European Food Safety Authority

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SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 203, Revision 1 (FGE.203Rev1): α,β-Unsaturated aliphatic aldehydes and precursors from chemical subgroup 1.1.4 of FGE.19 with two or more conjugated double-bonds and with or without additional non-conjugated double-bonds[1]

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)[2, 3]

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ABSTRACT

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids of the European Food Safety Authority was requested to evaluate the genotoxic potential of flavouring substances from subgroup 1.1.4 of FGE.19 in the Flavouring Group Evaluation 203, Revision 1 (FGE.203Rev1). The Flavour Industry has provided additional genotoxicity studies for one representative substance in FGE.203, namely 2,4-decadienal [FL-no: 05.140]. Based on the available data, on newly submitted studies and on the scientific evidence from the literature, the Panel concluded that the genotoxic potential cannot be ruled out for the flavouring substances in this FGE.

KEY WORDS

α,β-unsaturated aldehydes, straight chain, FGE.203, α,β-unsaturated conjugated double-bonds, FGE.19, subgroup 1.1.4

[1] On request from the European Commission, Question No EFSA-Q-2013-00373 to 00392, adopted on 26 March 2014.

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SUMMARY

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

The Flavouring Group Evaluation 203 (FGE.203) concerned 17 substances, corresponding to subgroup 1.1.4 of FGE.19. Twelve of these substances are α,β-unsaturated aldehydes with two or more conjugated double-bonds with and without additional non-conjugated double-bonds [FL-no: 05.057, 05.064, 05.071, 05.084, 05.101, 05.108, 05.125, 05.127, 05.140, 05.141, 05.173 and 05.196] and five are precursors for such aldehydes [FL-no: 02.139, 02.153, 02.162, 02.188 and 09.573]. Since FGE.203 was published, three additional substances have been included in the subgroup 1.1.4 of FGE.19 (2,4-decadienal [FL-no: 05.081], 2,4-octadienal [FL-no: 05.186] and tr-2, tr-4-nonadienal [FL-no: 05.194]), accordingly the present FGE concerns the genotoxicity evaluation of 20 flavouring substances.

New data on the representative substance 2,4-decadienal [FL-no: 05.140] and literature data on the representative substance hexa-2(trans),4(trans)-dienal [FL-no: 05.057] which have been submitted by Industry were considered in this revision of FGE.203, i.e. FGE.203Rev1.

On the basis of the genotoxicity data of 2,4-decadienal showing some indication for genotoxicity *in vivo* and considering the evidence from *in vitro* studies for the induction of different types of DNA damage (oxidised DNA bases and bulky adducts) a non-threshold mechanism of genotoxicity cannot be excluded for 2,4-decadienal.

Based on the evidence from publications reporting the induction of DNA adducts in different systems *in vitro* and *in vivo* and of the IARC classification of 2,4-hexadienal as "possible carcinogen to humans" and considering the conclusion drawn by IARC that "mechanistic data provide additional support for the relevance of the animal carcinogenicity data to humans" and that "there is a moderate evidence that tumour induction occurs via a genotoxic mechanism" the Panel confirms the safety concern for 2,4-hexadienal.

Overall, the Panel considered that a non-threshold mechanism of action cannot be excluded for both representative substances based on the data available and the Panel concluded that the safety concern cannot be ruled out for hexa-2(trans),4(trans)-dienal [FL-no: 05.057] and for 2,4-decadienal [FL-no: 05.140]. This conclusion is likewise applicable to the other substances of this FGE.

Therefore, the substances of this FGE cannot be evaluated through the Procedure.

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BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The use of flavouring is regulated under Regulation (EC) No 1334/2008[4] 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[5]. The list contains flavouring substances for which the scientific evaluation should be completed in accordance with Commission Regulation (EC) No 1565/2000[6].

EFSA has evaluated 17 flavouring substances, which correspond to subgroup 1.1.4 of FGE.19, in its evaluation of the flavouring group 203 (FGE.203). The opinion was adopted on 27 November 2008.

EFSA concluded that there is a safety concern for hexa-2(trans),4(trans)-dienal [FL-no: 05.057] since a non-threshold mechanism cannot be excluded. Therefore, the substances of this FGE cannot be evaluated through the Procedure and requested data which clarify whether the carcinogenic effects were based on a thresholded mechanism.

Information on two representative materials hexa-2(trans),4(trans)-dienal [FL-no: 05.057] and deca-2(trans),4(trans)-dienal [FL-no: 05.140] has now been submitted by the European Flavour Association. This information is intended to cover the re-evaluation of the above mentioned substances and of the following 18 substances from FGE.19 subgroup 1.1.4:

- Deca-2,4-dien-1-ol [FL-no: 02.139]
- Hepta-2,4-dien-1-ol [FL-no: 02.153]
- Hexa-2,4-dien-1-ol [FL-no: 02.162]
- Nona-2,4-dien-1-ol [FL-no: 02.188]
- Trideca-2(trans),4(cis),7(cis)-trienal [FL-no: 05.064]
- Nona-2,4-dienal [FL-no: 05.071]
- 2,4-Decadienal [FL-no: 05.081]
- Hepta-2,4-dienal [FL-no: 05.084]
- Penta-2,4-dienal [FL-no: 05.101]
- Undeca-2,4-dienal [FL-no: 05.108]
- Dodeca-2,4-dienal [FL-no: 05.125]
- Octa-2(trans),4(trans)-dienal [FL-no: 05.127]
- Deca-2,4,7-trienal [FL-no: 05.141]
- Nona-2,4,6-trienal [FL-no: 05.173]
- 2,4-Octadienal [FL-no: 05.186]
- tr-2, tr-4-Undecadienal [FL-no: 05.196]
- tr-2, tr-4-Nonadienal [FL-no: 05.194]
- Hexa-2,4-dienyl acetate [FL-no: 09.573].

The Commission asks EFSA to evaluate this new information and depending on the outcome proceed to the full evaluation of the flavouring substances.

[4] 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.

[5] 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.

[6] 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 of the European Parliament and of the Council. OJ L 180, 19.7.2000, p. 8-16.

TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The European Commission requests the European Food Safety Authority to carry out a safety assessment on the following 20 flavouring substances: deca-2,4-dien-1-ol [FL-no: 02.139], hepta-2,4-dien-1-ol [FL-no: 02.153], hexa-2,4-dien-1-ol [FL-no: 02.162], nona-2,4-dien-1-ol [FL-no: 02.188], hexa-2(trans),4(trans)-dienal [FL-no: 05.057], trideca-2(trans),4(cis),7(cis)-trienal [FL-no: 05.064], nona-2,4-dienal [FL-no: 05.071], 2,4-decadienal [FL-no: 05.081], hepta-2,4-dienal [FL-no: 05.084], penta-2,4-dienal [FL-no: 05.101], undeca-2,4-dienal [FL-no: 05.108], dodeca-2,4-dienal [FL-no: 05.125], octa-2(trans),4(trans)-dienal [FL-no: 05.127], deca-2(trans),4(trans)-dienal [FL-no: 05.140], deca-2,4,7-trienal [FL-no: 05.141], nona-2,4,6-trienal [FL-no: 05.173], 2,4-octadienal [FL-no: 05.186], tr-2, tr-4-undecadienal [FL-no: 05.196], tr-2, tr-4-nonadienal [FL-no: 05.194], hexa-2,4-dienyl acetate [FL-no: 09.573], in accordance with Commission Regulation (EC) No 1565/2000.

HISTORY OF FGE.19

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 (EFSA, 2008a). The Panel noted that there were limited genotoxicity data on these flavouring substances but that positive genotoxicity studies were identified for some substances in the group.

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

The Panel noted that for most of these models internal and external validation has been performed, but considered that the outcome of these validations was not always extensive enough to appreciate the validity of the predictions of these models for these 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; Benigni and Netzeva, 2007b) 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 Procedure due to concern with respect to genotoxicity. Corresponding to these subgroups, 15 Flavouring Group Evaluations (FGEs) were established: FGE.200, 204, 205, 206, 207, 208, 209, 211, 215, 219, 221, 222, 223, 224 and 225.

For 11 subgroups the 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 will be 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). 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.

ASSESSMENT

1. History of the Evaluation of the Substances in Subgroup 1.1.4

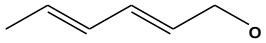
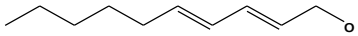
In November 2008, the Panel concluded based on the *in vitro* and *in vivo* genotoxicity data and carcinogenicity data available at that time as well as on the outcome of the (Q)SAR predictions that there is a safety concern for hexa-2(trans),4(trans)-dienal [FL-no: 05.057] since a non-threshold mechanism cannot be excluded. The Panel requested data which clarify whether the carcinogenic effects were based on a threshold mechanism. This conclusion also applies to the other substances of this FGE.203 (EFSA, 2009).

The Panel identified two substances in FGE.19 subgroup 1.1.4 (hexa-2(trans),4(trans)-dienal [FL-no: 05.057] and deca-2(trans),4(trans)-dienal [FL-no: 05.140]) as representative substances (EFSA, 2008c) to be tested in accordance with the conditions set out in the "Genotoxicity Test Strategy for Substances belonging to Subgroups of FGE.19" (EFSA, 2008b), and in accordance with the conclusion in FGE.203. The representative substances for subgroup 1.1.4 are shown in Table 1.

FGE	Adopted by EFSA	Link	No. of Substances
FGE.203	27 November 2008	http://www.efsa.europa.eu/en/efsajournal/pub/877.htm	17
FGE.203Rev1			20

The present Revision 1 of FGE.203 (FGE.203Rev1), concerns the evaluation of additional data submitted by Industry (EFFA, 2013) in response to the requested genotoxicity data in FGE.203 on representative substances for subgroup 1.1.4 (see Table 1). Since FGE.203 was published, three additional substances have been included in the subgroup 1.1.4 of FGE.19 (2,4-decadienal [FL-no: 05.081], 2,4-octadienal [FL-no: 05.186] and tr-2, tr-4-nonadienal [FL-no: 05.194]), accordingly the present FGE concerns the genotoxicity evaluation of 20 flavouring substances.

Table 1: Representative Substances for Subgroup 1.1.4 of FGE.19 (EFSA, 2008c)

FL-no	EU Register name	Structural formula	Comments
05.057	Hexa-2(trans),4(trans)-dienal		
05.140	Deca-2(trans),4(trans)-dienal		

2. Presentation of the Substances Belonging to FGE.203Rev1

FGE.203Rev1 concerns 20 substances, corresponding to subgroup 1.1.4 of FGE.19. Fifteen of these substances are α,β-unsaturated aldehydes with two or more conjugated double bonds with and without additional non-conjugated double bonds [FL-no: 05.057, 05.064, 05.071, 05.081, 05.084, 05.101, 05.108, 05.125, 05.127, 05.140, 05.141, 05.173, 05.186, 05.194 and 05.196] and five are precursors for such aldehydes [FL-no: 02.139, 02.153, 02.162, 02.188 and 09.573] (see Table 3).

A summary of their current evaluation status by JECFA is given in Table 4 (JECFA, 2004). Four substances [FL-no: 05.081, 05.186, 05.194 and 05.196] have not been previously evaluated by the JECFA.

The Panel has also taken into consideration the outcome of the predictions from five selected (Q)SAR models (Benigni and Netzeva, 2007a; Gry et al., 2007; Nikolov et al., 2007) on 13 aldehydes [FL-no:

05.057, 05.064, 05.071, 05.081, 05.084, 05.101, 05.108, 05.125, 05.127, 05.140, 05.141, 05.173 and 05.196]. The 13 aldehydes and their (Q)SAR predictions are shown in Table 10.

Section 3 reports the same information that was presented in FGE.203. Section 4 describes additional data submitted by the Industry in response to the data requested in FGE.203.

3. Toxicity

3.1. (Q)SAR Predictions

In Table 10 the outcomes of the (Q)SAR predictions for possible genotoxic activity in five *in vitro* (Q)SAR models (ISS-Local Model-Ames test, DTU-NFI MultiCASE-Ames test, Chromosomal aberration test (CHO), Chromosomal aberration test (CHL) and Mouse lymphoma test) are presented.

Ten out of 13 substances were predicted as positive by the ISS Local Model for the Ames test (TA100). By using the MultiCASE for the Ames test, one positive prediction (hexa-2(trans),4(trans)-dienal [FL-no: 05.057]), nine equivocal predictions, two negative predictions and one out of domain were obtained. All substances were predicted as "out of domain" by the MultiCASE model for the Mouse lymphoma test. All substances were predicted as negative by the MultiCASE model for the Chromosomal aberration test both in CHO and CHL cells.

3.2. Carcinogenicity Studies

Groups of 50 male and 50 female F344/N rats were administered 2,4-hexadienal (89 % trans,trans-isomer, 11 % cis,trans-isomer) in corn oil by gavage at dose levels of 0 (controls), 22.5, 45 or 90 mg/kg body weight (bw) per day, five times per week for up to 105 weeks. The survival of the dosed animals was not affected by the treatment. The mean body weights of the high dose males were generally lower than that of the controls. The incidences of squamous cell papillomas of the forestomach occurred with a statistically significant positive trend in male and female rats (males: 0/50; 3/50; 10/50; 29/50; females: 0/50; 1/50; 5/50; 17/50). Squamous cell carcinomas were found in one male at 45 mg/kg bw per day and in two males at the highest dose group (males papillomas and carcinomas: 0/50; 3/50; 11/50; 29/50). Incidence of epithelial hyperplasia were statistically significantly increased in rats at all dose levels (males: 3/50; 19/50; 42/50; 50/50; females: 2/50; 16/50; 37/50; 41/50) (NTP, 2003).

Groups of 50 male and 50 female B6C3F1 mice were administered 2,4-hexadienal in corn oil by gavage at dose levels of 0 (controls), 30, 60, or 120 mg/kg bw/day, five times per week for 105 weeks. The survival and the mean body weights of the dosed animals were not affected by the treatment. The incidences of squamous cell papillomas of the forestomach occurred with a statistically significant positive trend in male and female mice (males: 2/50; 4/50; 5/50; 8/50; females: 2/50; 2/50; 11/50; 13/50). Squamous cell carcinomas were found in males and females at the highest dose group (males carcinomas: 0/50; 1/50; 0/50; 2/50; males papillomas and carcinomas: 2/50; 4/50; 5/50; 10/50; females carcinomas: 0/50; 0/49; 0/50; 7/50; females papillomas and carcinomas: 2/50; 2/49; 11/50; 18/50). Epithelial hyperplasia occurred in mice of either sex at the highest dose level (males: 14/50; 7/50; 9/50; 26/50; females: 4/50; 8/49; 12/50; 31/50). Two males from the highest dose group had squamous cell carcinoma of the tongue (NTP, 2003). Although not statistically significantly increased relative to the controls, this increase exceeded historical incidences in controls.

Additional studies were performed by NTP (NTP, 2003) in order "*to evaluate whether oral administration of 2,4-hexadienal to F344/N rats induces the formation of the lipid peroxidation product malondialdehyde in the forestomach and/or affects the defensive antioxidant glutathione system. Forestomach samples were collected from groups of 10 male and 10 female F344/N rats administered 0, 90, or 120 mg/kg 2,4-hexadienal in corn oil by gavage for 28 days to measure the concentrations of reduced glutathione (GSH), oxidized glutathione (GSSG), and malondialdehyde (MDA). The concentration of GSH increased significantly in males at 1 and 4 hours postdosing and in females at 4 and 24 hours postdosing. The concentration of GSSG increased significantly in males at*

all three timepoints and in females at 4 and 24 hours postdosing. The concentration of GSH + GSSG increased significantly in males at 4 hours postdosing and in females at 4 and 24 hours postdosing. There was a significant reduction of the GSH/GSSG ratio in males at 4 hours postdosing and no significant trend at other times. No statistically significant changes in the concentration of MDA were observed in the forestomach of male or female rats".

The hypothesis that treatment with this dienal can result in an increase in the endogenous formation of acrolein and crotonaldehyde-derived cyclic DNA adducts in the target tissues was also investigated by NTP (NTP, 2003): "*DNA adduct analysis was performed on samples of liver and forestomach tissue from male F344/N rats and forestomach tissue from B6C3F1 mice administered 0, 90 (rats only), or 120 (mice only) mg 2,4-hexadienal/kg body weight by gavage. Vehicle control male rats were treated for 118 days; all other rats and mice were treated for 90 days.*

Following 90 days of administration, there was no significant difference in the concentration of DNA adducts detected in liver samples of vehicle control and 90 mg/kg male rats. In rat forestomach samples, Acr-dG 3 concentrations appeared to be greater in the treated group than in the vehicle control group, although the difference was not significant (P=0.079). While neither Cro-dG 1 nor Cro-dG 2 were detected in forestomach tissue from vehicle control rats, Cro-dG 2 was present in tissue from rats dosed with 90 mg/kg. These results suggest that treatment with 2,4-hexadienal may increase cyclic adduct formation in rat forestomach DNA via a lipid peroxidation pathway. In mouse forestomach tissue, no significant change in concentration of the Acr-dG 3 adduct was detected following 90 days of exposure to 120 mg/kg 2,4-hexadienal. Cro-dG adduct concentrations appeared to be greater in samples from the vehicle control group than in those from the 120 mg/kg group (P=0.0010 for Cro-dG 1; P=0.0011 for Cro-dG 2)".

Overall, the authors of the NTP report concluded (NTP, 2003):

"*Under the conditions of these 2-year gavage studies, there was clear evidence of carcinogenic activity of 2,4-hexadienal in male and female F344/N rats and male and female B6C3F1 mice based on increased incidences of squamous cell neoplasms of the forestomach. The occurrence of squamous cell carcinoma of the oral cavity (tongue) in male B6C3F1 mice may have been related to the administration of 2,4-hexadienal. Hyperplasia of the forestomach in male and female rats and mice was associated with administration of 2,4-hexadienal".*

At its 61st meeting the JECFA has discussed the occurrence of forestomach effects in rodents:

"*The occurrence of forestomach hyperplasia and squamous cell tumours in rodents is common in bioassay studies by the National Toxicology Program in which a high concentration of an irritating material in corn oil is delivered daily by gavage into the forestomach for 2 years. High concentrations of aldehydes (e.g. malonaldehyde, furfural, benzaldehyde and trans,trans-2,4-hexadienal (National Toxicology Program, 1988, 1990, 1993, 2001, respectively) and other irritating substances (e.g. dihydrocoumarin, coumarin (National Toxicology Program, 1990, 1992, respectively)) delivered in corn oil by gavage are consistently associated with these phenomena in the forestomach of rodents.*

Trans,trans-2,4-Hexadienal produced some positive results in short-term tests for genotoxicity in vitro, but was inactive in tests in vivo. Thus, although it may be genotoxic under some conditions, this is not believed to be the basis for its effects in the rodent forestomach. There was evidence of treatment-related injury to the forestomach epithelium and this is believed to be the primary cause of the neoplastic development. In the bioassays, development of hyperplasia in mice and rats receiving test substance by gavage in corn oil, and a low incidence of adenoma observed in mice reflect the sensitivity of the forestomach to irritation. The forestomach was the only site at which an increased incidence of neoplasia was observed in treated animals.

The relevance of the development of forestomach tumours in rodents to potential carcinogenic targets in humans has been the subject of much investigation (Grice, 1988; Wester & Kroes, 1988; Clayson et

al., 1990). An International Agency for Research on Cancer Working Group (IARC, 2003) concluded that in order to evaluate the relevance of the induction of forestomach tumours in rodents to cancer in humans, the exposure conditions used in these experiments have to be considered. The exposure conditions during oral administration are unusual (particularly if dosing is effected by gavage) in that physical effects may result in high local concentrations of test substances in the forestomach and prolonged exposure of the epithelial tissue. Agents that only produce tumours in the forestomach in rodents after prolonged treatment and via mechanisms that do not involve reaction with DNA may be of less relevance to humans, since human exposure to such agents would need to surpass time-integrated dose thresholds in order to elicit the carcinogenic response.

Therefore, the appearance of these lesions in the 2-year bioassay in rodents given trans,trans-2,4-hexadienal at a high concentration by gavage has no relevance to humans, given that the results are due to the irritating effect of high bolus doses of trans,trans-2,4-hexadienal delivered to the contact site (the forestomach) by gavage and not the effects of systemic concentrations in the whole animal." (JECFA, 2004).

Study validation and results are presented in Table 5.

3.3. Genotoxicity Studies

In subgroup 1.1.4 there are five *in vitro* studies and two *in vivo* studies on hexa-2(trans),4(trans)-dienal [FL-no: 05.057] and two *in vitro* studies on nona-2,4-dienal [FL-no: 05.071] available.

Hexa-2(*trans*),4(*trans*)-dienal [FL-no: 05.057] was found positive in three valid studies with *S. typhimurium* TA100 strain (Eder et al., 1992; NTP, 2003) and TA104 strain (Marnett et al., 1985). Two valid *in vivo* bone marrow micronucleus assays in mice and rats which have been considered as inconclusive by NTP (NTP, 2003) were considered weakly positive by the Panel. Negative results were reported in a 14-week mouse peripheral blood micronucleus assay (NTP, 2003), considered of limited relevance due to limitations in the experimental protocol. Of limited relevance, due to several shortcomings of the studies, are considered the positive results of a SOS chromotest in *E. coli* PQ37, the induction of DNA-strand breaks in mouse leukemia cells and the *in vitro* (nucleosides) induction of DNA adducts (Eder et al., 1993).

Nona-2,4-dienal [FL-no: 05.071] was found negative in a valid study with *S. typhimurium* TA104 strain (Marnett et al., 1985). The negative results of a SOS chromotest in *E. coli* PQ37, as well as the positive results in a test for DNA-strand breaks in mouse leukemia cells (Eder et al., 1993) were considered of limited relevance due to several shortcomings of these studies.

Study validation and results are presented in Tables 6 and 7.

3.4. Conclusion on Genotoxicity and Carcinogenicity

The Panel concluded that 2,4-hexadienal [FL-no: 05.057] increased the incidence of neoplasms in the forestomach of male and female rats and mice. In addition, squamous cell carcinoma of the tongue was observed in two mice of the high dose group. Based on the data available a non-threshold genotoxic mechanism cannot be excluded. This conclusion also applies to the other substances in this FGE likewise.

3.5. Conclusions for FGE.203

Based on the available data on carcinogenicity and genotoxicity there is a safety concern for hexa-2(trans),4(trans)-dienal [FL-no: 05.057] since a non-threshold mechanism cannot be excluded. Therefore, the substances of this FGE cannot be evaluated through the Procedure. The Panel requests data which clarify whether the carcinogenic effects were based on a threshold mechanism.

4. Additional genotoxicity Data Submitted by Industry for Subgroup 1.1.4

In response to the EFSA request in FGE.203 for additional genotoxicity data for subgroup 1.1.4 the Flavour Industry (EFFA, 2013; IOFI, 2013) has submitted genotoxicity data on deca-2(trans),4(trans)-dienal [05.140] (Table 2).

Table 2: Overview of New Data Submitted for Subgroup 1.1.4

Test substance	Additional data submitted	Reference
Deca-2(trans),4(trans)-dienal [FL-no: 05.140]	Ames test. *S. typhimurium* TA97, TA98, TA100, TA102, TA104 and TA1535. Dosed from 0.1 to 1000 µg/plate ± S9-mix	NTP, 2011
	Micronucleus Induction. Male rat bone marrow polychromatic erythrocytes. Dosed from 100 to 600 mg/kg bw	
	Micronucleus Induction. Male and female mice bone marrow and peripheral blood polychromatic erythrocytes. Dosed from 25 to 600 mg/kg bw.	
Hexa-2(trans),4(trans)-dienal [FL-no: 05.057]	Data review. Cytotoxicity, genotoxicity carcinogenicity	IARC, 2012

4.1. *In Vitro* Data

Bacterial reverse mutation assay

2,4-Decadienal was tested independently in two laboratories in *S. typhimurium* TA97, TA98, TA100, TA102, TA104 and TA1535 in the absence and presence of rat or hamster S9-mix, using the pre-incubation method. Concentrations from 0.3 to 666 µg/plate in strains TA97 and TA1535 and from 0.3 to 1000 µg/plate in TA 98 and TA 100 were tested in the first study and from 0.1 to 100 µg/plate in strains TA97, TA98, TA100, TA102, TA104, TA1535 were evaluated in the second study. The test (NTP, 2011) was performed according the OECD Guideline 471 (OECD, 1997a), following the GLP principles. In the absence of S9-mix, evidence of toxicity above 10 µg/plate and cell killing at 33 µg/plate or above was observed in all tester strains. In the presence of S9-mix signs of toxicity were observed starting from 1000 in strain TA98 (with 30 % hamster S9-mix) and from 333 or 666 µg/plate in the other tester strains. The vehicle and positive control substances produced appropriate responses. No evidence of mutagenicity was observed in any of the tester strains.

Study validation and results are presented in Table 8.

4.2. *In Vivo* Data

Micronucleus assay

2,4-Decadienal was evaluated in a micronucleus assay in bone marrow PCEs for its ability to induce chromosomal damage in rats. 2,4-Decadienal dissolved in corn oil as a carrier was administered by a single intraperitoneal injection to F344/N rats (5 males/dose) at doses of 100, 200, 400 and 600 mg/kg bw. Cyclophosphamide (25 mg/kg bw) was given as the positive control. Rats from all dose groups were sampled 24 hours after dosing. At least 1000 PCEs were scored for each animal for micronuclei (MN). No cytotoxic effects were observed at any dose, as determined by a reduction in the number of PCEs versus vehicle controls. Statistically significant increase in micronuclei frequency was observed in the groups dosed with 100 to 400 mg 2,4-decadienal/kg bw (up to 6-fold compared to control) but not for the highest dose 600 mg/kg bw, which produced marked clinical toxicity (NTP, 2011). The p-value for the trend test was not significant for this study due to the downturn in micronuclei induction at the highest dose.

In a parallel study, 2,4-decadienal dissolved in corn oil as a carrier was administered to mice (5 males/dose) by 3 intraperitoneal injections at 24 hours intervals, at doses of 25, 50, 100 and 200 mg/kg bw. Cyclophosphamide (25 mg/kg bw) was given as the positive control. Mice from all groups were sampled 24 hours after the final dosing. Only 1000 PCEs were scored for each animal for MN instead of 2000 as recommended in OECD Guidelines 474 (OECD, 1997b). A trend of increase in micronuclei frequency is evident in the range of doses 25 - 200 mg/kg bw, but no statistically significant difference with respect to the control was observed at any dose level of 2,4-decadienal. It should be noted that the mean micronuclei frequency in control group (1.2 per 1000 cells) is twofold compared with the value at the lowest dose tested (NTP, 2011).

In a second experiment of the above study, mice (5 males/dose) were administered a single intraperitoneal injection of 400 or 600 mg/kg bw of 2,4-decadienal dissolved in corn oil. Bone marrow and peripheral blood were sampled 48 hours post dosing. A statistically significant increase in micronucleated polychromatic erythrocytes was observed for the 600 mg/kg bw group (3.5 fold compared to control). Analysis of peripheral blood polychromatic erythrocytes in these same mice did not show a statistically significant increase in the frequency of micronucleated cells.

The evaluation of the peripheral blood sampled from male and female mice at the end of a 90-day gavage toxicity study at doses of 0, 50, 100, 200, 400, or 800 mg/kg, 5 days per week for 14 weeks, by the same laboratory, showed no increase in the frequency of micronucleated reticulocytes in treated groups compared with controls. No relevant treatment-related hematological effects were described with the exception of a minimal treatment-related, but not dose-related, decreases in hematocrit values, hemoglobin concentrations and erythrocyte counts occurred in the higher-dosed male and/or female mice. No data on clinical signs, bone marrow toxicity and blood analysis are available to demonstrate the systemic exposure (NTP, 2011).

Overall, the Panel noted that a statistically significant increase of micronucleated polychromatic erythrocytes was observed in both rats and mice up to 6-fold and 3.5-fold compared to control, respectively. Therefore, the Panel considered that 2,4-decadienal cannot be considered non-genotoxic *in vivo* after intraperitoneal injection.

Study validation and results are presented in Table 9.

5. Literature data on hexa-2(trans),4(trans)-dienal [FL-no: 05.057] and 2,4-Decadienal [FL-no: 05.140]

For hexa-2(trans),4(trans)-dienal [FL-no: 05.057] no new experimental data have been submitted by Industry, but additional data from literature including a IARC monograph (IARC, 2012).

Hexa-2(trans),4(trans)-dienal [FL-no: 05.057] tested in V79 and in Caco-2 cells through a comet assay, induced a concentration-dependent induction of DNA damage, in association with a depletion of glutathione levels (Glaab et al., 2001). The production of oxidative DNA damage (FPG-sensitive sites detected by comet assay) by 2,4-hexadienal was demonstrated to be the consequence of the glutathione depletion in V79 cells (Janzowski et al., 2003). 2,4-hexadienal produced 1,*N*2-cyclic-deoxyguanosine and 7,8-cyclic-guanosine adducts in a cell-free system (Eder et al., 1993). Crotonaldehyde-deoxyguanosine-2 adduct levels determined by a ^{32}P-postlabeling technique were increased in forestomach but not in liver of rats exposed to 2,4-hexadienal at a dose of 90 mg/kg bw by gavage for 90 days (NTP, 2003). These results suggest that treatment with 2,4-hexadienal may increase cyclic adduct formation in rat forestomach DNA via a lipid peroxidation pathway (NTP, 2003). Reactive oxygen species can cause DNA damage in forestomach in the form of 8-hydroxydeoxy-guanosine. According to IARC (2012), the increase in chronic inflammation of the forestomach and the presence of forestomach ulcers observed in the high-dose group of male rodents in the 2-year study (NTP, 2003) does not support the hypothesis that the dose-related increases in forestomach neoplasms in male and female rodents is due only to 2,4-hexadienal cytotoxicity. IARC classified 2,4-hexadienal as possible carcinogen to humans and concluded that "mechanistic data

provide additional support for the relevance of the animal carcinogenicity data to humans" and that "there is a moderate evidence that tumour induction occurs via a genotoxic mechanism".

A number of papers are also available in the scientific literature related to the mechanism of action of the genotoxic damage induced by 2,4-decadienal.

The reaction of 2,4-decadienal with 2-deoxyguanosine results in the production of a number of base derivatives. Six different stable DNA adducts (hydroxyl-etheno-dGua derivatives) were isolated by reverse-phase HPLC and fully characterised with spectroscopic measurements, following *in vitro* treatment of calf thymus DNA with 2,4-decadienal (Loureiro et al., 2000 and 2004).

A number of studies report the induction of DNA damage in human cells in culture.

Treatment of human erythroleukemia cell line (HEL cells) with 2,4-decadienal leads to a marked variation of the cellular glutathione level (GSH) and induces DNA fragmentation, as revealed by the presence of low molecular weight DNA fragments upon electrophoresis (Nappez et al., 1996).

It has been shown that 2,4-decadienal induces intracellular ROS, (determined by dichlorofluorescein assay) and causes significant oxidative damage of the 8-hydroxy-2'-deoxyguanosine in lung adenocarcinoma cell line A549 at concentrations from 50 to 200 µM (Wu and Yen, 2004).

Significant induction of DNA strand breaks, detected by comet assay, was observed *in vitro* in human bronchiolar epithelial cells (BEAS-2B) after 4 hours of exposure to 1 µM of 2,4-decadienal. The extent of DNA fragmentation was significantly reduced by the co-treatment with antioxidants, such as NAC, SOD and catalase, indicating that an oxidative stress is involved in the process of DNA breakage.

A significant enhancement of the DNA damage induced by the treatment with 2,4-decadienal was observed through an *in vitro* challenge with Endo III/Fpg (a group of repair enzymes that specifically recognize and repair oxidised purines and pyrimidines) after 1 hour of treatment, and with NER (nucleotide excision repair) enzymes after 4 hours of treatment (Young et al., 2010). These results reveal that 2,4-decadienal induces two different types of DNA damage: oxidised DNA bases and formation of bulky adducts. The results indicate that, in addition to early oxidative DNA damage, non-oxidative DNA damage, such as bulky adduct formation, was also induced by 2,4-decadienal (Young et al., 2010).

6. Discussion of available data

In FGE.203, the Panel noted that 2,4-hexadienal [FL-no: 05.057] increased the incidence of neoplasms in the forestomach of male and female rats and mice in a 2-year carcinogenicity study. In addition, squamous cell carcinoma of the tongue has been observed in two mice of the high dose group (NTP, 2003). The Panel noted that tongue cancer is generally rare in laboratory animals and that it could be relevant for humans.

On the basis of the evidence from the additional papers reporting the induction of DNA adducts in different systems *in vitro* and *in vivo* and of the IARC classification of 2,4-hexadienal as "possible carcinogen to humans" and considering the conclusion drawn by IARC that "mechanistic data provide additional support for the relevance of the animal carcinogenicity data to humans" and that "there is a moderate evidence that tumour induction occurs via a genotoxic mechanism" the Panel confirms the safety concern for 2,4-hexadienal.

2,4-Decadienal was tested for genotoxicity in a NTP study (NTP, 2011). No increase in revertants was observed in any of the several strains of *S. typhimurium* tested with and without liver S9 activation enzymes. According to the authors of the NTP report, the *in vivo* micronucleus tests in rats and mice produced mixed results. The conclusion of the NTP study report is that 2,4-decadienal was not mutagenic *in vitro* or *in vivo*. The Panel, however, noted that statistically significant increases in the

frequency of micronuclei in PCE were observed with 2,4-decadienal up to 6-fold in rats without a dose-response relationship and in mice at a single dose level (3.5-fold compared to controls), after intraperitoneal injection in the NTP study. The Panel also noted that the negative result of the micronucleus assay performed in the 90-day study by gavage, without any evidence of a systemic exposure, cannot overrule the effects observed in rats and mice after an acute exposure. Based on these considerations, the Panel did not agree with the authors of the NTP report and concluded that 2,4-decadienal cannot be considered non-genotoxic *in vivo* in rats and mice after intraperitoneal injection.

On the basis of the overall evaluation of the genotoxicity data of 2,4-decadienal showing some indication for genotoxicity *in vivo* and considering the evidence from *in vitro* studies for the induction of different types of DNA damage (oxidised DNA bases and bulky adducts) a non-threshold mechanism of genotoxicity cannot be excluded for 2,4-decadienal.

CONCLUSION

The Panel considered that a non-threshold mechanism of action cannot be excluded for both representative substances based on the data available. The Panel concluded that the safety concern cannot be ruled out for the representative substances hexa-2(trans),4(trans)-dienal [FL-no: 05.057] and for 2,4-decadienal [FL-no: 05.140]. Therefore, the substances of this FGE cannot be evaluated through the Procedure.

SPECIFICATION SUMMARY OF THE SUBSTANCES IN THE FLAVOURING GROUP EVALUATION 203REV1

Table 3: Specification Summary of the Substances in the Present Group Evaluation

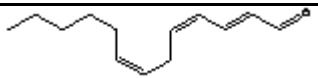
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C [c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]
02.139 1189	Deca-2,4-dien-1-ol		3911 11748 18409-21-7	Liquid $C_{10}H_{18}O$ 154.25	Insoluble Soluble	112 (13 hPa) IR NMR 92 %	1.485-1.495 0.861-0.871
02.153 1784	Hepta-2,4-dien-1-ol		 33467-79-7	Liquid $C_7H_{12}O$ 112.17	 Freely soluble	80 (19 hPa) MS 95 %	1.487-1.493
02.162 1174	Hexa-2,4-dien-1-ol		3922 111-28-4	Solid $C_6H_{10}O$ 98.16	Insoluble Soluble	n.a. 24-33 IR NMR 98 %	n.a. n.a.
02.188 1183	Nona-2,4-dien-1-ol		3951 11802 62488-56-6	Liquid $C_9H_{16}O$ 140.23	Insoluble Soluble	85 (0.7 hPa) IR NMR 92 %	1.486-1.496 0.862-0.872
05.057 1175	Hexa-2(trans),4(trans)-dienal		3429 640 142-83-6	Liquid C_6H_8O 96.13	Slightly soluble Soluble	64 (20 hPa) MS 97 %	1.538-1.543 0.896-0.902 (20°)
05.064 1198	Trideca-2(trans),4(cis),7(cis)-trienal		3638 685 13552-96-0	Liquid $C_{13}H_{20}O$ 192.30	Insoluble Soluble	138 (0.4 hPa) NMR 71 %	1.472-1.478 0.801-0.809
05.071 1185	Nona-2,4-dienal		3212 732 6750-03-4	Liquid $C_9H_{14}O$ 138.21	Insoluble Soluble	97 (13 hPa) IR 89 %	1.522-1.525 0.850-0.870
05.081	2,4-Decadienal		3135 2120 2363-88-4	Liquid $C_{10}H_{16}O$ 152.24	Insoluble Soluble	104 MS 89 %	1.512-1.517 0.866-0.876

Table 3: Specification Summary of the Substances in the Present Group Evaluation

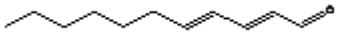
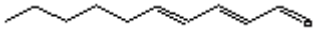
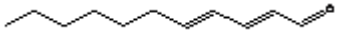
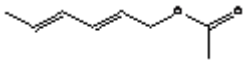
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility [a] Solubility in ethanol [b]	Boiling point, °C [c] Melting point, °C ID test Assay minimum	Refrac. Index [d] Spec.gravity [e]
05.084 1179	Hepta-2,4-dienal		3164 729 4313-03-5	Liquid $C_7H_{10}O$ 110.16	Insoluble Soluble	84 (1 hPa) IR 92 %	1.478-1.480 0.822-0.828
05.101 1173	Penta-2,4-dienal		3217 11695 764-40-9	Liquid C_5H_6O 82.13	n.a. Soluble	60 (91 hPa) NMR 98 %	1.525-1.532 0.801-0.809
05.108 1195	Undeca-2,4-dienal		3422 10385 13162-46-4	Liquid $C_{11}H_{18}O$ 166.26	Insoluble Soluble	129 (17 hPa) NMR 99 %	1.500-1.505 0.896-0.906
05.125 1196	Dodeca-2,4-dienal		3670 11758 21662-16-8	 180.28			
05.127 1181	Octa-2(trans),4(trans)-dienal		3721 11805 30361-28-5	Liquid $C_8H_{12}O$ 124.18	Insoluble Soluble	105-106 (10hPa) IR NMR 99 %	1.519-1.525 0.832-0.839
05.140 1190	Deca-2(trans),4(trans)-dienal		3135 2120 25152-84-5	Liquid $C_{10}H_{16}O$ 152.24	Insoluble Soluble	104 IR 89 %	1.512-1.517 0.866-0.876
05.141 1786	Deca-2,4,7-trienal		4089 51325-37-2	Liquid $C_{10}H_{14}O$ 150.22	Very slightly soluble Very soluble	233 n.a. IR NMR MS 98 %	1.538-1.544 0.898-0.905
05.173 1785	Nona-2,4,6-trienal		4187 57018-53-8	Liquid $C_9H_{12}O$ 136.19	 Freely soluble	194 MS 95 %	 0.867-0.873
05.186	2,4-Octadienal		3721 11805	Liquid $C_8H_{12}O$	Insoluble Soluble	106 (1.1 hPa)	1.519-1.525 0.832-0.839

Table 3: 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)]
			5577-44-6	124.18		MS 99 %	
05.194	tr-2, tr-4-Nonadienal		3212 732 5910-87-2	Liquid $C_9H_{14}O$ 138.21	Insoluble Soluble	97 (1.3 hPa) MS 89 %	1.522-1.525 0.850-0.870
05.196	tr-2, tr-4-Undecadienal		3422 10385 30361-29-6	Liquid $C_{11}H_{18}O$ 166.26	Practically insoluble or insoluble Freely soluble	129 (1.73 hPa) NMR 99 %	1.500-1.505 0.896-0.906
09.573 1780	Hexa-2,4-dienyl acetate		10675 1516-17-2	Liquid $C_{10}H_{20}O_2$ 140.18	Freely soluble	80 (20 hPa) MS 95 %	1.470-1.476 0.908-0.914

(a): Solubility in water, if not otherwise stated.
(b): Solubility in 95 % ethanol, if not otherwise stated.
(c): At 1013.25 hPa, if not otherwise stated.
(d): At 20°C, if not otherwise stated.
(e): At 25°C, if not otherwise stated.

SUMMARY OF SAFETY EVALUATION APPLYING THE PROCEDURE

Table 4: Summary of Safety Evaluation of the JECFA Substances in the Present Group

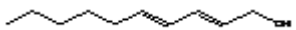
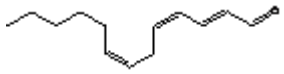
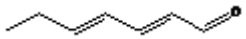
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [(a)] US MSDI (μg/*capita*/day)	Class [(b)] Evaluation procedure path [(c)]	JECFA Outcome on the named compound [(d) or (e)]	EFSA conclusion on the named compound
02.139 1189	Deca-2,4-dien-1-ol		ND 26	Class I A3: Intake below threshold	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
02.162 1174	Hexa-2,4-dien-1-ol		ND 0.4	Class I A3: Intake below threshold	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
02.188 1183	Nona-2,4-dien-1-ol		ND 26	Class I A3: Intake below threshold	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
05.057 1175	Hexa-2(trans),4(trans)-dienal		0.97 0.1	Class I A3: Intake below threshold	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
05.064 1198	Trideca-2(trans),4(cis),7(cis)-trienal		0.18 0.009	Class I A3: Intake below threshold	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
05.071 1185	Nona-2,4-dienal		1.5 0.7	Class I A3: Intake below threshold	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
05.084 1179	Hepta-2,4-dienal		3.0 23	Class I A3: Intake below threshold	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
05.101 1173	Penta-2,4-dienal		0.12 0.2	Class I A3: Intake below threshold	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available

Table 4: Summary of Safety Evaluation of the JECFA Substances in the Present Group

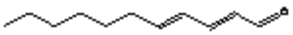
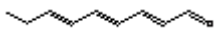
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [(a)] US MSDI (μg/*capita*/day)	Class [(b)] Evaluation procedure path [(c)]	JECFA Outcome on the named compound [(d) or (e)]	EFSA conclusion on the named compound
						could not rule out the concern for genotoxicity.
05.108 1195	Undeca-2,4-dienal		3.2 0.4	Class I A3: Intake below threshold	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
05.125 1196	Dodeca-2,4-dienal		0.57 0.1	Class I A3: Intake below threshold	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
05.127 1181	Octa-2(trans),4(trans)-dienal		0.55 0.007	Class I A3: Intake below threshold	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
05.140 1190	Deca-2(trans),4(trans)-dienal		22 70	Class I A3: Intake below threshold	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
02.153 1784	Hepta-2,4-dien-1-ol		0.061 0.01	Class I B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
05.141 1786	Deca-2,4,7-trienal		0.12 0.01	Class I B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
05.173 1785	Nona-2,4,6-trienal		0.0012 ND	Class I B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
09.573 1780	Hexa-2,4-dienyl acetate		0.61 0.01	Class I B3: Intake below threshold,	(d)	Evaluated in FGE.203Rev1. The genotoxicity data available

Table 4: Summary of Safety Evaluation of the JECFA Substances in the Present Group

FL-no JECFA-no	EU Register name	Structural formula	EU MSDI [(a)] US MSDI (µg/*capita*/day)	Class [(b)] Evaluation procedure path [(c)]	JECFA Outcome on the named compound [(d) or (e)]	EFSA conclusion on the named compound
				B4: Adequate NOAEL exists		could not rule out the concern for genotoxicity.
05.081	2,4-Decadienal		27	No evaluation	Not evaluated by the JECFA.	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
05.186	2,4-Octadienal		0.65	No evaluation	Not evaluated by the JECFA.	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
05.194	tr-2, tr-4-Nonadienal		2.9	No evaluation	Not evaluated by the JECFA.	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.
05.196	tr-2, tr-4-Undecadienal		3.2	No evaluation	Not evaluated by the JECFA.	Evaluated in FGE.203Rev1. The genotoxicity data available could not rule out the concern for genotoxicity.

(a): EU MSDI: Amount added to food as flavour in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita/day.
(b): Thresholds of concern: Class I = 1800 µg/person/day, Class II = 540 µg/person/day, Class III = 90 µg/person/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.
ND: not determined.

TOXICITY DATA

Table 5: Carcinogenicity Studies Considered by the Panel in FGE.203

Register Name [FL-no]	Species; Sex No./Group	Route	Dose levels	Duration	Results	Reference	Comments
Hexa-2(trans),4(trans)-dienal [05.057]	Rats; Male , Female 50/sex/group	Gavage in corn oil	0 (controls), 22.5, 45 or 90 mg/kg bw/day, five times per week	105 weeks	Males: Positive trend in increased squamous cell papillomas of the forestomach. 1 squamous cell carcinoma of the forestomach was seen in the mid dose group and 2 in the high dose group. Females: Positive trend in increased squamous cell papillomas of the forestomach. No carcinomas were seen.	(NTP, 2003)	Valid study. Males: The carcinomas of the forestomach were preceeded by epithelial hyperplasia and papillomas. Females: Squamous cell papillomas and epithelial hyperplasia were increased at the two highest doses.
	Mice; Male, Female 50/sex/group	Gavage in corn oil	0 (controls), 30, 60, or 120 mg/kg bw/day, five times per week	105 weeks	Males and females: Increased incidences of squamous cell papillomas and carcinomas of the forestomach in the high dose groups.	(NTP, 2003)	Valid study. The carcinomas of the forestomach were preceeded by epithelial hyperplasia and squamous cell papillomas.

Table 6: Genotoxicity Data (*in vitro*) Considered by the Panel in FGE.203

Register Name [FL-no]	Test System	Test Object	Concentration	Reported Result	Reference	Comments [(f)]
Hexa-2(trans),4(trans)-dienal [05.057]	Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, and TA1537	3 mmol/plate (288 µg/plate)	Negative [(a,b)]	(Florin et al., 1980)	Insufficient validity (spot test, not according to OECD guideline, methods and results insufficiently reported).
		S. typhimurium TA104	<1 µmol/plate (96 µg/plate)	Positive	(Marnett et al., 1985)	Valid. Published non-GLP study carried out only in the absence of S9; for the purpose of the study the result is considered valid.
		S. typhimurium TA102	Not reported	Negative[(c)]	(Marnett et al., 1985)	Limited validity. The result is reported without details.
		S. typhimurium TA100	0.01 – 0.75 µl/plate (8.95 – 671.3 µg/plate)	Positive [(c)]	(Eder et al., 1992)	Valid.
		S. typhimurium TA1535, TA98	0 - 1500 µg/plate	Negative [(d)]	(NTP, 2003)	Valid. With metabolic activation in two testing centers.
		S. typhimurium TA98	0 - 150 µg/plate	Negative [(c)]	(NTP, 2003)	Valid. Without metabolic activation in two testing centers.
		S. typhimurium TA1535	0 - 166 µg/plate	Negative [(c)]	(NTP, 2003)	Valid. Without metabolic activation in two testing centers.
		S. typhimurium TA100	0 - 333 µg/plate	Positive [(c)]	(NTP, 2003)	Valid. Without metabolic activation, Positive in 1 of 2 testing centres.
		S. typhimurium TA100	0 - 1500 µg/plate	Positive [(d)]	(NTP, 2003)	Valid. With metabolic activation in 2 testing centers.
	SOS chromotest	*E. coli* PQ37 and PQ243	<590 nmol	Negative	(Eder et al., 1992)	Limited validity (only without S9-mix).
		E. coli PQ37	Not reported	Positive	(Eder et al., 1993)	Limited validity (results poorly reported, concentrations and bacteriotoxicity not reported).
	DNA strand breaks	L1210 mouse leukaemia cells	20 µmol/ml (1 923 µg/ml) 300 and 500 µmol/ml (28839 and 48065 µg/ml)	Negative Positive	(Eder et al., 1993)	Limited validity (results poorly reported).
	DNA adducts	Nucleosides	100 mmol/L	Positive	(Eder et al., 1993)	Validity cannot be evaluated (result poorly reported).

Register Name [FL-no]	Test System	Test Object	Concentration	Reported Result	Reference	Comments [f]
Nona-2,4-dienal [05.071]	Reverse mutation	*S. typhimurium* TA104	<0.4 µmol/plate (<55 µg/plate)	Negative [c]	(Marnett et al., 1985)	Valid. Published non-GLP study, considered valid.
		S. typhimurium TA102	Not reported	Negative [c]	(Marnett et al., 1985)	Limited validity.
	SOS chromotest	*E. coli* PQ37	Not reported	Negative	(Eder et al., 1993)	Limited validity.
	DNA strand breaks	L1210 mouse leukaemia cells	400 µmol/ml (55284 µg/ml)	Negative [e]	(Eder et al., 1993)	Limited validity.
			500 µmol/ml (69105 µg/ml)	Positive		

(a): Spot test method.
(b): With and without metabolic activation.
(c): Without metabolic activation.
(d): With metabolic activation.
(e): Results demonstrated in the presence of cytotoxicity.
(f): 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 and/or inappropriate test system).
Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

Table 7: Genotoxicity Data (*in vivo*) Considered by the Panel in FGE.203

Register Name [FL-no]	Test System	Test Object	Route	Dose	Result	Reference	Comments [(a)]
Hexa-2(trans),4(trans)-dienal [05.057]	Micronucleus formation	Mouse bone marrow	Administered three times by intraperitoneal injection at 24-hour intervals	40, 80, 120 or 160 mg/kg	Inconclusive	(NTP, 2003)	Valid. Administered three times 24 hrs intervals. Bone marrow studied at 24 hrs after the last dosing. A very weak positive response was observed at the highest dose level in conjunction with a slight decrease in PCE/NCE ratio. Technically the study is not flawed. The test was not repeated. Despite the presence of a significant positive trend, NTP decided that the study was inconclusive.
		Mouse	Administered by gavage for 14 weeks	7.5, 15, 30, 60 or 120 mg/kg	Negative		Limited validity. Administered by gavage for 14 weeks. No increase in MN-NCEs was observed. PCE/ NCE ratios were not affected either. The study is of limited validity, due to shortcomings n the experimental protocol (no-standard assay).
		Rat bone marrow	Administered as a single i.p. injection	50, 100, 150 or 200 mg/kg	Inconclusive		Valid. Administered once. Bone marrow studied at 24 hrs post dosing. A very weak non-significant positive response was observed at the highest dose level but no decrease in PCE/NCE ratio. Technically the study is not flawed. The test was not repeated. Despite the presence of a significant positive trend, NTP decided that the study was inconclusive.

(a): 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 and/or inappropriate test system).
Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).
i.p. Intraperitoneal.

Table 8: Additional Genotoxicity Data (*in vitro*) Considered by the Panel in FGE.203Rev1

Register name [FL-no]	Test System	Test Object	Concentration	Result	Reference	Comments
Deca-2(trans),4(trans)-dienal [05.140]	Reverse Mutation	*S. typhimurium* TA1535, TA97	0.3, 1.0, 3.0, 10.0, 16.0, 33.0, 100.0, 166.0, 333.0 and 666.0 µg/plate [1,2]	Negative	NTP, 2011	Valid. The test was performed in two testing centres. Study design complies with OECD Guideline 471, and GLP principles The highest concentration tested is limited by the toxicity.
		S. typhimurium TA98, TA100	0.3, 1.0, 3.0, 10.0, 16.0, 33.0, 100.0, 333.0 and 1000.0 µg/plate [1,2]	Negative		
		S. typhimurium TA100, TA102, TA104, TA1535, TA97, TA98	0.1, 0.3, 1.0, 3.0, 10.0, 33.0 and 100.0 µg/plate [1,2]	Negative		

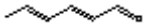
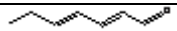
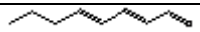
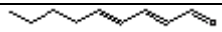
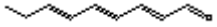
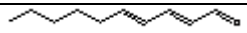
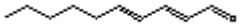
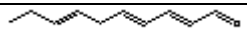
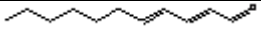
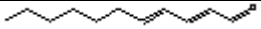
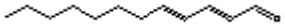
[1] With and without S-9 metabolic activation
[2] Pre-incubation method

Table 9: Additional Genotoxicity Data (*in vivo*) Considered by the Panel in FGE.203Rev1

Register name [FL-no]	Test System *in vivo*	Test Object	Route	Dose	Result	Reference	Comments
Deca-2(trans),4(trans)-dienal [05.140]	Micronucleus Induction	Male rat bone marrow polychromatic erythrocytes	i.p.	100, 200, 400 and 600 mg/kg bw	Positive[(a)]	NTP, 2011	Study design complies with OECD Guideline 474.
		Male mouse bone marrow polychromatic erythrocytes	i.p.	25, 50, 100 and 200 mg/kg bw	Equivocal[(b)]		A trend of increase but not statistically significant. Study design complies with OECD Guideline 474.
		Male mouse bone marrow polychromatic erythrocytes	i.p.	400 and 600 mg/kg bw	Positive[(a)]		Significant increase only at the highest dose. Study design complies with OECD Guideline 474.
		Male mouse peripheral blood polychromatic erythrocytes	i.p.	400 and 600 mg/kg bw	Negative[(a)]		No statistically significant increase of micronucleated cells was observed. Study design complies with OECD Guideline 474.
		Mouse peripheral blood reticulocytes	gavage	50, 100, 200, 400 and 800 mg/kg bw/day	Negative[(c)]		No statistically significant increase of micronucleated cells was observed. Study design complies with OECD Guideline 474.

(a): Administered as a single intraperitoneal injection.
(b): Administered 3x by intraperitoneal injection at 24-hour intervals.
(c): Administered by gavage for a period of 14 weeks.

Table 10: (Q)SAR Predictions on Mutagenicity for 13 Aldehydes from Subgroup 1.1.4

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]
05.101 1173	Penta-2,4-dienal		POS	OD	OD	NEG	NEG
05.057 1175	Hexa-2(trans),4(trans)-dienal		POS	POS	OD	NEG	NEG
05.084 1179	Hepta-2,4-dienal		POS	EQU	OD	NEG	NEG
05.127 1181	Octa-2(trans),4(trans)-dienal		POS	EQU	OD	NEG	NEG
05.071 1185	Nona-2,4-dienal		POS	EQU	OD	NEG	NEG
05.173	Nona-2,4,6-trienal		NEG	EQU	OD	NEG	NEG
05.081	2,4-Decadienal		POS	NEG	OD	NEG	NEG
05.140 1190	Deca-2(trans),4(trans)-dienal		POS	NEG	OD	NEG	NEG
05.141	Deca-2,4,7-trienal		NEG	EQU	OD	NEG	NEG
05.108 1195	Undeca-2,4-dienal		POS	EQU	OD	NEG	NEG
05.196	tr-2, tr-4-Undecadienal		POS	EQU	OD	NEG	NEG
05.125 1196?	Dodeca-2,4-dienal		POS	EQU	OD	NEG	NEG
05.064 1198	Trideca-2(trans),4(cis),7(cis)-trienal		NEG	EQU	OD	NEG	NEG

(a): Structure group 1.1.4: α,β-Unsaturated aliphatic aldehydes with one or more conjugated doublebonds
(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 Lymphona 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).

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ABBREVIATIONS

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
dGuo	2-deoxyguanosine
DNA	Deoxyribonucleic acid
EFFA	European Flavour Association
EFSA	The European Food Safety Authority
ENDOIII	Endonuclease III
EU	European Union
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
FPG	Formamidopyrimidine DNA Glycosylase
GLP	Good Laboratory Practice
GSH	Glutathione
GSSG	oxidised glutathione
HPLC	High-Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
ID	Identity
IOFI	International Organization of the Flavor Industry
i.p.	intraperitoneal
IR	Infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MN	Micronuclei

MNBN	MicroNucleated BiNucleate cells
MS	Mass spectra
MSDI	Maximised Survey-derived Daily Intake
NAC	N-acetylcysteine
NER	Nucleotide Excision Repair
NMR	Nuclear Magnetic Resonance
No	Number
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development
PCE	Polychromatic Erythrocytes
(Q)SAR	(Quantitative) Structure Activity Relationship
ROS	Reactive Oxygen Species
SCF	Scientific Committee on Food
SOD	Superoxide Dismutase
WHO	World Health Organisation