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EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF); Scientific Opinion on Flavouring Group Evaluation 218, Revision 1 (FGE.218Rev1): alpha,beta-Unsaturated aldehydes and precursors from subgroup 4.2 of FGE.19: Furfural derivatives

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SCIENTIFIC OPINION

Scientific Opinion on Flavouring Group Evaluation 218, Revision 1 (FGE.218Rev1):

alpha,beta-Unsaturated aldehydes and precursors from subgroup 4.2 of FGE.19: Furfural derivatives.[1]

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

SUMMARY

The European Food Safety Authority (EFSA) asked the Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (the Panel) to provide scientific advice to the Commission on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Panel was requested to evaluate flavouring substances using the Procedure as referred to in the Commission Regulation (EC) No 1565/2000.

In the present revision of FGE.218, FGE.218Rev1, there has been a reassessment of one candidate substance, 5-methylfurfural [FL-no: 13.001], for which there was a request for genotoxicity data in FGE.218.

Flavouring Group Evaluation 218 (FGE.218) consists of furfural [FL-no: 13.018] and seven substances structurally related to furfural, 5-methylfurfural [FL-no: 13.001], furfuryl alcohol [FL-no: 13.019] and five esters of furfuryl alcohol and aliphatic saturated carboxylic acids [FL-no: 13.057, 13.062, 13.067, 13.068 and 13.128].

In the previous version of this Opinion, FGE.218, the Panel had expressed the following view.

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1 On request from the Commission, Question No EFSA-Q-2009-01083, adopted on 30 September 2010.

2 Panel members Arturo Anadon, Mona-Lise Binderup, Wilfried Bursch, Laurence Castle, Riccardo Crebelli, Karl-Heinz Engel, Roland Franz, Nathalie Gontard, Thomas Haertle, Trine Husøy, Klaus-Dieter Jany, Catherine Leclercq, Jean Claude Lhuguenot, Wim Mennes, Maria Rosaria Milana, Karla Pfaff, Kettil Svensson, Fidel Toldra, Rosemary Waring, Detlef Wölfle. Correspondence: cef-unit@efsa.europa.eu

3 Acknowledgement: The Panel wishes to thank the members of the Working Groups on Flavourings for the preparation of this Opinion: Ulla Beckman Sundh, Vibe Beltoft, Wilfried Bursch, Angelo Carere, Karl-Heinz Engel, Henrik Frandsen, Rainer Gürtler, Frances Hill, Trine Husøy, John Christian Larsen, Pia Lund, Wim Mennes, Gerard Mulder, Karin Nørby, Gerard Pascal, Iona Pratt, Gerrit Speijers, Harriet Wallin and EFSA's staff members Anna Frederica Castoldi and Kim Rygaard Nielsen for the preparatory work on this scientific Opinion.

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The five furfuryl esters are anticipated to be hydrolysed to furfuryl alcohol (and carboxylic acids). Furfuryl alcohol is expected to be oxidised to the alpha,beta-unsaturated aldehyde furfural. However, based on the data then available the Panel concluded that furfural is not of concern with respect to genotoxicity. Furthermore, the Panel concluded that not only furfural but also the structurally related furfuryl alcohol and the five furfuryl esters are not of concern with respect to genotoxicity. Accordingly these seven substances can be evaluated through the Procedure in FGE.66.

In the FGE.218 Opinion of 2008 the Panel also expressed its view on 5-hydroxymethylfurfural and 5-methylfurfural. It is anticipated that 5-methylfurfural [FL-no: 13.001] can be oxidised to the primary alcohol 5-hydroxymethylfurfural [FL-no: 13.139]. 5-Hydroxymethylfurfural has been evaluated by EFSA in FGE.13 dealing with furfuryl and furan derivatives. In the latter Opinion, it was concluded that since 5-hydroxymethylfurfural may be metabolised to 5-[(sulphoxy)methyl]furfural which shows genotoxic potential *in vitro,* 5-hydroxymethylfurfural could not be evaluated through the Procedure. Accordingly, the Panel concluded that 5-methylfurfural could not be evaluated through the Procedure either.

Industry has submitted additional data on the 5-hydroxymethylfurfural including metabolism, genotoxicity and carcinogenicity data. Based on these data and further genotoxicity studies identified by EFSA, the Panel concluded that, notwithstanding the indications of *in vitro* genotoxicity in conditions that favour the formation of 5-[(sulphoxy)methyl]furfural and the limited *in vivo* genotoxicity study, the essentially negative results of the carcinogenicity study in rats and mice indicate that 5-hydroxymethylfurfural is of no concern under the conditions of intended use. This conclusion is also applicable to 5-methylfurfural, a candidate substance in the current FGE.218Rev1, because this substance may be metabolised to 5-hydroxymethylfurfural. Accordingly, both 5-hydroxymethylfurfural [FL no: 13.001] and 5-methylfurfural [FL-no: 13.139] can be evaluated through the Procedure.

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KEYWORDS

alpha,beta-Unsaturated aldehydes, furfural, furfuryl alcohol, furfurylesters, flavouring substances, safety evaluation.

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BACKGROUND

Regulation (EC) No 2232/96 of the European Parliament and the Council (EC, 1996a) lays down a Procedure for the establishment of a list of flavouring substances the use of which will be authorised to the exclusion of all other substances in the EU. In application of that Regulation, a Register of flavouring substances used in or on foodstuffs in the Member States was adopted by Commission Decision 1999/217/EC (EC, 1999a), as last amended by Commission Decision 2009/163/EC (EC, 2009a). Each flavouring substance is attributed a FLAVIS-number (FL-number) and all substances are divided into 34 chemical groups. Substances within a group should have some metabolic and biological behaviour in common.

Substances which are listed in the Register are to be evaluated according to the evaluation programme laid down in Commission Regulation (EC) No 1565/2000 (EC, 2000a), which is broadly based on the Opinion of the Scientific Committee on Food (SCF, 1999a). For the submission of data by the manufacturer, deadlines have been established by Commission Regulation (EC) No 622/2002 (EC, 2002b).

After the completion of the evaluation programme the Union List of flavouring substances for use in or on foods in the EU shall be adopted (Article 5 (1) of Regulation (EC) No 2232/96) (EC, 1996a).

TERMS OF REFERENCE

The European Food Safety Authority (EFSA) is requested to carry out a risk assessment on flavouring substances in the Register prior to their authorisation and inclusion in a Union List according to Commission Regulation (EC) No 1565/2000 (EC, 2000a).

In addition, in a letter of 11 September 2009 the Commission requested EFSA to carry out a re-evaluation of 5-methylfurfural [FL-no: 13.001] in accordance with Commission Regulation (EC) No 1565/2000 (EC, 2000a), if possible by the end of the evaluation programme, if not, within nine month from the finalisation of that programme. The deadline of the Terms of Reference was negotiated to 30 November 2010.

HISTORY OF THE EVALUATION OF THE SUBSTANCES IN THE PRESENT FGE

EFSA has considered a group of furfural and seven furfural derivatives. The eight substances have been evaluated by the JECFA at their 55th meeting (JECFA, 2001a).

FGE	Adopted	Link	No. of Candidate Substances
FGE.218	9 July 2008	http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1211902310134.htm	8
FGE.218Rev1			8

In the present revision of FGE.218, FGE.218Rev1, there has been a reassessment of one candidate substance, 5-methylfurfural [FL-no: 13.001], for which there was a request for genotoxicity data in FGE.218. The additional genotoxicity data submitted by Industry is on a structurally related substance, 5-hydroxymethylfurfural [FL-no: 13.139] evaluated in FGE.13 (EFSA, 2005c). These new data are described, evaluated and discussed in Section 4 of the present version of FGE.218.

ASSESSMENT

1. Presentation of the Substances in Flavouring Group Evaluation 218

1.1. Description

The present Flavouring Group Evaluation (FGE) concerns eight flavouring substances, furfural, 5-methylfurfural, furfuryl alcohol and five furfuryl esters which all are alpha,beta-unsaturated aldehydes or precursors for alpha,beta-unsaturated aldehydes, corresponding to subgroup 4.2 of FGE.19 (EFSA, 2008b).

The alpha,beta-unsaturated aldehyde and ketone structures are considered by the Panel to be structural alerts for genotoxicity (EFSA, 2008b), and accordingly these eight substances [FL-no: 13.001, 13.018, 13.019, 13.057, 13.062, 13.067, 13.068 and 13.128] will be considered in this FGE, especially with respect to the available data on genotoxic or carcinogenic activity.

In the evaluation of the genotoxic potential of the substances in FGE.218, the Panel has taken into consideration the Opinion of the Scientific Committee on Food (SCF) on furfural and furfural diethylacetal expressed in December 2002 (SCF, 2003a). In its later updated version of Opinion of June 2004 (EFSA, 2004c), EFSA considered additional data on the potential genotoxicity of furfural which was not available at the time of the SCF Opinion. Furthermore, the Panel took into account the evaluation by the joint FAO/WHO Expert Committee on Food Additives (the JECFA) on furfural and furfuryl alcohol and related flavouring substances at its 55th meeting (JECFA, 2001b).

The eight substances under consideration in the present evaluation are listed in Table 1 and a summary of their current evaluation status by JECFA is given in Table 2.

2. Toxicity

Genotoxicity and carcinogenicity studies from FGE.218 are summarised in Tables 3, 4, 5 and 6. Additional genotoxicity data are summarised in Table 7 and 8.

2.1. Genotoxicity / Carcinogenicity - Text Taken from the SCF Opinion on Furfural and Furfural Diethylacetal (SCF, 2003a)

2.1.1. Carcinogenicity Studies

“In a two-year study in $B6C3F_1$ mice, 50 animals of each sex received doses of 0, 50, 100 or 175 mg/kg bw by gavage in corn oil on five days per week. At termination, there was no significant effect of furfural on body weight or survival. Histological examination showed chronic inflammation, necrosis and pigmentation in the liver of males in the two highest dose groups and in females of the top dose group only. Hepatocellular adenomas and carcinomas were observed in all dose groups, including controls but these tumours occurred with significantly increased incidence only in males of the top dose group. The incidence of carcinomas was similar in high dose females and controls. Tumours of other organs occurred only with low incidence and with no dose response relationship. Slight increases in the incidence of hyperplasia and papillomas in the forestomach of female mice were considered by the authors to be due to the irritating effect of gavage administration and not of toxicological significance; none of the animals had malignant lesions of the forestomach (NTP, 1990a).

In a similar study in Fischer 344/N rats, furfural was administered at doses of 0, 30 or 60 mg/kg bw by gavage in corn oil to 50 animals of each sex. Mild centrilobular hepatocellular necrosis occurred in all groups but the incidence did not appear to be dose related, particularly in females where the incidence

was inversely related to dose. Bile duct hyperplasia occurred with high incidence in all groups, including controls and did not appear to be treatment related. Focal bile duct dysplasia was seen in one male at the intermediate dose level and bile duct hyperplasia accompanied by fibrosis occurred in two males in the top dose group. One control male had a hepatocellular adenoma and two males in the high dose group had a cholangiocarcinoma (a rare tumour in historical controls). It was considered by the authors that although the incidence of this lesion was not statistically significant it offered some evidence of carcinogenicity (NTP, 1990a)."

2.1.2. Genotoxicity Studies

"Negative or weakly positive results have been obtained for most bacterial tests for genotoxicity. In particular, positive results were obtained in three out of several assays for reverse mutation in *Salmonella typhimurium* at relatively high concentrations in the absence of metabolic activation. Furfural was found to be clearly genotoxic in cultured mammalian cells at the gene and chromosome level in the absence of metabolic activation. It induced SCE in cultured CHO cells and human lymphocytes. It was genotoxic in Drosophila in somatic cells (Wing spot test by inhalation) and germ cells (sex-chromosome loss by injection). It did not induce reciprocal translocations and sex-linked recessive lethal mutations, with only a doubtful increase in one study in Drosophila. Furfural was not genotoxic in any *in vivo* mammalian assays for chromosome aberrations, SCE or UDS."

2.1.3. Conclusion

"The Committee was of the opinion that the data were not totally convincing in demonstrating that the carcinogenicity of furfural was mediated via a thresholded mechanism and hence was unable to allocate an ADI to furfural at the present time. It was aware that a study in transgenic mice of the potential of furfural to induce gene mutations *in vivo* was in progress. The results were expected to be available in the near future and the Committee would wish to re-evaluate furfural in the light of the results of this study."

2.2. Genotoxicity / Carcinogenicity (Text Taken from the EFSA Opinion on Furfural and Furfural Diethylacetal (EFSA, 2004c)

2.2.1. Genotoxicity

"In a new study not previously evaluated by the SCF, furfural was examined for its potential to induce gene mutations of the λ*lacZ*-gene *in vivo* in the liver of male transgenic mice (CD2F$_1$(BALB/c x DBA/2) strain 40.6, with *lacZ*-genes as reporter genes). The study was carried out under GLP. As formal technical guidelines for this type of study are not available, the study protocol was designed in conformity with principles for transgenic studies identified by international expert groups (Gorelick & Mirsalis, 1996; Heddle et al., 2000). The study was conducted in five groups, three of which received furfural by gavage in corn oil, one negative control group received vehicle alone and one positive control group received ethylnitrosourea (ENU). The furfural and negative control groups each comprised 13 mice plus 2 back-up animals; the positive control group comprised 8 mice plus 2 reserves. The furfural groups were given doses of 75, 150 or 300 mg furfural/kg bw in corn oil by gavage for 28 consecutive days; ENU was given to the positive control group by intraperitoneal injection in saline on days 5-9 of the study at a dose of 50 mg/kg bw/day. On day 28, three animals from each of the furfural and negative control groups were sacrificed for assessment of hepatotoxicity by clinical chemistry and histological examination. In addition, organ and body weights were monitored throughout. After a manifestation period of 34-35 days (days 62-63 of the study), the livers and samples of gastrointestinal tract tissues were fixed for mutation analysis. Mutation analysis was carried out on livers of eight animals per group. At least 5000 (preferably 120,000) plaque-forming units (PFU) were examined (one PFU corresponding to one recovered copy of the λ*gt10lacZ* shuttle vector).

There were three early decedents in the highest furfural dose group; two during treatment with no clinical signs, and one during the manifestation period. One animal from the low-dose group died during the manifestation period. The cause of death could not be ascertained.

Body weights in the furfural-treated groups showed a dose related increase compared to negative controls during the first week of treatment. In the post-treatment period the difference between control and two lower dose groups disappeared but the body weight of the group treated with 300 mg furfural/kg bw remained higher.

Evaluation of the clinical chemistry and gross and histopathology of the liver of the treated animals sacrificed at the end of the treatment period showed an increase in blood triglycerides, increased liver weight and centrilobular hypertrophy. This was interpreted by the authors as some evidence of hepatotoxicity. These changes did not persist until the end of the manifestation period, 34-35 days after the last dose.

The mutation frequency in DNA extracted from the livers of the negative control group was similar to historical data. There was no significant difference in mutation frequency between negative controls and the furfural-treated groups; the positive control group showed a significant increase in mutation frequency. It was concluded that oral administration of furfural in corn oil at levels of up to 300 mg/kg bw/day is not associated with an increase in the induction of mutations in liver cells of *λlacZ* transgenic mice (CIVO-TNO, 2003).

Negative or weakly positive results have been obtained for most bacterial tests for genotoxicity. In particular, positive results were obtained in three out of several assays for reverse mutation in *Salmonella typhimurium* at relatively high concentrations in the absence of metabolic activation. Furfural was found to be clearly genotoxic in cultured mammalian cells at the gene and chromosome level in the absence of metabolic activation. It induced Sister Chromatid Exchange (SCE) in cultured Chinese Hamster Ovary (CHO) cells and human lymphocytes. It was genotoxic in *Drosophila* in somatic cells (Wing spot test by inhalation) and germ cells (sex-chromosome loss by injection). It did not induce reciprocal translocations and sex-linked recessive lethal mutations, with only a doubtful increase in one study in *Drosophila*. Furfural was not genotoxic in any *in vivo* mammalian assays for chromosome aberrations, SCE or Unscheduled DNA Synthesis (UDS) and the study in transgenic mice confirms that furfural does not induce gene mutations *in vivo*."

2.2.2. Discussion

"The Panel noted the metabolic and toxicity data previously reviewed by the SCF together with the new results of the genotoxicity study in transgenic mice *in vivo*.

Furfural was negative in the *in vivo* genotoxicity assay and this corroborated earlier negative *in vivo* studies at the chromosome level and in a UDS assay.

In view of the absence of genotoxicity *in vivo,* the tumours observed in the long-term toxicity/carcinogenicity studies in male, but not female mice, are considered to arise as a consequence of chronic hepatotoxicity (hepatocellular necrosis) which was more marked in male animals. An increased tumour incidence was only observed at the highest dose level and at a dose higher than the minimal hepatotoxic dose.

It should be noted that no hepatocellular tumours were seen in the long-term rat study. However, liver toxicity was seen in this study (see SCF Opinion, Appendix 1) and the rat was considered more sensitive to liver toxicity. The hepatotoxicity of furfural is dose-dependent but a NOEL was not established in the long-term studies. However, the short-term (90-day) study in rats was conducted to establish a NOEL for hepatotoxicity, which was determined to be 54 mg/kg bw The Panel noted that because of possible formulation (corn oil) and dose regimen (bolus dose) effects observed in the gavage studies, the dietary administration studies were more appropriate for identifying a NOAEL.

The Panel concluded that the NOAEL of 54 mg/kg bw/day for hepatic changes from the 90 day dietary study was appropriate and noted that the effects observed with doses up to threefold higher were of doubtful toxicological relevance. Therefore the Panel concluded that a safety factor of 100 would be sufficient in establishing an ADI from this subchronic study (see SCF Opinion)."

2.2.3. Conclusion and Recommendation

"The Panel concluded that furfural did not exhibit genotoxicity *in vivo* in male mice, the species and sex which displayed an increased tumour incidence in long-term studies and that the tumours arose by a secondary mechanism consequent on hepatotoxicity, which is dose dependent, displays a threshold and is seen in both rats and mice. It was therefore considered that the NOEL for hepatotoxicity in the rat could be used to derive an ADI for furfural.

An ADI for furfural was established at 0.5 mg/kg bw based on the NOEL of 54 mg/kg bw from the 90-day rat study to which a 100 fold safety factor was applied. Since furfural diethylacetal is rapidly converted to furfural at physiological pH, the ADI applies also to the furfural component of furfural diethylacetal since furfural is readily liberated from the acetal *in vivo*."

2.3. EFSA Remark on Carcinogenicity Studies

The hepatocellular tumours induced in $B6C3F_1$ mice in the NTP study (NTP, 1990a) are not considered relevant for humans. Indeed, it was clearly demonstrated that such tumours arise in this strain of mice, which is highly susceptible to hepatocarcinogenicity due to the presence of various strain-species-specific genes (hcs). Thus, the study does not give rise to concern with respect to carcinogenicity in humans.

2.4. EFSA Remark on Genotoxicity of 5-Hydroxymethylfurfural – Text taken from FGE.218[4] (EFSA, 2009s)

"It is anticipated that 5-methylfurfural [FL-no: 13.001] can be oxidised to the primary alcohol 5-hydroxymethylfurfural [FL-no: 13.139]. 5-Hydryxymethylfurfural was evaluated by EFSA in FGE.13 dealing with furfuryl and furan derivatives (EFSA, 2005c). As 5-hydroxymethylfurfural may be metabolised to 5-[(sulphoxy)methyl]furfural which shows genotoxic potential *in vitro*, it was concluded that 5-hydroxymethylfurfural could not be evaluated through the Procedure (EFSA, 2005c). Accordingly 5-methylfurfural cannot be evaluated through the Procedure either."

2.5. Genotoxicity of Furfuryl Alcohol and Related Substances - Text Taken from JECFA (JECFA, 2001b)

No genotoxicity text was prepared by JECFA on the group of furfuryl alcohol and related substances – the studies are only given in table format (see Table 4).

One of the substances in the group of furfuryl alcohol and related substances is furfural [FL-no: 13.018], which also was considered separately at the 55th JECFA meeting where the following was stated:

"Furfural was evaluated previously by the Committee at its thirty-ninth and fifty-first meetings (JECFA, 1992a; JECFA, 2000a). An ADI was not established at either meeting because of concern about the finding of tumours in male mice given furfural in corn oil by gavage and the fact that no NOEL was identified for hepatotoxicity in male rats. In a study in mice, the combined incidence of

[4] The conclusion in section 2.4 is cited from the previous version of the present FGE, FGE.218. This conclusion is the basis for the request of additional genotoxicitydata in FGE.218.

adenomas and carcinomas was increased in males at the highest dose (175 mg/kg bw per day). In order to address its concern with regard to the formation of liver tumours in mice, the Committee at its fifty-first meeting requested the results of studies of DNA binding or adduct formation *in vivo* to clarify whether furfural interacts with DNA in the liver of mice, and also requested the results of a 90-day toxicity study in rats to identify a NOEL for hepatotoxicity (Annex 1, reference 137).

Since the last meeting, the results of a 14-day study to determine a dose range, a 90-day study of toxicity in rats, and an assay for unscheduled DNA synthesis in mice *in vivo* have become available. These data were reviewed and are summarised in the following monograph addendum."

"The ability of furfural to induce DNA repair in the hepatocytes of $B6C3F_1$ mice was assessed in an assay for unscheduled DNA synthesis. The maximum tolerated dose for animals of each sex was determined in a preliminary study to be 320 mg/kg bw. In the study of unscheduled DNA synthesis, doses of 50, 175, and 320 mg/kg bw were given to groups of three animals of each sex, and expression of DNA repair was measured 2–4 and 12–16 hours after treatment. N-Nitrosodimethylamine (20 mg/kg bw) was used to measure expression within 2–4 hours and aminoazotoluene (200 mg/kg bw) for expression within 12–16 hours, as positive controls.

The animals treated with furfural did not show increased UDS at either time after dosing, whereas the positive controls showed statistically significant increases in net nuclear grain counts. Little replicative DNA synthesis (0–0.4 %) was seen at either interval. The results provided no evidence that furfural damages DNA in mouse hepatocytes at doses up to 320 mg/kg bw (Edwards, 1999)."

"The results of an assay for unscheduled DNA synthesis in mice *in vivo* were reviewed by the Committee. This study, in which doses of up to 350 mg/kg bw were given, was particularly relevant since it addressed potential DNA repair in the cells in which tumours arose, namely hepatocytes. The negative results obtained in this assay were considered by the Committee to provide evidence that the liver tumours observed in the long-term study in mice were unlikely to have occurred through a genotoxic mechanism. The Committee considered that the concerns raised previously with respect to the liver tumours in mice were adequately addressed by this study and that a study of DNA binding was unnecessary."

3. EFSA Conclusions on Genotoxicity of Furfuryl Alcohol and Related Substances – Text taken from FGE.218[5] (EFSA, 2009s)

"The present group consists of furfural [FL-no: 13.018] and seven substances structurally related to furfural: 5-methylfurfural [FL-no: 13.001], furfuryl alcohol [FL-no: 13.019] and five esters of furfuryl alcohol and aliphatic saturated carboxylic acids [FL-no: 13.057, 13.062, 13.067, 13.068 and 13.128]. The five furfuryl esters are anticipated to be hydrolysed to furfuryl alcohol (and carboxylic acids), which is expected to be oxidised to the alpha,beta-unsaturated aldehyde furfural (EFSA, 2005c). Based on data available the Panel has previously concluded that furfural is not of concern with respect to genotoxicity (EFSA, 2004c). Furthermore, the Panel concluded that not only furfural but also the structurally related furfuryl alcohol and the five furfuryl esters are not of concern with respect to genotoxicity. Accordingly, these seven substances can be evaluated through the Procedure in FGE.66.

It is anticipated that 5-methylfurfural [FL-no: 13.001] can be oxidised to the primary alcohol 5-hydroxymethylfurfural [FL-no: 13.139]. 5-Hydroxymethylfurfural was evaluated by EFSA in FGE.13 dealing with furfuryl and furan derivatives (EFSA, 2005c). As 5-hydroxymethylfurfural may be metabolised to 5-[(sulphoxy)methyl]furfural which shows genotoxic potential *in vitro*, it was concluded that 5-hydroxymethylfurfural could not be evaluated through the Procedure. Accordingly 5-methylfurfural cannot be evaluated through the Procedure either."

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[5] The conclusion in Section 3 is cited from the previous version of the present FGE, FGE.218. This conclusion is the basis for the request of additional genotoxicitydata in FGE.218.

4. Additional Data Submitted by Industry

4.1. Background

As a response to the conclusions in FGE.13 (EFSA, 2005c) and FGE.218 (Adopted 9 July 2008), Industry has presented additional toxicology and metabolism data relevant to the safety evaluation of 5-hydroxymethylfurfural (5-HMF) [FL-no: 13.139] and 5-methylfurfural (5-MF) [FL-no: 13.001].

These data include:

- the NTP (2008-draft) carcinogenicity studies in mice and rats
- mutagenicity studies in *S. typhimurium* and *E. coli*,
- a 3-months mouse micronucleus assay (NTP, 2008b),
- an *in vitro* Comet assay (Durling et al., 2009) and
- metabolic data in mice (NTP, 2008b).

All of these studies have been carried out using 5-HMF as the testing substance.

Besides the new data submitted by Industry, additional studies (Dahlberg, 2004, Glatt et al., 2005, Glatt and Sommer, 2006, Monien et al., 2009) have been retrieved from the public literature by EFSA.

5-HMF has been shown to be bioactivated *in vitro* to 5-sulfoxy-methylfurfural (SMF) through sulphonation of its allylic hydroxymethyl group catalyzed by sulphotransferases (SULT). SMF can be transformed to a highly reactive electrophilic allyl carbocation, which can react with nucleophiles (e.g. DNA) producing mutagenic effects. With few exceptions, HMF was negative in most *in vitro* genotoxicity tests, very likely because the metabolic activation systems lacked SULT enzymes or the cofactor sulpho-group donor PAPS. 5-HMF was mutagenic in *Salmonella typhimurium* after addition of PAPS to the liver cytosol, while SMF was directly mutagenic. No *in vivo* genotoxicity data were reported in FGE 13. Even if the occurrence of the metabolic pathway through sulphonation by SULT enzymes *in vivo* cannot be ruled out, the data reported in FGE.13 (Godfrey et al., 1999) indicate that for 5-HMF the principal route in mice and rats is the oxidation to furoic acid, followed by conjugation with glycine and by rapid excretion in the urine.

4.2. Summaries and Evaluation of Additional Data

4.2.1. Carcinogenicity Studies

Groups of $B6C3F_1$ mice (50/sex/dose) were administered 0, 188, 375 or 750 mg/kg body weight (bw) per day of 5-HMF, five days per week for 104 weeks *via* aqueous gavage. Survival probabilities of male and female mice in the 750 mg/kg bw per day dose group were significantly lower than those of vehicle controls. Mean body weights of 750 mg/kg males were 14 % lower than those of the vehicle controls after week 26. Mean body weights of 375 and 750 mg/kg females were 9 % and 30 % lower, respectively, than those of the vehicle controls after week 36. Because of the reduced survival (30 % reduction) of the groups receiving 750 mg/kg bw per day 5-HMF, the groups of mice receiving this dose were not included in the evaluation of carcinogenic potential. Incidences of hepatocellular adenoma or carcinoma (combined) were significantly increased in the 188 and 375 mg/kg female mice when compared to vehicle controls, with increased incidences (about two-fold, not dose-related) of 53 % and 52 %, respectively. No carcinogenic effect was observed in male mice (NTP, 2008b). Historical control data for hepatocellular adenoma in female $B6C3F_1$ mice were: 20/50, 11/50, 8/50, 10/51, 13/50, 12/50, 3/50, 6/50, 17/60 (Average 21.7 %) (NTP, 2008b). Survival of both males and female mice in the 750 mg/kg group was significantly lower than that of the vehicle control group.

Groups of F344/N rats (50/sex/dose) were administered 0 (vehicle control), 188, 375 and 750 mg/kg bw per day of 5-HMF, five days per week for 104 weeks *via* aqueous gavage. Survival of the 188 and 750 mg/kg bw per day male groups was higher than that of vehicle controls and the remaining groups were comparable to vehicle controls. Mean body weights of all test groups were comparable to vehicle controls throughout the study. Males of the 188 and 375 mg/kg bw per day males showed increased incidences of calcitonin-producing parafollicular cell adenoma or carcinoma (combined) of the thyroid gland. No other carcinogenic effects were observed (NTP, 2008b).

The NTP concluded: "Under the conditions of this 2-year gavage study, there was no evidence of carcinogenic activity in male or female F344/N rats administered 188, 375 or 750 mg/kg/day. There was no evidence of carcinogenic activity in male B6C3F$_1$ mice administered 188 or 375 mg/kg. There was some evidence of carcinogenic activity of 5-(hydroxymethyl)-2-furfural in female B6C3F$_1$ mice, based on increased incidences of hepatocellular adenoma in the 188 and 375 mg/kg groups."

According to the Panel, the about two-fold increase (not dose-related) of hepatocellular tumours observed in B6C3F$_1$ female mice is not relevant for humans, in view of the recognized high genetic susceptibility of this strain to hepatocarcinogenesis. These studies do not give rise to concern with respect to the carcinogenic potential of 5-HMF.

4.2.2. Genotoxicity Studies

Weak mutagenic activity was reported in *S. typhimurium* TA100 strain in the absence of metabolic activation, while no mutagenicity was observed in strains TA97, TA98, TA102 and TA1535 in a range of concentrations of 100-10,000 micrograms/plate; however, negative results were reported in another study with TA98 and TA100 strain and *E.coli* WP2 uvrA/pKM101 in a range of concentrations of 1,500-10,000 micrograms/plate (NTP, 2008b).

At the end of a 3-month toxicity study, peripheral blood samples were obtained from male and female B6C3F$_1$ mice receiving 0, 47, 188, 375 or 750 mg/kg bw/day of 5-HMF *via* gavage. Slides were scanned to determine the frequency of micronuclei in 1,000 normochromatic erythrocytes (NCEs) in 10 animals per sex per treatment group. In addition, the percentage of polychromatic erythrocytes (PCE) in a population of 1,000 erythrocytes was determined as a measure of bone marrow toxicity. No increases in the frequency of micronucleated erythrocytes were observed; in addition, no significant dose-related changes in the percentage of immature PCE were observed, suggesting that the chemical did not exhibit bone marrow toxicity (NTP, 2008b).

The DNA-damaging potential of 5-HMF was tested *in vitro* in the Comet assay with the following five cell lines with various degree of SULT1A1 expression (Durling et al., 2009):

two human lines (Caco-2, no detectable 1A1 activity; HEK293, high 1A1 activity),

two cell lines from Chinese hamster (V79, no detectable 1A1 activity and V79-hp-PST, high 1A1 activity) and

one mouse lymphoma line (L5178Y, no detectable activity).

The cell lines were incubated with 0, 2.5, 7.5, 25, 50 or 100 mM *(ca.* 0, 0.3, 1.0, 3.3 6.3 or 12.6 mg/ml) of 5-HMF for three hours and subjected to a Comet assay to assess DNA damage. DNA damage was observed at the highest concentration (100 mM) in all cell lines, with significant reduction in cell viability (from 11 to 30 %). The concentration of 100 mM is 10 times higher than the highest concentration (10 mM or 5000 micrograms/ml) recommended by OECD guidelines for *in vitro* testing with mammalian cells. 100 mM was the lowest effective concentration for three cell lines, Caco-2, HEK293 and L5178Y. In the V79 (lowest SULT1A1) and V79-hp-PST (highest SULT1A1) DNA damage was induced also at lower concentrations (lowest effective concentration: 25 mM or 3193 micrograms/ml), without a reduction in cell viability. Surprisingly, the positive control (HMP,

0.01 mM) induced significant damage in Caco-2, V79 and V79-hp-PST cells, but not in HEK293. The authors (Durling et al., 2009) concluded that in all cell lines 5-HMF-induced DNA damage was unrelated to the expression of SULT1A1, but they mentioned that the SULT1A1 activities in these three cell lines (Caco-2, HEK293 and L5178Y) were much lower than those that can be found in human gut and liver. The possibility was left open that SULT1A1 activity was too low to efficiently bioactivate 5-HMF also in the cell line with highest SULT1A1 activity. In V79 cells without SULT1A1 activity and in V79-hp-PST with SULT1A1 activity at the same level as in human gut and liver, no difference in extent of DNA-damage could be observed. This would indicate absence of a significant contribution of sulphate conjugation in the DNA-damaging activity of 5-HMF.

These results are in conflict with the results of Glatt et al. (Glatt et al., 2005) who reported induction of SCE in 5-HMF-exposed genetically modified V79 cells expressing high levels of human CYP2E1 and SULT1A1. They are also in contrast with the observations by Sommer et al. (2003; abstract only) reporting the mutagenicity of 5-HMF in a genetically modified *S. typhimurium* strain expressing human SULT1A1. According to Durling et al. (2009), the reasons of these discrepancies are unknown. One possibility is the different sensitivity of the Comet assay compared to other systems. Durling et al. (2009) concluded that other important mechanisms for the observed DNA damage should be investigated, but that under the conditions of the test, 5-HMF is a rather weak DNA-damaging agent.

In a new publication by Severin et al. (2010), a dose dependent increase in DNA damage was observed in a Comet assay with HepG2 cells exposed to 5-HMF (0, 5.35, 7.87, 11.57, 17, 25, 36.6 mM) for 20 hours, with a significant increase from 7.87 to 36.6 mM 5-HMF. Cytotoxicity was observed at the two highest doses (25 and 36.6 mM), with an estimated IC_{50} of 38 mM. HepG2 cells express both CYP and SULT enzymes. In the same publication, no effect of 5-HMF was found in an *in vitro* micronucleous assay in the same cell line exposed to similar doses of 5-HMF (20 hours). 5-HMF was also tested in an Ames test performed according to the OECD guidelines 471. No increase in mutants was observed in *S. typhimurium* strains TA 98, TA 100, TA1535, TA 1537 exposed to 5-HMF at 0.5 µg/mL up to 5000 µg/mL with or without metabolic activation (S9). No additional PAPS was added to the test system, making activation by SULT less likely (Severin et al., 2010).

However, while 5-methylfurfural (5-HMF) was unable to induce micronuclei *in vivo*, in the NTP 3-months study in mice by gavage, and *in vitro*, using the Hep-G2 human cell line expressing both CYP and SULT enzymes, its metabolite 5-sulphoxy-methylfurfural (5-SMF) has been reported to induce micronuclei in peripheral erythrocytes in mice (Dahlberg, 2004).

According to Glatt and Sommer (2006), incubation of DNA with SMF in a cell-free system led to the formation of DNA adducts that could be detected by the ^{32}P-postlabelling technique. No adducts were formed after incubations with 5-HMF instead of 5-SMF. In subsequent experiments, the authors searched for these adducts in mammalian and bacterial cells treated with 5-SMF and in SULT-proficient cells treated with 5-HMF. Although mutations were induced, adducts were not seen in these cells under the same conditions. The authors hypothesized that the lack of DNA adducts might be due to technical problems, since generally DNA adducts are a more sensitive endpoint than mutations as observed with many other compounds (Glatt & Sommer, 2006).

The additional available genotoxicity studies are summarised in Tables 7 and 8.

4.2.3. New Metabolic Data

As a part of the NTP 3-week and 3-month sub-chronic toxicity studies program, male and female F344/N rats and B6C3F$_1$ mice were administered 1,500 mg/kg bw per day of 5-HMF. During this time the urine of the animals was collected and analyzed for the presence of metabolites. The two major metabolites detected were 5-(hydroxymethyl)-2-furoic acid and the corresponding glycine conjugate 5-(Hydroxymethyl)-furoyl glycine. Continuous exposure of mice to 5-HMF showed that a considerably larger amount of each metabolite was excreted in the urine at day 94 as compared to day 17. This would indicate that exposure to 5-HMF induces enzymes that facilitate oxidation to furoic

acid and conjugation to glycine. As expected due to its reactivity and short half-life, 5-(sulphoxy)-methylfurfural was not detected (see below) (NTP, 2008b).

Monien et al. (2009) have shown that 5-(sulphoxy)-methylfurfural (5-SMF) is formed *in vivo* in mice. Twelve week-old FVB/N mice (n=28) were given an intravenous injection of 100 mg/kg bw 5-HMF in isotonic saline. Two blood samples were taken from each animal, and blood samples were pooled from different animals to study 5-HMF and 5-SMF pharmacokinetics. 5-SMF was detected in plasma from animals given 5-HMF, and the half-life of 5-SMF was calculated to be 4.2 minutes. It should be noted that 5-SMF is very hydrophilic and therefore has limited capacity to cross cell membranes and of entering cells. Therefore 5-SMF would be expected to induce mutation at the site of formation, i.e mainly within the liver cell. Based on the plasma half-life of 4.2 minutes, it is not expected that this metabolite could reach the bone marrow (Monien et al., 2009).

4.3. Discussion of the newly submitted data

Taking into account all the presently available data the following scenario emerges:

5-HMF is negative in the conventional Ames test. Mutagenicity is observed only upon inclusion of PAPS, a sulpho-group donor and liver cytosol into the metabolic system, suggesting the formation of a sulphate-ester (5-SMF). In accordance, 5-SMF was mutagenic in the absence of any metabolic activation system. In an *in vitro* assay, 5-HMF induced dose-dependent increase in DNA damage (Comet assay), but this study has major drawbacks and inconsistencies that limit its validity. A major limit is the use of too high concentrations that can produce unpredictable effects, not related to the real genotoxic potential of 5-HMF, and this is particularly true for a test like the Comet assay. Furthermore, as also stated by the authors, DNA damage was unrelated to the expression of SULT1A1 activity. Also in another Comet assay in HepG2 cells, able to express both CYP and SULT enzymes, indications for DNA damage were observed, but the substance did not induce clastogenic or aneugenic effects in a micronucleus assay in the same cell system.

In vivo, a non-standard micronucleus assay in peripheral blood erythrocytes associated to a sub-chronic study in mice, provided no indication of a genotoxic potential, but this study has limited validity since no bone marrow cell toxicity was observed.

Metabolic studies in B6C3F$_1$ mice and rats indicate that *in vivo* the principal route of metabolism is oxidation of 5-HMF to furoic acid, followed by glycine conjugation and rapid elimination in the urine. However, a recent pharmacokinetic study in FVB/N mice has shown that SMF (half-life of 4.2 minutes) has been detected in plasma from animals given 5-HMF intravenously. This indicates that there is a competition for the substrate 5-HMF between the oxidation pathway leading to furoic acid and the sulphonation pathway leading to the 5-SMF metabolite. In rodents the formation of the SMF metabolite is too low to result in a carcinogenic response. Assuming that the ratio between the two competing pathways is not more favorable for the formation of 5-SMF in humans than in rodents, no genotoxicity or carcinogenicity is expected in humans either. As reported in FGE.13Rev1 (EFSA, 2009am), the limited data available in humans showed that furoylglycine and 2,5-furan dicarboxylic acid can be found in urine, and that these metabolites are derived from precursors in food e.g. fructose (Jellum et al., 1973). Thus, it can be anticipated that in humans, after oral administration, 5-HMF will also be rapidly converted into furoic acid derivatives which will be rapidly eliminated *via* the urine.

The results of NTP long-term carcinogenicity bioassays have shown that 5-HMF is not carcinogenic in male and female rats, as well as in male mice. The about two-fold increase (not-dose-related) of hepatocellular adenomas in B6C3F$_1$ female mice, a strain known to be genetically highly susceptible to liver tumours, is considered to be not relevant for humans.

4.4. Conclusion

Notwithstanding the indications of *in vitro* genotoxicity in conditions that favour the formation of 5-SMF and the limited *in vivo* genotoxicity study, the essentially negative results of the carcinogenicity study in rats and mice indicate therefore that 5-HMF is of no concern under the conditions of intended use (EFSA, 2005c). This conclusion is also applicable to 5-methylfurfural, a candidate substance in the current FGE, because this substance may be metabolised to 5-hydroxymethylfurfural. Accordingly, both 5-HMF [FL no: 13.001] and 5-MF [FL-no: 13.139] can be evaluated through the Procedure.

TABLE 1: SPECIFICATION SUMMARY OF THE SUBSTANCES IN THE FLAVOURING GROUP EVALUATION 218 (JECFA, 2001B)

Table 1: Specification Summary of the Substances in the Flavouring Group Evaluation 218 (JECFA, 2001b)

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility 1) Solubility in ethanol 2)	Boiling point, °C 3) Melting point, °C ID test Assay minimum	Refrac. Index 4) Spec.gravity 5)
13.001 745	5-Methylfurfural		2702 119 620-02-0	Liquid $C_6H_6O_2$ 110.11	Slightly soluble Miscible	187 IR 97 %	1.525-1.532 1.098-1.108
13.018 450	Furfural		2489 2014 98-01-1	Liquid $C_5H_4O_2$ 96.10	soluble miscible	161-162 IR 95 %	1.521-1.529 1.153-1.162
13.019 451	Furfuryl alcohol		2491 2023 98-00-0	Liquid $C_5H_6O_2$ 98.1	miscible miscible	169-171 IR 97 %	1.481-1.489 1.126-1.136
13.057 743	Furfuryl isovalerate		3283 10642 13678-60-9	Liquid $C_{10}H_{14}O_3$ 182.22	Insoluble Miscible	97-98 (14 hPa) IR 98 %	1.456-1.464 1.014-1.023
13.062 740	Furfuryl propionate		3346 10646 623-19-8	Liquid $C_8H_{10}O_3$ 154.17	Slightly soluble Miscible	195-196 IR 98 %	1.457-1.464 1.076-1.086
13.067 742	Furfuryl octanoate		3396 10645 39252-03-4	Liquid $C_{13}H_{20}O_3$ 224.30	Insoluble Miscible	139 (13 hPa) IR 98 %	1.456-1.464 0.980-0.989
13.068 741	Furfuryl valerate		3397 10647 36701-01-6	Liquid $C_{10}H_{14}O_3$ 182.22	Insoluble Miscible	228-229 IR 98 %	1.457-1.462 1.024-1.031
13.128 739	Furfuryl acetate		2490 2065 623-17-6	Liquid $C_7H_8O_3$ 140.14	Insoluble Miscible	175-177 IR 97 %	1.457-1.466 1.110-1.119

1) Solubility in water, if not otherwise stated.
2) Solubility in 95 % ethanol, if not otherwise stated.
3) At 1013.25 hPa, if not otherwise stated.
4) At 20°C, if not otherwise stated.
5) At 25°C, if not otherwise stated.

TABLE 2: SUMMARY OF SAFETY EVALUATION APPLYING THE PROCEDURE (BASED ON INTAKES CALCULATED BY THE MSDI APPROACH)

Table 2: Summary of Safety Evaluation Applying the Procedure as Applied by JECFA (JECFA, 2001a)

FL-no JECFA-no	EU Register name	Structural formula	MSDI 1) (µg/capita/day)	Class 2) Evaluation procedure path 3)	Outcome on the named compound [4) or 5]
13.001 745	5-Methylfurfural		180 25	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)
13.018 450	Furfural		440 460	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)
13.019 451	Furfuryl alcohol		180 24	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)
13.057 743	Furfuryl isovalerate		0.024 1	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)
13.062 740	Furfuryl propionate		1.7 5	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)
13.068 741	Furfuryl valerate		0.24 14	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)
13.128 739	Furfuryl acetate		16 21	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	4)
13.067 742	Furfuryl octanoate		0.012 6	Class III B3: Intake below threshold, B4: Adequate NOAEL exists	4)

1) EU MSDI: Amount added to food as flavour in (kg / year) x 10E9 / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita/day.

2) Thresholds of concern: Class I = 1800, Class II = 540, Class III = 90 µg/person/day.

3) Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.

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

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

TABLE 3: CARCINOGENICITY STUDIES (SCF, 2003A)

One carcinogenicity study is available for the substances in subgroup 4.2.

Table 3: Carcinogenicity Studies

Chemical Name [FL-no] [JECFA-no]	Species; Sex No./Group	Route	Dose levels	Duration	Results (EFSA interpretation)	Reference	EFSA Comments
Furfural [13.018] [450]	Rats; Male , Female 50/sex/group	Gavage	0, 30 and 60 mg/kg bw/day in corn oil for 5 days/week	2 year	Male: Centrilobular necrosis of the liver was seen at increased incidences in the dosed male rats (control, 3/50; low dose, 9/50; high dose, 12/50. Two high dose males had bile duct dysplasia with fibrosis, and two had cholangiosarcomas. Female: No increases in tumour incidences	(NTP, 1990a)	The Panel agrees with the authors that for male rats there is some evidence of carcinogenic activity and for females no evidence of carcinogenic activity.
	Mice; Male, Female 50/sex/group	Gavage	0, 50, 100 and 175 mg/kg bw/day in corn oil for 5 day/week	2 year	Male: Increased incidence of hepatocellular adenomas and carcinomas in the high dose group Female: Increased incidence of hepatocellular adenomas and carcinomas in the high dose group. Forestomach hyperplasia and squamous cell papillomas were increased in the high dose group.	(NTP, 1990a)	1) The Panel agrees with the authors that for male mice there is clear evidence of carcinogenic activity and for females some evidence of carcinogenic activity.

1) The Panel noted the strain/species specific susceptibility of the tested mice to hepatocarcinogenesis. These tumours are not relevant for the evaluation of carcinogenic effect for humans.

TABLE 4: GENOTOXICITY (*IN VITRO* AND *IN VIVO*) (JECFA, 2001B)

Table 4: Summary of genotoxicity data of furfuryl derivatives evaluated by JECFA (JECFA, 2001b)

FL-no JECFA-no	EU Register name JECFA name	Structural formula	End-point	Test system	Concentration	Results	Reference
13.019 451	Furfuryl alcohol		Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	294 µg/plate	Negative[a,b]	(Florin et al., 1980)
			Reverse mutation	*S. typhimurium* TA98, TA100, TA1535	Up to 10 000 µg/plate	Negative[a,b]	(Mortelmans et al., 1986)
			Reverse mutation	*S. typhimurium* TA100	2500–12 500µg/ml	Negative[a,b]	(Stich et al., 1981a)
			Reverse mutation	*S. typhimurium* TA98, TA100, TA102	Up to 198 000 µg/plate	Negative[a,b]	(Aeschbacher et al., 1989)
			Reverse mutation	*S. typhimurium* TA98, TA100	81–323 µg/plate	Negative[a,b]	(Shinohara et al., 1986)
			Reverse mutation	*S. typhimurium* TA1535, TA100, TA1537 (modified assay)	200 000 µg/ml	Positive[a,b]	(McGregor et al., 1981)
			DNA repair and H17 (rec+)	*B. subtilis* M45 (rec–) µg/disc	2000–20 000	Positive[a,b]	(Shinohara et al., 1986)
			Sister chromatid exchange	Chinese hamster ovary cells	245 µg/ml	Positive[a,b]	(Stich et al., 1981b)
			Sister chomatid exchange	Human lymphocytes	Up to 196 µg/ml	Negative	(Jansson et al., 1986)
			Siser chromatid exchange	Human lymphocytes	Up to 970 µg/ml	Negative	(Gomez-Arroyo & Souza, 1985)
			Chromosomal aberration	Chinese hamster ovary cells	2000 µg/ml	Positive	(Stich et al., 1981b)
			Gene conversion	*S. cerevisiae* strain D7	13 500–16 000 µg/ml	Positive[a]	(Stich et al., 1981a)
			Sex-linked recessive lethal mutation	*D. melanogaster*	6500 ppm by injection	Negative	(Rodriquez-Arnaiz et al., 1989)
			Sister chromatid exchange	Adult human lymphocytes	32 300 mg/m3 in occupational atmosphere	Negative	(Gomez-Arroyo & Souza, 1985)
			Sister chomatid exchange	Adult human lymphocytes	32 300 mg/m3 in occupational atmosphere	Negative	(Gomez-Arroyo & Souza, 1985)
			Chromosomal aberration	Mouse bone marrow cells	0.5 mg/kg bw in drinking water 1–2 mg/kg bw in drinking water	Negative Positive	(Sujatha & Subramanyam, 1994)
13.128 739	Furfuryl acetate		Reverse mutation	*S. typhimurium* TA1535, TA98, TA100	33–666 µg/plate	Positive[a,b]	(Mortelmans et al., 1986)
13.018 450	Furfural		Reverse mutation	*S. typhimurium* TA1535, TA100, TA1537, TA1538, TA98	0.1–1000 µg/ml	Negative[a,b]	(McMahon et al., 1979)
			Reverse mutation	*S. typhimurium* TA100, TA98, TA1535	Up to 3460 µg/plate 5766 µg/plate	Negative[a,b] Positive[a] (weak)	(Loquet et al., 1981)
			Reverse mutation	*S. typhimurium* TA100, TA98, TA102	Up to 115 320 µg/plate	Negative[a,b]	(Aeschbacher et al., 1989)
			Reverse mutation	*S. typhimurium* TA100, TA98	15–63 µg/plate	Negative[a,b]	(Shinohara et al., 1986)
			Reverse mutation	*S. typhimurium* TA104	5–500 µg/plate	Positive[b]	(Shane et al., 1988)
			Reverse mutation	*S. typhimurium* TA100, TA102	5–500 µg/plate	Negative[b]	(Shane et al., 1988)
			Reverse mutation	*S. typhimurium* TA104, TA102	96 µg/plate	Negative	(Marnett et al., 1985a)

Table 4: Summary of genotoxicity data of furfuryl derivatives evaluated by JECFA (JECFA, 2001b)

FL-no JECFA-no	EU Register name JECFA name	Structural formula	End-point	Test system	Concentration	Results	Reference
			Reverse mutation	*S. typhimurium* TA98, TA100, TA1535	Up to 6667 µg/plate	Negative[a,b]	(Mortelmans et al., 1986)
13.018 450	Furfural (cont.)		Reverse mutation	*S. typhimurium* TA98, TA100	Up to 1000 µg	Negative[a]	(Osawa & Namiki, 1982)
			Reverse mutation	*S. typhimurium* TA98, TA100, TA1535, TA1537	33– 6666 µg/plate	Negative[a,b] Equivocal in TA100[a]	(NTP, 1990a)
			Reverse mutation	*S. typhimurium* TA100	8000 µg/plate	Positive[a,b]	(Zdzienicka et al., 1978)
			Reverse mutation	*S. typhimurium* TA98	8000 µg/plate	Negative[a,b]	(Zdzienicka et al., 1978)
			Reverse mutation	*S. typhimurium* TA100, TA102	100–10 000 µg/plate	Negative[a]	(Dillon et al., 1998)
			Reverse mutation	*S. typhimurium* TA104	100–10 000 µg/plate	Equivocal[a]	(Dillon et al., 1998)
			Reverse mutation	*S. typhimurium* TA102, TA104	100–10 000 µg/plate	Negative[b]	(Dillon et al., 1998)
			Reverse mutation	*S. typhimurium* TA100	100–10 000 µg/plate	Equivocal[b]	(Dillon et al., 1998)
			Reverse mutation	*S. typhimurium* TA100 (modified assay)	426 µg/plate	Negative[a,b]	(Kim et al., 1987b)
			Reverse mutation	*S. typhimurium* TA100, TA1535, TA1537 (modified assay)	200 000 µg/ml	Negative	(McGregor et al., 1981)
			Reverse mutation	*E. coli* WP2, WP2 uvrA (modified assay)	0.1–1000 µg/ml	Negative[a,b]	(McMahon et al., 1979)
			SOS induction	*S. typhimurium* TA1535/ pSK1002	1932 µg/ml	Negative[a,b]	(Nakamura et al., 1987)
			DNA repair	*B. subtilis* H17 (rec+) and M45 (rec–)	Up to 1000 µg	Negative[a]	(Osawa & Namiki, 1982)
			DNA repair	*B. subtilis* H17 (rec+) and M45 (rec–)	0.6 ml	Negative[a,b]	(Matsui et al., 1989)
			DNA repair	*B. subtilis* H17 (rec+) and M45 (rec–)	1700–17 000 µg/disc	Positive[a,b]	(Shinohara et al., 1986)
			Forward mutation	*L5178Y* mouse lymphoma cells, Tk+/– locus	25–100 µg/ml 200 µg/ml	Negative[a] Positive[a]	(McGregor et al., 1988b)
			Sister chromatid exchange	Chinese hamster ovary cells	2500–4000 µg/ml	Positive[a,b]	(Stich et al., 1981b)
			Sister chromatid exchange	Chinese hamster ovary cells	Up to 1170 µg/ml	Positive[a,b]	(NTP, 1990a)
			Sister chromatid exchange	Human lymphocytes	Up to 0.035 mmol/L[a] 0.07– 0.14 mmol/L[c]	Negative[a,b] Positive[a,b]	(Gomez-Arroyo & Souza, 1985)
			Chromosomal aberration	Chinese hamster ovary cells	500 µg/ml 1000–2000 µg/ml	Negative Positive	(Nishi et al., 1989)
			Chromosomal aberration	Chinese hamster ovary cells	Up to 40 mmol/L (3840 mg)	Positive[a,b]	(Stich et al., 1981b)
			Chromosomal aberration	Chinese hamster ovary cells	3000 µg/ml	Positive	(Stich et al., 1981a)
			Chromosomal aberration	Chinese hamster ovary cells	Up to 1230 µg/ml	Positive[a,b]	(NTP, 1990a)
			Unscheduled DNA synthesis	Human liver slices	0.005–10 mmol/L	Negative	(Adams et al., 1998b)
			Sex-linked recessive lethal mutation	*D. melanogaster*	1000 mg/kg of diet	Negative	(Woodruff et al., 1985)
			Sex-linked recessive lethal mutation	*D. melanogaster*	100 mg/kg by injection	Positive	(Woodruff et al., 1985)
			Sex-linked recessive lethal mutation	*D. melanogaster*	Up to 6500 mg/kg by injection	Negative	(Rodriquez-Arnaiz et al., 1989)
			Chromosomal loss	*D. melanogaster*	Oral or injected dose of 3750–5000 mg/kg of diet. Mated with repair-proficient females	Negative	(Rodriquez-Arnaiz et al., 1992)
			Chromosomal loss	*D. melanogaster*	Oral or injected dose of 3750–5000 mg/kg of diet. Mated	Positive	(Rodriquez-Arnaiz et al., 1992)

Table 4: Summary of genotoxicity data of furfuryl derivatives evaluated by JECFA (JECFA, 2001b)

FL-no JECFA-no	EU Register name JECFA name	Structural formula	End-point	Test system	Concentration	Results	Reference
					with repair-deficient females		
			Reciprocal translocation	*D. melanogaster*	100 mg/kg by injection	Negative	(Woodruff et al., 1985)
13.018 450	Furfural (cont.)		Sister chromatid exchange	Mouse bone-marrow cells	50–200 mg/kg bw by injection	Negative	(NTP, 1990a)
			Spermhead abnormalities	Mice	4000 mg/kg of diet daily for 5 weeks	Negative	(Subramanyam et al., 1989)
			Somatic chromosomal mutation	Swiss albino mouse bonemarrow cells	1000–2000 mg/kg of diet 4000 mg/kg bw for 5 days	Negative Positive	(Subramanyam et al., 1989)
			Sister chromatid exchange	Adult human lymphocytes	9454 mg/m^3 in occupational atmosphere	Negative	(Gomez-Arroyo & Souza, 1985)
			Chromosomal aberration	Adult human lymphocytes	9454 mg/m^{3}in occupational atmosphere	Negative	(Gomez-Arroyo & Souza, 1985)
			Unscheduled DNA synthesis	B6C3F1 mice	50–320 mg/kg bw orally	Negative	(Edwards, 1999)
			Unscheduled DNA synthesis	Fischer 344 rats	5–50 mg/kg bw orally	Negative	(Phillips et al., 1997)
			Reverse mutation	*S. typhimurium* TA1537, TA100, TA1535	288 µg/plate	Negative[a,b]	(Florin et al., 1980)
13.001 745	5-Methylfurfural		Reverse mutation	*S. typhimurium* TA98, TA100, TA102	96,100 µg/plate	Negative[a,b]	(Aeschbacher et al., 1989)
			Reverse mutation	*S. typhimurium* TA98, TA100	79–316 µg/plate	Negative[a,b]	(Shinohara et al., 1986)
			DNA repair	*B. subtilis* H17 (rec+) and M45 (rec–)	0.55–5500 µg/disk	Positive[a,b]	(Shinohara et al., 1986)
			Sister chromatid exchange	Chinese hamster ovary cells	2200–4070 µg/ml	Positive[a,b]	(Stich et al., 1981b)

NR=Not Reported.

[1]With and without S9 metabolic activation.

[2] Without S9 metabolic activation.

[3] With S9 metabolic activation.

[4] Concentration that was added to the culture.

[5] Significant increases in % DNA-protein cross-links occurred only when cell viability was 40 % or less (i.e. high incidence of cell death).

[6] TA98 with S9 metabolic activation; TA100 without S9 metabolic activation.

[7] 5-Hydroxymethylfurfuraldehyde with 0.05 mol L-tryptophan without the presence of nitrite treatment.

[8] 5-Hydroxymethylfurfuraldehyde with 0.05 mol L-tryptophan treated with nitrite.

[9] At concentrations of 12 mmol and greater, positive results were obtained without S9 metabolic activation. The dose dependent results were noted at concentrations known to be cytotoxic.

[10] Metabolic activation not reported.

[11] Effects occurred at concentrations inhibiting cellular growth.

[12] Dose levels above 300 microgram/ml were cytotoxic.

TABLE 5: GENOTOXICITY OF FURFURAL, SCF OPINION ON FURFURAL AND FURFURAL DIETHYLACETAL (SCF, 2003A)

Table 5: Genotoxicity on Furfural, SCF Opinion on Furfural and Furfuraldiethylacetal (SCF, 2003a)

Substance	End-point	Test object	Concentration	Result	Reference
In vitro					
Furfural	Reverse mutation	*S.typhimurium* TA100, TA98, TA1535	0.05-60 µmol/plate	Weakly positive (TA100)[b]	(Loquet et al., 1981)
		S.typhimurium TA100, TA98, TA102	≤1.2 mmol/plate	Negative[a]	(Aeschbacher et al., 1989)
		S.typhimurium TA100, TA98	0.165-0.660 µmol/plate	Negative[a]	(Shinohara et al., 1986)
		S.typhimurium TA102, TA104	5-500 µg/plate	Positive (TA104)	(Shane et al., 1988)
		S.typhimurium TA98, TA100,TA1535, TA1537	33.3-6666 µmol/plate	Negative[a]	(Mortelmans et al., 1986)
		S.typhimurium TA98, TA100	1-15 µL/plate	Positive[a] (TA100)	(Zdzienicka et al., 1978)
		S.typhimurium TA98, TA100	7 µL/plate	Negative[a]	(Sasaki & Endo, 1978)
		S.typhimurium TA100	4.44 µmol/plate	Negative[a]	(Osawa & Namiki, 1982)
		S.typhimurium TA98, TA100, TA104, E.coliWP2uvrA/PKM101	20 µL/plate	Negative[a]	(McMahon et al., 1979)
		S.typhimurium TA104	1 µmol (max. non-toxic dose)	Negative[b]	(Marnett et al., 1985a)
	Umu gene expression	*S.typhimurium* TA1535/pSK/002	1932 µg/mL	Negative[a]	(Nakamura et al., 1987)
	Rec assay	*B.subtilis* H17, M45	1.7-17 mg/disk	Positive[a]	(Shinohara et al., 1986)
		B.subtilis H17, M45	1 mg/disk	Negative[a]	(Matsui et al., 1989)
	Forward mutation	L5178Ytk+/- mouse lymphoma cells	25-800 µg/mL	Positive[b]	(McGregor et al., 1988b)
	Chromosomal aberration	Chinese hamster ovary cells	10-40 mM	Positive[a]	(Stich et al., 1981a; Stich et al., 1981b)
		Chinese hamster ovary cells	200-1230 µg/mL	Positive[a]	(Galloway et al., 1985)
		Chinese hamster ovary cells	1.5-5000 µg/mL	Positive[a]	(Gudi & Schadly, 1996)
		Chinese hamster V79 cells	500-2000 µg/mL	Positive[a]	(Nishi et al., 1989)
	Sister chromatid exchange	Chinese hamster ovary cells	11.7-3890 µg/mL	Positive[a]	(Galloway et al., 1985)
		Human peripheral lymphocytes	3.5-14x10-5 M	Positive[b]	(Gomez-Arroyo & Souza, 1985)
	Unscheduled DNA synthesis	Human liver slices	0.14 mmol/L 0-25 mmol/L	Negative	(Lake, 1998)
In vivo					
Furfural	Sex-linked recessive lethal mutation	*D.melanogaster*	1000 ppm, in diet	Negative	(Woodruff et al., 1985)
		D.melanogaster	100 ppm, by injection	Positive	(Woodruff et al., 1985)
	Wing spot test	*D.melanogaster*	3750-7500 ppm by aerial exposure	Positive	(Rodriquez-Arnaiz et al., 1989)
	Sex-chromosome loss	*D.melanogaster*	3750-5000 ppm, in diet and by injection	Positive on injection	(Rodriquez-Arnaiz et al., 1989; Rodriquez-Arnaiz et al., 1992)
	Reciprocal translocation	*D.melanogaster*	1000 ppm, in diet	Negative	(Woodruff et al., 1985)
	Sister chromatid exchange/ chromosomal aberration	B6C3F1 mouse bone marrow cells	50-200 mg/kg bw, once i.p.	Negative	(NTP, 1990a)
	Somatic chromosomal aberration	Swiss albino mouse bone marrow cells	4000 ppm for 5 days, in diet	Negative	(Subramanyam et al., 1989)+
	Spermhead abnormalities	Swiss albino mouse	4000 ppm for 5 weeks, in diet	Negative	(Subramanyam et al., 1989)+
	Unscheduled DNA synthesis	Fischer 344 rat hepatocytes	5.0, 16.7 or 50 mg/kg bw, orally	Negative	(Phillips et al., 1997)
		B6C3F$_1$ mouse hepatocytes	50, 175 or 320 mg/kg bw, orally	Negative	(Edwards, 1999)

a With and without metabolic activation.
a Without metabolic activation.

c With metabolic activation.
+ Abstract only; no details available.

TABLE 6: SUMMARY OF ADDITIONAL GENOTOXICITY DATA CONSIDERED BY EFSA (EFSA, 2004C)

Table 6: GENOTOXICITY (*in vivo*) (EFSA, 2004c)

Chemical Name [FL-no]	Test System	Test Object	Route	Dose	Result	Reference	Comments
Furfural [13.018]	Gene mutation λ*lacZ*	CD2F1(BALB/c x DBA/2	Gavage	0, 75, 150, 300 mg/kg bw/d	No increase in λ*lacZ* gene mutation in liver DNA	(CIVO-TNO, 2003)	This study is considered valid.

TABLE 7: SUMMARY OF ADDITIONAL GENOTOXICITY DATA ON 5-HMF (*IN VITRO*)

Table 7: Summary of Additional Genotoxicity data on 5-hydroxymethylfurfural (*in vitro*)

Chemical name [Fl.-no.]	Test system	Test object	Concentration	Result	Reference
5-Hydroxymethylfurfural [13.139]	Ames	*S. typhimurium* TA97, TA98, TA102, TA1535	100-10,000 µg/plate	Negative[1]	(NTP, 2008b)
	Ames	*S. typhimurium* TA100	100-10,000 µg/plate	Weakly positive[2]	(NTP, 2008b)
	Ames	*S. typhimurium* TA100 and TA98	1,500-10,000 µg/plate	Negative[1]	(NTP, 2008b)
	Ames	*S. typhimurium* TA98, TA100, TA1535, TA1537	at 0.5 µg/mL up to 5000 µg/mL	Negative[1]	(Severin et al., 2010)
	Reverse mutation	*E. coli* WP2 uvrA/pKM101	1,500-10,000 µg/plate	Negative[1]	(NTP, 2008b)
	Micronucleus assay	HepG2 cells	0, 5.35, 7.87, 11.57, 17, 25, 36.6 mM	Negative[6]	(Severin et al., 2010)
	SCE induction	V79-hCYP2E1-hSULT1A1 cells	19.8–3808 µM	Positive	(Glatt et al., 2005)
	SCE induction	V79-Mz cells	238 - 3808 µM,	Positive[7]	(Glatt et al., 2005)
	Comet Assay	HepG2 cells	0, 5.35, 7.87, 11.57, 17, 25, 36.6 mM	Positive[5, 6]	(Severin et al., 2010)
	Comet Assay	Human Caco-2 cells	3,153-12,611 µg/mL (25-100 mM)	Positive[3]	(Durling et al., 2009)
	Comet Assay	Human HEK293 cells	3,153-12,611 µg/mL (25-100 mM)	Positive[3]	(Durling et al., 2009)
	Comet Assay	Mouse lymphoma L5178Y cells	3,153-12,611 µg/mL (25-100 mM)	Positive[3]	(Durling et al., 2009)
	Comet Assay	Chinese hamster V-79 cells	315-12,611 µg/mL (2.5-100 mM)	Positive[4]	(Durling et al., 2009)
	Comet Assay	Chinese hamster V-79-hP-PST cells	315-12,611 µg/mL (2.5-100 mM)	Positive[4]	(Durling et al., 2009)
	Micronucleus assay	Mouse peripheral blood cells	47, 94, 188, 375 or 750 mg/kg bw/day	Negative	(NTP, 2008b)

[1] With and without S9 metabolic activation.
[2] Without S9 metabolic activation.
[3] Positive only at the highest concentration tested with significant decrease in cell viability.
[4] Positive at high concentration with significantly reduced cell viability.
[5] Cytotoxic at the two highest doses.
[6] 20 hours of exposure.
[7] Weakly positive but statistically significant at each concentration.

TABLE 8: SUMMARY OF ADDITIONAL GENOTOXICITY DATA ON 5-HMF (*IN VIVO*)

Table 8: Summary of Additional Genotoxicity data on 5-hydroxymethylfurfural (*in vivo*)

Chemical name [Fl.-no.]	Test system	Test object	Route	Concentration	Result	Reference	EFSA Comments
5-Hydroxymethylfurfural [13.139]	Micronucleus assay	Mouse peripheral blood cells	Gavage	47, 94, 188, 375 or 750 mg/kg bw/d	Negative	(NTP, 2008b)	3-months micronucleus assay.

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Abbreviations

ADI	Acceptable Daily Intake
BW	Body Weight
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids Chemical Abstract Service
CHO	Chinese hamster ovary (cells)
CoE	Council of Europe
DNA	Deoxyribonucleic acid
EC	European Commission
EFFA	European Flavour and Fragrance Association
EFSA	European Food Safety Authority
ENU	Ethylnitrosourea
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
GLP	Good Laboratory Practice
HMF	Hydroxymethylfurfural
ID	Identity
IOFI	International Organization of the Flavour Industry
IR	Infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LD_{50}	Lethal Dose, 50 %; Median lethal dose
MF	Methylfurfural
MS	Mass spectrometry
MSDI	Maximised Survey-derived Daily Intake
mTAMDI	Modified Theoretical Added Maximum Daily Intake
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NCE	Normochromatic erythrocytes
No	Number
NOAEL	No Observed Adverse Effect Level
NOEL	No Observed Effect Level
NTP	National Toxicology Program
OECD	Organisation for Economic Co-operation and Development

PAPS	3'-Phosphoadenosine-5'-phosphosulphate
PCE	Polychromatic erythrocytes
PFU	Plaque-forming units
SCE	Sister Chromatid Exchange
SCF	Scientific Committee on Food
SMART	Somatic Mutation and Recombination Test
SMF	5-Sulphoxy-methylfurfural
SULT	Sulphotransferases
TAMDI	Theoretical Added Maximum Daily Intake
UDS	Unscheduled DNA Synthesis
WHO	World Health Organisation