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Scientific Opinion on Flavouring Group Evaluation 217 Revision 2 (FGE.217Rev2), consideration of genotoxic potential for α,β-unsaturated ketones and precursors from chemical subgroup 4.1 of FGE.19: lactones

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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 12 flavouring substances from subgroup 4.1 of FGE.19 in the Flavouring Group Evaluation 217 (FGE.217). Based on experimental data, in previous versions of this FGE (FGE.217 and FGE217Rev1), for 6-methylcoumarin [FL-no: 13.012] and 5-ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [FL-no: 10.023] the concern for genotoxicity was ruled out. 6-Methylcoumarin was evaluated using the Procedure in FGE.80Rev1. For 5-ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [FL-no: 10.023] and the structurally related substance 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one [FL-no: 10.030], no further EFSA considerations were needed because these substances were evaluated by JECFA before 2000. Also based on experimental data, in FGE217Rev1, the concern for genotoxicity could not be ruled out for furan-2(5*H*)-one [FL-no: 10.066] and 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042], which later substance represents the following flavourings: [FL-no: 10.034, 10.036, 10.043, 10.046, 10.054, 10.057, 10.060 and 10.170]. In the current revision of this FGE (FGE217Rev2), based on the results of additional genotoxicity studies, the FAF Panel concluded that [FL-no: 10.066] is genotoxic *in vivo*. Therefore, furan-2(5*H*)-one [FL-no: 10.066] cannot be evaluated according to the Procedure. For [FL-no: 10.042] in order to rule out a concern for clastogenicity at site of first contact, the FAF Panel requests results from an *in vivo* comet assay in duodenum. In addition, [FL-no: 10.042] has also been identified as an aneugenic substance *in vitro*. Until the concern for clastogenicity at site of first contact for [FL-no: 10.042] and the concern for aneugenicity can be ruled out, this substance and [FL-no: 10.034, 10.036, 10.043, 10.046, 10.054, 10.057, 10.060 and 10.170] cannot be evaluated through the Procedure.

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1. Introduction

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

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

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

On 4 July 2013, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids adopted an opinion on Flavouring Group Evaluation 217, Revision 1 (FGE.217Rev1). Consideration of genotoxic potential for α,β-unsaturated ketones and precursors from chemical subgroup 4.1 of FGE.19: Lactones.[4]

The Panel concluded that, for the representative substances, 3,4-dimethyl-5-pentylidenefuran-2 (5H)-one [FL-no: 10.042] and furan-2(5H)-one [FL-no: 10.066], the test results from the studies in mammalian test systems raise concern with respect to genotoxicity *in vitro* and accordingly, these two substances [FL-no: 10.042 and 10.066] and seven substances [FL-no: 10.034, 10.036, 10.043, 10.046,10.054, 10.057 and 10.060] for which these two substances were representatives cannot be evaluated using the Procedure until additional *in vivo* genotoxicity data will become available. According to the recommendations of EFSA Scientific Committee (EFSA Scientific Committee, 2011), a combined micronucleus and Comet assay should be considered. The Comet assay should be performed at least in the liver.

The applicant has submitted additional data in response to this EFSA evaluation.

In addition, the re-evaluation of the flavouring substance [FL-no: 10.170] from FGE.10Rev3, which is not a single substance but a mixture containing 33% of [FL-no: 10.054] and which is currently on hold until additional genotoxicity data for [FL-no: 10.054] has been submitted, can resume as soon as the evaluation of the additional genotoxicity data submitted on the above-mentioned substances from FGE.217 (FGE.19, subgroup 4.1) has been finalised.

1.1.1. Terms of Reference

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

1.1.2. Interpretation of the Terms of Reference

In the most recent requests from the European Commission related to the evaluation of genotoxicity data submitted for the substances belonging to FGE.19, if a genotoxicity concern is identified, the Panel is requested to include information on the natural occurrence in food and on the exposure of the substance of concern. In line with these most recent EC requests the Panel has reported information on occurrence in food and on exposure (Appendix G).

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

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

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

[4] EFSA Journal 2013; 11(7): 3304.

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

The Panel took note of the (Q)SAR predictions by using two ISS Local Models (Benigni and Netzeva, 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 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 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 judgment was applied. In each subgroup, the representative substances were selected taking into account chain length, chain branching, lipophilicity and 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. Presentation of the substances in flavouring group evaluation 217

The FGE.217 concerns 12 substances, which are presented in Appendix A, Table A.1. These 12 substances correspond to subgroup 4.1 of FGE.19 (EFSA, 2008b). Ten of the substances are α,β-unsaturated lactones [FL-no: 10.023, 10.030, 10.034, 10.036, 10.042, 10.046, 10.054, 10.060, 10.066 and 13.012], which by hydrolysis and oxidation give rise to α,β-unsaturated ketones, and two substances [FL-no: 10.043 and 10.057] are precursors for the two α,β-unsaturated ketones 2,7-dimethyl-4-oxo-oct-5,7-dienoic acid and 3-methyl-6-(1-carboxyethyl)-2-cyclohexen-1-one, respectively. Of these 12 substances, 6-methylcoumarin [FL-no: 13.012] is the only substance in which the double bond in the α,β-position is conjugated with an aromatic ring.

Ten of the substances [FL-no: 10.023, 10.030, 10.034, 10.036, 10.042, 10.054, 10.057, 10.066, 10.170 and 13.012] have been evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). A summary of their current evaluation status by JECFA is given in Appendix B, Table B.1 (JECFA, 1998, 2004, 2008, 2011, 2016b).

In FGE.10 Revision 3 (EFSA CEF Panel, 2012), the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF Panel) noted that 5-pentyl-3*H*-furan-2-one [FL-no: 10.170] is a mixture of two structural isomers: 2/3 is the named compound (5-pentyl-3*H*-furan-2-one) and 1/3 is the structural isomer 5-pentyl-5*H*-furan-2-one. This latter isomer is identical to [FL-no: 10.054], which is an alpha,beta-unsaturated alcohol (after hydrolysis of the lactone), allocated to subgroup 4.1 of FGE.19 (FGE.217). The CEF Panel concluded that 5-pentyl-3*H*-furan-2-one [FL-no: 10.170] should not be evaluated through the Procedure until the additional genotoxicity data for [FL-no: 10.054] are available. Therefore, the evaluation of the potential genotoxicity of 5-pentyl-3*H*-furan-2-one [FL-no: 10.170] is included in the present revision of FGE.217 (FGE.217Rev2).

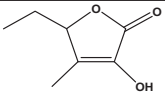
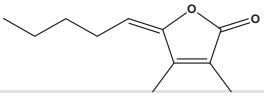
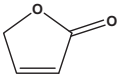
The Panel has also taken into consideration the outcome of the predictions from five selected (Q) SAR models (Benigni & Netzeva, 2007a; Gry et al., 2007; Nikolov et al., 2007) on 10 of the 12 lactones [FL-no: 10.023, 10.030, 10.034, 10.036, 10.042, 10.046, 10.054, 10.060, 10.066 and 13.012] and the two α,β-unsaturated ketones (2,7-dimethyl-4-oxo-oct-5,7-dienoic acid and 3-methyl-6-(1-carboxyethyl)-2-cyclohexen-1-one – both non-Register substances) anticipated to be metabolism products formed from the two remaining lactones [FL-no: 10.043 and 10.057]. The 10 lactones and the two ketones and their (Q)SAR predictions are shown in Appendix C, Table C.1.

2.3. History of the evaluation of the substances in subgroup 4.1

In the first scientific opinion on FGE.217 (EFSA CEF Panel, 2009), the CEF Panel concluded that additional genotoxicity data were required for 11 of the 12 α,β-unsaturated lactones considered in the FGE. For one substance, 6-methylcoumarin [FL-no: 13.012], the concern for genotoxicity could be ruled out and accordingly the substance could be evaluated using the Procedure in FGE.80Rev1. As 6-methylcoumarin is the only substance in FGE.217 with the α,β-ketone grouping in conjugation with an aromatic ring, the genotoxicity data for this substance could not be used for reading across to any of the remaining α,β-unsaturated lactones in this subgroup.

In the EFSA opinion 'List of α,β-unsaturated aldehydes and ketones representative of FGE.19 substances for genotoxicity testing' (EFSA, 2008c), three representative flavouring substances have been selected (Table 1) for the remaining 11 substances of FGE.19, subgroup 4.1, corresponding to FGE.217. 5-Ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [FL-no: 10.023] is a representative for the structurally related substance 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one [FL-no: 10.030], furan-2(5*H*)-one [FL-no 10.066] is considered as a stand-alone substance, while 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no 10.042] is representative of the remaining eight substances [FL-no: 10.034, 10.036, 10.043, 10.046, 10.054, 10.057, 10.060 and 10.170].

Table 1: Representative substances selected by EFSA for FGE.19 Subgroup 4.1 (EFSA, 2008c)

FL-no JECFA-no	EU Register name	Structural formula
10.023 222	5-Ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one	
10.042 2002	3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one	
10.066 2000	Furan-2(5*H*)-one	

FL-no: FLAVIS number; FLAVIS: Flavour Information System (database); JECFA: the Joint FAO/WHO Expert Committee on Food Additives.

The CEF Panel reviewed the previous JECFA evaluations (JECFA, 1998, 2004) (Appendix B, Table B.1) and in FGE.217 (EFSA CEF Panel, 2009) reached the conclusions based on the data available at that time. These included a (Q)SAR prediction analysis (Appendix C, Table C.1), a

carcinogenicity study on 6-methylcoumarin [FL-no: 13.012], four *in vitro* studies and three *in vivo* studies on 6-methylcoumarin [FL-no: 13.012] (Appendix D, Tables D.1, D.2 and D.3).

In FGE.217, the CEF Panel concluded that the data available do not indicate a genotoxic or carcinogenic potential for 6-methylcoumarin [FL-no: 13.012]. However, 6-methylcoumarin is the only substance in FGE.217 with the α,β-ketone grouping in conjugation with an aromatic ring; therefore, this substance would not be considered a representative for the remaining α,β-unsaturated lactones in this group.

Based on the data previously available, a genotoxic potential of the remaining 11 substances in FGE.217 [FL-no: 10.023, 10.030, 10.034, 10.036, 10.042, 10.043, 10.046, 10.054, 10.057, 10.060 and 10.066] could not be excluded. Therefore, the CEF Panel concluded that additional data on genotoxicity for representative substances of this subgroup should be provided according to the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19 (EFSA, 2008b).

A revision of FGE.217 (FGE.217Rev1) was prepared due to additional data submitted by industry (IOFI, 2012a,b) for the three representative substances 5-ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [FL-no: 10.023], dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] and furan-2(5*H*)-one [FL-no: 10.066]. Based on the new data, the CEF Panel concluded that the genotoxicity concern could be ruled out for [FL-no: 10.023] and the one structurally related substance [FL-no: 10.030] for which it is a representative. Since these two substances were evaluated by JECFA before the year 2000 (JECFA, 1998), there is no need for EFSA to further evaluate these substances following the Procedure (see also Appendix B, Table B.1) For the representative substances 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] and furan-2(5*H*)-one [FL-no: 10.066], the concern for genotoxicity could not be ruled out and a combined micronucleus and comet assay was requested for these two substances, covering the remaining seven substances [FL-no: 10.034, 10.036, 10.043, 10.046, 10.054, 10.057 and 10.060] (EFSA CEF Panel, 2013).

FGE	Adopted by EFSA	Link	No. of substances
FGE.217	24 May 2012	http://www.efsa.europa.eu/en/efsajournal/pub/1068	12
FGE.217Rev1	4 July 2013	https://www.efsa.europa.eu/en/efsajournal/pub/3304	12
FGE.217Rev2	11 December 2018	http://www.efsa.europa.eu/en/efsajournal/pub/5568	10

FGE: Flavouring Group Evaluation.

The requested data have been provided by industry for the two representative substances, 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] and furan-2(5*H*)-one [FL-no: 10.066] that include two combined *in vivo* micronucleus and comet assays (Covance, 2014a,b, 2015) and four *in vitro* micronucleus assays (Covance, 2013a,b; BioReliance, 2018a,b). These data are evaluated in the present revision 2 of FGE.217 (FGE.217Rev2).

Sections 2.4 and 2.5 of this opinion report the same information that was presented in FGE.217 and FGE.217Rev1, respectively. Section 3 reports the evaluation of the new data submitted by industry.

2.4. Data evaluated by the CEF Panel in FGE.217[5]

2.4.1. (Q)SAR predictions

In Appendix C (Table C.1), 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 in Chinese hamster ovary cells (CHO), chromosomal aberration test in Chinese hamster lung cells (CHL) and mouse lymphoma test) are presented.

For all of the substances, the (Q)SAR models predict negative or out of domain results for the Ames test system except for one positive prediction for 6-methylcoumarin [FL-no: 13.012].

For the predictions in the mouse lymphoma test and the chromosomal aberration test in CHO and CHL, the results are inhomogeneous (in most cases, either negative, out of domain or equivocal). The only positive predictions are seen in the mouse lymphoma test for the furan-2(5*H*)-one [FL-no: 10.066] and in the chromosomal aberration test for hex-2-eno-1,4-lactone [FL-no: 10.046].

[5] The data presented in Section 2.4 are cited from the first version of FGE.217. These data are the basis for the conclusions in FGE.217 requesting additional genotoxicity data.

2.4.2. Carcinogenicity studies

Groups of 25 male and 25 female weanling Osborne–Mendel rats were fed diets containing 0, 500, 1,000, 3,500, 5,000, 7,500 or 15,000 mg/kg body weight (bw)/day 6-methylcoumarin [FL-no: 13.012] for 2 years, corresponding to 0, 25, 50, 175, 250, 375 or 750 mg 6-methylcoumarin/kg bw per day.

Growth depression was observed in males at 375 mg 6-methylcoumarin/kg bw per day (moderate effect) and at 750 mg/kg bw per day (severe effect) paralleled by decreased food intake. In the liver, slight fatty metamorphosis and very slight bile duct proliferation was observed at the highest dose level. In addition, moderate testicular atrophy was seen in the high-dose males, presumably due to the severe growth depression. No other toxicological effects, including carcinogenicity, were seen. The CEF Panel noted that in parallel studies the same research group was able to clearly demonstrate the liver carcinogenicity of safrole after dietary administration to rats (Hagan et al., 1967).

The CEF Panel also noted that this study was performed before OECD test guidelines 451/453 (1981) were established and that it does not meet the criteria of these OECD test guidelines with respect to the number of animals. However, the CEF Panel agreed with the conclusion of the authors that 6- methylcoumarin was not carcinogenic in rats under the study conditions.

Study validation and results are presented in Appendix D, Table D.1.

2.4.3. Genotoxicity studies

In the subgroup 4.1, studies are available for one substance, 6-methylcoumarin [FL-no: 13.012], for which four *in vitro* and two *in vivo* studies have been evaluated.

6-Methylcoumarin was found negative in two valid Ames tests (Brusick, 1982; Haworth et al., 1983); equivocal results were obtained in a valid study with strain TA100 (Wild et al., 1983). It was found negative in a valid mouse lymphoma Tk assay (Cifone, 1982). Furthermore, it was found negative in the following three *in vivo* studies considered of limited validity: a *Drosophila melanogaster* sex-linked recessive lethal test (Wild et al., 1983), a mouse bone marrow micronucleus assay (Wild et al., 1983) and a mouse peripheral blood micronucleus 90-day assay reported by Witt et al. (2000).

Overall, the CEF Panel concluded that the data available do not indicate a genotoxic potential for 6-methylcoumarin.

For the remaining 11 substances in FGE.217, no genotoxicity studies are available. Therefore, the genotoxic potential of these substances cannot be evaluated.

Study validation and results are presented in Appendix D, Tables D.2 and D.3.

2.4.4. Conclusion on genotoxicity and carcinogenicity

The data available do not indicate a genotoxic or carcinogenic potential for 6-methylcoumarin. As the alpha,beta-unsaturated lactone 6-methylcoumarin is the only substance in this FGE with the alpha,beta-ketone grouping in conjugation with an aromatic ring, this substance would not be considered a representative for the remaining lactones in this group. The genotoxic potential of the other substances in this FGE cannot be evaluated.

2.4.5. Conclusions for FGE.217

6-Methylcoumarin [FL-no: 13.012] is not considered genotoxic and will therefore be allocated to FGE.80Rev1 for evaluation through the Procedure.

Based on the data available, a genotoxic potential of the remaining substances in the present FGE [FL-no: 10.023, 10.030, 10.034, 10.036, 10.042, 10.043, 10.046, 10.054, 10.057, 10.060 and 10.066] cannot be excluded. Therefore, the CEF Panel concluded that they presently cannot be evaluated through the Procedure. Additional data on genotoxicity for representative substances of this subgroup should be provided according to the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19 (EFSA, 2008b).

2.5. Additional genotoxicity data evaluated by the CEF Panel in FGE.217Rev1[6]

Based on Panel request described in Section 2.4, additional data were provided by Industry (IOFI, 2012a,b) for the three representative substances, 5-ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [FL-no: 10.023], dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] and furan-2(5*H*)-one [FL-no: 10.066] (Table 2), as requested by EFSA. FGE.217, Revision 1 (FGE.217Rev1), includes the assessment of these additional genotoxicity data. The study types provided are shown below:

Table 2: Overview of Data Submitted for Subgroup 4.1 and evaluated in FGE.217Rev1

Test substance	Ames test	Micronucleus test
5-Ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [FL-no: 10.023]	Bowen (2011a)	Lloyd (2011)
3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042]	Bowen (2011b)	Whitwell (2012a)
Furan-2(5*H*)-one [FL-no: 10.066]	Bowen (2011c)	Whitwell (2012b)

FL-no: FLAVIS number; FGE: Flavouring Group Evaluation.

2.5.1. *In Vitro* data

2.5.1.1. Bacterial reverse mutation assay

5-Ethyl-3-hydroxy-4-methylfuran-2(5H)-one [FL-no: 10.023]

5-Ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [FL-no: 10.023] was tested for mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *Salmonella* Typhimurium, both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S9-mix), in two separate experiments. An initial toxicity range-finding experiment was carried out in the absence and in the presence of the S9-mix in strain TA100 (Bowen, 2011a).

In experiment 1, treatments were performed in all tester strains in the absence and in the presence of S9-mix, at concentrations of 1.6, 8, 40, 200, 1,000 and 5,000 μg/plate. Following these treatments, evidence of toxicity was observed in strain TA1537 in the presence of S9-mix at 5,000 μg/plate and in strain TA102 in the presence of S9-mix at 200 μg/plate and above. Further evidence of toxicity in the form of a reduction in revertant numbers was observed in strain TA1535 in the presence of S9-mix and in strain TA102 in the absence of S9-mix at 5,000 μg/plate.

In experiment 2, treatments were performed in all the tester strains in the absence and in the presence of S9-mix, using more narrow concentration intervals covering the range 156.3–5,000 μg/plate. In addition, all treatments in the presence of S9-mix were further modified by the inclusion of a pre-incubation step. The maximum test concentration of 5,000 μg/plate was retained for all strains. Following these treatments, evidence of toxicity was observed in the presence of S9-mix in strains TA1537 and TA102 at 2,500 μg/plate and above. Further evidence of toxicity in the form of a reduction in revertant numbers was observed in strain TA98 in the presence of S9-mix at 5,000 μg/plate and in strains TA98 and TA102 in the absence of S9-mix at 5,000 and 2,500 μg/plate, respectively.

No statistically significant increases in revertant numbers were observed following 5-ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one treatments in any of the test strains, either in the absence or presence of S9-mix, in either experiment.

The CEF Panel concluded that 5-ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [FL-no: 10.023] did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of S. Typhimurium when tested under the conditions of this study. These conditions included treatments at concentrations up to 5,000 μg/plate, in the absence and in the presence of a rat liver metabolic activation system (S9-mix).

3,4-Dimethyl-5-pentylidenefuran-2(5H)-one [FL-no: 10.042]

3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] was tested for mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *S.* Typhimurium, both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver

[6] The data presented in Section 2.5 are cited from the Scientific Opinion FGE.217Rev1. These data are the basis for the conclusions in FGE.217 requesting additional genotoxicity data.

post-mitochondrial fraction (S9-mix), in two separate experiments and a third experiment performed in TA1537 (Bowen, 2011b).

In experiment 1, treatments were performed in all tester strains in the absence and in the presence of S9-mix, at concentrations of 0.32, 1.6, 8, 40, 200, 1,000 and 5,000 μg/plate. Following these treatments, evidence of toxicity was observed in all strains at the highest, second highest, and/or third highest concentrations in both the presence and absence of S9-mix metabolic activation.

In experiment 2, treatments were performed in all the tester strains in the absence and in the presence of S9-mix, using more narrow concentration intervals. For strains TA98, TA1535 and TA102, the range in both the absence and presence of S9-mix was 78.13–5,000 μg/plate. For strain TA100, the concentration ranges were 78.13–5,000 μg/plate in the presence of S9-mix and 19.53–1,250 μg/plate in the absence of S9-mix. For strain TA1537, the concentration ranges were 9.76–1,250 μg/plate in the absence of S9-mix and 78.13–5,000 μg/plate in the presence of S9-mix. In this experiment, all treatments done in the presence of S9-mix utilised a pre-incubation step. After incubation, evidence of toxicity was observed for all strains at 312.5 or 625 μg/plate and higher, except for strain TA102 in the presence of S9-mix where the toxicity was only observed at 1,250 μg/plate and above. No increases in revertant numbers were observed in any strains in the presence or absence of S9-mix. For strain TA1537, there were too few non-toxic concentrations to fully assess the mutagenic potential in the presence of S9-mix. Therefore, a third experiment in the presence of S9-mix was carried out using the pre-incubation methodology at a concentration range of 19.53–1,250 μg/plate. Evidence of toxicity was observed at 156.3 μg/plate and above. Thus, the study design complied with current recommendations from OECD Test Guideline 471 (OECD, 1997a). No statistically significant increases in revertant numbers were observed.

The CEF Panel concluded that 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of S. Typhimurium when tested under the conditions of this study. These conditions included treatments up to toxic concentrations, in the absence and in the presence of a rat liver metabolic activation system (S9-mix).

Furan-2(5H)-one [FL-no: 10.066]

Furan-2(5*H*)-one [FL-no: 10.066] was tested for mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *S.* Typhimurium, both in the absence and in the presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S9-mix), in two separate experiments (Bowen, 2011c).

In experiment 1, treatments were performed in all tester strains in the absence and in the presence of S9-mix, at concentrations of 0.32, 1.6, 8, 40, 200, 1,000 and 5,000 μg/plate. Following these treatments, evidence of toxicity was observed in all strains at 5,000 μg/plate with the exception of TA100 in the presence of S9-mix activation and TA1535 in the absence of S9-mix. No increases in revertant numbers were observed in any strains in the presence or absence of S9-mix.

In experiment 2, treatments were performed in all the tester strains in the absence and in the presence of S9-mix, using a narrower concentration range of 156.3–5,000 μg/plate. In this experiment, all treatments were done in the presence of S9-mix utilised a pre-incubation step. Evidence of toxicity was observed for all strains in the presence and absence of S9-mix at 2,500 and/or 5,000 μg/plate. Thus, the study design complied with current recommendations from OECD Test Guideline 471 (OECD, 1997a). No increases in revertant numbers were observed in any strains in the presence or absence of S9-mix.

The CEF Panel concluded that furan-2(5*H*)-one [FL-no: 10.066] did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *S.* Typhimurium when tested under the conditions of this study. These conditions included treatments up to toxic concentrations, in the absence and in the presence of a rat liver metabolic activation system (S9-mix).

2.5.1.2. Micronucleus assay

5-Ethyl-3-hydroxy-4-methylfuran-2(5H)-one [FL-no: 10.023]

5-Ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [FL-no: 10.023] was tested for the induction of chromosome damage and potential aneugenic effects in an *in vitro* micronucleus assay using duplicate human peripheral blood lymphocytes prepared from pooled blood from two healthy male volunteers in a single experiment. Treatments were performed both in the absence and presence of Aroclor 1254 induced rat liver S9-mix (Lloyd, 2011).

Treatment with 5-ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one was conducted 48 h after culture initiation (stimulation by phytohaemagglutinin (PHA)).

A preliminary toxicity range-finding experiment was conducted with and without S9-mix for 3 h treatment and 21 h of recovery (3 + 21 h) and without S9-mix for 24 h treatment. Toxicity was evaluated as the effect of treatment on the replication index (RI). Ten concentrations from 14.33 to 1,422 μg/mL were tested. The concentrations selected for the main experiments were based on toxicity data from this preliminary test.

5-Ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one was tested at concentrations 1,000, 1,200 and 1,422 μg/mL (equivalent to 10 mM), in the absence and presence of S9-mix, resulting in frequencies of micronucleated binucleate cells (MNBN), which were similar to those observed in concurrent vehicle controls for all concentrations analysed, and fell within historical vehicle control (normal) ranges (Lloyd, 2011). The above treatment concentrations induced maximum cytotoxicity (reduction in replication index) of 10% in the absence of S9-mix activation and 23% in the presence of S9-mix activation. Thus, the study design complies with current recommendations (including OECD Test Guideline 487 (OECD, 2010)). No increases in MNBN cells were observed following continuous 24 h treatment in the absence of S9-mix at concentrations of 500, 750 and 900 μg/mL, the top concentration inducing 53% cytotoxicity. These data indicated the absence of induction of MNBN cells as a result of treatment with 5-ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one at concentrations either reaching 10 mM or inducing 50–60% toxicity.

The CEF Panel concluded that 5-ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [FL-no: 10.023] does not induce micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence or in the presence of S9-mix. All values were within historical vehicle control ranges in all parts of the study and were not significantly different from concurrent controls.

3,4-Dimethyl-5-pentylidenefuran-2(5H)-one [FL-no: 10.042]

3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] was tested for the induction of chromosome damage and potential aneugenic effects in an *in vitro* micronucleus assay using duplicate human peripheral blood lymphocytes prepared from pooled blood from two healthy female volunteers in a single experiment. Treatments were performed both in the absence and presence of Aroclor 1254 induced rat liver S9-mix (Whitwell, 2012a).

A preliminary toxicity range-finding experiment was conducted with and without S9-mix for 3 h treatment followed by 21 h recovery period and without S9-mix for 24 h treatment. Toxicity was evaluated as the effect of treatment on the RI. Twelve concentrations from 7.256 to 2,000 μg/mL were tested. The concentrations selected for the main experiments were based on toxicity data from this preliminary test (Whitwell, 2012a).

Cells were stimulated for 48 h with PHA to produce exponentially growing cells, and then treated for 3 h (followed by 21 h recovery) with 0, 40, 60, 70 and 90 μg/mL of 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one in the absence of S9-mix and 0, 60, 90, 110 and 140 μg/mL in the presence of S9-mix. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 57% and 56%, respectively. In a parallel assay, cells were treated for 24 h with 0, 10, 13 and 15 μg/mL of 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one in the absence of S9-mix with no recovery period. The top concentration induced 57% cytotoxicity. There were two replicate cultures per treatment, and 1,000 binucleate cells per replicate were scored for micronuclei. Thus the study design complies with current recommendations (OECD Test Guideline 487 (OECD, 2010)).

Treatment of cells with 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one for 3 h with 21 h recovery period showed an increase in the frequency of MNBN cells at concentration levels of 70 and 90 μg/mL ($p \leq 0.05$) in the absence of S9-mix, but these were significantly below the 95% confidence interval of the normal control range (0.10–1.60%) and are not considered biologically relevant by the applicant. In the presence of S9-mix, treatment of cells with 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one for 3 + 21 h showed an increase in the frequency of MNBN cells at concentration levels of 60 ($p \leq 0.01$), 90, 110 and 140 μg/mL ($p \leq 0.001$). No significant increases in MNBN frequencies were observed at any concentration after treatment for 24 h with no recovery period. It was concluded that 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] induced micronuclei when assayed in cultured human peripheral lymphocytes for 3 + 21 h in the presence of S9-mix (Whitwell, 2012a).

Furan-2(5H)-one [FL-no: 10.066]

Furan-2(5*H*)-one [FL-no: 10.066] was tested for the induction of chromosome damage and potential aneugenic effects in an *in vitro* micronucleus assay using duplicate human peripheral blood

lymphocytes prepared from pooled blood from two healthy male volunteers in a single experiment. Treatments were performed both in the absence and presence of Aroclor 1254 induced rat liver S9-mix (Whitwell, 2012b).

A preliminary toxicity range-finding experiment was conducted with and without S9-mix for 3 h treatment and 21 h recovery (3 + 21 h) and without S9-mix for 24 h treatment. Toxicity was evaluated as the effect of treatment on the RI. Twelve concentrations from 3.047 to 840 μg/mL were tested. The concentrations selected for the main experiments were based on toxicity data from this preliminary test (Whitwell, 2012b).

Cells were stimulated for 48 h with PHA to produce exponentially growing cells, and then treated for 3 + 21 h with 0, 200, 350, 425, 450 and 475 μg/mL furan-2(5*H*)-one in the absence of S9-mix and 0, 100, 250, 425, 450 and 475 μg/mL in the presence of S9-mix. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 53% and 51% respectively. In a parallel assay, cells were treated for 24 h with 0, 10, 50, 60, 67.5 and 72.5 μg/mL of furan-2(5*H*)-one in the absence of S9-mix with no recovery period. The top concentration induced 61% cytotoxicity. There were two replicate cultures per treatment, and 1,000 binucleate cells per replicate were scored for micronuclei. Thus the study design complies with current recommendations (OECD Test Guideline 487 (OECD, 2010)).

Treatment of cells with furan-2(5*H*)-one for 3 + 21 h showed an increase in the frequency of MNBN cells at a concentration of 450 μg/mL ($p \leq 0.05$) in the absence of S9-mix, but it was associated with 64% cytotoxicity to the cells and is not considered biologically relevant by the applicant. In the presence of S9-mix, treatment of cells with furan-2(5*H*)-one for 3 + 21 h showed an increase in the frequency of MNBN cells at the three top concentrations ($p \leq 0.001$), and all were significantly above the 95% confidence interval of the normal control range (0.10–1.10%). Treatment for 24 h with no recovery period showed an increase in MNBN frequencies at the top-dose only, but it was lower than the 95% confidence interval of the historical control range and was associated with high cytotoxicity (61%) by the applicant.

The CEF Panel concluded that furan-2(5*H*)-one induces micronuclei when assayed in cultured human peripheral lymphocytes for 3 + 21 h in the presence of S9-mix (Whitwell, 2012b).

The results of the additional *in vitro* studies are summarised in Appendix E, Table E.1.

2.5.2. Additional available data

In more recent literature, the only reference to the potential of furanone compounds to induce DNA damage has been reported in association with the reduction of trivalent copper in an *in vitro* DNA damage assay (Murakami et al., 2007). Of three furanone analogues tested, 2,5-furanone (furaneol, 4-hydroxy-2,5-dimethyl-furan-3-one [FL-no: 13.010] in FGE.220), 4,5-furanone (4,5-dimethyl-3-hydroxy-2(5*H*)-furanone; [FL-no: 10.030]) and cyclotene (2-hydroxy-3-methyl-2-cyclopenten-1-one [07.056] in FGE.213 – not a furanone), only the first produced 8-hydroxy-2′-deoxyguanosine in DNA and strand breaks. These were associated with the generation of reactive oxygen species (superoxide radical) through the reduction of trivalent cupric to divalent cuprous ions. In contrast, to 2,5-furanone, the 4,5-analogue [FL-no: 10.030], which is one of the 12 substances evaluated in this group, did not produce a similar effect. These observations indicate that genotoxicity associated with members of the nine substances in group 4.1 is likely to be indirect and mediated via oxidative stress.

2.5.3. Conclusion (FGE.217Rev.1)

The FGE.217 concerned 12 substances, corresponding to subgroup 4.1 of FGE.19. The 12 substances are α,β-unsaturated lactones [FL-no: 10.023, 10.030, 10.034, 10.036, 10.042, 10.043, 10.046, 10.054, 10.057, 10.060, 10.066 and 13.012], which by hydrolysis and oxidation gives rise to α, β-unsaturated ketones, which is a structural alert for genotoxicity.

In FGE.217, 6-methylcoumarin [FL-no: 13.012] was not considered genotoxic and was therefore allocated to FGE.80Rev1 for evaluation through the Procedure. For the remaining 11 substances, the CEF Panel concluded that based on the data available, a genotoxic potential could not be excluded and accordingly they could not be evaluated through the Procedure. Additional data on genotoxicity for three representative substances, 5-ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [FL-no: 10.023], 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] and furan-2(5*H*)-one [FL-no: 10.066], of this subgroup, should be provided according to the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19. The present revision of FGE.217 (FGE.217Rev1) deals with additional data submitted by the Industry in response to the EFSA request expressed in FGE.217.

In vitro data in bacteria and mammalian test systems have now been provided for the three representative substances [FL-no: 10.023, 10.042 and 10.066] selected by the EFSA.

The three representative substances 5-ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [FL-no: 10.023], 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] and furan-2(5*H*)-one [FL-no: 10.066] did not induce mutations in bacterial reverse mutation assays. In an *in vitro* micronucleus assay, 5-ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [FL-no: 10.023] also did not reveal genotoxic effects under all test conditions according to OECD Test Guideline 487 (OECD, 2010). The CEF Panel therefore concluded that the genotoxic concern could be ruled out for 5-ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [FL-no: 10.023] and accordingly this substance and the one structurally related substance, 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one [FL-no: 10.030] for which it is a representative, can be evaluated using the Procedure.[7]

In the *in vitro* micronucleus assay 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] was negative in the 24 + 0 h protocol, but equivocal results were obtained with 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] as well as for furan-2(5*H*)-one [FL-no: 10.066] in the 3 + 21 h protocol in the absence of the S9-mix. Furthermore, in the presence of the S9-mix these two substances unequivocally induced micronuclei.

The CEF Panel therefore concluded that 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] and furan-2(5*H*)-one [FL-no: 10.066] raise concern with respect to genotoxicity *in vitro* and accordingly, these two substances [FL-no: 10.042 and 10.066] and the seven substances [FL-no: 10.034, 10.036, 10.043, 10.046, 10.054, 10.057 and 10.060] of subgroup 4.1 for which these two substances were representatives cannot be evaluated using the Procedure until additional *in vivo* genotoxicity data will become available. According to the recommendations of EFSA Scientific Committee (EFSA Scientific Committee, 2011), a combined micronucleus and comet assay should be considered. The comet assay should be performed at least in the liver.

3. Assessment

3.1. Additional data evaluated by the Panel in FGE.217Rev.2

In response to the EFSA request for further genotoxicity data for the two representative substances of subgroup 4.1 (3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] and furan-2(5*H*)-one [FL-no: 10.066]), as described in FGE.217Rev1, the industry has submitted the requested studies: two combined bone marrow micronucleus and comet assays with liver analysis of the treated rats (Table 3 and Appendix F, Table F.1).

In the *in vivo* studies (received after the request in FGE.217Rev1), the exposure of the bone marrow had not been demonstrated; therefore the Working Group (WG) on Genotoxicity of the CEF Panel requested to provide evidence of bone marrow exposure to furan-2(5*H*)-one and 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one by plasma analysis as recommended in the OECD TG 474 (EFSA letter dated 13/1/2015). In the same letter, the WG requested to provide the statistical analysis on the raw data of the comet assay with furan-2(5*H*)-one as recommended in the OECD TG 489.

Following this request by the WG, a technical hearing was held with the applicant on 19 January 2016 (EFSA, 2016) to clarify the challenges observed by the applicant in performing the plasma analysis and demonstrating bone marrow exposure.

As follow-up of the technical hearing, the WG requested additional information on the plasma analysis already performed (EFSA letter dated 2/2/2016). After reviewing these data, the WG suggested to suspend the activities related to plasma analysis and requested to test both representative substances in an *in vitro* micronucleus assay with centromere analysis, in order to investigate the mechanism inducing MN *in vitro* (clastogenicity or aneugenicity) (EFSA letter dated 26/4/2016).

Following this request (EFSA letter dated 26/4/2016), a second technical hearing was held with the applicant on 24 January 2017 (EFSA, 2017) to clarify the applicant's proposal to test the representative substances with a new method instead of the *in vitro* micronucleus with centromere analysis.

Following the second technical hearing, the WG reiterated the request for an *in vitro* micronucleus assay with centromere analysis for investigating the mode of action of the representative substances (EFSA letter dated 10/4/2017). The applicant provided the requested data on 10 April 2018 (EFFA, 2018, see Documentation provided to EFSA n.17) that are listed in Table 3 and evaluated in the present revision of FGE.217 (FGE.217Rev2).

[7] JECFA have already evaluated [FL-no: 10.023 and 10.030] before the year 2000 (JECFA, 1998). Therefore, no further consideration of these two flavouring substances according to the Procedure is needed.

Since the clarification of the mechanism of action would allow to identify the most appropriate follow-up study, industry submitted an *in vitro* micronucleus assay in human lymphocytes and *in vitro* micronucleus assay in TK6 cells with kinetochores staining (CREST staining), for both the representative flavouring substances [FL-no: 10.042] and [FL-no: 10.066].

Table 3: List of genotoxicity studies evaluated in FGE.217Rev2

Test substance	Additional data submitted	Reference
3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042]	*In vivo* combined bone marrow micronucleus test and comet assay in liver	Covance (2014a)
	In vitro micronucleus assay in human peripheral blood lymphocytes	Covance (2013a)
	In vitro micronucleus assay with CREST staining in TK6 cells	BioReliance (2018a)
Furan-2(5*H*)-one [FL-no: 10.066]	*In vivo* combined bone marrow micronucleus test and comet assay in liver	Covance (2014b, 2015)
	In vitro micronucleus assay in human peripheral blood lymphocytes	Covance (2013b)
	In vitro micronucleus assay without CREST staining in TK6 cells	BioReliance (2018b)

3.2. Data on furan-2(5*H*)-one [FL-no: 10.066]

3.2.1. Furan-2(5*H*)-one [FL-no: 10.066] – combined bone marrow micronucleus test and comet assay

The genotoxic potential of furan-2(5*H*)-one [FL-no: 10.066] (purity > 98%) was assessed *in vivo* using the bone marrow micronucleus assay combined with the comet assay in liver of rats (Covance, 2014b, 2015). The micronucleus study was conducted in accordance with Good Laboratory Practice (GLP) and OECD TG 474 (OECD, 1997b). The comet study was conducted before the publication of the first relevant OECD test guideline (OECD TG 489, 2014b), but it was based on the guidance provided by the Comet Workshop (Tice et al., 2000; Hartmann et al., 2003), International Workshops on Genotoxicity Testing (Burlinson et al., 2007), the international validation of the *in vivo* comet assay by the Japanese Center for the Validation of Alternative Methods (JaCVAM) and literature available at that time (Hartmann et al., 2004; Smith et al., 2008).

In a dose range-finding assay, groups of three male and three female Han Wistar rats were given three administrations by gavage (at 0, 24 and 45 h) of furan-2(5*H*)-one, at 250, 350, 500 and 1,000 mg/kg bw per day. Mortality was observed at doses above 350 mg/kg bw per day. At 250 mg/kg bw per day, piloerection, hunched posture and/or staining around the mouth were observed in one male and all female animals on day 2 and all animals on day 3. Minor loss of body weight was recorded in females dosed at 250 mg/kg bw per day.

Based on this study, a Maximum Tolerated Dose (MTD) of 250 mg/kg bw per day was established. As no sex specific effects were seen, only male rats were used in the main study.

Groups of 6 male Han Wistar rats per dose group were administered doses by gavage of 0 (corn oil), 62.5, 125, or 250 mg/kg bw of furan-2(5*H*)-one on three consecutive days (0, 24 and 45 h). A positive control group of six male rats were given doses of 150 mg ethyl methanesulfonate (EMS)/kg bw at the same time intervals as the dosed groups.

After 48 h (i.e. 3 h after the final administration), bone marrow and liver were sampled from the same animals for micronucleus assay and comet assay respectively. Due to a procedural error, no bone marrow filtrate was collected from the first groups of animals, therefore additional groups of animals were treated and sampled for bone marrow analysis.

No clinical signs of toxicity were observed in any animal following treatments with furan-2(5*H*)-one, vehicle or the positive control (EMS). Reduced body weight gain and loss of body weight were observed in all groups dosed with furan-2(5*H*)-one.

Except small decreases in group mean aspartate aminotransferase observed in all furan-2(5*H*)-one treated groups, no other changes in clinical chemistry were observed.

On microscopic examination, in the liver of animals administered with the highest dose, decreased glycogen vacuolation was observed. In the duodenum, villous tip necrosis was observed in animals dosed at 250 mg/kg bw per day. In the macroscopic examination, duodenum was described as pale in the high-dose group.

Micronucleus assay

Bone marrow from the femurs was prepared for micronucleus scoring. A total of at least 500 polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) were scored to calculate the degree of bone marrow toxicity by the relative decrease in PCE. For MN analysis, 2,000 PCE per animal were scored for the presence of MN.

Rats treated with furan-2(5*H*)-one exhibited group mean frequencies of micronucleated polychromatic erythrocytes (MNPCE) that were similar to and not statistically significantly different from those observed in the concurrent vehicle control for all dose groups. The treatment with furan-2(5*H*)-one did not reduce the percentage of PCE, therefore no indication of bone marrow toxicity was observed.

Under the conditions of this test furan-2(5*H*)-one did not induce micronuclei in bone marrow of rats tested up to 250 mg/kg bw per day (MTD), by gavage. However, there was no evidence that bone marrow was exposed. Therefore, the Panel considered the results from this assay as inconclusive.

Comet assay

Liver cells were prepared for comet analysis. Tail moment and tail intensity (%) of a total of 150 cells per animal, split over three slides, were recorded.

No dose-related increase in %clouds was observed following treatment with furan-2(5*H*)-one demonstrating that treatment did not cause excessive DNA damage that could have interfered with comet analysis.

Group mean %tail intensity and tail moment values for animals treated with furan-2(5*H*)-one at 62.5 and 125 mg/kg bw per day were similar to the group mean vehicle control data. At the highest dose (250 mg/kg bw per day), a statistically significant increase (almost twofold increase) in tail intensity was observed compared to the vehicle control (4.50 $\pm$ 0.21 and 2.43 $\pm$ 0.40, respectively). Moreover, a statistically significant dose–response was observed.

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

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

The Panel considered that the third criterion ('any of the results are outside the distribution of the historical negative control data for a given species, vehicle, route, tissue, and number of administrations') mentioned in the OECD TG 489 was not applicable in this case because of the very wide range for historical negative controls reported (95% reference range for the vehicle control ranging from 0.02 to 11.39; 95% reference range for the positive control ranging from 7.15 to 65.07).

The response in the comet assay was consistent across all animals within the 250 mg/kg bw per day group, which supports its genotoxic potential expressed *in vitro* in the MN assay in human lymphocytes in the presence of metabolic activation.

Therefore, the Panel concluded that furan-2(5*H*)-one [FL-no: 10.066] is genotoxic in this *in vivo* comet assay in the liver of rats.

Study results are summarised in Appendix F, Table F.1.

3.2.2. Furan-2(5*H*)-one [FL-no: 10.066] – *in vitro* micronucleus test in human lymphocytes

Human peripheral blood lymphocytes, stimulated with PHA, were treated with furan-2(5*H*)-one [FL-no: 10.066] (purity > 98%) for 3 h with 21 h of recovery period (3 + 21 h) in the absence or presence of S9-mix or for 24 h (without recovery period) in the absence of S9-mix.

Furan-2(5*H*)-one [FL-no: 10.066] was tested in a cytotoxicity range-finding assay performed at concentrations ranging from 3.0 to 840.7 μg/mL for 3 + 21 h with and without S9-mix and 24 h without S9-mix. In the 24-h treatment, precipitation was observed at the highest concentration tested at the termination of the treatment period (Covance, 2013b). The *in vitro* micronucleus assay was carried out according to OECD TG 487 (OECD, 2010) and following GLP principles.

Based on the cytotoxicity range-finding results, duplicate cultures of lymphocytes were treated with the test article 48 h after culture initiation at concentrations ranging from 50 to 600 μg/mL for 3 h with and without S9-mix and at concentrations ranging from 5 to 100 μg/mL for the 24-h treatment.

Cytochalasin B (final concentration of 6 μg/mL) was added to each culture after the 3-h treatment period, while in the 24-h treatment cultures were treated with the test article in the presence of cytochalasin B.

Appropriate vehicle (DMSO) and positive controls were used (mitomycin C and vinblastine (VB) in the absence of S9-mix, cyclophosphamide (CP) in the presence of S9-mix). All positive control compounds induced a statistically significant increase of micronuclei (MN) frequency and the system was considered sensitive and valid.

Two thousand cells were scored per concentration. Based on the level of cytotoxicity observed, four concentrations were selected for MN analysis in each experimental condition: (i) 10, 45, 60 and 65 μg/mL, 24 h treatment (3, 25, 43 and 57% cytotoxicity, respectively); (ii) 100, 200, 300 and 350 μg/mL, 3 + 21 h treatment without S9-mix (6, 25, 39 and 57% cytotoxicity, respectively); and (iii) 100, 300, 350 and 400 μg/mL, 3 + 21 h treatment with S9-mix (7, 35, 43 and 53% cytotoxicity, respectively).

The MN frequency observed in the treatments for 3 + 21 h and for 24 h in the absence of metabolic activation was similar to the concurrent vehicle control and in the range of the historical vehicle control.

Statistically significant increase in the frequency of micronuclei was observed after treatment with furan-2(5*H*)-one for 3 h in the presence of S9-mix, at the highest concentrations analysed (300, 350 and 400 μg/mL). The MNBN cell frequency of both treated cultures at 400 μg/mL exceeded the historical vehicle control range. A concentration-related increase of MNBN was observed.

The Panel noted that this study confirms the increase of MN observed in the short-term treatment in the presence of S9-mix in the *in vitro* micronucleus study (Whitwell, 2012b) evaluated in FGE.217Rev1. The Panel concluded that furan-2(5*H*)-one [FL-no: 10.066] induces chromosomal damage in the presence of metabolic activation.

3.2.3. Furan-2(5*H*)-one [FL-no: 10.066] – *in vitro* micronucleus test without CREST staining in TK6 cells

Furan-2(5*H*)-one (purity > 98%) was tested in an *in vitro* micronucleus assay in the human lymphoblastoid cell line TK6 cells (BioReliance, 2018b), with the purpose of evaluating the aneugenic and clastogenic potential of the tested substance. DMSO was used as the vehicle. The study was performed according to GLP and OECD TG 487 (OECD, 2014a).

TK6 cells were treated for 4 h with 23 h of recovery period (4 + 23 h) in the absence or presence of S9-mix (from Aroclor 1254-induced rats) or for 27 h (without recovery period) in the absence of S9-mix. Positive controls were: CP and VB.

In the preliminary cytotoxicity test, concentrations between 0.0841 and 840 μg/mL in DMSO were tested. Cytotoxicity (more than 50% decrease in relative population doubling (RPD) compared to the vehicle control) was observed at concentrations above 252 μg/mL for the 4 + 23 h treatment both in the absence and in the presence of S9-mix, and at concentrations above 84 μg/mL for the 27-h treatment in the absence of S9-mix.

Based on the results of the preliminary toxicity test the following concentrations were tested:

- for the 4 + 23 h treatment in the absence of metabolic activation 25, 75, 100, 125, 150, 175, 200, 225 μg/mL;
- for the 4 + 23 h treatment in the presence of metabolic activation 25, 75, 100, 125, 150, 175, 200, 225 μg/mL;
- for the 27-h treatment in the absence of metabolic activation 5, 25, 35, 45, 55, 65, 75, 85 μg/mL.

Each concentration was tested in duplicate cultures, for each culture 1,000 mononucleated cells were analysed for MN (a total of 2,000 cells per concentration). Due to cytotoxicity of approximately 50%, the highest concentrations evaluated for MN induction were 150 μg/mL (for the 4 + 23 h treatment both in the absence and in the presence of S9-mix) and 55 μg/mL (for the 27-h treatment in the absence S9-mix).

After the 4 + 23 h treatment, both in the absence of S9-mix (at 25, 125 and 150 μg/mL) and in the presence of S9-mix (at 25, 75 and 150 μg/mL) no statistically significant increase in MN was observed.

After the 27-h treatment, in the absence of S9-mix (at 5, 35, 55 μg/mL) no statistically significant increase in MN was observed.

The authors concluded that under the conditions of this study, furan-2(5*H*)-one did not induce micronuclei in TK6 cells.

The Panel noted that the negative results observed in this study in TK6 cells for all treatment conditions are in contrast with results obtained in two *in vitro* micronucleus studies in human peripheral blood lymphocytes. In these studies, furan-2(5*H*)-one increased the frequency of MN in the short-term treatment in the presence of S9-mix (Whitwell, 2012b; Covance, 2013b).

The data on furan-2(5*H*)-one [FL-no: 10.066] described above are discussed in section 3.4 and the Panel's conclusion is reported in section 3.5.

3.3. Data on 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042]

3.3.1. 3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] – combined bone marrow micronucleus test and comet assay

The genotoxic potential of 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] (purity > 95%) was assessed *in vivo* using the bone marrow micronucleus assay combined with the comet assay in liver of rats (Covance, 2014a). The micronucleus study was conducted in accordance with GLP and OECD TG 474 (OECD, 1997b). The comet study was conducted before the publication of the first relevant OECD test guideline (OECD, 2014b) but it was based on the guidance provided by the Comet Workshop (Tice et al., 2000; Hartmann et al., 2003), International Workshops on Genotoxicity Testing (Burlinson et al., 2007), the international validation of the *in vivo* comet assay by the JaCVAM and literature available at that time (Hartmann et al., 2004; Smith et al., 2008).

In a dose range-finding assay, groups of three male and three female Han Wistar rats were given three administrations by gavage (at 0, 24 and 45 h) of 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one, at 500, 700 and 1,000 mg/kg bw per day. At 1,000 mg/kg bw per day, piloerection, decreased activity and ataxia were observed on day 1. On the second day, the toxicity effects observed were more severe (including ataxia, abnormal breathing, rales and piloerection) and animals were killed in extremis.

At 700 mg/kg bw per day, clinical signs of toxicity were observed including piloerection, ataxia and decreased activity.

At 500 mg/kg bw per day, more moderate clinical signs of toxicity were observed (decreased activity and piloerection), no mortality was observed at the two lower doses tested.

Based on this study, a MTD of 500 mg/kg bw per day was established. As no sex-specific effects were seen, only male rats were used in the main study.

Groups of six male Han Wistar rats per dose group were administered doses by gavage of 0 (corn oil), 125, 250 or 500 mg/kg bw of 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one on three consecutive days (0, 24 and 45 h). A positive control group of six male rats were given doses of 150 mg EMS/kg bw at the same time intervals as the dosed groups.

During the experiment bone marrow and liver were sampled from the same animals for micronucleus assay and comet assay, respectively, but the comet analysis was invalidated and the experiment was repeated. Only results of the second analysis were reported in the study.

No clinical signs of toxicity were observed in any animal following treatments with 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one, vehicle or the positive control (EMS). A dose-related decrease in body weight gain was observed. Dose-related increases in aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase were observed. The authors of the study considered these changes as an early indicator of liver injury/stress. On microscopic examination, decreased glycogen hepatocellular vacuolation and hepatocyte vacuolation were observed in the liver of animals administered with the highest dose. There were no macroscopic findings related to administration of 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one.

Micronucleus assay

Bone marrow from the femurs was prepared for micronucleus scoring. 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. For MN analysis, 2,000 PCE per animal were scored for the presence of MN.

Rats treated with 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one exhibited group mean frequencies of MNPCE that were similar to and not statistically different from those observed in the concurrent vehicle control for all dose groups. The treatment with 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one did not reduce the percentage of PCE; therefore, no indication of bone marrow toxicity was observed.

Under the conditions of this study, 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one did not induce micronuclei in bone marrow of rats tested up to 500 mg/kg bw per day (MTD), by gavage.

Considerations on the micronucleus assay

In the *in vivo* micronucleus assay, 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] did not prove to induce micronuclei in bone marrow erythrocytes of male rats following gavage administration up to 500 mg/kg bw per day (an estimate of the MTD for this study).

Considering that the percentage of PCE in the bone marrow of treated animals was not changed compared to the concurrent vehicle control group, it was unclear whether bone marrow exposure had occurred and therefore additional evidence of systemic exposure through plasma analysis was requested to the applicant in order to conclude on the *in vivo* micronucleus assay.

However, in view of technical problems reported by the applicant with the plasma analysis, the Panel decided to waive its initial request for additional evidence of systemic exposure, and requested the applicant to investigate the mechanism of MN induction (clastogenicity or aneugenicity) through an *in vitro* micronucleus assay with centromere analysis. The clarification of the mechanism of genotoxicity would allow to interpret the already available *in vivo* data or to identify an appropriate *in vivo* follow-up study.

Comet assay

Liver cells were prepared for comet analysis. Tail moment and tail intensity (%) of a total of 150 cells per animal, split over three slides, were recorded.

No dose-related increase in %clouds was observed following treatment with 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one demonstrating that treatment did not cause excessive DNA damage that could have interfered with comet analysis.

No statistically significant increase in group mean tail intensity and tail moment values were observed in any test substance treatment group compared to the vehicle control treatment group.

Considerations on the Comet assay

Results of the comet assay in liver were negative. Based on increased activities of aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase, there is indication that the liver was exposed, therefore the Panel concluded that 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] did not induce primary DNA damage in the liver of rats after oral administration.

Study results are summarised in Appendix F, Table F.1.

3.3.2. 3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] – *in vitro* micronucleus test in human lymphocytes

Human peripheral blood lymphocytes, stimulated with PHA, were treated with 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] (purity > 95%) for 3 h with 21 h of recovery period (3 + 21 h) in the absence or presence of S9-mix or for 24 h in the absence of S9-mix (Covance, 2013a). The *in vitro* micronucleus assay was carried out according to OECD TG 487 (OECD, 2010) and GLP principles.

3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one was tested in a cytotoxicity range-finding assay performed at concentrations ranging from 6.5 to 1,803 μg/mL for 3 + 21 h with and without S9-mix and 24 h without S9-mix. For the 3 + 21 h treatment, precipitate was observed from 233.7 μg/mL already at the beginning of the treatment. For the 24-h treatment, precipitate was observed from 84.1 μg/mL already at the beginning of the treatment. At the termination of the treatment period, precipitate and cytotoxicity were observed at concentrations ≥ 1,000 μg/mL and ≥ 400 μg/mL, respectively, in all three treatment conditions.

Based on the dose range-finding results, duplicate cultures of lymphocytes were treated with the test article 48 h after culture initiation at concentrations ranging from 20 to 200 μg/mL for 3 h without S9-mix and from 10 to 140 μg/mL for 3 h with S9-mix; for the 24-h treatment, the concentrations tested ranged from 10 to 100 μg/mL.

Cytochalasin B (final concentration of 6 μg/mL) was added to each culture after the 3-h treatment period, while in the 24-h treatment cultures were treated with the test article in the presence of cytochalasin B.

Appropriate vehicle (DMSO) and positive controls were used (mitomycin C and VB in the absence of S9-mix, cyclophosphamide in the presence of S9-mix). All positive control compounds induced a

statistically significant increase of MN frequency therefore the system was considered sensitive and valid.

Two thousand cells were scored per concentration. Based on the level of cytotoxicity observed, three or four concentrations were selected for MN analysis in each experimental condition: (i) 10, 30, 45 and 50 μg/mL for the 24-h treatment (5, 30, 44 and 56% cytotoxicity, respectively); (ii) 90, 130 and 140 μg/mL for the 3 + 21 h treatment without S9-mix (4, 10 and 57% cytotoxicity, respectively); (iii) 40, 75 and 140 μg/mL for the 3 + 21 h treatment with S9-mix (4, 23 and 52% cytotoxicity, respectively).

Statistically significant increase in the frequency of micronuclei was observed after the 3-h treatment in the presence of metabolic activation at 75 and 140 μg/mL, but only with the highest concentration both replicates exceeded the historical vehicle control range. A concentration-related increase in MNBN was observed.

In the 3 + 21 h treatment, in the absence of metabolic activation, sporadic increases in the MNBN cell frequency, above the historical vehicle control range (95% reference range 0.1–1.0), were observed in single cultures of the vehicle control and of the intermediate concentration analysed, 130 μg/mL, from the initial analysis. To determine the biological relevance of these increases, a further 1,000 binucleate cells were scored from the test article treated cultures and controls. These data show that the MNBN cell frequency increase was not reproduced within or between replicate cultures. The increases appear sporadic with no evidence of a concentration-related response and the mean MNBN cell frequency for all concentrations analysed fall within the normal range. Therefore, they were considered as not biologically relevant.

Treatment of cells for 24 h in the absence of S9-mix resulted in frequencies of MNBN that were generally similar to those observed in concurrent vehicle controls for all concentrations analysed. The MNBN cell frequency of a single sampling of 1,000 binucleate cells in one replicate culture at 30 μg/mL marginally exceeded the normal range (1.2% compared to the normal range 0.1–1.1%), but this was not reproduced in the sampling of a further 1,000 binucleate cells within the same replicate culture or in any other culture analysed. In addition, the mean MNBN cell frequencies of all concentrations analysed fell within the normal range. Therefore, the Panel considered the isolated increase as not biologically relevant.

The Panel noted that this study confirms the increase of MN observed in the short-term treatment in the presence of S9-mix in the *in vitro* micronucleus study (Whitwell, 2012a) evaluated in FGE.217Rev1. The Panel concluded that 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no:10.042] induces chromosomal damage *in vitro* in the presence of metabolic activation. Therefore clarification is needed on the mechanism of formation of MN.

3.3.3. 3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] -*in vitro* micronucleus test with CREST staining in TK6 cells

3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one (purity 96%) was tested in an *in vitro* micronucleus assay with kinetochores staining in the human lymphoblastoid cell line TK6 cells (BioReliance, 2018a), with the purpose of evaluating the aneugenic and clastogenic potential of the tested substance. DMSO was used as the vehicle. The study was performed according to GLP and OECD TG 487 (OECD, 2014a).

TK6 cells were treated for 4 h with 23 h of recovery period (4 + 23 h) in the absence or presence of S9-mix (from Aroclor 1254-induced rats) or for 27 h in the absence of S9-mix. Positive controls were: CP and VB.

In the initial micronucleus test, the following concentrations were tested:

- for the treatment 4 + 23 h in the absence of metabolic activation 15, 30, 60, 80, 90, 100, 110 μg/mL;
- for the treatment 4 + 23 h in the presence of metabolic activation 15, 30, 50, 60, 70 80 μg/mL;
- for the treatment 27 h in the absence of metabolic activation 15, 30, 60, 65, 70, 75, 80 μg/mL.

Each concentration was tested in duplicate cultures, for each culture 1,000 mononucleated cells were analysed for MN (a total of 2,000 cells per concentration). Due to cytotoxicity of approximatively 50%, the highest concentrations evaluated for MN induction were: 50 μg/mL for the 4 + 23 h treatment in the presence of S9-mix and 60 μg/mL for both the 4 + 23 h and 27 h treatment in the absence of S9-mix.

After the 4 + 23 h treatment, in the absence of S9-mix, at 15 and 30 μg/mL, a statistically significant increase in MN induction (1.05% and 1.65%, respectively) was observed, but this increase was not concentration related.

After the 4 + 23 h treatment, in the presence of S9-mix, at 15 and 50 μg/mL, a statistically significant increase in MN induction (1.20%) was observed, but this increase was not concentration related.

After the 27-h treatment, in the absence of S9-mix, at 30 μg/mL, a statistically significant increase in MN induction (1.60%) was observed, but this increase was not concentration related.

The study authors considered the results of the first experiment as equivocal, and the micronucleus assay was repeated including additional concentrations:

- for the 4 + 23 h treatment in the absence of metabolic activation: 10, 15,20, 25, 30, 35, 55, 60, 65, 80 μg/mL;
- for the 4 + 23 h in the presence of metabolic activation: 10, 15, 20, 30, 35, 40, 45, 50, 55, 60 μg/mL;
- for the 27 h treatment in the absence of metabolic activation: 5, 15, 20, 25, 30, 35, 55, 60, 65, 70 μg/mL.

The highest concentrations tested were selected based on the cytotoxicity (more than 50% decrease in RPD compared to the vehicle control).

After the 4 + 23 h treatment in the absence of S9-mix, at concentrations 15, 20, 25, 30, 35 and 60 μg/mL, a statistically significant and concentration related increase in MN induction (1.40%, 1.35%, 1.90%, 1.80%, 1.70% and 3.45%, respectively) was observed.

After the 4 + 23 h treatment, in the presence of S9-mix, at concentrations 20, 30, 35 and 60 μg/mL, a statistically significant and concentration related increase in MN induction (1.45%, 1.60%, 1.45% and 1.65%, respectively) was observed.

After the 27 h treatment, in the absence of S9-mix, at concentrations 15, 25, 30, 35 and 70 μg/mL, a statistically significant and concentration related increase in MN induction (1.15%, 1.70%, 2.35%, 3.55% and 3.25%, respectively) was observed.

Since positive responses were observed, kinetochores staining (CREST staining) was applied in order to determine the mechanism of action (aneugenicity or clastogenicity).

Three concentrations of 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one from the repeated assay (60 μg/mL from the 4 + 23 h treatment both in the absence and in the presence of S9-mix), and 70 μg/mL from the 27 h treatment in the absence of S9-mix) and positive controls (CP and VB) were analysed via CREST staining.

In the 4 + 23 h treatment both in the absence and presence of S9-mix, the percentage of micronucleated cells positive for kinetochore staining (K+MN) was 67%. In the 27-h treatment in the absence of S9-mix, the percentage of K+MN was 64%.

The clastogen positive control (CP) showed 30% K+MN, while the aneugenic positive control (VB) induced 82% of K+MN. Comparing the results of the CREST staining of the 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one treated cells with the positive controls, the study authors suggest that MN were induced by mixed clastogenic and aneugenic mechanisms.

The Panel considered that 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one induces MN in TK6 cells, both in the presence and absence of metabolic activation, via mixed mechanisms of action (clastogenic and aneugenic) with an apparent prevalence of aneugenicity.

The Panel noted that the statistically significant increase of MN frequency observed in TK6 cells for all treatment conditions is in contrast with results obtained in 2 *in vitro* micronucleus studies in human peripheral blood lymphocytes. In these studies, 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one increased the frequency of MN only in the short-term treatment in the presence of S9-mix (Whitwell, 2012a; Covance, 2013a).

3.4. Discussion

3.4.1. Furan-2(5*H*)-one [FL-no: 10.066]

In FGE.217Rev1, the CEF Panel considered that furan-2(5*H*)-one [FL-no: 10.066] did not induce mutations in bacterial reverse mutation assays.

In the *in vitro* micronucleus assay, furan-2(5*H*)-one [FL-no: 10.066] induced MN in the 3 + 21 h protocol in the presence of the S9-mix. In the absence of S9-mix, results were considered as equivocal by the CEF Panel.

In FGE.217Rev1, the CEF Panel concluded that furan-2(5*H*)-one [FL-no: 10.066] raise concern with respect to genotoxicity *in vitro.* Therefore, the CEF Panel requested to test [FL-no: 10.066] in an *in vivo* combined micronucleus and comet assay with scoring at least of the liver.

In response to this request for further genotoxicity data, industry submitted an *in vivo* combined bone marrow micronucleus test and comet assay in liver for [FL-no: 10.066].

The results of the *in vivo* micronucleus assay in bone marrow were negative, but with no evidence of bone marrow exposure, therefore these results are considered as inconclusive.

The Panel requested to provide evidence of bone marrow exposure to furan-2(5*H*)-one by plasma analysis as recommended in the OECD TG 474. Due to challenges in the plasma analysis, the Panel decided to waive this request, but requested to test the flavouring substance in an *in vitro* micronucleus assay with centromere analysis, in order to investigate the mechanism of MN induction (clastogenicity or aneugenicity). The clarification of the mechanism of action would allow to identify the most appropriate follow-up study.

Industry submitted an *in vitro* micronucleus assay in human peripheral blood lymphocytes and an *in vitro* micronucleus assay in TK6 cells for [FL-no: 10.066].

The *in vitro* micronucleus assay in human peripheral blood lymphocytes confirmed the results observed in the previously available study. Furan-2(5*H*)-one [FL-no: 10.066] increased the MN frequency in the short-term treatment in the presence of S9-mix.

In the *in vitro* micronucleus assay in TK6 cells, furan-2(5*H*)-one [FL-no: 10.066] did not induce micronuclei.

The Panel noted that the negative results reported in the assay conducted in TK6 cells could be related to a reduced sensitivity of the test due to the experimental conditions used. In particular, the protocol without application of cytochalasin B in the TK6 cells study, may have limited the detection of DNA damage. Since no cytokinesis block with cytochalasin B was applied (as it was performed in the studies on human lymphocytes) the analysis was not limited to cells that had divided only once after the treatment (binucleated cells).[8] Therefore, the Panel considered the results of the two *in vitro* micronucleus assays performed in human peripheral blood lymphocytes as more reliable, in which furan-2(5*H*)-one [FL-no: 10.066] clearly increased the frequency of MN in the presence of metabolic activation.

In the *in vivo* comet assay in the liver, furan-2(5*H*)-one [FL-no: 10.066] induced increases in DNA breakage, confirming the clastogenic activity observed *in vitro*. The Panel considered that this substance is genotoxic *in vivo*.

The most recent available data on production volume and use levels for furan-2(5*H*)-one [FL-no: 10.066], as chemically defined flavouring substance, are reported with the respective calculation of Maximised Survey-derived Daily Intake (MSDI) and modified Theoretical Added Maximum Daily Intake (mTAMDI) (Appendix G).

Data on occurrence of furan-2(5*H*)-one in food is scarce. Some examples are also given in Appendix G; they indicate that the flavouring compound appears to be mainly generated during food processing.

3.4.2. 3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042]

In FGE.217Rev1, the Panel considered that 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] did not induce mutations in the bacterial reverse mutation assay.

In the *in vitro* micronucleus assay, 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] induced MN in the 3 + 21 h protocol in the presence of the S9-mix. 3,4-Dimethyl-5-pentylidenefuran-2 (5*H*)-one [FL-no: 10.042] was negative in the 24 h protocol, but equivocal results were obtained in the 3 + 21 h protocol in the absence of the S9-mix.

In FGE.217Rev1, the Panel concluded that 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] raises concern with respect to genotoxicity *in vitro.* Therefore, the Panel requested to test [FL-no: 10.042] in an *in vivo* combined micronucleus and comet assay with scoring at least of the liver.

In response to this request for further genotoxicity data, industry submitted an *in vivo* combined bone marrow micronucleus test and comet assay in liver for [FL-no: 10.042].

The results of the *in vivo* micronucleus assay in bone marrow were negative, but with no evidence of bone marrow exposure, therefore these results are considered as inconclusive.

The Panel requested to provide evidence of bone marrow exposure to 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one by plasma analysis as recommended in the OECD TG 474. Due to

[8] Further division of cells would result in a lower frequency of MN. This would be prevented by the use of cytoB.

challenges in the plasma analysis, the Panel decided to waive this request, but requested to test the flavouring substance in an *in vitro* micronucleus assay with centromere analysis, in order to investigate the mechanism inducing MN *in vitro* (clastogenicity or aneugenicity). The clarification of the mechanism of action would allow to identify the most appropriate follow-up study.

Industry submitted an *in vitro* micronucleus assay in human peripheral blood lymphocytes and an *in vitro* micronucleus assay in TK6 cells with kinetochores staining for [FL-no: 10.042].

The *in vitro* micronucleus assay in human peripheral blood lymphocytes confirmed the results observed in the previously available study. 3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] increased the MN frequency in the short-term treatment in the presence of S9-mix.

In TK6 cells, 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] induced a statistically significant increase in MN frequency both in the presence and in the absence of metabolic activation, contrary to the results from the study in human lymphocytes, which showed increase of MN frequency only in the presence of metabolic activation. The CREST analysis indicates mixed mechanisms (both clastogen and aneugen) with an apparent prevalence of aneugenicity.

The *in vivo* comet assay in liver allows the Panel to conclude that 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] did not induce primary DNA damage in the liver of rats after oral administration, therefore the potential clastogenicity in the presence of metabolic activation is ruled out. However, in order to overrule the potential clastogenicity observed in the absence of metabolic activation, an *in vivo* comet assay in duodenum is needed. The Panel noted that such a study has been already performed and therefore the respective slides are available for analysis (Covance, 2014a).

3.5. Conclusions

3.5.1. Furan-2(5*H*)-one [FL-no: 10.066]

Based on the data available, the Panel concluded that furan-2(5*H*)-one [FL-no: 10.066] is genotoxic *in vivo.* Therefore, it cannot be evaluated according to the Procedure. The Panel considered that furan-2(5*H*)-one [FL-no: 10.066] should be assessed as a stand-alone substance; therefore, its evaluation will not affect the other substances in subgroup 4.1.

3.5.2. 3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042]

Based on the data available, the Panel concluded that the potential clastogenicity of 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042], in the presence of metabolic activation, can be ruled out whereas potential clastogenicity at the site of contact should be further investigated through an *in vivo* comet assay in duodenum. Therefore, the Panel requests the analysis of the respective duodenum slides that are already available from a previous study (Covance, 2014a).

Based on the available data, the Panel also concluded that 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] is also aneugenic *in vitro*. For such substances, there is currently no agreed follow-up strategy to finalise their safety assessment. The Panel is aware that the EFSA Scientific Committee is going to address this issue and a statement clarifying the assessment of *in vitro* aneugenic substances is expected by autumn 2019 (EFSA Scientific Committee, 2018).

The Panel concluded that 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042] and the other eight represented substances [FL-no: 10.034, 10.036, 10.043, 10.046, 10.054, 10.057, 10.060 and 10.170] cannot be evaluated through the Procedure.

Documentation provided to EFSA

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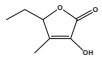
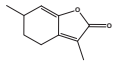
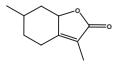
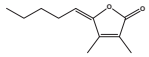
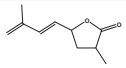
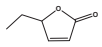
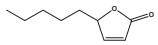
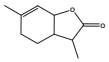
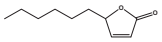
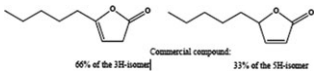
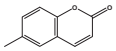
Abbreviations

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
CP	cyclophosphamide
CREST	staining anti-kinetochore antibody staining
DMSO	dimethyl sulfoxide
EFFA	European Flavour Association
EMS	ethyl methanesulfonate
FAF	Panel on Food Additives and Flavourings
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
GLP	Good Laboratory Practice
HPLC	high-performance liquid chromatography
ID	Identity
IOFI	International Organization of the Flavor Industry
i.p.	intraperitoneal
IR	infrared spectroscopy
JaCVAM	Japanese Center for the Validation of Alternative Methods
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MN	micronuclei
MNBN	micronucleated binucleate cells
MNPCE	micronucleated polychromatic erythrocytes
MS	mass spectra
mTAMDI	modified Theoretical Added Maximum Daily Intake
MSDI	Maximised Survey-derived Daily Intake
MTD	maximum tolerated dose
NOAEL	no-observed-adverse-effect-level
NMR	nuclear magnetic resonance
NCE	normochromatic erythrocytes
No	Number
OECD	Organisation for Economic Co-operation and Development
PCE	polychromatic erythrocytes
PHA	phytohaemagglutinin
(Q)SAR	(quantitative) structure–activity relationship
RI	replication index
RPD	relative population doubling
SCF	Scientific Committee on Food
WG	Working Group
WHO	World Health Organization

Appendix A – Specification Summary of the Substances in the Flavouring Group Evaluation 217Rev2

Table A.1: Specification Summary of the Substances in FGE.217Rev2 (JECFA 2000, 2003, 2010, 2016a)

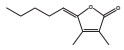
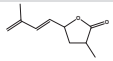
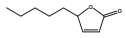
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys. form Mol. formula Mol. weight	Solubility[a] Solubility in ethanol[b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index[d] Spec. gravity[e]
10.023 222	5-Ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one		3153 2300 698-10-2	Liquid $C_7H_{10}O_3$ 142.15	Soluble	83–86 (1 hPa) IR 95%	1.486–1.493 1.134–1.144
10.030 243	3-Hydroxy-4,5-dimethylfuran-2(5*H*)-one		3634 11834 28664-35-9	Liquid $C_6H_8O_3$ 128.13		81 (8 hPa) 25 IR 97.5%	
10.034 1163	5,6-Dihydro-3,6-dimethylbenzofuran-2(4*H*)-one		3755 80417-97-6	Liquid $C_{10}H_{12}O_2$ 164.20	Slightly soluble Soluble	264–266 (13 hPa) IR NMR 95%	1.542–1.548 1.090–1.096
10.036 1162	5,6,7,7a-Tetrahydro-3,6-dimethylbenzofuran-2(4*H*)-one		3764 13341-72-5	Liquid $C_{10}H_{14}O_2$ 166.22	Slightly soluble Soluble	261–263 (8 hPa) IR NMR 98%	1.497–1.503 1.058–1.063
10.042 2002	3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one		4050 11873 774-64-1	Liquid $C_{11}H_{16}O_2$ 180.24	Insoluble Freely soluble	303 MS NMR 93%	1.560–1.575 0.930–0.980 (20°C)
10.043	2,7-Dimethylocta-5(trans),7-dieno-1,4-lactone		74183-60-1	Liquid $C_{10}H_{14}O_2$ 166.22	Practically insoluble or insoluble Freely soluble	132 (8 hPa) NMR 95%	1.453–1.459 0.977–0.983
10.046	Hex-2-eno-1,4-lactone		2407-43-4	Liquid $C_6H_8O_2$ 112.13	Soluble	93 (13 hPa) 95%	1.431–1.437 1.067–1.073
10.054 2001	Non-2-eno-1,4-lactone		4188 21963-26-8	Liquid $C_9H_{14}O_2$ 154.21	Practically insoluble or insoluble Freely soluble	230–233 NMR IR MS 97%	1.457–1.463 0.981–0.987

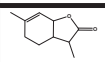
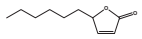
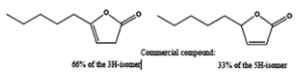
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys. form Mol. formula Mol. weight	Solubility[a] Solubility in ethanol[b]	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index[d] Spec. gravity[e]
10.057 2223	3a,4,5,7a-Tetrahydro-3,6-dimethylbenzofuran-2(3*H*)-one		4140 57743-63-2	Liquid $C_{10}H_{14}O_2$ 166.10	Practically insoluble or insoluble Freely soluble	231 13 MS IR NMR 95%	1.490–1.496 1.065–1.071
10.060	2-Decen-1,4-lactone		2518-53-8	Liquid $C_{10}H_{16}O_2$ 168.24	Practically insoluble Freely soluble	145 (13 hPa) MS 95%	1.457–1.463 0.976–0.981
10.066 2000	Furan-2(5*H*)-one		4138 497-23-4	Liquid $C_4H_4O_2$ 84.07	Soluble Freely soluble	214 NMR MS 95%	1.466–1.472 1.182–1.188
10.170 1989	5-Pentyl-3*H*-furan-2-one [f]		4323 51352-68-2	Liquid $C_9H_{14}O_2$ 154.21	Sparingly soluble Soluble	73 at 1.2 Torr IR NMR MS 95%	1.447–1.459 0.970–0.980
13.012 1172	6-Methylcoumarin		2699 579 92-48-8	Solid $C_{10}H_8O_2$ 160.17	Insoluble Soluble	73-79 IR 99%	n.a. n.a.

FL-no: FLAVIS number; FLAVIS: Flavour Information System (database); JECFA: The Joint FAO/WHO Expert Committee on Food Additives; FEMA: Flavor and Extract Manufacturers Association; CoE: Council of Europe; CAS: Chemical Abstract Service; ID: Identity; IR: infrared; 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.
(f): Mixture of 3*H*- and 5*H*-isomer (2:1) (IFF, 2010).

Appendix B – Summary of Safety Evaluation Applying the Procedure

Table B.1: Summary of Safety Evaluation Applying the Procedure (JECFA 1998, 2004, 2008, 2011, 2016b)

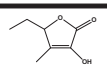
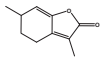
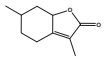
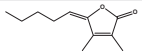
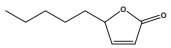
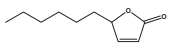
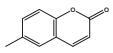
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI[(a)] US MSDI (μg/capita per day)	Class[(b)] Evaluation procedure path[(c)]	Outcome on the named compound [(d) or (e)]	EFSA conclusion on the named compound:(Procedure steps, intake estimates, NOAEL, genotoxicity)
10.023 222	5-Ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one		13 6.1	Class III B3: Intake below threshold, B4: Adequate NOAEL exists	d	Evaluated in FGE.217Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No further EFSA considerations needed
10.030 243	3-Hydroxy-4,5-dimethylfuran-2(5*H*)-one		2.1 0.1	Class III B3: Intake below threshold, B4: Adequate NOAEL exists	d	Evaluated in FGE.217Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No further EFSA considerations needed
10.034 1163	5,6-Dihydro-3,6-dimethylbenzofuran-2(4*H*)-one		1 0.01	Class III A3: Intake below threshold	d	Evaluated in FGE.217Rev2, additional genotoxicity data required
10.036 1162	5,6,7,7a-Tetrahydro-3,6-dimethylbenzofuran-2(4*H*)-one		8 1	Class III A3: Intake below threshold	d	Evaluated in FGE.217Rev2, additional genotoxicity data required
10.042 2002	3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one		0.01	Class III B3: Intake below threshold, B4: Adequate NOAEL exists	d	Evaluated in FGE.217Rev2, additional genotoxicity data required
10.043	2,7-Dimethylocta-5(trans),7-dieno-1,4-lactone		0.0012	Class I No evaluation		Evaluated in FGE.217Rev2, additional genotoxicity data required
10.046	Hex-2-eno-1,4-lactone		0.0024	No evaluation		Evaluated in FGE.217Rev2, additional genotoxicity data required
10.054 2001	Non-2-eno-1,4-lactone		0.01	Class III A3: Intake below threshold	d	Evaluated in FGE.217Rev2, additional genotoxicity data required

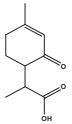
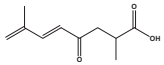
FL-no JECFA-no	EU Register name	Structural formula	EU MSDI[(a)] US MSDI (μg/capita per day)	Class[(b)] Evaluation procedure path[(c)]	Outcome on the named compound [[(d)] or [(e)]]	EFSA conclusion on the named compound:(Procedure steps, intake estimates, NOAEL, genotoxicity)
10.057 2223	3a,4,5,7a-Tetrahydro-3,6-dimethylbenzofuran-2(3*H*)-one		0.012	Class III A3: Intake above the threshold, A4: metabolites not endogenous, A5: adequate NOAEL exists	d	Evaluated in FGE.217Rev2, additional genotoxicity data required
10.060	2-Decen-1,4-lactone		0.037	Class III No evaluation		Evaluated in FGE.217Rev2, additional genotoxicity data required
10.066 2000	Furan-2(5*H*)-one		0.01	Class III A3: Intake above the threshold, A4: metabolites not endogenous, A5: adequate NOAEL exists	d	Evaluated in FGE.217Rev2 as of genotoxicity concern
10.170 1989	5-Pentyl-3*H*-furan-2-one		1.2	Class II A3: Intake below threshold	d	Evaluated in FGE10Rev3, additional genotoxicity and specification data required. 1/3 of the named compound correspond to FL-no: 10.054. Evaluated in FGE.217Rev2, additional genotoxicity data required
13.012 1172	6-Methylcoumarin		250 96	Class III B3: Intake above threshold	e	Evaluated in FGE.217. Genotoxicity concern could be ruled out. Evaluated using the Procedure in FGE.80Rev1: No safety concern at the estimated level of intake based on the MSDI approach

FL-no: FLAVIS number; FLAVIS: Flavour Information System (database); JECFA: the Joint FAO/WHO Expert Committee on Food Additives; MSDI: Maximised Survey-derived Daily Intake; NOAEL: no-observed-adverse-effect-level; FGE: Flavouring Group Evaluation.
(a): EU MSDI: Amount added to food as flavour in (kg/year) × 10E9/(0.1 × population in Europe (= 375 × 10E6) × 0.6 × 365) = μg/capita per day.
(b): Thresholds of concern: Class I = 1,800 μg/person per day, Class II = 540 μg/person per day, Class III = 90 μg/person per day.
(c): Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.
(d): No safety concern based on intake calculated by the MSDI approach of the named compound.
(e): Data must be available on the substance or closely related substances to perform a safety evaluation.

Appendix C – (Q)SAR Predictions on Mutagenicity

Table C.1: (Q)SAR Predictions on Mutagenicity for 10 Lactones from subgroup 4.1 and two precursors

FL-no JECFA-no	Subgroup	EU Register name	Structural formula[a]	FEMA no CoE no CAS no	ISS local Model Ames Test TA100[b]	MultiCASE Ames test[c]	MultiCASE mouse lymphoma test[d]	MultiCASE chromosomal aberration test in CHO[e]	MultiCASE chromosomal aberration test in CHL[f]
10.023 222	4.1	5-Ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one		3153 2300 698-10-2	OD	NEG	NEG	NEG	NEG
10.030 243	4.1	3-Hydroxy-4,5-dimethylfuran-2(5*H*)-one		3634 11834 28664-35-9	OD	NEG	NEG	NEG	NEG
10.034 1163	4.1	5,6-Dihydro-3,6-dimethylbenzofuran-2(4*H*)-one		3755 – 80417-97-6	OD	NEG	OD	OD	OD
10.036 1162	4.1	5,6,7,7a-Tetrahydro-3,6-dimethylbenzofuran-2(4*H*)-one		3764 – 13341-72-5	OD	NEG	OD	OD	OD
10.042 2002	4.1	3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one		4050 11873 774-64-1	OD	OD	OD	OD	OD
10.046	4.1	Hex-2-eno-1,4-lactone		– – 2407-43-4	OD	NEG	OD	POS	OD
10.054 2001	4.1	Non-2-eno-1,4-lactone		4188 – 21963-26-8	OD	NEG	OD	EQU	OD
10.060	4.1	2-Decen-1,4-lactone		– – 2518-53-8	OD	NEG	OD	EQU	OD
10.066 2000	4.1	Furan-2(5*H*)-one		4138 –	OD	NEG	POS	EQU	EQU
13.012 1172	4.1	6-Methylcoumarin		2699 579 92-48-8	OD	POS	OD	OD	OD

FL-no JECFA-no	Subgroup	EU Register name	Structural formula[a]	FEMA no CoE no CAS no	ISS local Model Ames Test TA100[b]	MultiCASE Ames test[c]	MultiCASE mouse lymphoma test[d]	MultiCASE chromosomal aberration test in CHO[e]	MultiCASE chromosomal aberration test in CHL[f]
Not in Register	2.6	3-Methyl-6-(1-carboxyethyl)-2-cyclohexen-1-one			OD	NEG	OD	NEG	EQU
Not in Register	1.2.4	2,7-Dimethyl-4-oxo-oct-5,7-dienoic acid			NYA	NYA	NYA	NYA	NYA

(Q)SAR: (Quantitative) Structure–Activity Relationship; FL-no: FLAVIS number; FLAVIS: Flavour Information System (database); JECFA: The Joint FAO/WHO Expert Committee on Food Additives; FEMA: Flavor and Extract Manufacturers Association; CoE: Council of Europe; CAS: Chemical Abstract Service; CHO: Chinese hamster ovary (cells); CHL: Chinese hamster lung (cells); OD: Out of domain (out of applicability domain: not matching the range of conditions where a reliable prediction can be obtained in this model. These conditions may be physicochemical, structural, biological etc.); POS: Positive; NEG: Negative; EQU: Equivocal; NYA: not yet assessed.
(a): Structural formula of substances in FGE.19 subgroup 4.1.
(b): Local model on aldehydes and ketones, Ames TA100.
(c): MultiCase Ames test.
(d): MultiCase mouse lymphoma test.
(e): MultiCase chromosomal aberration in CHO.
(f): MultiCase chromosomal aberration in CHL.

Appendix D – Genotoxicity and carcinogenicity studies evaluated in FGE.217

Table D.1: Carcinogenicity Study

Chemical name [FL-no]	Species; sex No./group	Route	Dose levels	Duration	Results	Reference	Comments
6-Methylcoumarin [13.012]	Rat; Male, Female 25/sex per group	Diet	0, 25, 50, 175, 250, 375 or 750 mg/kg bw per day	2 years	Males and females: No increases in tumour incidences	Hagan et al. (1967)	The study is not in accordance with OECD Guidelines or current standards. Under the condition of the study, the negative result is considered valid. The NOAEL was 250 mg/kg bw per day based on growth depression and slight liver changes particularly in males at the higher dose levels. The study is reported together with the results of studies of many more flavouring substances with and without related structures. Therefore, no detailed description of the findings is given

FL-no: FLAVIS number; FLAVIS: Flavour Information System (database); bw: body weight; NOAEL: no-observed-adverse-effect-level; OECD: Organisation for Economic Co-operation and Development.

Table D.2: Genotoxicity (*in vitro*)

Chemical name [FL-no]	Test system	Test object	Concentration	Result	Reference	Comments[(d)]
6-methylcoumarin [13.012]	Reverse mutation	*Salmonella* Typhimurium TA100	5 concentrations up to cytotoxicity or max. 3,600 μg/plate	Marginally positive[(c)]	Wild et al. (1983)	Valid, however the results are considered equivocal (+ S9: dose–response showed positive trend, but was never above twice control frequency; – S9: negative)
	Reverse mutation	*S.* Typhimurium TA98, TA1535, TA1537, and TA1538	5 concentrations up to cytotoxicity or max. 300 μg/plate	Negative[(a)]	Wild et al. (1983)	Valid
	Reverse mutation	*S.* Typhimurium TA98, TA100, TA1535, and TA1537	33–3,333 μg/plate	Negative[(a),(b)]	Haworth et al. (1983)	Valid
	Reverse mutation	S. Typhimurium TA98, TA100, TA1535, TA1537 and TA1538	1–5,000 μg/plate	Negative[(a)]	Brusick (1982)	Valid. Unpublished GLP study carried out according to current OECD guideline; result is considered as valid
	Forward mutation	Mouse lymphoma L5178Y $Tk^{+/-}$ cells	6.25–100 μg/mL	Negative[(c)]	Cifone (1982)	Valid. Unpublished GLP study carried out according to current OECD guideline; result is considered as valid
	Forward mutation	Mouse lymphoma L5178Y $Tk^{+/-}$ cells	15.6 – 250 μg/mL	Negative	Cifone (1982)	Valid. Unpublished GLP study carried out according to current OECD guideline; result is considered as valid

FL-no: FLAVIS number; FLAVIS: Flavour Information System (database); GLP: Good Laboratory Practice; OECD: Organisation for Economic Co-operation and Development.
(a): With and without metabolic activation.
(b): Pre-incubation method.
(c): With metabolic activation.
(d): 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).

Genotoxicity (*in vivo*) Considered by the Panel in FGE.217

Table D.3: Genotoxicity (*in vivo*)

Chemical name [FL-no]	Test system	Test object	Route	Dose	Result	Reference	Comments[(a)]
6-Methylcoumarin [13.012]	Sex-linked recessive lethal mutation	*Drosophila melanogaster*	Feed	10 mmol/l (1,602 μg/mL)	Negative	Wild et al. (1983)	Limited validity (limited reporting, study system considered of limited relevance)
	Micronucleus formation	Mouse peripheral blood cells	Gavage	200 and 400 mg/kg for 90 days	Equivocal (M) Negative (F)	Witt et al. (2000)	Limited validity (not a standard protocol; exposure for 90 days; no information on cytotoxicity; no positive controls)
	Micronucleus formation	Mouse bone marrow cells	i.p.	160, 240 and 320 mg/kg	Negative	Wild et al. (1983)	Limited validity (only analysis at one time point; no PCE/NCE ratio reported)

FL-no: FLAVIS number; FLAVIS: Flavour Information System (database); FGE: Flavouring Group Evaluation; PCE: polychromatic erythrocytes; NCE: normochromatic erythrocytes; i.p.: intraperitoneal.
(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).

Appendix E – Genotoxicity studies evaluated in FGE.217Rev1

In vitro Genotoxicity Data Considered by the Panel in FGE.217Rev1

Table E.1: Summary of *in vitro* Genotoxicity Data for [FL-no: 10.023, 10.042 and 10.066] of subgroup 4.1

Chemical name [FL-no:]	Test system	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
5-ethyl-3-hydroxy-4-methylfuran-2(5*H*)-one [10.023]	Reverse Mutation	*Salmonella* Typhimurium TA98, TA100, TA1535, TA1537 and TA102	1.6, 8, 40, 200, 1,000 and 5,000 μg/plate[(a),(b)]	Negative	Bowen (2011a)	Valid study in accordance with OECD Guideline 471 and in compliance with GLP. Evidence of toxicity was observed in strain TA1537 in the presence of S9-mix at 5,000 μg/plate and in strain TA102 in the presence of S9-mix at 200 μg/plate and above. Further evidence of toxicity in the form of a reduction in revertant numbers was observed in strain TA1535 in the presence of S9-mix and in strain TA102 in the absence of S9-mix at 5,000 μg/plate
		S. Typhimurium TA98, TA100, TA1535, TA1537 and TA102	156.3, 312.5, 625, 1250, 2,500 and 5,000 μg/plate[(b),(c)]	Negative		Evidence of toxicity was observed in the presence of S9-mix in strains TA1537 and TA102 at 2,500 μg/plate and above. Further evidence of toxicity in the form of a reduction in revertant numbers was observed in strains TA98 in the presence of S9-mix at 5,000 μg/plate and in strains TA98 and TA102 in the absence of S9-mix at 5,000 and 2,500 μg/plate, respectively
			156.3, 312.5, 625, 1250, 2,500 and 5,000 μg/plate[(d),(e)]	Negative		
	Micronucleus Assay	Human peripheral blood lymphocytes	1,000, 1,200 and 1,422 μg/mL (equivalent to 10 mM)[(a),(f)]	Negative	Lloyd (2011)	Valid study in accordance with draft OECD Guideline 487 (OECD, 2010) and in compliance with GLP. A top concentration of 10 mM was employed or an acceptable level of cytotoxicity was achieved at the top concentration used in the continuous treatment schedule
			500, 750 and 900 μg/mL[(c),(g)]	Negative		

Chemical name [FL-no:]	Test system	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one [10.042]	Reverse Mutation	*S.* Typhimurium TA98, TA100, TA1535, TA1537, and TA102	0.32, 1.6, 8, 40, 200, 1,000 and 5,000 μg/plate[(a),(b)]	Negative	Bowen (2011b)	Valid study in accordance with OECD Guideline 471 (OECD, 1997a,b) and in compliance with GLP. Evidence of toxicity was observed in all strains in the absence and presence of S-9 at 200 μg/plate and above
		S. Typhimurium TA98, TA102, TA1535	78.13–5,000 μg/plate[(d),(e)]	Negative		Evidence of toxicity was observed in strain TA102 in the presence of S-9 at 1,250 μg/plate and above, and for all other treatment conditions at either 312.5 or 625 μg/plate and above
		S. Typhimurium TA100	19.53–1,250[(b),(c)] 78.13–5,000[(d),(e)] μg/plate			
		S. Typhimurium TA1537	9.76–1,250[(b),(c)] 78.13–5,000 μg/plate[(d),(e)]			
		S. Typhimurium TA1537	19.53–1,250 μg/plate[(d),(e)]	Negative		Evidence of toxicity was observed at 156.3 μg/plate and above
	Micronucleus induction	Human peripheral blood lymphocytes	40, 60, 70 and 90 μg/mL[(c),(f)]	Equivocal	Whitwell (2012a)	Valid study in accordance with OECD Guideline 487 (OECD, 2010) and in compliance with GLP
			60, 90, 110 and 140 μg/mL[(e),(f)]	Positive		
			10, 13, and 15 μg/mL[(c),(g)]	Negative		
Furan-2(5*H*)-one [10.066]	Reverse Mutation	*S.* Typhimurium TA98, TA100, TA1535, TA1537 and TA102	0.32, 1.6, 8, 40, 200, 1,000 and 5,000 μg/plate[(a),(b)]	Negative	Bowen (2011c)	Valid study in accordance with OECD Guideline 471 (OECD, 1997a) and in compliance with GLP. Evidence of toxicity was observed in all treatment conditions in the absence and presence of S9 at 5,000 μg/plate, with the exception of TA100 in the presence of S9 and strain TA1535 in the absence of S9

Chemical name [FL-no:]	Test system	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
			156.3, 312.5, 625, 1250, 2,500 and 5,000 μg/plate[(b),(c)] 156.3, 312.5, 625, 1,250, 2,500 and 5,000 μg/plate[(d),(e)]	Negative		Evidence of toxicity was observed in all treatment conditions in the absence and presence of S9 at 2,500 and 5,000 μg/plate
	Micronucleus induction	Human peripheral blood lymphocytes	200, 350, 425 450 and 475 μg/mL[(c),(f)]	Equivocal	Whitwell (2012b)	Valid study in accordance with OECD Guideline 487 (OECD, 2010) and in compliance with GLP
			100, 250, 425, 450 and 475 μg/mL[(e),(f)]	Positive		
			10, 50, 60, 67.5 and 72.5 μg/mL[(c),(g)]	Equivocal		

FL-no: FLAVIS number; FLAVIS: Flavour Information System (database); FGE: Flavouring Group Evaluation; GLP: Good Laboratory Practice; OECD: Organisation for Economic Co-operation and Development.
(a): With and without S9 metabolic activation.
(b): Plate incorporation method.
(c): Without S9 metabolic activation.
(d): Pre-incubation method.
(e): With S9 metabolic activation.
(f): 3-h incubation with 21-h recovery period.
(g): 24-h incubation with no recovery period.

Appendix F – Genotoxicity studies evaluated in FGE.217Rev2

Table F.1: Summary of *in vivo* Genotoxicity Data for [FL-no:10.042 and 10.066] evaluated in FGE.217Rev2

Name [FL-no]	Test system	Test object	Route	Dose	Result	Reference	Comments
3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one [10.042]	Micronucleus assay (bone marrow)	Han Wistar Rat; M	Gavage	125, 250 and 500 mg/kg bw per day[(a)]	Negative	Covance (2014a)	Reliable with restrictions. Study performed in compliance with GLP and OECD TG 474. No clear evidence of bone marrow exposure
	Comet assay (liver)	Han Wistar Rat; M	Gavage		Negative		Reliable without restrictions. The study was performed in compliance with recommendations of the Comet and IWGT workshop, Japanese Center for the Validation of Alternative Methods (JaCVAM) and current literature
Furan-2(5*H*)-one [10.066]	Micronucleus assay (bone marrow)	Han Wistar Rat; M	Gavage	62.5, 125 and 250 mg/kg bw per day[(a)]	Negative	Covance (2014b, 2015)	Reliable with restrictions. Study performed in compliance with GLP and OECD TG 474. No clear evidence of bone marrow exposure
	Comet assay (liver)	Han Wistar Rat; M	Gavage		Positive		Reliable without restrictions. Statistically significant increase in mean tail intensity ($p \leq 0.001$) observed at the highest dose. The study was performed in compliance with recommendations of the Comet and IWGT workshop, Japanese Center for the Validation of Alternative Methods (JaCVAM) and current literature

FL-no: FLAVIS number; FGE: Flavouring Group Evaluation; bw: body weight; GLP: Good Laboratory Practice; OECD: Organisation for Economic Co-operation and Development; M: male.
(a): Administered via gavage in 3 doses at times 0, 24 and 45 h with sacrifice and harvest at 48 h.

Table F.2: Summary of Additional *in vitro* Genotoxicity Data Submitted for [FL-no:10.042 and 10.066] and evaluated in FGE.217Rev2

Name [FL-no]	Test system	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
3,4-Dimethyl-5-pentylidenefuran-2(5*H*)-one [10.042]	Micronucleus Assay with CREST staining	Mammalian TK6 cells	15, 30, 60, 80, 90, 100, 110 µg/mL[(a)]	Positive	BioReliance (2018a)	Reliable without restrictions. Study performed in compliance with GLP and OECD TG 487. CREST analysis indicates that 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one induced micronuclei by mixed clastogenic and aneugenic mechanisms of action
			15, 30, 50, 60, 70, 80 µg/mL[(b)]			
			15, 30, 60, 65, 70, 75, 80 µg/mL[(c)]			
			10, 15, 20, 25, 30, 35, 55, 60, 65, 80 µg/mL[(a),(b)]			
			10, 15, 20, 30, 35, 40, 45, 50, 55, 60 µg/mL[(b),(d)]			
			5, 15, 20, 25, 30, 35, 55, 60, 65, 70 µg/mL[(c),(d)]			
	Micronucleus assay	Human blood lymphocytes	90, 130 and 140 µg/mL[(e)]	Positive[(f)]	Covance (2013a)	Reliable without restrictions. Study performed in compliance with GLP and OECD TG 487
			40, 75 and 140 µg/mL[(f)]			
			10, 30, 45 and 50 µg/mL[(g)]			
Furan-2(5*H*)-one [10.066]	Micronucleus assay	Mammalian TK6 cells	25, 125, 150 µg/mL[(a),(b)]	Negative	BioReliance (2018b)	Reliable with restrictions. Study performed in compliance with GLP and OECD TG 487
			5, 35, 55 µg/mL[(c)]			
	Micronucleus assay	Human blood lymphocytes	100, 200, 300 and 350 µg/mL[(e)]	Positive[(f)]	Covance (2013b)	Reliable without restrictions. Study performed in compliance with GLP and OECD TG 487
			100, 300, 350 and 400 µg/mL[(f)]			
			10, 45, 60 and 65 µg/mL[(g)]			

FL-no: FLAVIS number; FLAVIS: Flavour Information System (database); FGE: Flavouring Group Evaluation; GLP: Good Laboratory Practice; OECD: Organisation for Economic Co-operation and Development.
(a): Without S9 metabolic activation, 4 + 23 h treatment.
(b): With S9 metabolic activation, 4 + 23 h treatment.
(c): Without S9 metabolic activation, 27 h treatment.
(d): Repeated concentrations tested due to equivocal results in the previous MN assays.
(e): Without S9 metabolic activation, 3 + 21 h treatment.
(f): With S9 metabolic activation, 3 + 21 h treatment.
(g): Without S9 metabolic activation, 24 h treatment.

Appendix G – Exposure

Presence of furan-2(5*H*)-one in food

According to the VCF database (Triskelion, 2018), the candidate substance furan-2(5*H*)-one is reported to be present in food (Table G.1).

Table G.1: Examples of furan-2(5*H*)-one occurrence in food

Food	Sample description	Qualitative	Quantitative (mg/kg)	Reference
Allium species	Onions heated in butter	Yes		Ledl (1975)
Beer	10 L of Pilsner beer (spalt hops)		0.01	Tressl & Renner (1975)
Coffee	1) Compound isolated by vapour phase chromatography 2) Roasting, cooking or pressure cooking of mixtures	Yes		1) Gianturco et al. (1966) 2) Baltes & Bochmann (1987)
Filbert, Hazelnut (*Corylus avellano*)	Roasted Filberts	Yes		Kinlin et al. (1972)
Fish	Cooked wild Grey Mullet from Turkey		0.231	Cayhan & Selli (2010)
Honey	Different honey varieties from Poland: rape, acacia, linden, buckwheat, heather, honey-dew and polyfloral honeys	Yes		Plutowska et al. (2011)
Licorice (*Glycyrrhiza species*)	Dried licorice root from China		0.09	Tanaka et al. (2008)
Peanut (*Arachis hypogaea* L.)	Freshly roasted peanuts	Yes		Ho et al. (1982)
Pomegranate juice (*Punica granatum* L.)	Fresh juice extracted by squeezing	Yes		Mayuoni-kirshinbaum et al. (2012)
Rooibos tea (*Aspalathus linearis*)	Tea brewed in 700 mL of boiling water for 10 min	Yes		Kawakami et al. (1993)
Saffron (*Crocus sativus* L.)	16 g of saffron homogenised in 100 mL of diethyl ether for 2 min, extracted and then used for gas chromatographic analysis	Yes		Zarghami (1971)
Wheaten bread	1) White bread fragmented, immersed in liquid nitrogen and extracted by hexane, pentane-ether and ether extraction. 2) Bread loaf baked for 40–45 min as Coburgs	Yes		1) Mulders & Dhont (1972) 2) Folkes & Gramshaw (1977)

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Intended use and use levels as provided by the Flavour Industry

Use levels in the different food categories reported in Annex III of Reg. (EC) 1565/2000[9] have been submitted by the flavour industry and are reported in Table G.2 (EFFA, 2017).

Table G.2: Use levels of furan-2(5*H*)-one [FL-no: 10.066] in food categories listed in Annex III of Reg. (EC) 1565/2000 (EFFA, 2017)

FL-no	Food categories																	
	Normal use levels (mg/kg)[a] Maximum use levels (mg/kg)																	
	01.0	**02.0**	**03.0**	**04.2**	**05.0**	**05.3[b]**	**06.0**	**07.0**	**08.0**	**09.0**	**10.0**	**11.0**	**12.0**	**13.0**	**14.1**	**14.2**	**15.0**	**18.0[C]**
10.066	3.87 14.99	1.00 2.38	1.00 150.5	0.55 0.9	7.64 21.05	1.10 7.05	5.33 14.99	4.08 15.06	0.08 2.13	– –	– –	– –	1.20 5.00	– –	1.90 5.41	0.81 3.10	0.05 0.27	0.09 –

FL-no: FLAVIS number; mTAMDI: modified Theoretical Added Maximum Daily Intake.
(a): 'Normal use' is defined as the average of reported usages and 'maximum use' is defined as the 95th percentile of reported usages (EFFA, 2002).
(b): Additional food category 05.3 (chewing-gum as per Annex II part D of Reg. (EC) 1333/2008) for which EFFA submitted use levels (EFFA, 2017). These have been considered in the calculation of mTAMDI.
(c): Instead of food category 16.0 (see Table G.3) EFFA (EFFA, 2017) provided use levels on food category 18.0 (processed food as per Annex II part D of Reg. (EC) 1333/2008) which have been considered in the calculation of mTAMDI.

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

Table G.3: Distribution of the 18 food categories listed in Commission Regulation (EC) No 1565/2000[9] into the seven SCF food categories used for TAMDI calculation (SCF, 1995)

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

(a): For explanation of exceptions see SCF (1995).

Intake data from intended use

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

Use levels for furan-2(5*H*)-one [FL-no: 10.066] provided by industry (EFFA, 2017) are listed in Table G.4. These data have been used to calculate the 'modified Theoretical Added Maximum Daily Intake' (mTAMDI).[10]

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

Table G.4: Exposure to furan-2(5*H*)-one [FL-no: 10.066]

FL-no	Name	EU MSDI µg/capita per day	mTAMDI µg/person per day
10.066	Furan-2(5*H*)-one	0.01[(a)] 0[(b)]	1,576[(c)]

FL-no: FLAVIS number; MSDI: Maximised Survey-derived Daily Intake; mTAMDI: modified Theoretical Added Maximum Daily Intake.
(a): Based on EU poundage of 0.1 kg (JECFA, 2011).
(b): Based on poundage survey of 2015; according to this survey this substance is not produced in the EU (EFFA, 2017).
(c): Based on use levels data from survey of 2015 (documentation provided by EFFA, 2017).

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