al., 2008). In this study, it was shown that when hex-2(*trans*)-enal is incubated with S9-mix fractions of rat liver and rat small intestine, in the presence of NAD^+, both fractions predominantly convert the substrate to 2-hexenoic acid which does not readily form DNA conjugates and is efficiently eliminated from the urine in the form of glucuronic acid conjugates. This model predicts that the conversion of *trans*-2-hexenal at doses of 0.04 mg/kg bw (predicted human dietary exposure) and 200 mg/kg bw (dose at which DNA adduct formation in the liver was reported in rats, by Schuler and Eder, 1999) is complete within 3 h. At 0.04 mg/kg bw, GSH concentration is not affected both in liver and small intestine. At 200 mg/kg bw, GSH concentration in the small intestine (predicted as the most important detoxification pathway in this tissue) dropped rapidly and amounted to only 65% of the initial level after 24 h; also in the liver, GSH concentration is depleted, but restored within 24 h. The model suggests that at low doses of *trans*-2-hexenal, protective levels of GSH are unaffected, while at high doses significant GSH depletion occurs. The model predicts that at doses below 80 mg/kg bw all the three pathways contribute to *trans*-2-hexenal detoxification in the liver. The PBK/D model predicts that hex-2(*trans*)-enal is readily detoxified through GSH conjugation at 30 mg/kg bw and below. The same model was further developed to examine dose-dependent detoxification and DNA adducts formation in humans upon dietary exposure (Kiwamoto et al., 2013). In this study, the kinetic parameters were derived from literature or calculated through *in vitro* reactions using human tissue fractions, taking into account interindividual differences. The model reveals that rapid *in vivo* detoxification of hex-2(*trans*)-enal at levels of average dietary exposure (0.04 mg/kg bw) makes DNA adduct formation negligible (Kiwamoto et al., 2013). Additionally, EFFA estimated a daily exposure of 0.01 mg/kg bw per day for hex-2(*trans*)-enal (EFFA, 2010) which is below the concentrations predicted to induce DNA adduct formation.

The Panel noted that all the metabolic parameters were obtained from *in vitro* studies using rat (Kiwamoto et al., 2012) or human (Kiwamoto et al., 2013) liver S9-mix or small intestine S9-mix and cofactors or liver mitochondrial fraction to determine the kinetic constants for ALDH-mediated oxidation, AR-mediated reduction and GST-catalysed conjugation of GSH with *trans*-2-hexenal in these different tissue fractions. Due to the fact that data were obtained only *in vitro*, such a model is limited. The Panel noted that for these reasons, this model should be considered with cautions.

2.4.5. Discussion of mutagenicity/genotoxicity and related relevant data

In Ames assays, positive results in TA100 and TA104, were reported for several of the substances in subgroup 1.1.1, particularly when pre-incubation conditions were used. Slight concentration-dependent increase in revertant colony numbers was observed with hex-2(*trans*)-enal [FL-no: 05.073] and pent-2-enal [FL-no: 05.102] but not with nona-2(*trans*),6(*cis*)-dienal [FL-no: 05.058] and 2-octenal [FL-no: 05.060]. When using a threefold bacterial cell density, pent-2-enal and hex-2(*trans*)-enal were clearly mutagenic with and without metabolic activation; hept-2(*trans*)-enal induced a weak and concentration-dependent mutagenic effect with metabolic activation and it was clearly mutagenic without S9-mix. In this assay, it was demonstrated that mutagenicity decreased and toxicity increased with increasing length of the alkyl chain in β-position (Eder et al., 1992). The authors suggested that the dependence of cell toxicity on the increasing β-chain length could be related to the increasing lipophilicity. A double bound in the β-alkyl chain conjugated with that of the acrolein moiety exerted a special effect: it increases the mutagenicity significantly (Eder et al., 1992). This has been confirmed in recent GLP studies (Sokolowski, 2007a,b).

Five alk-2-enals, penta-2-enal, hex-2-enal, hept-2-enals, oct-2-enal and non-2-enal (isomers not specified), were tested for mutagenic activity in V79 Chinese hamster cells. All five alk-2-enals induced a concentration-dependent increase of 6-thioguanine (TG)-resistant mutants with a statistically significant increase at 0.3 mM for penta-2-enal and hex-2-enal, at 0.1 mM for hept-2-enal and oct-2-enal and at 0.01 mM for non-2-enal. The authors reported that a significant increase in mutation frequency is caused by alkyl-2-enal concentrations that caused cytotoxicity. Both mutagenicity and cytotoxicity seems directly related to the chain length of the compound. Only hept-2-enal induced a statistically significant increase in the frequency of mutations to ouabain resistance in the same cell line (Canonero et al., 1990). This study was considered of limited validity because there is no information about the cytotoxicity levels at each concentration tested, the number of tested concentrations is limited (2 or 3) and the criteria for their choices not clearly presented.

Hex-2(*trans*)-enal [FL-no: 05.073] and *trans*-2-nonenal [FL-no: 05.072] were positive in an *in vitro* unscheduled DNA synthesis (UDS) assay performed in primary cultures of rat hepatocytes. Concentrations of both compounds from 60 to 600 nmol/10^6 cells (equal to 70–700 nmol/mL) showed a concentration-dependent increase of cells positive for UDS (Griffin and Segall, 1986).

In the study by Eder et al. (1992), it is shown that in the presence of S9-mix there is a shift in toxicity toward higher chemical concentrations, suggesting that S9-mix could lead to partial detoxification. Also, Marnett et al. (1985) reported that toxicity is an important factor in the detection of enals as mutagens. The authors observed positive results only in the presence of GSH and attributed this effect to a partial detoxification that allows survival of bacteria and the growth of revertant colonies.

In the TA104 strain (which carry one non-sense mutation TAA in the main DNA and not on a plasmid like TA102 strain), 2-hexenal [FL-no: 05.189] was mutagenic, but 2-heptenal [FL-no: 05.070], 2-octenal [FL-no: 05.060] and 2-nonenal [FL-no: 05.171] were not mutagenic. No mutagenic activity was observed in the TA102 strain (Marnett et al., 1985).

Positive evidence of genotoxicity was also reported in other assays (sister chromatid exchange (SCE), chromosomal aberrations (ABS), MN, hypoxanthine guanine ribosyl transferase (HPRT) mutations and UDS) in mammalian cells, but more particularly in cell lines that have low detoxification capacity, e.g. Namalva cells and V79 cells (Griffin and Segall, 1986; Canonero et al., 1990; Esterbauer et al., 1990; Eckl et al., 1993).

Hex-2(*trans*)-enal [FL-no: 05.073] (concentrations tested from 5 to 250 μM) and nona-2(*trans*),6(*cis*)-dienal [FL-no: 05.058] (concentrations tested from 5 to 40 or 50 μM) were tested in a human lymphoblastoid Namalva cell line and in human lymphocytes for SCE, ABS and MN induction without metabolic activation. Both aldehydes increased the frequency of SCE in the two cell types. The treatment with hex-2(*trans*)-enal induced a statistically significant increase in SCE from 40 μM on lymphocytes and 20 μM for Namalva cells. Nona-2(*trans*),6(*cis*)-dienal induced a statistically significant increase in SCE from 20 μM on lymphocytes and 10 μM for Namalva cells. Nona-2(*trans*),6(*cis*)-dienal was more cytotoxic than hex-2(*trans*)-enal. In human lymphocytes, neither hex-2(*trans*)-enal nor nona-2(*trans*),6(*cis*)-dienal induced statistically significant increase of structural chromosomal aberrations. On the contrary, in Namalva cells, both hex-2(*trans*)-enal and nona-2(*trans*),6(*cis*)-dienal induced structural chromosomal aberrations from 100 μM and 5 μM respectively. Hex-2(*trans*)-enal and nona-2(*trans*),6(*cis*)-dienal induced aneuploidies in human lymphocytes from 40μM. Hex-2(*trans*)-enal increased the frequencies of MN both in lymphocytes and in Namalva cells in a concentration-dependent manner. The increase of MN frequency, induced by hex-2(*trans*)-enal, was statistically significant in lymphocytes from 50 μM and in Namalva cells from 150 μM, while for nona-2(*trans*),6(*cis*)-dienal a statistically significant increase of MN frequency was observed from 20 μM in lymphocytes and from 40 μM in Namalva cells. Using fluorescent in situ hybridisation, both lymphocytes and Namalva cells showed significantly enhanced frequencies of centromere-positive MN for both hex-2(*trans*)-enal and nona-2(*trans*),6(*cis*)-dienal, which is coherent with the observation of aneuploidy inductions in the cytogenetic assay. This study shows that both hex-2(*trans*)-enal and nona-2(*trans*),6(*cis*)-dienal gave equivocal results in lymphocytes and positive results in Namalva cells, for structural aberrations. While for aneugenicity hex-2(*trans*)-enal and nona-2(*trans*),6(*cis*)-dienal were positive in both cell types. The Namalva cells were generally more sensitive than lymphocytes. These cells have been found poor or even totally deficient in many detoxifying enzymes and they also contain only rather low concentrations of GSH and of glutathione-related enzymes (Dittberner et al., 1995).

Using an alkaline elution method, Eisenbrand et al. (1995) demonstrated that Namalva cells were significantly more sensitive than primary rat hepatocytes to the induction of DNA strand breaks by hexenal. In hepatocytes, about 3–5 times higher concentrations of aldehydes were necessary to induce significant effects compared to Namalva cells. The authors explained this difference by the better enzymatic activity (GSH transferase, ALDH) in primary rat hepatocytes compared to Namalva cells. In this study, the authors demonstrated that hexenal induced DNA binding in a range of doses from 1 to 5 mM (Eisenbrand et al., 1995).

Dittberner et al. (1997) performed studies on exfoliated cells of human oral mucosa. Seven healthy non-smoking volunteers rinsed their mouth four times per day for 3 days with 100 mL of hex-2(*trans*)-enal [FL-no: 05.073] solution at the concentration of 10 ppm, which represents a possible concentration in food. Results showed at least a doubling of MN frequency in exfoliated cells of human oral mucosa during one of the next 4 days, then the MN number dropped down to nearly the control level. In a second study, seven other volunteers were observed before and after eating 3–6 bananas that contained 35 ppm hex-2(*trans*)-enal. Six of the seven volunteers showed at least a doubling of the MN frequency during one of the next 6 days (Dittberner et al., 1997). The Panel noted that the

results were statistically significant and that the protocol was consistent with standard protocols recently developed for biomonitoring studies. Therefore, the results are considered reliable. However, the Panel also noted that this kind of studies is not validated for regulatory purposes.

Primary rat hepatocytes were treated for 3 h with 0, 0.1, 1.0, 10 and 100 μM of *trans*-2-nonenal [FL-no: 05.072] followed by a 48-h recovery period. *trans*-2-Nonenal induced an increase ($p < 0.01$) in MN at 10 and 100 μM. At a concentration of 100 μM, the mean value of chromosomal aberrations was 2.7-fold higher than in the controls, but due to the high standard deviations, these increases were not statistically significant (Esterbauer et al., 1990).

Primary rat hepatocytes were seeded and after 20 h treated with *trans*-2-nonenal [FL-no: 05.072] at 0, 0.1, 1, 10 or 100 μM for 3 h. Then the culture medium was replaced by fresh medium added with epidermal growth factor (EGF) and bromodeoxyuridine (BrdU) (Eckl et al., 1993). Forty-eight hours after the end of the treatment, cells were treated with colcemid, and sampled 3 h later. Slides treated with Hoechst 33258 were used for determination of SCE and the other for chromosomal aberration. *trans*-2-Nonenal induced no significant toxicity at the highest concentration tested. *trans*-2-Nonenal increased neither chromosomal aberrations nor the frequency of micronuclei. The Panel noted that in this study, EGF was added to induce cell division, but cells were not in division during the period of treatment, this deviation could result in a bias compared to recommended protocols. The Panel noted that cells used to determine the induction of chromosomal aberrations and micronuclei were pre-treated with BrdU which weakens the chromosomes. The testing for chromosomal aberrations and micronuclei was done after a short treatment, followed by a long recovery time which does not appear to be an optimum protocol and is a deviation from the OECD Guidelines. Moreover, hepatocytes do not divide all since the mitotic index in control cultures ranged from 0.41% to 1.94%, and no method (such as the addition of cytochalasin B) was used to determine the frequency of micronuclei only in cells that divided which reduces the sensitivity of this test (Eckl et al., 1993).

Chung et al. (1999) reported that formation of cyclic propano adducts are common products from reactions of enals with DNA bases. Enals derive from lipid peroxidation of cell membrane, but the contribution from environmental sources, cannot be excluded. The mutagenicity of enals and the mutations observed in site-specific mutagenesis studies, using a model for Hex-PdG adducts, suggest that these adducts are potential promutagenic lesions. The authors showed that tissue GSH plays an important role in protecting DNA from cyclic adduction by enals.

Coles and Ketterer (1990) reported that 4-hydroxynon-2-enal is a substrate of different classes of rat glutathione transferases that detoxified this compound. But the authors concluded that these enzymes do not provide a perfect protection and cytotoxic or genotoxic damage cannot always be avoided.

Kelson et al. (1997) isolated and characterised a human microsomal fatty ALDH, which is a distinct human ALDH isozyme that acts on a variety of medium- and long-chain aliphatic substrates with a high activity towards saturated and unsaturated aliphatic aldehydes ranging from 6 to 24 carbons in length.

In cell lines poor in detoxification capacity, there is an opportunity for high concentrations (20–40 μM) of α,β-unsaturated aldehydes to either interact directly with DNA or indirectly forming DNA adducts due to oxidative stress, leading to single DNA strand breaks but no cross-linking of DNA. The depletion of GSH by high concentrations of α,β-unsaturated aldehydes is known to lead to oxidative stress and to the release of nucleocytolytic enzymes, causing DNA fragmentation, cellular damage and apoptosis (see Sections 2.3 and 2.4). Hex-2(*trans*)-enal, 2-nonenal and 2-dodecenal did not induce MN in mice in robust GLP studies (Honarvar, 2007a, 2007b, 2008). However, only for hex-2(*trans*)-enal exposure of the target tissue was demonstrated. In addition, hex-2(*trans*)-enal did not induce unscheduled DNA synthesis in rat hepatocytes at doses up to 500 mg/kg bw in a GLP study (Durward, 2009).

However, this type of assays does not address the potential clastogenicity in the gastrointestinal tract. While such studies have not been conducted, due to structure similarity it seems to be possible that lifetime gavage administration of high concentrations of hex-2(*trans*)-enal [FL-no: 05.073] to rats might result in carcinogenicity in the forestomach or oesophagus, similar to that observed for 2,4-hexadienal (subgroup 1.1.4 of FGE.19, EFSA CEF Panel, 2014a,b). It is also likely that the ulcerative and necrotising lesions and consequent regenerative cell proliferation in the forestomach produced under these unique conditions would be associated with increased DNA adducts, as was observed in the hexenal DNA adduct study (Stout et al., 2008). However, production of exocyclic guanine adducts following GSH depletion may be involved, but the evidence from the 2-hexenal and 2,4-hexadienal studies suggests that these events are associated with significant tissue damage (ulceration, inflammation and hyperplasia) related to high bolus dosing by gavage. The inflammation and tissue damage could affect the normal biochemical processes involved in the metabolism of α,β-unsaturated

aldehydes and their detoxification. The reduced metabolic activity could increase the probability of a direct reaction between aldehydes and DNA nucleotides.

A recent PBK/D model shows that 2-hexenal is rapidly detoxified predominantly by conjugation with GSH by GSTs, and that the rapid detoxification of 2-hexenal reduces the risk arising from 2-hexenal exposure through the diet. Thus, dietary exposure to doses that do not deplete GSH, and therefore do not lead either to tissue damage or DNA adducts would not be expected to pose a mutagenic or carcinogenic hazard (Kiwamoto et al., 2012, 2013). However, the Panel considered that PBK/D studies are not sufficient due to the lack of validation.

Hex-2(*trans*)-enal induced weak gene mutations in bacteria. When tested in the Muta™Mouse assay up to the MTD of 350 mg/kg bw per day, hex-2(*trans*)-enal was not mutagenic in the tissues of the duodenum, presumably the first point of contact for the test material upon transit from the glandular stomach. These results are also supported by no indication of mutagenic activity in the liver, primary point of metabolism.

Hex-2(*trans*)-enal tested for chromosomal aberrations in mammalian cell lines showed positive results, but resulted negative in the *in vivo* micronucleus test performed in peripheral blood reticulocytes (Beevers, 2013) and in bone marrow (Honavar, 2007a).

In summary, the available data indicate that at high concentrations of hex-2(*trans*)-enal no gene mutations were induced in the liver and duodenum of transgenic mice after a daily treatment for 28 days up to 350 mg/kg bw per day (Beevers, 2013). DNA adducts were detected in the forestomach, liver, oesophagus and kidneys of rats treated with hex-2(*trans*)-enal by gavage at relatively high single doses, i.e. 200 and 500 mg/kg bw and at 50 mg/kg in the oesophagus. DNA adducts were not quantifiable in forestomach DNA of rats after exposure to 10 or 30 mg/kg bw for 1 or 4 weeks (Schuler and Eder, 1999). However, in the same experimental condition, DNA adducts were detected locally (duodenum and oesophagus) and systemically (kidney and liver) at doses lower than the dose that proved no induction of gene mutation. The Panel noted that overall the available experimental data from animals and humans, while not showing an induction of gene mutations, do not allow to assess the potential clastogenic activity of hex-2(*trans*)-enal at the first site of contact and in the liver, where high levels of DNA adducts were observed.

2.4.6. Conclusion

The new data submitted are related only to one representative substance of subgroup 1.1.1, hex-2 (*trans*)-enal [FL-no: 05.073].

For hex-2(*trans*)-enal [FL-no: 05.073], gene mutations were observed *in vitro* in *S*. Typhimurium TA100 and TA104, and chromosomal aberrations were observed *in vitro* likewise. In addition, a biomonitoring study in human buccal cells showed a statistically significant increase in the frequency of micronuclei at concentrations that might be relevant for the use of hex-2(*trans*)-enal as flavouring substances. The new submitted study performed on a Muta™Mouse model does not cover these endpoints adequately. The Panel noted that overall the available experimental data from animals and humans, while not showing an induction of gene mutations, do not allow to assess the potential clastogenic activity of hex-2(*trans*)-enal at the first site of contact and in the liver where higher levels of DNA adducts were observed than in other tissues investigated. Therefore, the new data provided by the Industry do not rule out the genotoxicity concern for the substances of subgroup 1.1.1.

For both 2-dodecenal and 2-nonenal tested through micronucleus assays in mouse bone marrow PCE (Honarvar, 2007b, 2008), there was no direct confirmation that the bone marrow was exposed, as no toxicokinetic measures of the test substance in plasma were made.

Under these conditions, the Panel confirms, the need for an *in vivo* Comet assay performed in duodenum and liver for hex-2(*trans*)-enal [FL-no: 05.073]. For the two other representative substances of subgroup 1.1.1 (nona-2(*trans*),6(*cis*)-dienal [FL-no: 05.058] and oct-2-enal [FL-no: 05.060]), a combined *in vivo* Comet assay and micronucleus assay would be required. For the latter, evidence of target tissue exposure should be provided.

3. Assessment

3.1. New data evaluated in FGE. 200 Revision 1

The applicant has submitted data from published studies and new *in vivo* genotoxicity studies for: hex-2(*trans*)-enal [FL-no: 05.073], *trans*-2-octenal [FL-no: 05.190], nona-2(*trans*)-6(*cis*)-dienal [FL-no: 05.058]. Industry submitted genotoxicity studies on *trans*-2-octenal [FL-no: 05.190], instead of oct-2-

enal [FL-no: 05.060] (configuration not specified); however, the Panel considered it acceptable because it is not expected that the different isomer will affect the outcome of the genotoxicity studies. The studies submitted are evaluated in the present revision of FGE.200 (FGE.200 Rev1). A summary of results is reported in Appendix E, Table E.1. All these studies were performed in accordance with respective OECD guidelines and in compliance with GLP.

Table 3: List of additional genotoxicity studies evaluated in FGE.200Rev1

Chemical name [FL-no]:	Test system *in vivo*	Reference
Hex-2(*trans*)-enal [FL-no: 05.073]	Two Comet assays in liver and duodenum	Keig-Shevlin (2017)
	Comet assay in liver with or without hOGG1	
	Micronucleus assay in bone marrow	
Trans-2-Octenal [FL-no: 05.190]	Micronucleus assay in bone marrow	Beevers (2015a)
	Comet assay in liver	
Nona-2(*trans*), 6(*cis*)-dienal [FL-no: 05.058]	Micronucleus assay in bone marrow	Beevers (2015b)
	Comet assay in liver	

3.2. Hex-2(*trans*)-enal [FL-no: 05.073] - *in vivo* combined Comet and Micronucleus assay

Hex-2(*trans*)-enal [FL-no: 05.073] was tested in a combined micronucleus assay in bone marrow and comet assay in duodenum and liver of Han Wistar rats by gavage (Keig-Shevlin, 2017). Hex-2 (*trans*)-enal [FL-no: 05.073] was stored at 15–25°C, under nitrogen and protected from light. Two different batches were tested, purity was 99.5% and 99.2%, respectively. The study was performed in accordance with GLP, OECD TG 474 (OECD, 2014a) and OECD TG 489 (OECD, 2016).

In a dose range-finding study, groups of three males and three females were given three administrations at 0 (day 1), 24 h (day 2) and 45 h (day 3) of hex-2(*trans*)-enal [FL-no: 05.073] at 500 and 350 mg/kg bw per day, via oral gavage. Clinical signs of toxicity (e.g. piloerection, decreased activity, ptosis and hunched posture) were observed at the highest dose tested, following the second and the third administration. Following the third administration, at 4 h post-dose, one female was found dead. In animals dosed at 350 mg/kg bw per day, signs of toxicity including piloerection and anogenital soiling were observed after the third administration.

Based on this dose range-finding experiment, where no differences in response between female and male rats were seen, a MTD of 350 mg/kg bw per day was established; the two lower doses tested were 175 and 87.5 mg/kg bw per day.

Hex-2(*trans*)-enal [FL-no: 05.073] was tested in three main experiments where the following assays were conducted: in the first experiment, micronucleus assay in bone marrow and comet assay in liver and duodenum; in the second experiment, comet assay in liver and duodenum; in the third experiment, comet assay in liver including a modified comet assay with human 8-hydroxyguanine DNA-glycosylase 1 (hOGG1) treatment.

In the main experiment, male rats (six animals per group) were dosed at 0 (day 1), 24 h (day 2) and 45 h (day 3) – by oral gavage at dose levels of 0 (corn oil), 87.5, 175 and 350 mg/kg bw per day. Animals were sampled at 48 h. Three male rats were given 150 mg/kg bw per day ethyl methanesulfonate (EMS) as a positive control. Corn oil was used as a vehicle following the same treatment schedule.

No clinical signs of toxicity where observed in the main experiments.

In experiments 1 and 2, dose-related decreases in bodyweight were observed at 350 mg/kg bw per day. No findings in clinical chemistry and histopathology were observed.

A total of at least 500 PCE and NCE was scored to calculate the degree of bone marrow toxicity by the relative decrease in PCE. Four thousand PCE per animal were scored for the presence of MN. Although a slight decrease in PCE was observed in the bone marrow of rats treated with hex-2(*trans*)-enal [FL-no: 05.073], this was not marked enough to indicate bone marrow toxicity. Group mean results of MN frequencies were similar to the concurrent vehicle control and no statistically significant increases in MN frequency were seen for any of the dose groups. The positive control group showed statistically significant increases in MN frequencies. Negative and positive control values were within the laboratory's historical control data.

Comet assay in liver – experiment 1

In the groups of animals administered with hex-2(*trans*)-enal, the mean tail intensity was higher than the vehicle control, but the increase was not statistically significant and it was not dose-related. All values for the mean tail intensity were within the range of the vehicle historical control (95% reference range 0.04–5.50). The authors of the study noted that in each group there were one or two animals displaying elevated %tail intensity and tail moment values which increased the group mean, therefore, the experiment was repeated.

Comet assay in liver – experiment 2

In the second experiment, it was observed that the group mean %tail intensity values for all groups treated with hex-2(*trans*)-enal exceeded the group mean vehicle control data. At 350 mg/kg bw per day, these increases were found to be statistically significant ($p \leq 0.05$) and there was also a statistically significant linear trend ($p \leq 0.01$). Individual animal %tail intensities in the 87.5 mg/kg bw per day dose group were in the range of 0.21–1.46, the 175 mg/kg bw per day dose group were in the range of 0.43–1.16 and the 350 mg/kg bw per day dose group were in the range of 0.25–2.68 compared to the vehicle control range of 0.08–1.03 (the 95% reference range of the historical control data was 0.04–5.50). At least three animals in each test article treated group were found to overlap with the concurrent vehicle control and all animals fell within the laboratory's historical control data.

In order to investigate the potential oxidative DNA damage of hex-2(*trans*)-enal, a modified Comet assay was conducted in a third experiment.

Comet assay in liver – experiment 3

The third comet assay was conducted with and without hOGG1. In the experiment without hOGG1, %tail intensities and tail moments were similar to the concurrent vehicle control group and fell within the laboratory's historical vehicle control data. There were no statistically significant increases in %tail intensity for any of the groups administered with hex-2(*trans*)-enal compared to the concurrent vehicle control.

In the experiment with hOGG1 modification, no statistically significant increase in mean tail intensity was observed in any groups treated with hex-2(*trans*)-enal compared to the concurrent vehicle control group confirming that hex-2(*trans*)-enal did not induce oxidative DNA damage.

The authors of the study considered that small increases in %tail intensity were observed in sporadic animals over two separate experiments in the liver, but only achieved statistical significance at one dose in one of these experiments. In the third experiment, no increases in %tail intensity were observed and hOGG1 modification did not reveal any evidence of oxidative DNA damage. The increases were considered by the authors as isolated not reproducible and therefore not biologically relevant. The Panel agrees with the conclusions by the authors.

Comet assay in duodenum – experiment 1

In the groups of animals administered with hex-2(*trans*)-enal, the mean %tail intensity values were lower than the vehicle control, but the decrease was not statistically significant and it was not clearly dose related. All individual animal data at all dose levels were generally consistent with the vehicle control animals and fell within the laboratory's historical control data. The only exceptions to this were one animal at 175 mg/kg bw per day and two animals at 350 mg/kg bw per day which fell below the laboratory's historical control 95% reference range. However, mean %tail intensity values for all test article treated groups were within the laboratory's historical vehicle ranges.

Comet assay in duodenum – experiment 2

In the groups of animals administered with hex-2(*trans*)-enal, group mean %tail intensity values were comparable with the group mean vehicle control data. There were no statistically significant differences in %tail intensity between treated groups and the vehicle control group. All individual animal data at all dose levels were generally consistent with the vehicle control animals and fell within the laboratory's historical control data. The only exceptions to this were three animals in the dose group of 87.5 mg/kg bw per day and one animal in the dose group of 350 mg/kg bw per day which fell below the laboratory's historical control 95% reference range, but one animal in the control group was also below. However, mean %tail intensity values for all test article treated groups were within the laboratory's historical vehicle ranges.

This result that did not confirm the decrease of tail intensity in animals treated with hex-2(*trans*)-enal observed in experiment 1, allowed to conclude that this compound did not induce DNA damage in the duodenum of treated male rats.

The Panel noted that the clinical signs of toxicity observed in the dose range-finding test (e.g. piloerection, hunched posture and decreased activity), can be considered as lines of evidence for a systemic exposure of rats after oral administration. Moreover, the Panel considered additional evidence of systemic exposure from toxicokinetic studies already evaluated in FGE.200 (see Sections 2.4.2–2.4.4). Therefore, the Panel concluded that hex-2(*trans*)-enal [FL-no: 05.073] induced no chromosomal damage in the rat bone marrow as demonstrated in the *in vivo* micronucleus assay. In the comet assay in the liver, the first experiment was negative and the second one equivocal. The third experiment was negative and no DNA strand breaks and no primary DNA damage due to oxidative damage were induced. The Panel concluded that hex-2(*trans*)-enal [FL-no: 05.073] induced no primary DNA damage neither in liver nor in duodenum of rats treated by oral route.

3.3. *trans*-2-Octenal [FL-no: 05.190] - *in vivo* Combined Comet and Micronucleus assay

trans-2-Octenal [FL-no: 05.190] (purity 97.8%) was tested in a combined micronucleus assay in bone marrow and comet assay in liver of Han Wistar rats by gavage (Beevers, 2015a). The study was performed in accordance with GLP, OECD TG 474 (OECD, 2014a) and OECD TG 489 (OECD, 2014b).

In a dose range-finding study, groups of three males and three females were given three administrations at 0 (day 1), 24 h (day 2) and 45 h (day 3) of *trans*-2-octenal, at 2,000 and 1,400 mg/kg bw per day, via oral gavage. Clinical signs of toxicity (e.g. piloerection, ptosis and hunched posture) were observed at the highest dose tested. On day 3, one female animal was found dead prior to dosing. All other animals showed piloerection and hunched posture. In animals dosed at 1,400 mg/kg bw per day, signs of toxicity including decreased activity, piloerection, hunched posture and ptosis were observed. Body weight loss was recorded in all animals.

Based on this dose range-finding experiment, where no differences in response between female and male rats were seen, a MTD of 1,000 mg/kg bw per day was established; the two lower doses tested were 500 and 250 mg/kg bw per day.

In the main experiment, male rats (six animals per group) were dosed at 0 (day 1), 24 h (day 2) and 45 h (day 3) by oral gavage at dose levels of 0 (corn oil), 250, 500 and 1,000 mg/kg bw per day. Animals were sampled at 48 h. Three male rats were given 150 mg/kg bw per day EMS, as a positive control. Corn oil was used as a vehicle following the same treatment schedule.

Test animals were examined daily for signs of toxicity. No clinical signs of toxicity were seen at any of the test conditions applied in the main experiment. Dose-related decreases in body weight gain were observed, resulting in body weight loss at 1,000 mg/kg bw per day. At this dose, it was observed an increase in total protein due to an increase in globulins, resulting in a small decrease in the albumin:globulin ratio. It was observed a small decrease in sodium concentration and an increase in phosphate and creatinine. An increase in urea was observed in animals administered with the two higher doses. Glucose was increased in animals given 1,000 mg/kg bw per day, and to a lesser extent in animals given 500 mg/kg bw per day.

In the liver, it was observed a decrease in glycogen vacuolation in animals dosed with 500 and 1,000 mg/kg bw per day.

A total of at least 500 PCE and NCE were scored to calculate the degree of bone marrow toxicity by the relative decrease in PCE. Four thousand PCE per animal were scored for the presence of MN.

No decrease in PCE was observed in the bone marrow of rats treated with *trans*-2-octenal [FL-no: 05.190], indicating no evidence of bone marrow toxicity.

Group mean results of MN frequencies were similar to the concurrent vehicle control and no statistically significant increases in MN frequency were seen for any of the dose groups. The positive control group showed statistically significant increases in MN frequencies.

Negative and positive control values were within the laboratory's historical control data.

The comet assay in liver did not show statistically significant differences in tail intensity and tail moment between the treated and control groups. All individual animal data at all dose levels were consistent with the vehicle control animals and fell within the laboratory's historical control data.

The authors of the study concluded that *trans*-2-octenal [FL-no: 05.190] did not induce micronuclei in bone marrow and it did not induce DNA damage in the liver of rats.

The Panel noted that the clinical signs of toxicity observed in the dose range-finding test and modification of clinical biochemistry and histopathological parameters observed in the main experiment in rats, can be considered as lines of evidence of systemic bioavailability of *trans*-2-octenal [FL-no: 05.190] after oral administration. The Panel concluded that *trans*-2-octenal [FL-no: 05.190] induced no primary DNA damage in the liver of rats when administered up to 1,000 mg/kg bw per day, by oral route, as demonstrated in the comet assay and that it induced no chromosomal damage in the rat bone marrow as demonstrated by the *in vivo* micronucleus assay.

3.4. Nona-2(*trans*),6(*cis*)-dienal [FL-no: 05.058] - *in vivo* Combined Comet and Micronucleus assay

Nona-2(*trans*),6(*cis*)-dienal [FL-no: 05.058] (purity 97.92%) was tested in a combined micronucleus assay in bone marrow and comet assay in liver of Han Wistar rats by gavage (Beevers, 2015b). The study was performed in accordance with GLP, OECD TG 474 (OECD, 2014a) and OECD TG 489 (OECD, 2014b).

Based on an oral gavage dose range-finding experiment with doses up to 2,000 mg/kg bw per day, where no differences in response between female and male rats were seen, a MTD of 700 mg/kg bw per day was established; the two lower doses tested were 350 and 150 mg/kg bw per day. In this dose range-finding experiment, clinical signs of toxicity (e.g. decreased activity, piloerection and hunched posture) were observed at the highest dose tested. Approximately 2 h after the second dose administrations all animals were found dead or humanely killed due to the severity of their observations. Also in animals, dosed at 1,000 mg/kg bw per day signs of toxicity including decreased activity, piloerection, hunched posture, arched gait and diarrhoea were observed. Although no mortality was observed, the animal conditions deteriorated after each administration. After the first administration at 700 mg/kg bw per day, male animals showed similar signs of toxicity. After the second and third administration, clinical signs of toxicity in both males and females were limited to soiling and soft faeces; no mortality occurred.

In the main experiment, male rats (six animals per group) were dosed at 0 (day 1), 24 h (day 2) and 45 h (day 3) – by oral gavage at dose levels of 0 (corn oil), 175, 350 and 700 mg/kg bw per day. Animals were sampled at 48 h. Three male rats were given 150 mg/kg bw per day of EMS, as a positive control. Corn oil was used as a vehicle following the same treatment schedule.

Test animals were examined daily for signs of toxicity. No clinical signs of toxicity were seen at any of the test conditions applied in the main experiment. At 700 mg/kg bw per day, only soft/loose brown faces were observed. Dose-related decreases in body weight gain were seen. The authors noted small decrease in calcium and increase in phosphate in animals dosed at 700 mg/kg bw per day. Small increase in urea was observed in animals dosed at the two higher doses. There was an increase in glucose concentration in one animal given 700 mg/kg per day. Lipaemia was observed in several serum samples. In the liver, a dose-related decrease in glycogen vacuolation was observed in all animals dosed with nona-2(*trans*),6(*cis*)-dienal.

A total of at least 500 PCE and NCE were scored to calculate the degree of bone marrow toxicity by the relative decrease in PCE. Four thousand PCE per animal were scored for the presence of MN.

No decrease in PCE was observed in the bone marrow of rats treated with nona-2(*trans*),6(*cis*)-dienal, indicating no evidence of bone marrow toxicity.

Group mean results of MN frequencies were similar to the concurrent vehicle control and no statistically significant increases in MN frequency were seen for any of the dose groups. There was no significant linear trend. The positive control group showed statistically significant increases in MN frequencies.

Negative and positive control values were within the laboratory's historical control data. Data are summarised in Appendix E, Table E.1.

The authors of the study noted that two animals treated at 350 and 700 mg/kg bw per day, respectively, had individual hedgehog values that exceeded weakly the 95% reference range of the historical control data. These were considered to be chance events that did not affect data interpretation.

The comet assay in liver did not show statistically significant differences in tail intensity between treated and control groups. All individual animal data at all dose levels were consistent with the vehicle control animals and fell within the laboratory's historical control data.

The authors of the study concluded that nona-2(*trans*),6(*cis*)-dienal [FL-no: 05.058] did not induce micronuclei in bone marrow and it did not induce DNA damage in the liver of rats.

The Panel noted that the clinical signs of toxicity observed in the dose range-finding test and the modification of clinical biochemistry and histopathological parameters observed in the main experiment in rats, can be considered as lines of evidence of systemic bioavailability of nona-2(*trans*),6(*cis*)-dienal after oral administration. The Panel concluded that nona-2(*trans*),6(*cis*)-dienal induced no primary DNA damage in the liver of rats when administered up to 700 mg/kg per day by oral route as demonstrated in the comet assay and that it induced no chromosomal damage in the rat bone marrow as demonstrated by the *in vivo* micronucleus assay.

3.5. Additional studies from literature

2-Hexenal was tested in an Ames test with pre-incubation and in the absence of metabolic activation. To decrease the toxicity of long-chain enals, GSH was added following the preincubation period and before plating (Grúz et al., 2017). A parallel experiment with *S*. Typhimurium strains TA100, TA104, TA2637, TA97, TA98 deficient of DNA polymerase RI (DNA Pol RI) was carried out in order to investigate its role in the potential mutagenic activity. 2-Hexenal induced base substitutions in the *S*. Typhimurium strains TA100 and TA104 in a concentration dependent manner. This mutagenic activity was dependent by the DNA Pol RI activity, because it was not observed in strains deficient of DNA Pol RI. The authors considered that probably the DNA Pol RI activity is needed to replicate DNA despite the possible presence of DNA adducts. No mutagenic activity was demonstrated in all the other tested strains under the same testing conditions.

Induction of oxidative DNA damage was investigated in mammalian cells (V79 and Caco-2), as a consequence of GSH depletion induced by 2-alkenals, including *E*-(2)-hexenal (Janzowski et al., 2003). Oxidative DNA breakage was studied through *in vitro* Comet assay, using treatment with formamidopyrimidine DNA glycosylase (FPG). After 1 h, incubation of V79 cells with *E*-(2)-hexenal (100 μM), the total cellular glutathione (tGSH) was depleted (< 20% compared to control; viability > 85%). During 3-h (post-treatment) of cell incubation without *E*-(2)-hexenal, a significant increase in tGSH was observed only in the cells treated with the lowest concentration tested (30 μM). After 1h of incubation, DNA damage was observed only at the highest concentration tested (100 μM); FPG-sensitive sites were not observed. During 3-h (without test compound), direct DNA damage was reduced and FPG-sensitives sites were detectable at 100 μM. Direct DNA breakage was markedly diminished, most probably by repair processes, and tGSH concentrations were observed to increase again within 3-h post-treatment. In Caco-2 cells, *E*-(2)-hexenal induced less direct DNA damage compared with V79, but after 3-h post-treatment, direct DNA damage in Caco-2 cells had increased compared to V79 cells. These results suggest that alkenal-mediated oxidative stress can contribute to cytotoxic/genotoxic cell damage.

(*E*)-2-Hexenal, (*E*)-2-octenal, (*E*)-2-nonenal and (2*E*,6*Z*)-2,6-nonadienal were tested in mammalian cell lines (V79 and Caco-2) and in primary human colon cells (Glaab et al., 2001). Inhibition of cell growth (IC_{50}) and cytotoxicity (LC_{50}) were measured in both V79 and Caco-2 cells showing similar results. Cytotoxicity was observed after 1 h incubation in V79 cells with LC_{50} of 3,666, 545, 271 and 270 μM for (*E*)-2-hexenal, (*E*)-2-octenal (*E*)-2-nonenal and (2*E*,6*Z*)-2,6-nonadienal, respectively. If the incubation time was prolonged up to 24 h, an IC_{50} of 17, 6, 9 and 5 μM was obtained for (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-2-nonenal and (2*E*,6*Z*)-2,6-nonadienal, respectively. DNA damage (both strand breaks and oxidised purines) was investigated through the *in vitro* comet assay.

With (*E*)-2-hexenal and (*E*)-2-octenal, concentration-dependent DNA damage was induced in V79 cells and in Caco-2 cells. In both cell lines, (*E*)-2-nonenal did not induce DNA damage up to the cytotoxic concentration limit (viability 70%), while (2*E*,6*Z*)-2,6-nonadienal was the most genotoxic. This substance tested in primary human colon cells showed a potency at least twofold lower compared to V79 and Caco-2 cells.

When tested in a modified comet assay, no induction of FPG sensitive sites in V79 cells were identified at concentrations that depleted up to 25% GSH. In primary human colon cells, (*E*)-2-hexenal did not induce direct DNA damage, neither FPG sensitive sites.

In V79 cells, all the substances induced depletion of total GSH down to about 20% of control after 1 h incubation at concentrations up to 50 or 100 μM. In Caco-2 cells and in primary human colon cells, about fivefold higher concentrations were needed to observe similar GSH depletion.

(*E*)-2-Hexenal, additionally tested in primary human colon cells, depleted GSH and increased the sensitivity towards oxidative stress.

The authors of this study concluded that an elongation of alkyl chain length of 2-alkenals results in an increase of cytotoxicity which might be related to lipophilicity. This correlation was less evident for the DNA damage. No DNA oxidative damage was noted in this study despite the decrease of cell glutathione.

3.6. Discussion

In FGE.200 (EFSA CEF Panel, 2014a), the Panel concluded that the gene mutation induced *in vitro* by hex-2(*trans*)-enal [FL-no: 05.073] was not expressed *in vivo*. However, the available data did not allow to assess the potential clastogenic activity.

Studies from literature suggest that the toxicity and mutagenicity of 2-alkenals are related to the chain length (Canonero et al., 1990; Eder et al., 1992; Glaab et al., 2001). The mechanism of genotoxicity seems to be related to the formation of DNA adducts (Schuler and Eder, 1999; Schuler et al., 1999; Stout et al., 2008) and probably to base substitution (Grúz et al., 2017). Genotoxic activity has been observed especially in cells that have low detoxification capacity showing also a decrease in GSH (Eisenbrand et al., 1995; Glaab et al., 2001; Janzowski et al., 2003). Some studies suggest that the genotoxic effects are due to DNA oxidative damage, but results are inconsistent (Glaab et al., 2001; Janzowski et al., 2003).

In the study by Dittberner et al. (1995), it is suggested an aneugenic mechanism for both hex-2(*trans*)-enal and nona-2(*trans*),6(*cis*)-dienal. However, the Panel noted that MN are observed in a range of concentrations too wide for an aneugenic substance; in addition, the lowest concentration at which a statistically significant increase in MN is observed (50 μM for hexenal and 20 μM for nonadienal) is not the same at which the increase of aneuploidy is observed (40 μM for both hexenal and nonadienal). Therefore, the Panel concluded that the mechanism of genotoxicity was not adequately investigated in this study and that the available data suggest more a clastogenic than an aneugenic activity.

Based on the new available results from a combined *in vivo* micronucleus assay in bone marrow and comet assay in duodenum and liver of Han Wistar rats, the Panel considered that for hex-2(*trans*)-enal [FL-no: 05.073] no chromosomal damage was observed in the micronucleus assay in the bone marrow and no primary DNA damage in the comet assay in duodenum. In addition, no direct DNA strand breaks and no primary DNA damage due to oxidative damage were observed in the comet assay performed in liver of rats administered via oral gavage. Proof of systemic exposure was provided.

For *trans*-2-octenal [FL-no: 05.190], no genotoxic effect was observed in the micronucleus assay in bone marrow and no DNA damage was noted in the comet assay in liver. The changes of clinical biochemistry and histopathological parameters can be considered as lines of evidence of systemic exposure of rats after oral administration.

For nona-2(*trans*),6(*cis*)-dienal [FL-no: 05.058], no genotoxic effect was observed in the micronucleus assay in bone marrow and no DNA damage was noted in the comet assay in liver. The changes of clinical biochemistry and histopathological parameters can be considered as lines of evidence of systemic exposure of rats after oral administration.

The extrapolation from 3 representative substances to 71 candidate substances is intrinsically associated with the introduction of uncertainty. In this opinion, only one toxicological endpoint is addressed (genotoxicity) related to one well defined structural alert, therefore the uncertainty is considered to be limited. Other toxicological endpoints will be addressed in separate opinions.

3.7. Conclusions

Based on these data, the Panel concluded that the concern for genotoxicity can be ruled out for the representative substances hex-2(*trans*)-enal [FL-no: 05.073], *trans*-2-octenal [FL-no: 05.190], nona-2(*trans*),6(*cis*)-dienal [FL-no: 05.058], therefore all the 74 substances [FL-no: 02.020, 02.049, 02.050, 02.090, 02.112, 02.137, 02.156, 02.192, 02.210, 02.231, 05.037, 05.058, 05.060, 05.070, 05.072, 05.073, 05.076, 05.078, 05.102, 05.109, 05.111, 05.114, 05.120, 05.144, 05.150, 05.171, 05.172, 05.179, 05.184, 05.189, 05.190, 05.191, 05.195, 06.025, 06.031, 06.072, 09.054, 09.097, 09.109, 09.119, 09.146, 09.233, 09.244, 09.247, 09.276, 09.277, 09.303, 09.312, 09.385, 09.394, 09.395, 09.396, 09.397, 09.398, 09.399, 09.400, 09.410, 09.411, 09.469, 09.482, 09.489, 09.492, 09.493, 09.498, 09.678, 09.701, 09.719, 09.741, 09.790, 09.841, 09.866, 09.947, 09.948, 13.004] can be evaluated through the Procedure for flavouring substances.

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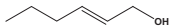
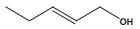
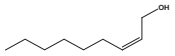
Abbreviations

ABS	chromosomal aberrations
ALDH	aldehyde dehydrogenase
AR	aldose reductase
BrdU	bromodeoxyuridine
bw	body weight
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CHL	Chinese hamster lung (cells)
CHO	Chinese hamster ovary (cells)
CoE	Council of Europe
DNA	Pol RI DNA polymerase RI
EFFA	European Flavour Association
EGF	epidermal growth factor
EMS	ethyl methanesulfonate
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	(FL) Flavour Information System (database)
FPG	formamidopyrimidine DNA glycosylase
GLP	Good Laboratory Practice
GSH	glutathione
GST	glutathione *S*-transferase
Hex-PdG	1,N^2-propanodeoxyguanosine
HPLC	high-performance liquid chromatography
HPRT	hypoxanthine guanine ribosyl transferase
hOGG1	human 8-hydroxyguanine DNA-glycosylase 1
ID	Identity
IOFI	International Organization of the Flavor Industry
IR	infrared spectroscopy
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC–MS/MS	liquid chromatography with tandem mass spectrometry
MF	mutation frequency
MN	micronuclei
MS	mass spectra
MTD	maximum tolerated dose
MSDI	maximised survey-derived daily intake
NAD^+	(oxidised) β-nicotinamide adenine dinucleotide phosphate
NCE	normochromatic erythrocytes
NMR	nuclear magnetic resonance
NOAEL	no-observed-adverse-effect-level
No	Number
OECD	Organisation for Economic Co-operation and Development
PBK/D	physiologically based kinetic/dynamic
PCE	polychromatic erythrocytes
PFU	plaque-forming unit
(Q)SAR	(Quantitative) Structure Activity Relationship
RET	reticulocytes
SCE	sister chromatid exchange
SOD	superoxide dismutase
TG	6-thioguanine
tGSH	total cellular glutathione
TLC	thin-layer chromatography
UDS	unscheduled DNA synthesis
WHO	World Health Organization

Appendix A – Specification summary of the substances in the Flavouring Group Evaluation 200Rev1

Table A.1: Specification Summary of the Substances in the Present Group Evaluation

FL-no JECFA-no	EU register name	Structural formula	FEMA no CoE no CAS no	Phys. form Mol. formula Mol. weight	Solubility[a] Solubility in ethanol[b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index[d] Spec. gravity[e]
02.020 1354	Hex-2-en-1-ol		2562 69 2305-21-7	Liquid $C_6H_{12}O$ 100.16	Very slightly soluble Soluble	158–160 IR 95%	1.437–1.442 0.836–0.841
02.049 1184	Nona-2,6-dien-1-ol		2780 589 7786-44-9	Liquid $C_9H_{16}O$ 140.23	Insoluble Soluble	196 IR NMR MS 95%	1.463–1.465 0.860–0.880
02.050 1793	Pent-2-en-1-ol		665 20273-24-9	Liquid $C_5H_{10}O$ 86.13	Freely soluble	141 MS 95%	1.427–1.433 0.844–0.850
02.090 1365	Non-2(*trans*)-en-1-ol		3379 10292 31502-14-4	Liquid $C_9H_{18}O$ 142.23	Insoluble Soluble	105 (16 hPa) IR 95%	1.444–1.448 0.835–0.845
02.112 1369	Non-2(*cis*)-en-1-ol		3720 10292 41453-56-9	Liquid $C_9H_{18}O$ 142.23	Slightly soluble Soluble	96 (13 hPa) NMR 96%	1.447–1.453 0.841–0.847
02.137 1794	Dec-2-en-1-ol		11750 22104-80-9	Liquid $C_{10}H_{20}O$ 156.27	Freely soluble	117 (19 hPa) MS 95%	1.446–1.452 0.842–0.848
02.156 1374	Hex-2(*cis*)-en-1-ol		3924 69 928-94-9	Liquid $C_6H_{12}O$ 100.16	Insoluble Soluble	65 (0.7 hPa) NMR 92%	1.437–1.445 0.845–0.853

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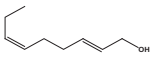
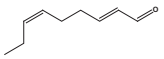
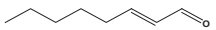
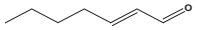
02.192	Oct-2-en-1-ol		3887 11804 22104-78-5	Liquid $C_8H_{16}O$ 128	Insoluble Soluble	88 (hPa) MS 96%	1.4371–1.4571 0.8384–0.8584
02.210 1384	Undec-2-en-1-ol		4068 37617-03-1	Liquid $C_{11}H_{22}O$ 170.30	Insoluble Soluble	100–102 (3 hPa) IR 95%	1.447–1.453 0.838–0.848
02.231	*trans*-2, *cis*-6-Nonadien-1-ol		2780 589 28069-72-9	Liquid $C_9H_{16}O$ 140.23	Insoluble Soluble	196 MS 95%	1.463–1.465 0.860–0.880
05.037 1350	2-Dodecenal		2402 124 4826-62-4	Liquid $C_{12}H_{22}O$ 182.31	Practically insoluble or insoluble Freely soluble	272 IR 93%	1.452–1.458 0.839–0.849
05.058 1186	Nona-2(*trans*),6(*cis*)-dienal		3377 659 557-48-2	Liquid $C_9H_{14}O$ 138.21	Insoluble Soluble	94 IR 92%	1.470–1.475 0.850–0.870
05.060 1363	Oct-2-enal		3215 663 2363-89-5	Liquid $C_8H_{14}O$ 126.20	Slightly soluble Soluble	84–86 (25 hPa) IR 92%	1.449–1.455 0.835–0.845
05.070 1360	2-Heptenal		3165 730 2463-63-0	Liquid $C_7H_{12}O$ 112.17	Practically insoluble or insoluble Freely soluble	166 IR MS 97%	1.428–1.434 0857–0.863
05.072 1362	*trans*-2-Nonenal		3213 733 18829-56-6	Liquid $C_9H_{16}O$ 140.22	Practically insoluble or insoluble Freely soluble	90 (1,2T) 1.333 IR MS 92%	1.454–1.460 0.855–0.865

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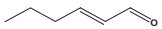
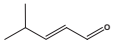
05.073 1353	Hex-2(*trans*)-enal		2560 748 6728-26-3	Liquid $C_6H_{10}O$ 98.14	Very slightly soluble Freely soluble	47 (1.7T) 2.266 NMR MS 92%	1.443–1.449 0.841–0.848
05.076 1349	Dec-2-enal		2366 2009 3913-71-1	Liquid $C_{10}H_{18}O$ 154.25	Insoluble Soluble	229 IR 92%	1.452–1.458 0.836–0.846
05.078 1359	Tridec-2-enal		3082 2011 7774-82-5	Liquid $C_{13}H_{24}O$ 196.33	Insoluble Soluble	115–118 (13 hPa) IR 92%	1.455–1.461 0.842–0.862
05.102 1364	Pent-2-enal		3218 10375 764-39-6	Liquid C_5H_8O 84.11	Insoluble Soluble	124 NMR 98%	1.440–1.447 (21°) 0.850–0.856 (21°)
05.109 1366	2-Undecenal		3423 11827 2463-77-6	Liquid $C_{11}H_{20}O$ 168.27	Insoluble Soluble	115 (13 hPa) NMR 98%	1.452–1.459 0.837–0.847
05.111 1182	Octa-2(*trans*),6(*trans*)-dienal		3466 10371 56767-18-1	Liquid $C_8H_{12}O$ 124.19	Insoluble Soluble	97–99 (5 hPa) IR NMR 96%	1.469–1.475 0.835–0.841
05.114 1208	4-Methylpent-2-enal		3510 10364 5362-56-1	Liquid $C_6H_{10}O$ 98.14	Slightly soluble Soluble	126–130 IR NMR 97%	1.435–1.445 0.858–0.866
05.120 1197	Dodeca-2,6-dienal		3637 21662-13-5	Liquid $C_{12}H_{20}O$ 180.28	Insoluble Soluble	130 (7 hPa) NMR 97.5%	1.425–1.431 0.987–0.993

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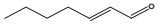
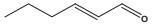
05.144	Dodec-2(*trans*)-enal		2402 20407-84-5	Liquid $C_{12}H_{22}O$ 182.31			
05.150 1360	Hept-2(*trans*)-enal		3165 730 18829-55-5	Liquid $C_7H_{12}O$ 112.17	Insoluble Soluble	165–167 IR 97%	1.428–1.434 0.857–0.863
05.171 1362	Non-2-enal		3213 733 2463-53-8	Liquid $C_9H_{16}O$ 140.22	Insoluble Soluble	88–90 (16 hPa) IR 92%	1.454–1.460 0.855–0.865
05.172 1187	Nona-2(*trans*),6(*trans*)-dienal		3766 17587-33-6	Liquid $C_9H_{14}O$ 138.21	Insoluble Soluble	88 (14 hPa) NMR 97%	1.439–1.445 0.856–0.864
05.179 1803	Tetradec-2-enal		4209 51534-36-2	Solid $C_{14}H_{26}O$ 210.36	Freely soluble	88 (0.3 hPa) 35 MS 95%	1.455–1.562 n.a.
05.184	Undec-2(*trans*)-enal		3423 11827 53448-07-0	Liquid $C_{11}H_{20}O$ 168.27	Insoluble Soluble	115 (1.3 hPa) MS 98%	1.452–1.459 0.837–0.847
05.189	2-Hexenal		 748 505-57-7	Liquid $C_6H_{10}O$ 98.14			
05.190	*trans*-2-Octenal		3215 2548-87-0	Liquid $C_8H_{14}O$ 126.2	Soluble Soluble	96 (2.5 hPa) MS 92%	1.449–1.455 0.835–0.845
05.191	*trans*-2-Decenal		2366 3913-81-3	Liquid $C_{10}H_{18}O$ 154.25			

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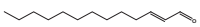
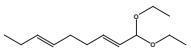
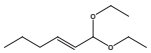
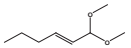
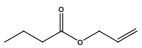
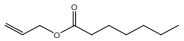
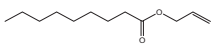
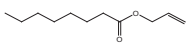
05.195	*trans*-2-Tridecenal		3082 7069-41-2	Liquid $C_{13}H_{24}O$ 196.33	Insoluble Soluble	117 (1.3 hPa) MS 92%	1.455–1.462 0.842–0.862
06.025 946	1,1-Diethoxynona-2,6-diene		3378 660 67674-36-6	Liquid $C_{13}H_{24}O_2$ 212.33	Insoluble Miscible	125 (5 hPa) IR 90%	1.441–1.448 0.860–0.868
06.031 1383	1,1-Diethoxyhex-2-ene		4047 2135 54306-00-2	Liquid $C_{10}H_{20}O_2$ 172.27	Practically insoluble or insoluble Freely soluble	66 (8T) 10.6657 MS 95%	1.418–1.426 0.843–0.849
06.072 1728	1,1-Dimethoxyhex-2 (*trans*)-ene		 18318-83-7	Liquid $C_8H_{16}O_2$ 144.21	Freely soluble	158 NMR 95%	1.420–1.424 0.867–0.871
09.054 2	Allyl butyrate		2021 280 2051-78-7	Liquid $C_7H_{12}O_2$ 128.17	Insoluble Soluble	44–45 (20 hPa) IR 98%	1.412–1.418 0.897–0.902
09.097 4	Allyl heptanoate		2031 369 142-19-8	Liquid $C_{10}H_{18}O_2$ 170.25	Freely soluble	210 IR 97%	1.426–1.430 0.880–0.885
09.109 6	Allyl nonanoate		2036 390 7493-72-3	Liquid $C_{12}H_{22}O_2$ 198.31	Insoluble Soluble	241–242 IR 96.5%	1.430–1.436 0.872–0.880
09.119 5	Allyl octanoate		2037 400 4230-97-1	Liquid $C_{11}H_{20}O_2$ 184.28	Insoluble Soluble	222 IR 97%	1.432–1.434 0.872–0.880

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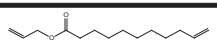
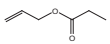
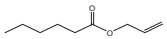
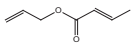
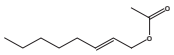
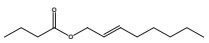
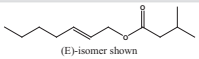
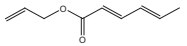
09.146 9	Allyl undec-10-enoate		2044 441 7493-76-7	Liquid $C_{14}H_{24}O_2$ 224.34	Insoluble Soluble	180 (39 hPa) IR 98%	1.448 at 30° 0.8802 at 30°
09.233 1	Allyl propionate		2040 2094 2408-20-0	Liquid $C_6H_{12}O_2$ 114.15		122–123 IR 99%	1.4105 0.914 at 20°
09.244 3	Allyl hexanoate		2032 2181 123-68-2	Liquid $C_9H_{16}O_2$ 156.22	Insoluble 1 mL in 6 mL 70% ethanol	185 IR 98%	1.422–1.426 0.884–0.890
09.247	Allyl crotonate		4072 2222 20474-93-5	Liquid $C_7H_{10}O_2$ 126.15	Freely soluble	146 MS 95%	0.932–0.937
09.276 1367	Oct-2-enyl acetate		3516 11906 3913-80-2	 $C_{10}H_{18}O_2$ 170.25			
09.277 1368	Oct-2(*trans*)-enyl butyrate		3517 11907 84642-60-4	Liquid $C_{12}H_{22}O_2$ 198.30	Insoluble Soluble	112–113 (10 hPa) IR NMR MS 96%	1.433–1.439 0.890–0.896
09.303 1799	Hept-2-enyl isovalerate	 (E)-isomer shown	4126 10664 253596-70-2	Liquid $C_{12}H_{22}O_2$ 198.30	Freely soluble	263 NMR 95%	0.868–0.873
09.312 8	Allyl hexa-2,4-dienoate		2041 2182 7493-75-6	Liquid $C_9H_{12}O_2$ 152.19	Soluble	67 IR 99%	1.506 0.945–0.947

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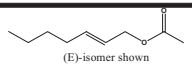
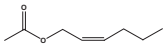
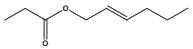
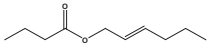
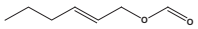
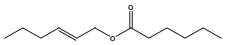
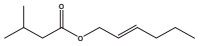
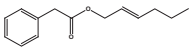
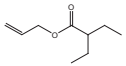
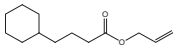
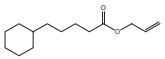
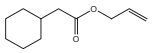
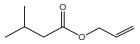
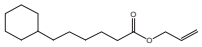
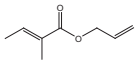
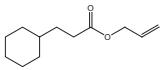
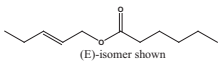
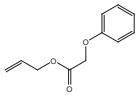
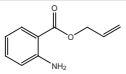
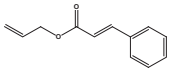
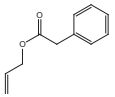
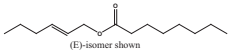
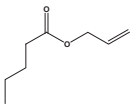
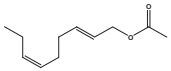
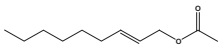
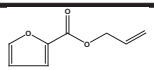
09.385 1798	Hept-2-enyl acetate	 (E)-isomer shown	4125 10661 16939-73-4	Liquid $C_9H_{16}O_2$ 156.22	Freely soluble	193 MS 95%	1.428–1.434 0.889–0.895
09.394 1355	Hex-2(*trans*)-enyl acetate		2564 643 2497-18-9	Liquid $C_8H_{14}O_2$ 142.20	Very slightly soluble Soluble	165–166 IR 90%	1.424–1.430 0.890–0.897
09.395 1378	Hex-2(*trans*)-enyl propionate		3932 11830 53398-80-4	Liquid $C_9H_{16}O_2$ 156.23	Insoluble Soluble	91 (26 hPa) NMR 95%	1.426–1.433 0.885–0.895
09.396 1375	Hex-2-enyl butyrate		3926 53398-83-7	$C_{10}H_{18}O_2$ 170.25			
09.397 1376	Hex-2-enyl formate		3927 11858 53398-78-0	$C_7H_{12}O_2$ 128.17			
09.398 1381	Hex-(2*E*)-enyl hexanoate		3983 53398-86-0	$C_{12}H_{22}O_2$ 198.31			
09.399 1377	(2*E*)-Hexenyl isovalerate		3930 68698-59-9	Liquid $C_{11}H_{20}O_2$ 184.28	Insoluble Soluble	105 (26 hPa) NMR 96%	1.425–1.435 0.875–0.885
09.400	Hex-2-enyl phenylacetate		 68133-78-8	Solid $C_{14}H_{18}O_2$ 218.29	Practically insoluble or insoluble Freely soluble	336 37 NMR 95%	n.a. n.a.
09.410 11	Allyl 2-ethylbutyrate		2029 281 7493-69-8	Liquid $C_9H_{16}O_2$ 156.23	Insoluble Soluble	165–167 IR 99%	1.422–1.427 0.882–0.887

FL-no JECFA-no	EU register name	Structural formula	FEMA no CoE no CAS no	Phys. form Mol. formula Mol. weight	Solubility[a] Solubility in ethanol[b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index[d] Spec. gravity[e]
09.411 14	Allyl cyclohexanebutyrate		2024 283 7493-65-4	Liquid $C_{13}H_{22}O_2$ 210.31	Insoluble Soluble	104 (1 hPa) NMR 98%	1.4608 at 20.5° 0.943–0.949
09.469 15	Allyl cyclohexanevalerate		2027 474 7493-68-7	Liquid $C_{14}H_{24}O_2$ 224.34	Insoluble Soluble	119 (1 hPa) IR 98%	1.4605 at 22° 0.942–0.947
09.482 12	Allyl cyclohexaneacetate		2023 2070 4728-82-9	Liquid $C_{11}H_{18}O_2$ 182.26	Soluble	60 (1 hPa) NMR 96%	1.455–1.499 0.945–0.965
09.489 7	Allyl isovalerate		2045 2098 2835-39-4	Liquid $C_8H_{14}O_2$ 142.20	Insoluble Freely soluble	155 IR 98%	1.413–1.418 0.879–0.884
09.492 16	Allyl cyclohexanehexanoate		2025 2180 7493-66-5	Liquid $C_{14}H_{28}O_2$ 238.37	Insoluble Soluble	128 (2 hPa) NMR 98%	1.462 0.941–0.947
09.493 10	Allyl 2-methylcrotonate		2043 2183 7493-71-2	Liquid $C_8H_{12}O_2$ 140.18	Slightly soluble	153 IR 98%	1.451–1.454 0.939–0.943
09.498 13	Allyl cyclohexanepropionate		2026 2223 2705-87-5	Liquid $C_{12}H_{20}O_2$ 196.29	Insoluble 1 mL in 4 mL 80% ethanol	91 (1 hPa) IR 98%	1.457–1.462 0.945–0.950
09.678 1795	Pent-2-enyl hexanoate		4191 74298-89-8	Liquid $C_{11}H_{20}O_2$ 184.28	Freely soluble	241 MS 95%	1.425–1.435 0.885–0.895

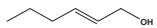
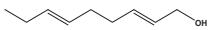
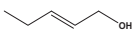
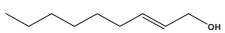
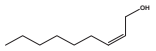
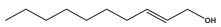
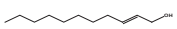
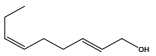
FL-no JECFA-no	EU register name	Structural formula	FEMA no CoE no CAS no	Phys. form Mol. formula Mol. weight	Solubility[a] Solubility in ethanol[b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index[d] Spec. gravity[e]
09.701 18	Allyl phenoxyacetate		2038 228 7493-74-5	Liquid $C_{11}H_{12}O_3$ 192.22		100–102 (1 hPa) IR 97.5%	1.512–1.519 1.00–1.11
09.719 20	Allyl anthranilate		2020 254 7493-63-2	Liquid $C_{10}H_{11}O_2N$ 177.21	Almost insoluble	105 (3 hPa) IR 98%	1.569–1.577 1.12
09.741 19	Allyl cinnamate		2022 334 1866-31-5	Liquid $C_{12}H_{12}O_2$ 188.22	Insoluble Miscible	286 IR 97%	1.562–1.569 1.050–1.056
09.790 17	Allyl phenylacetate		2039 2162 1797-74-6	Liquid $C_{11}H_{12}O_2$ 176.22		89–93 (4 hPa) IR 99%	1.5122 at 13.5° 1.033–1.041
09.841 1796	2-Hexenyl octanoate		4135 85554-72-9	Liquid $C_{14}H_{26}O_2$ 226.36	Freely soluble	309 MS 95%	
09.866	Allyl valerate		4074 6321-45-5	Liquid $C_8H_{14}O_2$ 142.20	Freely soluble	58 (16 hPa) MS 95%	0.999–1.005
09.947 1188	(*E*,*Z*)-2,6-Nonadienyl acetate		3952 68555-65-7	Liquid $C_{11}H_{18}O_2$ 182.26	Sparingly soluble Soluble	231 IR NMR MS 95%	1.448–1.458 0.905–0.907
09.948	(2*E*)-2-Nonenyl acetate		4552 30418-89-4	Liquid $C_{11}H_{20}O_2$ 184.79	Sparingly soluble Very soluble	228 IR NMR MS 98%	1.4325–1.4425 0.874–0.894

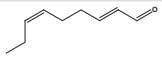
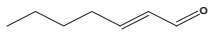
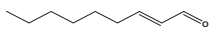
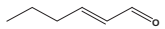
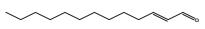
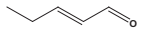
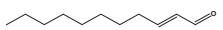
FL-no JECFA-no	EU register name	Structural formula	FEMA no CoE no CAS no	Phys. form Mol. formula Mol. weight	Solubility[a] Solubility in ethanol[b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index[d] Spec. gravity[e]
13.004 21	Allyl 2-furoate		2030 360 4208-49-5	Liquid $C_8H_8O_3$ 152.15		206–209 IR 98%	1.4945 1.181 (23°)

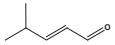
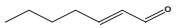
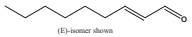
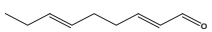
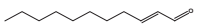
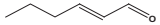
FL-no: FLAVIS number; JECFA: Joint FAO/WHO Expert Committee on Food Additives; FEMA: Flavor and Extract Manufacturers Association; CoE: Council of Europe; CAS: Chemical Abstract Service; ID: Identity; IR: infrared; NMR: nuclear magnetic resonance; MS: mass spectra.
(a): Solubility in water, if not otherwise stated.
(b): Solubility in 95% ethanol, if not otherwise stated.
(c): At 1,013.25 hPa, if not otherwise stated.
(d): At 20°C, if not otherwise stated.
(e): At 25°C, if not otherwise stated.

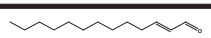
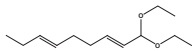
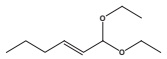
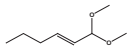
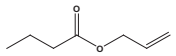
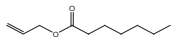
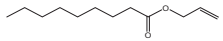
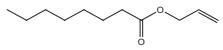
Appendix B – Summary of safety evaluation applying the Procedure

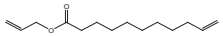
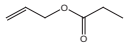
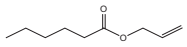
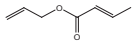
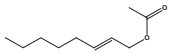
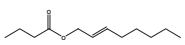
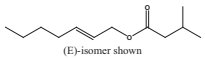
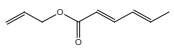
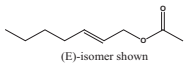
Table B.1: Summary of Safety Evaluation of the JECFA Substances in the Present Group (JECFA, 1996, 1999, 2001, 2003, 2005, 2007, 2009)

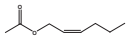
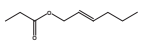
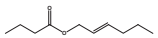
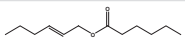
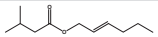
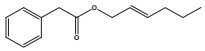
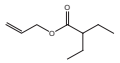
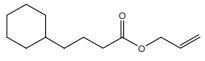
FL-no JECFA-no	EU register name	Structural formula	EU MSDI[a] US MSDI (μg/*capita* per day)	Class[b] Evaluation procedure path[c]	JECFA outcome on the named compound[d] or[e]	EFSA conclusion on the named compound
02.020 1354	Hex-2-en-1-ol		653.48 291	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
02.049 1184	Nona-2,6-dien-1-ol		9.07 1	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
02.050 1793	Pent-2-en-1-ol		2.44	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
02.090 1365	Non-2(*trans*)-en-1-ol		0.02 0.03	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
02.112 1369	Non-2(*cis*)-en-1-ol		0.01 2	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
02.137 1794	Dec-2-en-1-ol		0.01	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
02.156 1374	Hex-2(*cis*)-en-1-ol		0.01 10	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
02.192	Oct-2-en-1-ol		7.71	No evaluation	Not evaluated by JECFA	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
02.210 1384	Undec-2-en-1-ol		0.01 1	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
02.231	*trans*-2, *cis*-6-Nonadien-1-ol		8.73	No evaluation	Not evaluated by JECFA	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure

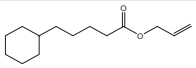
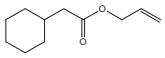
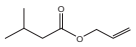
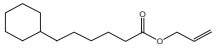
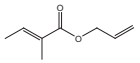
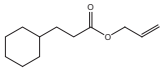
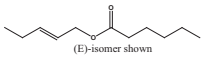
FL-no JECFA-no	EU register name	Structural formula	EU MSDI[(a)] US MSDI (μg/*capita* per day)	Class[(b)] Evaluation procedure path[(c)]	JECFA outcome on the named compound[(d)] or[(e)]	EFSA conclusion on the named compound
05.037 1350	2-Dodecenal		1.19 2	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.058 1186	Nona-2(*trans*),6(*cis*)-dienal		15.78 24	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.060 1363	Oct-2-enal		0.84 0.9	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.070 1360	2-Heptenal		8.17	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.072 1362	*trans*-2-Nonenal		1.70 0.12	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.073 1353	Hex-2(*trans*)-enal		2761 409	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.076 1349	Dec-2-enal		12.94 6	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.078 1359	Tridec-2-enal		0.97 0.7	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.102 1364	Pent-2-enal		0.37 0.1	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.109 1366	2-Undecenal		0.65 0.4	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.111 1182	Octa-2(*trans*),6(*trans*)-dienal		0.01 0.007	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure

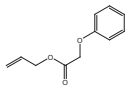
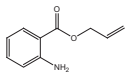
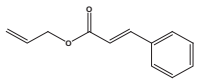
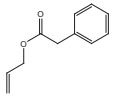
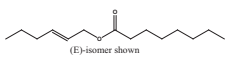
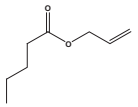
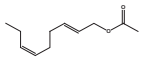
FL-no JECFA-no	EU register name	Structural formula	EU MSDI[(a)] US MSDI (μg/*capita* per day)	Class[(b)] Evaluation procedure path[(c)]	JECFA outcome on the named compound[(d)] or[(e)]	EFSA conclusion on the named compound
05.114 1208	4-Methylpent-2-enal		0.01 0.2	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.120 1197	Dodeca-2,6-dienal		0.01 0.009	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.144	Dodec-2(*trans*)-enal		0.75	No evaluation	Not evaluated by the JECFA	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.150 1360	Hept-2(*trans*)-enal		16.27 30	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.171 1362	Non-2-enal	 (E)-isomer shown	9.89 0.4	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.172 1187	Nona-2(*trans*),6(*trans*)-dienal		6.52 0.007	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.179 1803	Tetradec-2-enal		0.01	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.184	Undec-2(*trans*)-enal		0.84 ND	No evaluation	Not evaluated by JECFA	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.189	2-Hexenal		1.22 409	Class I No evaluation	Not evaluated by JECFA	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure.
05.190	*trans*-2-Octenal		0.79	No evaluation	Not evaluated by JECFA	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
05.191	*trans*-2-Decenal		8.10	No evaluation	Not evaluated by JECFA	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure

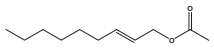
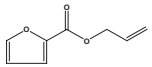
FL-no JECFA-no	EU register name	Structural formula	EU MSDI[(a)] US MSDI (μg/*capita* per day)	Class[(b)] Evaluation procedure path[(c)]	JECFA outcome on the named compound[(d)] or[(e)]	EFSA conclusion on the named compound
05.195	*trans*-2-Tridecenal		0.12	No evaluation	Not evaluated by JECFA	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
06.025 946	1,1-Diethoxynona-2,6-diene		0.01 0.01	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
06.031 1383	1,1-Diethoxyhex-2-ene		0.01	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
06.072 1728	1,1-Dimethoxyhex-2 (*trans*)-ene		0.01 ND	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
09.054 2	Allyl butyrate		0.01 < 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.097 4	Allyl heptanoate		12.92 28	Class II A3: Intake above threshold, A4: Endogenous	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.109 6	Allyl nonanoate		0.01 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.119 5	Allyl octanoate		0.01 1.3	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000

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 conclusion on the named compound
09.146 9	Allyl undec-10-enoate		0.01 < 0.01	Class II A3: Intake above threshold, A4: Endogenous	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.233 1	Allyl propionate		0.57 < 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.244 3	Allyl hexanoate		3583.8 820	Class II B3: Intake above threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.247	Allyl crotonate		0.04	No evaluation	Not evaluated by JECFA	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
09.276 1367	Oct-2-enyl acetate		0.03 0.7	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
09.277 1368	Oct-2(*trans*)-enyl butyrate		0.15 0.7	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
09.303 1799	Hept-2-enyl isovalerate	 (E)-isomer shown	0.01	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
09.312 8	Allyl hexa-2,4-dienoate		0.01 < 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.385 1798	Hept-2-enyl acetate	 (E)-isomer shown	0.01	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure

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 conclusion on the named compound
09.394 1355	Hex-2(*trans*)-enyl acetate		272.73 56	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
09.395 1378	Hex-2(*trans*)-enyl propionate		0.08 4	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
09.396 1375	Hex-2-enyl butyrate		5.62 4	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
09.397 1376	Hex-2-enyl formate		0.01 7	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
09.398 1381	Hex-2-enyl hexanoate		1.36 0.09	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
09.399 1377	Hex-2-enyl isovalerate		1.44 4	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
09.400	Hex-2-enyl phenylacetate		0.01	Class I No evaluation	Not evaluated by JECFA	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
09.410 11	Allyl 2-ethylbutyrate		0.01 0.02	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.411 14	Allyl cyclohexanebutyrate		0.01 < 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000

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 conclusion on the named compound
09.469 15	Allyl cyclohexanevalerate		0.01 < 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.482 12	Allyl cyclohexaneacetate		0.01 < 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.489 7	Allyl isovalerate		0.06 0.19	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.492 16	Allyl cyclohexanehexanoate		0.01 < 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.493 10	Allyl 2-methylcrotonate		0.01 < 0.01	Class II B3: intake below threshold B4: adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.498 13	Allyl cyclohexanepropionate		96.57 110	Class II B3: intake below threshold B4: adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.678 1795	Pent-2-enyl hexanoate	 (E)-isomer shown	0.01	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure

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 conclusion on the named compound
09.701 18	Allyl phenoxyacetate		9.93 2.5	Class III B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.719 20	Allyl anthranilate		0.01 0.09	Class III B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.741 19	Allyl cinnamate		0.01 0.28	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.790 17	Allyl phenylacetate		0.01 < 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. Evaluated by JECFA before 2000
09.841 1796	2-Hexenyl octanoate		0.01	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
09.866	Allyl valerate		0.01	No evaluation	Not evaluated by JECFA	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
09.947 1188	(*E*,*Z*)-2,6-Nonadienyl acetate		0.01	Class I A3: Intake below threshold	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure

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 conclusion on the named compound
09.948	(2*E*)-2-Nonenyl acetate		0.01	Class I No evaluation	Not evaluated by JECFA	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure
13.004 21	Allyl 2-furoate		0.01 < 0.01	Class III B3: Intake below threshold, B4: No adequate NOAEL JECFA evaluated at step B5: intake below 1.5 μg/person per day	(d)	Evaluated in FGE.200Rev1 as of no genotoxicity concern. The substance can be evaluated through the Procedure

JECFA: The Joint FAO/WHO Expert Committee on Food Additives; FL-no: FLAVIS number; MSDI: maximised survey-derived daily intake; FGE: Flavouring Group Evaluation; NOAEL: no-observed-adverse-effect-level; ND: not determined.

(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. EU MSDI values calculated based on the most recent EFFA poundage information (kg/year) for the year 2015 (EFFA, 2018).

(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.

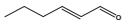
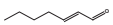
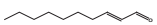
(c): Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.

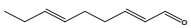
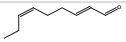
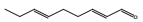
(d): No safety concern based on intake calculated by the MSDI approach of the named compound.

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

Appendix C – (Q)SAR predictions on mutagenicity for aldehydes from subgroup 1.1.1

Table C.1: QSAR Predictions on Mutagenicity for 25 Aldehydes from Subgroup 1.1.1

FL-no JECFA-no	EU register name	Structural formula[a]	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]
05.176	Prop-2-enal		POS	POS	OD	NEG	OD
05.102 1364	Pent-2-enal		POS	POS	OD	NEG	NEG
05.114 1208	4-Methylpent-2-enal		POS	NEG	OD	NEG	NEG
05.189 1353	2-Hexenal		POS	POS	OD	NEG	NEG
05.073	Hex-2(*trans*)-enal		POS	POS	OD	NEG	NEG
Not in Register	Hex-2(*cis*)-en-1-al		POS	POS	OD	NEG	NEG
05.150 1360	Hept-2(*trans*)-enal		POS	POS	OD	NEG	NEG
05.070	2-Heptenal		POS	POS	OD	NEG	NEG
05.060 1363	Oct-2-enal		POS	EQU	OD	NEG	NEG
05.190	*trans*-2-Octenal		POS	EQU	OD	NEG	NEG
05.171 1362	Non-2-enal		POS	EQU	OD	NEG	NEG
05.072	*trans*-2-Nonenal		POS	EQU	OD	NEG	NEG
Not in Register	Non-2(*cis*)-en-1-al		POS	EQU	OD	NEG	NEG
05.076 1349	Dec-2-enal		POS	EQU	OD	NEG	NEG
05.191	*trans*-2-Decenal		POS	EQU	OD	NEG	NEG
05.109 1366	2-Undecenal		POS	EQU	OD	NEG	NEG
05.144	Dodec-2(*trans*)-enal		POS	EQU	OD	NEG	NEG
05.037 1350	2-Dodecenal		POS	EQU	OD	NEG	NEG

FL-no JECFA-no	EU register name	Structural formula[a]	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]
05.078 1359	Tridec-2-enal		POS	EQU	OD	NEG	NEG
05.179	Tetradec-2-enal		POS	EQU	OD	NEG	NEG
05.111 1182	Octa-2(*trans*),6(*trans*)-dienal		NEG	EQU	OD	NEG	NEG
Not in register	Nona-2,6-dien-1-al		NEG	NEG	OD	NEG	NEG
05.058 1186	Nona-2(*trans*),6(*cis*)-dienal		NEG	NEG	OD	NEG	NEG
05.172 1187	Nona-2(*trans*),6(*trans*)-dienal		NEG	NEG	OD	NEG	NEG
05.120 1197	Dodeca-2,6-dienal		NEG	EQU	OD	NEG	NEG

FEMA: Flavor and Extract Manufacturers Association; CoE: Council of Europe; CAS: Chemical Abstract Service; CHL: Chinese hamster lung; CHO: Chinese hamster ovary.
(a): Structure subgroup.
(b): Local model on aldehydes and ketones, Ames TA100. (NEG: Negative; POS: Positive; OD: out of domain).
(c): MultiCase Ames test (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
(d): MultiCase Mouse lymphoma test (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
(e): MultiCase Chromosomal aberration in CHO (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).
(f): MultiCase Chromosomal aberration in CHL (OD: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

Appendix D – Genotoxicity studies evaluated in FGE.200

Table D.1: Genotoxicity data (*in vitro*) considered by the Panel

Register name [FL-no]	End-point	Test system	Concentration	Results	Reference	Remarks*
Nona-2 (*trans*),6 (*cis*)-dienal [05.058]	Reverse mutation	*Salmonella* Typhimurium TA100	0.01–0.1 μL/plate (8.6–86 μg/plate)[(a)] [4,1]	Negative	Eder et al. (1992)	Valid Standard bacterial density was used 30-min pre-incubation [(a)]Calculated using specific gravity = 0.850–0.870 g/mL (Food and Chemical Codex, 1996)
	Reverse mutation	*S.* Typhimurium TA100	0.005–0.15 μL/plate (4.3–129 μg/plate)[(a)] [4,1] 0.005–0.20 μL/plate (4.3–172 μg/plate)[(a)] [4,2]	Negative	Eder et al. (1992)	Valid Threefold bacterial cell density was used 90-min pre-incubation [(a)]Calculated using specific gravity = 0.850–0.870 g/mL (Food and Chemical Codex, 1996)
	SOS chromotest	*Escherichia coli* PQ37 and PQ243	5–80 nmol (0.69–11 μg/L)	Negative	Eder et al. (1992)	Valid
	Sister chromatid exchange	Human lymphoblastoid Namalva cell line	0–40 μM (0–5.5 μg/mL) [1]	Positive	Dittberner et al. (1995)	Valid
	Sister chromatid exchange	Primary human blood lymphocytes	0–50 μM (0–6.9 μg/mL) [1]	Positive	Dittberner et al. (1995)	Valid
	Structural chromosomal aberration test	Human lymphoblastoid Namalva cell line	0–40 μM (0–5.5 μg/mL) [1]	Positive	Dittberner et al. (1995)	Valid
	Structural chromosomal aberration test	Primary human blood lymphocytes	0–40 μM (0–5.5 μg/mL) [1]	Equivocal	Dittberner et al. (1995)	Valid
	Numerical chromosomal aberration test	Primary human blood lymphocytes	0–40 μM (0–5.5 μg/mL) [1]	Positive	Dittberner et al. (1995)	Valid
	Micronucleus formation	Primary human blood lymphocytes	0–50 μM (0–6.9 μg/mL) [1]	Positive	Dittberner et al. (1995)	Valid
	Micronucleus formation	Human lymphoblastoid Namalva cell line	0–50 μM (0–6.9 μg/mL) [1]	Positive	Dittberner et al. (1995)	Valid

Register name [FL-no]	End-point	Test system	Concentration	Results	Reference	Remarks*
Hex-2 (*trans*)-enal [05.073]	Reverse mutation	*S.* Typhimurium TA98, TA100, and TA104	Not reported [4,5]	Positive	Kato et al. (1989)	Validity cannot be evaluated. Abstract only According to the authors, 2-hexenal was 'suspected to be positive' (Kato et al., 1989); however, no further details were provided. Liquid pre-incubation was used
	Reverse mutation	*S.* Typhimurium TA100	0.05–0.35 μL/plate [4,1] 0.15–0.5 μL/plate [4,2]	Negative	Eder et al. (1992)	Valid Standard bacterial cell density was used 30-min pre-incubation
	Reverse mutation	*S.* Typhimurium TA100	0.01–0.15 μL/plate [4,1] 0.1–0.4 μL/plate [4,2]	Positive	Eder et al. (1992)	Valid Threefold bacterial cell density was used 90-min pre-incubation
	SOS chromotest	*E. coli* PQ37 and PQ243	70–435 nmol (6.9–42.7 μg)[(a)]	Negative	Eder et al. (1992)	Valid Cytotoxicity was observed at the highest dose tested [(a)]Calculated using the molecular weight of 2-hexenal = 98.14
	Mutation	*E. coli* WP2uvrA/pKM101	Not reported [5]	Positive	Kato et al. (1989)	Validity cannot be evaluated. Abstract only. According to the authors, 2-hexenal was 'suspected to be positive' (Kato et al., 1989); however, no further details were provided Liquid pre-incubation was used
	Micronucleus induction	Human blood lymphocytes	5–250 μM (0.5–24.5 μg/mL) [1]	Positive	Dittberner et al. (1995)	Valid
	Micronucleus induction	Lymphoblastoid Namalva cells	5–250 μM (0.5–24.5 μg/mL) [1]	Positive	Dittberner et al. (1995)	Valid
	Chromosomal aberration	Human blood lymphocytes	5–250 μM (0.5–24.5 μg/mL) [1]	Negative	Dittberner et al. (1995)	Valid
	Chromosomal aberration	Lymphoblastoid Namalva cells	5–150 μM (0.5–14.7 μg/mL) [1]	Positive	Dittberner et al. (1995)	Valid
	Sister chromatid exchange	Human blood lymphocytes	5–250 μM (0.5–24.5 μg/mL) [1]	Positive	Dittberner et al. (1995)	Valid
	Sister chromatid exchange	Lymphoblastoid Namalva cells	5–200 μM (0.5–19.6 μg/mL) [1]	Positive	Dittberner et al. (1995)	Valid

Register name [FL-no]	End-point	Test system	Concentration	Results	Reference	Remarks*
	DNA repair	Rat hepatocytes	60–600 nmol/10^6 cells (5.9–58.9 μmol)[(a)]	Positive	Griffin and Segall (1986)	Valid Study design complies with OECD Guideline 482. UDS clearly increased at two highest concentrations with only moderate toxicity [(a)]Calculated using the molecular weight of 2-hexenal = 98.14
Pent-2-enal [05.102]	Reverse mutation	*S.* Typhimurium TA100	0.075–0.5 μL/plate [4,1] 0.075–0.75 μL/plate [4,2]	Positive	Eder et al. (1992)	Valid Standard bacterial cell density was used 30-min pre-incubation
	Reverse mutation	*S.* Typhimurium TA100	0.01–0.25 μL/plate [4,1] 0.1–0.4 μL/plate [4,2]	Positive	Eder et al. (1992)	Valid Threefold bacterial cell density was used 90-min pre-incubation
	SOS chromotest	*E. coli* PQ37 and PQ243	60–435 nmol (5.0–36.7 μg)[(a)]	Negative	Eder et al. (1992)	Valid Cytotoxicity was observed at the highest dose tested [(a)]Calculated using the molecular weight of 2-pentenal = 84.12
	Mutation induction TG resistance	Chinese hamster V79 cells	0.03, 0.10 or 0.30 mM (2.5, 8.4 or 25.2 μg/mL)[(a)] [1]	Positive	Canonero et al. (1990)	Limited validity No data on cytotoxicity were provided, 2 or 3 doses were tested and unclear criteria to choose dose range. A dose-dependent increase in the number of 6-thioguanine mutants was observed [(a)]Calculated using the molecular weight of 2-pentenal = 84.12
	Mutation induction Ouabain resistance	Chinese hamster V79 cells	0.03, 0.10 or 0.30 mM (2.5, 8.4 or 25.2 μg/mL)[(a)] [1]	Negative	Canonero et al. (1990)	Limited validity No data on cytotoxicity were provided, 2 or 3 doses were tested and unclear criteria to choose dose range [(a)]Calculated using the molecular weight of 2-pentenal = 84.12

Register name [FL-no]	End-point	Test system	Concentration	Results	Reference	Remarks*
	Alkaline elution DNA single strand break	Mouse leukaemia cells L1210	400, 600 or 800 μmol (33.648, 50.472 or 67.296 μg)[(a)]	Positive	Eder et al. (1993)	Limited validity Results were not reported in detail. However, the authors stated that pentenal was slightly positive at doses at which cytotoxicity was just starting. Cytotoxicity was observed at the highest dose tested [(a)]Calculated using the molecular weight of 2-pentenal = 84.12
2-Heptenal [05.070]	Reverse mutation	*S.* Typhimurium TA104	Up to 0.9 μmol/plate[(a)] (101 μg/plate)[(b)] [4,1]	Negative	Marnett et al. (1985)	Validity cannot be evaluated Results were not reported in detail. Liquid pre-incubation procedure was used [(a)]Maximum non-toxic dose [(b)]Calculated using the molecular weight of 2-heptenal = 112.17
	Reverse mutation	*S.* Typhimurium TA104	Up to 4.4 μmol/plate[(a)] (493.5 μg/plate)[(b)] [4,1]	Negative	Marnett et al. (1985)	Validity cannot be evaluated Results were not reported in detail. Liquid pre-incubation procedure was used. Addition of glutathione at 10 mM [(a)]Maximum non-toxic dose [(b)]Calculated using the molecular weight of 2-heptenal = 112.17
	Reverse mutation	*S.* Typhimurium TA100	0.01–0.15 μL/plate [4,1] 0.075–0.3 μL/plate [4,2]	Negative	Eder et al. (1992)	Valid Standard bacterial cell density was used. 30-min pre-incubation. Dose-dependent increases in mutation frequency were noted; however, these increases were never twofold higher than the spontaneous mutation frequency
	Reverse mutation	*S.* Typhimurium TA100	0.005–0.1 μL/plate [4,1] 0.025–0.3 μL/plate [4,2]	Negative	Eder et al. (1992)	Valid Threefold bacterial cell density assay was used. 90-min pre-incubation Dose-dependent increases in mutation frequency were noted; however, these increases were never two-fold higher than the spontaneous mutation frequency

Register name [FL-no]	End-point	Test system	Concentration	Results	Reference	Remarks*
	SOS chromotest	*E. coli* PQ37 and PQ243	35–270 nmol (3.9–30.3 μg)[(a)]	Negative	Eder et al. (1992)	Valid Cytotoxicity was observed at the highest dose tested [(a)]Calculated using the molecular weight of 2-heptenal = 112.17
	Mutation induction TG resistance	Chinese hamster V79 cells	0.01, 0.03 or 0.10 mM (1.1, 3.4 or 11.2 μg/mL)[(a)] [1]	Positive	Canonero et al. (1990)	Limited validity No data on cytotoxicity were provided, 2 or 3 doses were tested and unclear criteria to choose dose range. Dose-dependent increases in the number of 6-thioguanine and ouabain mutants were observed [(a)]Calculated using the molecular weight of 2-heptenal = 112.17
	Mutation induction Ouabain resistance	Chinese hamster V79 cells	0.01, 0.03 or 0.10 mM (1.1, 3.4 or 11.2 μg/mL)[(a)] [1]	Negative	Canonero et al. (1990)	Limited validity No data on cytotoxicity were provided, 2 or 3 doses were tested and unclear criteria to choose dose range [(a)]Calculated using the molecular weight of 2-heptenal = 112.17
	DNA single-strand break	Mouse leukaemia L1210 cells	200, 400 or 500 μmol (22.434, 44.868 or 56.085 μg)[(a)]	Positive	Eder et al. (1993)	Limited validity Results were not reported in detail. However, the authors stated that heptenal was positive at non-toxic doses [(a)]Calculated using the molecular weight of 2-heptenal = 112.17
trans-2-Nonenal [05.072]	Micronucleus formation	Rat hepatocytes	0.1, 1, 10 or 100 μM (0.01, 0.1, 1.4 or 14.0 μg/mL)	Positive	Esterbauer et al. (1990)	Limited validity Difficult to interpret since the result was expressed as number of cells with micronuclei per mitotic index 1.00 minus control. However, the result is considered valid. The authors of this study were the same as these from Eckl et al. (1993)
	Micronucleus formation	Rat hepatocytes	0.1, 10 or 100 μM (0.01, 1.4 or 14.0 μg/mL)	Equivocal	Eckl et al. (1993)	Limited validity Cells were not under cell division at the treatment time

Register name [FL-no]	End-point	Test system	Concentration	Results	Reference	Remarks*
	Chromosomal aberration	Rat hepatocytes	0.1, 1, 10 or 100 μM (0.01, 0.1, 1.4 or 14.0 μg/mL)	Negative	Esterbauer et al. (1990)	Validity cannot fully be evaluated since types of chromosomal aberrations were not reported
	Chromosomal aberration	Rat hepatocytes	0.1, 10 or 100 μM (0.01, 1.4 or 14.0 μg/mL)	Negative	Eckl et al. (1993)	Validity cannot fully be evaluated since types of chromosomal aberrations were not reported. Cells were not under cell divisions at the treatment time
	Sister chromatid exchange	Rat hepatocytes	0.1, 10 or 100 μM (0.01, 1.4 or 14.0 μg/mL)	Equivocal	Eckl et al., 1993;	Limited validity Cells were not under cell division at the treatment time
	DNA repair	Rat hepatocytes	60–600 nmol/10^6 cells (8.4–84.1 μg/plate)	Positive	Griffin and Segall (1986)	Valid. Study design complies with OECD Guideline 482. UDS clearly increased at four highest concentrations. Maximum at the mid-concentration with only moderate toxicity. At increased levels of toxicity also decline in the net grain counts
Non-2-enal [05.171]	Reverse mutation	*S.* Typhimurium TA104	Up to 0.007 μmol/plate[(a)] (1.0 μg/plate) [4,1]	Negative	Marnett et al. (1985)	Validity cannot be evaluated Results were not reported in detail Liquid pre-incubation procedure was used [(a)]Maximum non-toxic dose
	Mutation induction TG resistance	Chinese hamster V79 cells	0.003 or 0.01 mM (0.4 or 1.4 μg/mL) [1]	Positive	Canonero et al. (1990)	Limited validity No data on cytotoxicity were provided, 2 or 3 doses were tested and unclear criteria to choose dose range. A dose-dependent increase in the number of 6-thioguanine mutants was observed
	Mutation induction Ouabain resistance	Chinese hamster V79 cells	0.003 or 0.01 mM (0.4 or 1.4 μg/mL) [1]	Negative	Canonero et al. (1990)	Limited validity No data on cytotoxicity were provided, 2 or 3 doses were tested and unclear criteria to choose dose range
2-Hexenal [05.189]	Reverse mutation	*S.* Typhimurium TA98, TA100, TA1535 and TA1537	3 μmol/plate (294.4 μg/plate)[(a)] [5]	Negative	Florin et al. (1980)	Insufficient validity. Not in accordance with OECD Guideline 471 (inadequate study design, spot test, only one concentration tested) Isomeric composition of test substance not given [(a)]Calculated using the molecular weight of 2-hexenal = 98.14

Register name [FL-no]	End-point	Test system	Concentration	Results	Reference	Remarks*
	Reverse mutation	*S.* Typhimurium TA104	Up to 2 μmol/plate[(a)] (196.3 μg/plate)[(b)] [4,1]	Positive	Marnett et al. (1985)	Validity cannot be evaluated Results were not reported in detail Isomeric composition of test substance not given. Liquid pre-incubation procedure was used [(a)]Maximum non-toxic dose [(b)]Calculated using the molecular weight of 2-hexenal = 98.14
	Reverse mutation	*S.* Typhimurium TA104	5 μmol/plate[(a)] (> 490.7 μg/plate)[(b)] [4,1]	Positive	Marnett et al. (1985)	Validity cannot be evaluated Results were not reported in detail Isomeric composition of test substance not given. Liquid pre-incubation procedure was used. Addition of 10 mM glutathione [(a)]Maximum non-toxic dose [(b)]Calculated using the molecular weight of 2-hexenal = 98.14
	Reverse mutation	*S.* Typhimurium TA102	Up to 2 μmol/plate[(a)] (196.3 μg/plate)[(b)] [4,1]	Negative	Marnett et al. (1985)	Validity cannot be evaluated Results were not reported in detail Isomeric composition of test substance not given. Liquid pre-incubation procedure was used [(a)]Maximum non-toxic dose [(b)]Calculated using the molecular weight of 2-hexenal = 98.14
	Mutation induction TG resistance	Chinese hamster V79 cells	0.03, 0.10 or 0.30 mM (2.9, 9.8 or 29.4 μg/mL)[(a)] [1]	Positive	Canonero et al. (1990)	Limited validity No data on cytotoxicity were provided, 2 or 3 doses were tested and unclear criteria to choose dose range. A dose-dependent increase in the number of 6-thioguanine mutants was observed. [(a)]Calculated using the molecular weight of 2-hexenal = 98.14
	Mutation induction Ouabain resistance	Chinese hamster V79 cells	0.03, 0.10 or 0.30 mM (2.9, 9.8 or 29.4 μg/mL)[(a)] [1]	Negative	Canonero et al. (1990)	Limited validity No data on cytotoxicity were provided, 2 or 3 doses were tested and unclear criteria to choose dose range [(a)]Calculated using the molecular weight of 2-hexenal = 98.14

Register name [FL-no]	End-point	Test system	Concentration	Results	Reference	Remarks*
	DNA single-strand break	L1210 mouse leukaemia cells	100, 250 or 500 μmol (9.814, 24.535 or 49.070 μg)[(a)]	Positive	Eder et al. (1993)	Limited validity Results were not reported in detail. However, the authors stated that hexenal was positive at non-toxic doses. Cytotoxicity was observed at the highest dose tested. Isomeric composition of test substance not given [(a)]Calculated using the molecular weight of 2-hexenal = 98.14
2-Octenal [05.060]	Bacterial reverse mutation	*S.* Typhimurium TA104	Up to 0.8 μmol/plate[(a)] (101.0 μg/plate)[(b)] [4,1]	Negative	Marnett et al. (1985)	Validity cannot be evaluated Results were not reported in detail. Liquid pre-incubation procedure was used [(a)]Maximum non-toxic dose [(b)]Calculated using the molecular weight of 2-octenal = 126.20
		S. Typhimurium TA104	Up to 4 μmol/plate[(a)] (504.8 μg/plate)[(b)] [4,1]	Negative	Marnett et al. (1985)	Validity cannot be evaluated Results were not reported in detail. Liquid pre-incubation procedure was used Addition of 10 mM glutathione [(a)]Maximum non-toxic dose [(b)]Calculated using the molecular weight of 2-octenal = 126.20
	Mutation induction TG resistance Ouabain resistance	Chinese hamster V79 cells	0.01, 0.03 or 0.10 mM (1.3, 3.8 or 12.6 μg/mL) [1]	Positive (TG resistance: HPRT mutation) Negative (Ouabain resistance)	Canonero et al. (1990)	Limited validity No data on cytotoxicity were provided, 2 or 3 doses were tested and unclear criteria to choose dose range The test was performed only without metabolic activation. No significant increase relative to controls was observed in the number of ouabain mutants
	DNA single-strand breaks	L1210 mouse leukaemia cells	250, 350 μmol (44 mg/plate)	Positive	Eder et al. (1993)	Limited validity Results were not reported in detail. However, the authors stated that hexenal was positive at 350 μmol

OECD: Organisation for Economic Co-operation and Development; UDS: unscheduled DNA synthesis.
[1]: Without S9 metabolic activation.
[2]: With S9 metabolic activation.
[3]: Plate incorporation method.
[4]: Pre-incubation method.
[5]: With and without S9 metabolic activation.
*: Validity of genotoxicity studies:
Valid.
Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and/or limited documentation).
Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards inappropriate/not validated test system).
Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided, text not in a Community language).

Table D.2: Genotoxicity data (*in vivo*) considered by the Panel

Register name [FL-no]	End-point	Test system	Concentration	Results	Reference	Remarks
Hex-2(*trans*)-enal [05.073]	Micronucleus induction	Human buccal mucosa cells	10 mg/kg	Positive	Dittberner et al. (1997)	Valid Statistically significant increases in micronuclei were observed on days 4, 5, 6 and 7 post-administration

Table D.3: Additional genotoxicity data (*in vitro*) considered by the Panel in FGE.200

Register name [FL-no]	Test system	Test object	Concentration	Result	Reference	Comments
Hex-2(*trans*)-enal [05.073]	Reverse mutation	*Salmonella* Typhimurium TA98, TA100, TA102, TA1535, TA1537	3–5,000 μg/plate [3,5]	Negative	Sokolowski (2007a)	A moderate concentration –dependent increase in revertant colony number was observed in strain TA100, in the absence of S9-mix. Study design complies with OECD Guidelines 471
		S. Typhimurium TA100	1–2,500 μg/plate [4,5]	Positive [4,1]		
		S. Typhimurium TA100	25–200 μg/plate [4,5]	Positive [4,1]		
	Reverse mutation	*S.* Typhimurium TA98, TA100, TA102, TA1535, TA1537	3–5,000 μg/plate [3,5]	Negative	Bhatia et al. (2010)	Same test as Sokolowski (2007a)
		S. Typhimurium TA100	1–2,500 μg/plate [4,5]	Positive [4,1]		
		S. Typhimurium TA100	25–200 μg/plate [4,1]	Positive [4,1]		
2-Dodecenal [05.037]	Reverse mutation	*S.* Typhimurium TA98, TA100, TA102, TA1535, and TA1537	3–5,000 μg/plate [3,5]	Negative	Sokolowski (2007b)	Concentrations up to 5,000 μg/plate were used in a pre-experiment test. Toxic effects as a reduction in the number of revertants were observed at the higher concentrations
			0.1–100 μg/plate [3,1] 1–1,000 μg/plate [3,2]	Negative		
			0.3–1,000 μg/plate [4,5]	Negative		
	Reverse mutation	*S.* Typhimurium TA98, TA100, TA102, TA1535, and TA1537	1–1,000 μg/plate [3,2] 0.1–100 μg/plate [3,1]	Negative	Bhatia et al. (2010)	Same test as Sokolowski (2007b)

OECD: Organisation for Economic Co-operation and Development.
[1]: Without S9 metabolic activation.
[2]: With S9 metabolic activation.
[3]: Plate incorporation method.
[4]: Pre-incubation method.
[5]: With and without S9 metabolic activation.

Table D.4: Additional genotoxicity data (*in vivo*) considered by the Panel in FGE.200

Register name [FL-no]	Test system *in vivo*	Test object	Doses	Result	Reference	Comments
trans-2-Hexenal [05.073]	Micronucleus assay	Mouse bone marrow polychromatic erythrocytes	250, 500 and 1,000 mg/kg bw	Negative	Honarvar (2007a)	Study design complies with OECD Guideline 474
	Unscheduled DNA synthesis	Male rats hepatocytes	200 or 500 mg/kg bw	Negative	Durward (2009)	Purity and isomer were not specified. Study design complies with OECD Guideline 486
	Micronucleus assay	Transgenic Muta™Mouse (CD2-lacZ80/HazfBR) blood erythrocytes and reticulocytes	120, 235 and 350 mg/kg per day	Negative	Beevers (2013)	Mice were treated by gavage for 28 days. The dose of 350 mg/kg per day was identified as MTD. Deviations from OECD Guideline 474 were identified
	Induction of *lacZ*⁻ mutation	Transgenic Muta™Mice (CD2-lacZ80/HazfBR) liver and duodenum	120, 235 and 350 mg/kg per day	Negative	Beevers (2013)	Mice were treated by gavage for 28 days. The dose of 350 mg/kg per day was identified as MTD. Liver and duodenum were analysed. Study design complies with OECD Guideline 488
	Micronucleus assay	Mouse bone marrow polychromatic erythrocytes	250, 500 and 1,000 mg/kg bw	Negative	Bhatia et al. (2010)	Same test as Honarvar (2007a)
2-Nonenal [05.171]	Micronucleus assay	Mouse bone marrow polychromatic erythrocytes	500, 1,000 and 2,000 mg/kg bw	Negative	Honarvar (2008)	Study design complies with OECD Guideline 474
	Micronucleus assay	Mouse bone marrow polychromatic erythrocytes	500, 1,000 and, 2,000 mg/kg bw	Negative	Bhatia et al. (2010)	Same test as Honarvar (2008a)
2-Dodecenal [05.037]	Micronucleus assay	Mouse bone marrow polychromatic erythrocytes	500, 1,000 and 2,000 mg/kg bw	Negative	Honarvar (2007b)	Study design complies with OECD Guideline 474
	Micronucleus assay	Mouse bone marrow polychromatic erythrocytes	500, 1,000 and 2,000 mg/kg bw	Negative	Bhatia et al. (2010)	Same test as Honarvar (2007b)

OECD: Organisation for Economic Co-operation and Development; MTD: maximum tolerated dose.

Appendix E – Genotoxicity data evaluated in FGE.200Rev1

Table E.1: Genotoxicity data (*in vivo*) evaluated by the Panel in FGE.200Rev1

Register name [FL-no]	Test system *in vivo*	Test object	Route	Dose mg/kg bw per day	Result	Reference	Comments
Hex-2(*trans*)-enal 05.073	Micronucleus bone marrow	Male Han Wistar rats	Gavage	87.5, 175, 350	Negative	Keig-Shevlin (2017)	Reliable without restriction. Study performed in accordance with OECD TG 474
	Comet liver				Negative		Reliable without restriction. Study performed in accordance with OECD TG 489
	Comet duodenum				Negative		Reliable without restriction. Study performed in accordance with OECD TG 489
trans-2-Octenal 05.190	Micronucleus bone marrow	Male Han Wistar rats	Gavage	25, 500, 1,000	Negative	Beevers (2015a)	Reliable without restriction. Study performed in accordance with OECD TG 474
	Comet liver				Negative		Reliable without restriction. Study performed in accordance with OECD TG 489
Nona-2(*trans*), 6 (*cis*)-dienal 05.058	Micronucleus bone marrow	Male Han Wistar rats	Gavage	175, 350, 700	Negative	Beevers (2015b)	Reliable without restriction. Study performed in accordance with OECD TG 474
	Comet liver				Negative		Reliable without restriction. Study performed in accordance with OECD TG 489

OECD: Organisation for Economic Co-operation and Development.