SCIENTIFIC OPINION

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Scientific opinion on Prosmoke BW 01

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

The EFSA Panel on Food Additives and Flavourings (FAF) was requested to evaluate the safety of Prosmoke BW 01 as a new smoke flavouring primary product, in accordance with Regulation (EC) No 2065/2003. Prosmoke BW01 is produced by pyrolysis of beechwood (*Fagus sylvatica* L.) sawdust. Its water content is estimated at 56 wt%, the total identified volatile fraction accounts for 28 wt% of the primary product, corresponding to 64% of the solvent-free mass, while the unidentified fraction amounts to 16 wt% of the primary product. Analytical data provided for three batches demonstrated that their batch-to-batch-variability was sufficiently low. However, for the batch used for the toxicological studies, there were substantial deviations in the concentration of nearly all the constituents compared to the other three batches. The dietary exposure of Prosmoke BW 01 was estimated to be between 6.2 and 9.2 mg/kg body weight (bw) per day, respectively, using SMK-EPIC and SMK-TAMDI. Using the FAIM tool, the 95th percentile exposure estimates ranged from 3.2 mg/kg bw per day for the elderly to 17.9 mg/kg bw per day for children. The Panel noted that furan-2(5H)-one is present in all batches of the primary product at an average concentration of 0.88 wt%. This substance was evaluated by the FAF Panel as genotoxic *in vivo* after oral exposure. The Panel considered that the (geno)toxicity studies available on the whole mixture were not adequate to support the safety assessment, due to limitations in these studies and because they were performed with a batch which may not be representative for the material of commerce. Considering that the exposure estimates for furan-2(5H)-one are above the TTC value of 0.0025 μg/kg bw per day (or 0.15 μg/person per day) for DNA-reactive mutagens and/or carcinogens, the Panel concluded that Prosmoke BW 01 raises a concern with respect to genotoxicity.

Keywords: Prosmoke BW 01, smoke flavouring primary product, furan-2(5H)-one, genotoxicity

Requestor: European Commission

Question number: EFSA-Q-2019-00441

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1. Introduction

The present opinion deals with the safety evaluation of a new smoke flavouring primary product, Prosmoke BW 01.

1.1. Background and Terms of Reference

The European Food Safety Authority (EFSA) received from Hungarian Competent Authority (Ministry of Agriculture - Department of Food Economy and Origin Protection) an application for the authorisation as a new smoke flavouring primary of Prosmoke BW 01, submitted by M Profood Zrt under Article 7 of Regulation (EC) No 2065/2003[1] of the European Parliament and of the Council of 10 November 2003 on smoke flavourings used or intended for use in or on foods.

According to Article 8 of Regulation (EC) No 2065/2003, EFSA is requested to carry out a risk assessment and deliver a scientific opinion on the safety of Prosmoke BW 01 as a new smoke flavouring primary product.

2. Data and methodologies

2.1. Data

The present evaluation is based on the data on Prosmoke BW 01 provided by the applicant in a dossier submitted according to Article 7 Regulation (EC) No 2065/2003 for its authorisation as a new smoke flavouring primary product (Documentation provided to EFSA No. 1). A request for additional data was sent to the applicant by EFSA on 4 December 2019. Following this request, the applicant asked for a clarification teleconference with EFSA that was held on 20 December 2019, after which the applicant provided additional data on 3 November 2020 (Documentation provided to EFSA No. 2). A second request for additional data was sent by EFSA on 18 December 2020 and data were provided by the applicant on 17 December 2021 (Documentation provided to EFSA No. 3).

2.2. Methodologies

The safety assessment of the primary product Prosmoke BW01 was conducted in line with the principles described in the EFSA guidance on smoke flavourings (EFSA CEF Panel, 2005) and in the EFSA opinion on dietary exposure assessment methods for smoke flavouring primary products (EFSA CEF Panel, 2009a). In addition, the requirements laid down in cross section guidance documents developed by EFSA since 2005 and relevant for the assessment of smoke flavouring primary products have been taken into account in the risk assessment, i.e. the EFSA Scientific Committee's guidance documents on genotoxicity (EFSA Scientific Committee, 2011, 2017, 2019, 2021), the EFSA's dietary exposure assessment methodologies based on real food consumption data across different population groups through the EFSA Comprehensive European Food Consumption Database.[2]

3. Assessment

3.1. Characterisation of the primary product

3.1.1. Manufacturing process

3.1.1.1. Source materials

The source material of Prosmoke BW 01 is 100% beechwood (*Fagus sylvatica L.*) sawdust.

According to the applicant, the wood used has a water content of maximum 13% and it is not subjected to chemical treatment.

3.1.1.2. Method of manufacture

The production of the Primary Product comprises:

1) controlled thermal degradation (pyrolysis) of wood;
2) condensation of the vapour by indirect heat exchanger;

[1] Regulation (EC) No 2065/2003 of the European Parliament and of the Council of 10 November 2003 on smoke flavourings used or intended for use in or on foods. OJ L 309, 26.11.2003, p. 1–8.

[2] https://www.efsa.europa.eu/en/food-consumption/comprehensive-database

3) fractionation of the resulting liquid by filtration to remove remaining charcoal and the oily phase, and by a decantation step to remove the heavy tar.

According to the applicant, all process parameters are automatically fixed and monitored, and the process is performed in accordance with FSSC 22000 certification.

3.1.2. Identity of the primary product

3.1.2.1. Trade name of the primary product

The trade name of the primary product is Prosmoke BW 01. Another name referred to by the applicant in a product specification is 'Prosmoke Aqua'.

3.1.2.2. Information on existing evaluation from other regulatory bodies

The applicant indicated that '*Russian authority has already evaluated and authorized 3 of our liquid smoke products to be used in and on meat products in Russia*'. These products are all based on Prosmoke BW 01 containing different formulas (Documentation provided to EFSA No. 1).

3.1.2.3. Description of physical state and organoleptic properties

The primary product is a dark brown liquid with an average density of 1.12 g/mL. The pH (10% emulsion in water) is ranging between 5 and 7, and the staining index (at 440 nm) from 74 to 122 (Documentation provided to EFSA No. 1).

3.1.2.4. Chemical composition of the Primary Product

The compositional data provided by the applicant for four batches of the primary product in the original dossier and in response to the EFSA requests for additional information are summarised in Table 1 (Documentation provided to EFSA No. 1, 2 and 3). Batch #1 (100 224 144) was used for toxicological tests (see Section 3.3).

Table 1: Compositional data provided for four batches of the primary product (Documentation provided to EFSA No. 1, 2 and 3)

Density (g/L)	**#1 100 224 144 2017-02-27**		**#2 100 324 144 2018-04-03**		**#3 100 824 144 2019-03-13**		**#4 101 024 144 2019-04-30**		**Average**
	1,180		**1,030**		**1,150**		**1,110**		**1,117.50**
	(g/L)	**(wt%)**	**(g/L)**	**(wt%)**	**(g/L)**	**(wt%)**	**(g/L)**	**(wt%)**	
Acetic acid (HS-GC)[(a)]		13.52 ± 0.48		18.82 ± 1.73		12.28 ± 0.64		13.08 ± 1.06	14.43
Total volatiles (GC)[(b)]	128.15	10.86	233.91	22.71	245.07	21.31	353.54	31.85	21.68
Ident. volatiles (GC)[(c)]	74.08	6.28	148.31	14.40	145.48	12.65	234.13	21.09	13.60
Unident. volatiles (GC)[(d)]	54.07	4.58	85.60	8.31	99.59	8.66	119.41	10.76	8.08
Total volatile fraction[(e)]		24.38		41.53		33.59		44.93	36.11
Non-volatile fraction[(f)]		8.30		7.80		7.02		8.51	7.91
Water [(g)]		67.32		50.67		59.39		46.56	55.98
Solvent-free fraction[(h)]		32.68		49.33		40.61		53.44	44.02
Ident./quant. proportion of solvent-free fraction (wt%) (regul. limit: ≥ 50 wt%)	60.6[(i)]		67.36		61.4		63.9		64.22
Ident./quant. proportion of volatile fraction (wt%) (regul. limit: ≥ 80 wt%)	81.2[(j)]		80.0		74.2		76.1		76.77

(a): Determined by headspace analysis (triplicate) using external calibration.
(b): Determined by weighing the residue remaining after subjecting the primary product to evaporation, thus removing water and acetic acid.

(c): Determined by subjecting the residue described under footnote b to GC/FID and GC/MS analysis and calculating the sum of wt% of the identified volatiles based on the sum of their peak areas.
(d): Determined by subtracting the sum of peak areas for volatiles identified according to footnote [c] from the total peak area in the chromatogram and using the sum of remaining peak areas to calculate the wt% of unidentified volatiles.
(e): Sum of amounts of acetic acid (HS) and total volatiles (GC).
(f): Determined by weighing the residue obtained after subjecting the primary product to high-temperature distillation (350°C).
(g): Calculated: 100 wt% – total volatile fraction wt% – non-volatiles wt%.
(h): Calculated: Total volatile fraction wt% + non-volatile fraction wt%.
(i): Calculated: [identified volatiles (GC) (6.28) + acetic acid (13.52)] × 100/Solvent-free fraction (32.68).
(j): Calculated: [identified volatiles (GC) (6.28) + acetic acid (13.52)] × 100/Total volatile (24.38).

3.1.2.4.1. Chemical characterisation

Water functions as the solvent of the primary product (Documentation provided to EFSA No. 1). The applicant provided divergent results regarding the analytically determined water contents of the investigated four batches of the primary product. According to Karl Fischer titrations, the water content would only range from 11.1% to 13.0%; the applicant explained these low values by the impairment of the analytical methodology by the phenolic compounds contained in the primary product. In contrast, the water content determined by measuring the weight loss of the primary product upon drying at 103°C ranged from 76.3% to 78.4% (Documentation provided to EFSA No. 3). However, it has to be taken into account that this method not only results in the loss of water but also of other volatile constituents of the primary product and thus is expected to result in an overestimation of the water content. Finally, the applicant calculated the water content by subtracting the mass of total volatile constituents and the mass of non-volatile constituents from the total mass of the primary product, resulting in an average water content of 56 wt% (Documentation provided to EFSA No. 1) (Table 1).

The applicant provided data on the contents of major chemical classes in the primary product, i.e. phenols, acids and carbonyls (Table 2) (Documentation provided to EFSA No. 1).

Table 2: Phenols, acids and carbonyls in the primary product (values of the four batches presented in Table 1)

Parameter	#1	#2	#3	#4
Phenols, as syringol (wt %)[(a)]	1.24	1.79	2.02	4.27
Acids, as acetic acid (wt%)[(b)]	13.95	10.32	17.95	11.28
Carbonyls (wt%)[(a)]	2.39	5.77	5.14	4.36

(a): By GC-FID analysis.
(b): By HS-GC analysis.

Concentrations of arsenic, mercury, cadmium and lead as determined by ICP-MS have been reported for an additional batch of the primary product (Table 2) (Documentation provided to EFSA No. 1).

Table 3: Heavy metals reported for one batch of the primary product

Element	mg/kg
Lead	< 0.1
Cadmium	< 0.01
Mercury	< 0.01
Arsenic	< 0.1

3.1.2.4.2. Identification and quantification of volatile fraction

The applicant determined the amount of acetic acid, the major volatile constituent of the primary product, by triplicate headspace gas chromatography analysis using external calibration (Documentation provided to EFSA No. 3).

For determination of the other volatile constituents, the applicant removed water and acetic acid from the primary product by evaporation and analysed the remaining residue by GC/FID (for quantification) and GC-MS (for identification). The mass chromatograms, recorded in the total ion current (TIC) mode, revealed around 300 individual peaks (Documentation provided to EFSA No. 2).

By comparison of the chromatographic and mass spectrometric data to those of authentic reference substances, in total 48 compounds were fully identified (Documentation provided to EFSA No 2).

The volatile constituents were quantified based on the total weight of the residue subjected to GC analysis and on their GC-peak areas; no substance-specific GC response factors were taken into account. The concentrations in the residue were calculated back to provide the concentrations in the whole primary product (Table 4). The 20 quantitatively dominating volatile constituents determined by this approach are presented in Table 4; a complete list of the 48 fully identified volatile constituents is given in Appendix A – Table A.2.

The total amount (wt%) of unidentified volatiles was calculated based on the peak areas remaining after subtracting the sum of GC peak areas for the identified volatiles from the total peak area in the chromatogram.

Table 4: Principal volatile constituents[3] of the primary product Prosmoke BW (Documentation provided to EFSA No. 2 and 3)

CAS	Chemical name	Average concentration[(a)] (wt%)
64-19-7	Acetic acid	14.43
498-02-2	3-Methoxy-4-hydroxyphenylethanone	2.67
91-10-1	2,6-Dimethoxyphenol	1.36
134-96-3	4-Hydroxy-3,5-dimethoxybenzaldehyde	1.22
498-07-7	Levoglucosan (1,6-Anhydro-β-D-glucose)	1.01
497-23-4	2(5H)-Furanone	0.88
80-71-7	3-Methyl-1,2-cyclopentanedione	0.70
150-76-5	2,5-Dimethoxytetrahydrofuran[(b)] (II)	0.52
696-59-3	4-Methoxyphenol	0.51
1575-57-1	1-Acetoxy-2-butanone	0.51
696-59-3	2,5-Dimethoxytetrahydrofuran[(b)] (I)	0.42
121-33-5	Vanillin	0.40
90-05-1	2-Methoxyphenol	0.38
2478-38-8	4-Acetyl-2,6-dimethoxyphenol	0.34
2503-46-0	4-Hydroxy-3-methoxyphenyl-2-propanone	0.34
118-71-8	Maltol	0.31
2380-78-1	Homovanillyl alcohol	0.30
6627-88-9	2,6-Dimethoxy-4-(2-propenyl)phenol	0.25
2785-89-9	4-Ethyl-2-methoxyphenol	0.24
5932-68-3	2-Methoxy-4(E)-1-propenylphenol	0.20
93-51-6	2-Methoxy-4-methylphenol	0.20

(a): From the analysis of the four batches presented in Table 1.
(b): Roman numbering according to the GC elution order of the diastereoisomers.

3.1.2.4.3. Non-volatile fraction

The non-volatile fraction was determined by weighing the residue obtained after subjecting the primary product to high-temperature distillation (350°C) (Documentation provided to EFSA No. 1). The non-volatile fraction on average accounted for 7.9 wt%. No further investigations of this fraction were performed.

3.1.2.4.4. Unidentified fraction

The unidentified fraction of the primary product amounts to 16 wt% and comprises the unidentified volatile constituents (Section 3.1.2.4.2) and the non-volatile fraction (Section 3.1.2.4.3).

[3] Acetic acid was determined by HS-GC analysis using external calibration. The other listed volatile constituents represent the 20 quantitatively predominant components determined by GC-FID in the residue remaining after subjecting the primary product to evaporation.

3.1.2.5. Overall composition of the primary product

Based on the average values reported for four batches of the primary product (Table 1), the overall composition of the Prosmoke BW 01 (wt% of Primary Product) is shown in Figure 1 and the composition (wt%) of the solvent-free fraction of ProSmoke BW 01 in Figure 2.

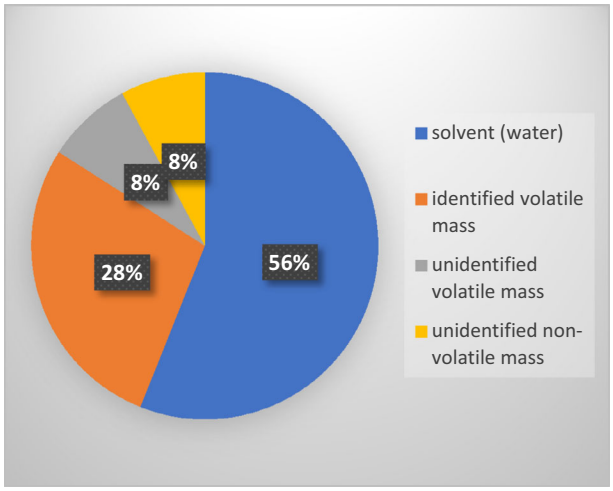

Figure 1: Overall composition of Prosmoke BW 01 (wt% of Primary Product)

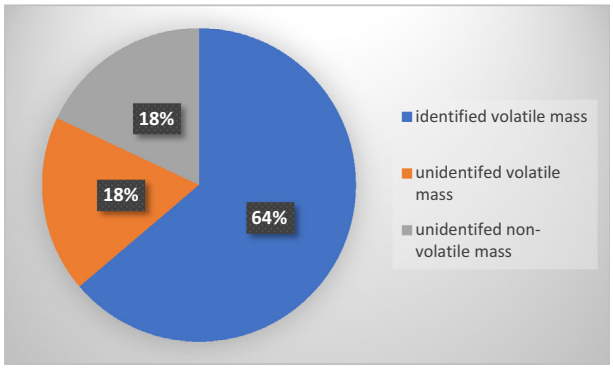

Figure 2: Composition (wt. %) of the solvent-free fraction of ProSmoke BW 01

The Panel noted that for all four investigated batches of the primary product, the identified and quantified proportion of the solvent-free fraction is higher than 50 wt%, and thus, it meets the legal requirement, i.e. at least 50% by mass (wt %) of the solvent-free fraction shall be identified and quantified (Commission Regulation (EC) No 627/2006[4]).

Regarding the identified and quantified proportion of the volatile fraction, two of the analysed batches (i.e. batches #1 and #2) comply with the legal requirement of having at least 80 wt% of the volatile fraction identified and quantified.[4]

For the other two batches (batches #3 and #4), the identified and quantified proportions of the volatile fraction are slightly below the legal limit. In this respect, the Panel noted that the analytical approaches applied by the applicant show several limitations. For instance, for the quantification of volatile constituents, no substance-specific GC response factors have been taken into account. Furthermore, the investigation of volatile constituents has been performed using a sample of the primary product subjected to evaporation prior to GC analysis. This evaporation step is expected to result not only in the intended removal of water and acetic acid but it might also lead to a partial loss particularly of highly volatile constituents and thus result in a shift of the proportion of volatile constituents that could have been identified and quantified in the volatile fraction of the primary

[4] Regulation (EC) No 627/2006 of 21 April 2006 implementing Regulation (EC) No 2065/2003 of the European Parliament and of the Council as regards quality criteria for validated analytical methods for sampling, identification and characterisation of primary smoke products. OJ L 109, 22.4.2006, p. 3–6.

product. Taking into account the limitations of the analytical procedure and the resulting uncertainties regarding the reported identified and quantified proportions of the volatile fraction, the panel considered that the slight deviations of these analytical values from the legal limits would not hamper the safety assessment of the primary product.

3.1.2.6. Polycyclic aromatic hydrocarbons (PAHs)

Analytical data on the contents of 15 PAHs were provided for one of the batches of the primary product listed in Table 1 (batch #1_100824144); the results are outlined in Table 5. The levels reported are consistently below the minimum required limits of quantification according to Commission Regulation (EC) No 627/2006. Other performance criteria of the analyses, which were performed in an external laboratory not accredited for determinations of these PAHs, were not provided.

Table 5: Concentrations of PAHs in the primary product Prosmoke BW 01 (batch #1_100824144) (documentation provided to EFSA No. 1)

Compound	µg/kg
Benz[a]anthracene	1.28
Benzo[b]fluoranthene	0.62
Benzo[j]fluoranthene	0.61
Benzo[k]fluoranthene	0.27
Benzo[ghi]perylene	0.53
Benzo[a]pyrene	0.87
Chrysene	1.26
Cyclopenta[cd]pyrene	0.73
Dibenz[a,h]anthracene	< 0.10
Dibenzo[a,e]pyrene	< 0.10
Dibenzo[a,h]pyrene	< 0.10
Dibenzo[a,i]pyrene	< 0.20
Dibenzo[a,l]pyrene	< 0.20
Indeno[1,2,3-cd]pyrene	0.30
5-Methylchrysene	< 0.10

In addition, the applicant provided analytical data, developed in an accredited laboratory, on the concentrations of four PAHs in an additional batch of the primary product (Table 6), (Documentation provided to EFSA No. 1). The concentrations of all analysed PAHs were below 0.5 µg/kg (the reported quantification limit). This limit of quantification fulfils the performance criterion as set in Commission Regulation (EC) No 627/2006. The levels of benzo(a)pyrene and benzo(a)anthracene are below their respective limits of 10 and 20 µg/kg as laid down in the Regulation (EC) No 2065/2003.

Table 6: Concentrations of four PAHs provided for an additional batch of the primary product Prosmoke BW 01 (Documentation provided to EFSA No. 1)

Compound	µg/kg
Benz[a]anthracene	< 0.5
Benzo[b]fluoranthene	< 0.5
Benzo[a]pyrene	< 0.5
Chrysene	< 0.5

3.1.3. Batch-to-batch variability

Batch-to-batch variability was demonstrated by GC/MS/FID data for the volatile fraction the same four batches presented in previous sections (see Table 1 on compositional data). In addition, the applicant provided data on the batch-to-batch variability as regard the content of acetic acid, determined by HS-GC analysis (Table 1). The four batches were manufactured between February 2017 and April 2019.

The Panel noted that batch-to-batch-variability of batches 2–4 is sufficiently low. Apart from acetic acid, batch #1 shows significant deviations from these batches for nearly all constituents (Table 7).

The higher content of levoglucosan (1,6-β-anhydroglucose) and the lower contents of further cellulose- and lignin-degradation products (e.g. maltol and vanillin) indicate that this batch has undergone a lower degree of pyrolysis than batches #2 to #4. This difference is also substantiated by the data for the chemical classes shown in Tables 1 and 2. The Panel noted that batch #1, which was used for the toxicity testing, may not be representative for the primary product of commerce. Alternatively, it may be concluded that if batch #1 does represent the primary product of commerce, the reproducibility of the production method might be questioned.

Table 7: Concentration (wt%) of main components in four batches of the primary product Prosmoke BW 01

Chemical name	Batch #1	Batch #2	Batch #3	Batch #4	Average	RSD%[(a)]	RSD%[(b)]
Acetic acid	13.52	18.82	12.28	13.08	14.43	20.62	24.22
3-Methoxy-4-hydroxyphenylethanone	0.07	1.92	3.62	5.05	2.67	80.75	44.39
2,6-Dimethoxyphenol	0.46	1.76	1.26	1.95	1.36	49.05	21.61
4-Hydroxy-3,5-dimethoxybenzaldehyde	0.27	1.49	1.23	1.88	1.22	56.28	21.47
Levoglucosan (1,6-Anhydro-β-D-glucose)	3.44	0.22	0.18	0.20	1.01	160.74	9.98
2(5H)-Furanone	0.35	1.21	0.88	1.07	0.88	43.33	16.03
3-Methyl-1,2-cyclopentanedione	0.33	0.91	0.71	0.85	0.70	37.38	12.04
2,5-Dimethoxytetrahydrofuran (II)[(c)]	0.11	0.75	0.40	0.81	0.52	63.52	34.20
4-Methoxyphenol	0.00	0.00	0.00	0.51	0.51	–	–
1-Acetoxy-2-butanone	0.15	0.50	0.42	0.95	0.51	65.77	45.41
2,5-Dimethoxytetrahydrofuran (I)[(c)]	0.09	0.60	0.31	0.68	0.42	63.70	36.27
Vanillin	0.09	0.50	0.39	0.63	0.40	56.65	23.24
2-Methoxyphenol	0.10	0.56	0.47	0.00	0.38	64.00	13.37
4-Acetyl-2,6-dimethoxyphenol	0.13	0.43	0.37	0.44	0.34	41.86	9.60
4-Hydroxy-3-methoxyphenyl-2-propanone	0.07	0.49	0.28	0.53	0.34	62.64	31.71
Maltol	0.08	0.23	0.20	0.73	0.31	93.59	77.08
Homovanillyl alcohol	0.04	0.40	0.34	0.44	0.30	59.78	13.06
2,6-Dimethoxy-4-(2-propenyl) phenol	0.04	0.22	0.22	0.51	0.25	78.08	52.88
4-Ethyl-2-methoxyphenol	0.04	0.23	0.18	0.49	0.24	78.60	54.71
2-Methoxy-4(*E*)-1-propenylphenol	0.01	0.06	0.03	0.70	0.20	168.26	144.09
2-Methoxy-4-methylphenol	0.05	0.34	0.24	0.16	0.20	62.71	38.36

(a): Relative standard deviation (RSD)% (i.e. ratio between standard deviation (SD) and the average) of batches #1–#4.
(b): Relative standard deviation* (RSD*)% of batches #2–#4 (without batch #1).
(c): Roman numbering according to the GC elution order of the diastereoisomers.

3.1.4. Specifications

The applicant provided a product specification data sheet used to trade the product (Documentation provided to EFSA No. 1). Information on parameters relevant for the specifications has been compiled by the Panel in Table 8.

Table 8: Relevant information for specifications of the primary product

Name of smoke flavouring primary product	**Prosmoke BW 01; Prosmoke Aqua**
Description	dark brown liquid
Source material:	100% beechwood (*Fagus sylvatica L.*) sawdust
Identity parameters:	
• Physico-chemical parameters:	
– pH	~ 1.5–3.0
– density	1.12 g/mL
– staining index	From 74 to 122
Chemical composition:	
• Chemical classes:	
– acids	10–20%
– carbonyls	4–6%
– phenols	2–5%
• 20 principal constituents of the volatile fraction	See Table 4
Purity:	
• Benzo(a)pyrene	Max. 10 ppb
• Benzo(a)anthracene	Max. 20 ppb
• Heavy metals:	
– Lead	< 10 mg/kg
– Arsenic	< 3 mg/kg
– Cadmium	< 1 mg/kg
– Mercury	< 1 mg/kg

The Panel noted that the analytical data for one batch (batch #1) indicated that actual concentrations of the two PAHs and heavy metals mentioned in Tables 3 and 6 are substantially lower than the proposed maximum limits in Table 8.

3.1.5. Stability

The applicant recommends storing the primary product in a dry, cool place, away from direct sunlight (Documentation provided to EFSA No. 1).

In the original dossier, the applicant provided GC-data on the contents of 12 volatile constituents determined in one batch of the primary product over approximately 4 months; there were no significant changes in the contents during this period (Documentation provided to EFSA No. 1).

Upon request from EFSA to provide information on storage stability from experimental conditions reflecting the intended shelf-life of the product, either in real-time settings or under forced, accelerated ageing, the applicant provided results from storage of a batch of the primary product under accelerated conditions (54 ± 2°C for 2 weeks) (Documentation provided to EFSA No. 2). GC/MS analyses were performed for three selected volatile constituents, 2,6-dimethoxyphenol, 3-methyl-1,2-cyclopentanedione and maltol. Under the applied storage conditions, their contents decreased by approximately 8%, 3% and 4%, respectively. Colour, odour and physical state of the primary product were not affected; there was a slight decrease of the pH.

The analytical data provided are limited; however, the Panel considered the stability of the primary product upon storage under the intended conditions not to be of concern.

3.2. Proposed uses and exposure assessment

3.2.1. Proposed use levels

The applicant is seeking an authorisation of the product Prosmoke BW 01 in the food categories presented in Table 9 at the maximum proposed use levels.

These use levels were used as indicated in Table 9, for the subsequent exposure estimates (see Section 3.2.2).

Table 9: Proposed uses and use levels of Prosmoke BW 01 (g/kg) according to food categories in Part D of Annex II of Regulation (EC) No 1333/2008[5]. (Documentation provided to EFSA No. 2)

Food category number	Food category name	Maximum proposed use levels of Prosmoke BW 01 (g/kg) – used for FAIM	Corresponding food categories (FC) used for SMK-TAMDI and SMK-EPIC*	Maximum proposed use levels of Prosmoke BW 01 (g/kg) – Used for SMK-TAMDI and SMK-EPIC
01.7.2	Ripened cheese	1.0	FC 1. Dairy products	1
01.7.5	Processed cheese	0.5		
08.2	Meat preparations as defined by Regulation (EC) No 853/2004[6]	0.5	FC 8 Meat and meat products	2
08.3	Meat Products	2.0		
09.2	Processed fish and fishery products including molluscs and crustaceans	2.0	FC 6 Fish and fish products	2
11.1	Sugars and syrups as defined by Directive 2011/111/EC[7]	0.5	FC 11 Sweeteners, including honey	0.5
12.1	Salt and salt substitutes	0.5	FC 12 Salts, spices, soups, sauces, salads, protein products, etc.	0.5
12.2	Herbs, spices, seasonings	0.5		
12.6	Sauces	0.5		
14.2.1	Beer and malt beverages	0.5	FC 14.2 Alcoholic beverages	0.5

*: The food categories used in FAIM tool are more specific than the broader ones used in SMK-TAMDI and SMK-EPIC.

3.2.2. Exposure assessment

In order to estimate dietary exposure to the primary product, the FAF Panel used different methodologies.

Two methodologies, the Smoke Theoretical Added Maximum Daily Intake (SMK-TAMDI) and the smoke flavouring EPIC model (SMK-EPIC), were developed by the CEF Panel specifically for smoke flavourings and are described in detail in the EFSA opinion on dietary exposure assessment methods for smoke flavouring primary products (EFSA CEF Panel, 2009a). These two methodologies were applied because the application for Prosmoke BW 01 was submitted prior to the publication of the EFSA guidance on smoke flavouring primary products (EFSA FAF Panel, 2021a). In line with this currently applicable guidance, a further approach was followed, using the FAIM tool to estimate chronic dietary exposure.

3.2.2.1. Exposure assessment using SMK-TAMDI and SMK-EPIC

SMK-TAMDI and SMK-EPIC assume that the hypothetical consumer will daily consume a fixed amount of flavoured solid foods and liquids. A single group 'Beverages' is used for liquids whereas solid foods are divided in 'traditionally smoked solid foods' and 'other solid foods not traditionally smoked'.

The SMK-TAMDI model is intended to represent the dietary exposure of a regular consumer of one flavoured product among each of these three food groups. It is calculated by summing the highest potential dietary exposure for each of the three food groups.

[5] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives. OJ L 354, 31.12.2008, p. 16–33.

[6] Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. OJ L 139, 30.4.2004, p. 55–205.

[7] 2011/111/EU: Commission Decision of 18 February 2011 authorising France, pursuant to Council Directive 92/66/EEC, to transport day-old chicks and ready-to-lay pullets outside the protection zone established due to an outbreak of Newcastle disease in the department of Côtes d'Armor (notified under document C(2011) 869). OJ L 46, 19.2.2011, p. 44–45.

The SMK-EPIC model assumes the hypothetical high consumer of smoked meat is also an average consumer of the other traditionally smoked foods and an occasional consumer of smoked foods or beverages from each of the other categories.

Details of the methodologies are described in the dietary exposure document (EFSA CEF Panel, 2009a).

Estimated dietary exposures from all sources were 9.2 and 6.2 mg/kg bw per day, according to the SMK-TAMDI and SMK-EPIC methodologies, respectively.

Table 10: Summary of the dietary exposure to Prosmoke calculated on the basis of the maximum use levels proposed by the applicant

		Dietary exposure (mg/kg bw per day)
SMK-TAMDI	Traditionally smoked food	3.3
	Other foods not traditionally smoked	3.3
	Beverages (alcoholic or non-alcoholic)	2.5
	Total dietary exposure	**9.2**
SMK-EPIC	Traditionally smoked food	5.3
	Other foods not traditionally smoked	0.5
	Beverages (alcoholic or non-alcoholic)	0.4
	Total dietary exposure	**6.2**

3.2.2.2. Exposure assessment using FAIM

FAIM tool is based on actual food consumption data from European dietary surveys available through the EFSA Comprehensive European Food Consumption Database (Comprehensive Database).

Consumption surveys from the Comprehensive database were taken into account in this assessment.[8] Data are available for six population groups: infants, toddlers, children, adolescents, adults and the elderly.

More details on the EFSA Comprehensive database can be seen in Appendix D.

Foods are recorded in the EFSA Comprehensive database according to the EFSA FoodEx2 nomenclature. FAIM tool[9] was built by reclassifying food records according to the food nomenclature from the Regulation (EC) No 1333/2008, Annex II, part D. This food nomenclature is also the one which Regulation (EC) 1334/2008[10] on flavourings is referring to, which has repealed Regulation (EU) 1565/2000[11].

Table 11: Summary of the dietary exposure to Prosmoke BW 01 (in mg/kg bw per day) calculated based on FAIM and the maximum use levels proposed by the applicant

	Infants (12 weeks–11 months)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (≥ 65 years)
• Mean	0.9–2.6	2.4–11.6	3.3–9.8	2.0–6.0	2.0–5.7	1.4–4.9
• 95th percentile	3.4–8.9	7.7–13.5	7.7–17.9	4.4–13.3	4.7–16.8	3.2–10.2

Mean dietary exposure to Prosmoke BW 01 ranged from 0.9 mg/kg bw per day in infants to 11.6 mg/kg bw per day for toddlers. At the high level, exposure to Prosmoke BW 01 ranged from 3.2 mg/kg bw per day for the elderly to 17.9 mg/kg bw per day for children.

[8] Available online: https://www.efsa.europa.eu/en/datexfoodcdb/datexfooddb.htm
[9] FAIM tool version 2.0.
[10] 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 of Regulations (EC) No. 2232/96 and (EC) No. 110/2008 and Directive 2000/13/EC. OJ L 354, 31.12.2008, p. 34-50.
[11] Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council. OJ L 180, 19.7.2000, p. 8–16.

3.2.2.3. Anticipated exposure to toxic elements from specification limits

The Panel calculated the potential exposure to the toxic elements from the uses and use levels of ProSmoke BW 01 assuming contamination of the primary product may be up to the specification limits for toxic elements (Table 8) and then by calculation pro-rata to the estimates of exposure to the primary product itself (95th percentile, toddlers; see Table 11 Section 3.2.2.2). This exposure estimate was obtained with the FAIM tool and it is the most accurate and conservative estimate available.

The resulting exposure estimates are then compared against the following reference points or health-based guidance values for the four elements (see Table 12).

Table 12: Reference points/health-based guidance value for toxic elements potentially present in ProSmoke

Element/HBGV/ RP (μg/kg bw)	Basis
Arsenic (As) 0.3–8 ($BMDL_{01}$)	The reference point is based on a range of benchmark dose lower confidence limit ($BMDL_{01}$) values between 0.3 and 8 μg/kg bw per day identified for cancers of the lung, skin and bladder, as well as skin lesions. In general, the MOS/MOE should be at least 10,000 if the reference point is based on carcinogenicity in animal studies. However, as the BMDL for As is derived from human studies, an interspecies extrapolation factor (i.e. 10) is not needed (EFSA CONTAM Panel, 2009a; EFSA Scientific Committee, 2012).
Lead (Pb) 0.5 ($BMDL_{01}$)	The reference point is based on a study demonstrating perturbation of intellectual development in children with the critical response size of 1 point reduction in IQ. The EFSA CONTAM Panel mentioned that a 1-point reduction in IQ is related to a 4.5% increase in the risk of failure to graduate from high school and that a 1 point reduction in IQ in children can be associated with a decrease of later productivity of about 2%. A risk cannot be excluded if the exposure exceeds the BMDL01 (MOS/MOE lower than 1) (EFSA CONTAM Panel, 2010).
Cadmium (Cd) 2.5 (TWI)	The derivation of the reference point is based on a meta-analysis to evaluate the dose–response relationship between selected urinary cadmium and urinary beta-2-microglobulin (B2 M) as the biomarker of tubular damage recognised as the most useful biomarker in relation to tubular effects. A group-based $BMDL_5$ of 4 μg Cd/g creatinine for humans was derived. A chemical-specific adjustment factor of 3.9 was applied to account for human variability in urinary cadmium within each dose subgroup in the analysis resulting in a reference point of 1.0 μg Cd per g creatinine. In order to remain below 1 μg Cd/g creatinine in urine in 95% of the population by age 50, the average daily dietary cadmium intake should not exceed 0.36 μg Cd/kg bw, corresponding to a weekly dietary intake of 2.5 μg Cd/kg bw (EFSA CONTAM Panel, 2009b).
Mercury (Hg) 4 (TWI)	The HBGV was set using kidney weight changes in male rats as the pivotal effect. Based on the $BMDL_{10}$ of 0.06 mg/kg bw per day, expressed as mercury, and an uncertainty factor of 100 to account for inter- and intraspecies differences, with conversion to a weekly basis and rounding to one significant figure, a TWI for inorganic mercury of 4 μg/kg bw, expressed as mercury was established (EFSA CONTAM Panel, 2012).

The risk assessment of the toxic elements helps informing whether there could be a possible health concern if these elements would be present at the proposed specification limits for Prosmoke BW 01.

Table 13: Risk assessment for toxic elements in ProSmoke based on the specification limits for toxic elements (Documentation provided to EFSA No. 1)

Exposure to ProSmoke (mg/kg bw per day)	As exposure (μg/kg bw per day)	MOE for As at 3 mg/kg	Pb exposure (μg/kg bw per day)	MOE for Pb at 10 mg/kg	Hg exposure (μg/kg bw per day)	% TWI for Hg at 1 mg/kg	Cd exposure (μg/kg bw per day)	% TWI for Cd at 1 mg/kg
17.9[(a)]	0.0537	5.6–149	0.18	2.8	0.018	3.1	0.018	5

(a): 95th percentile children (Table 11).

For Hg and Cd, the resulting estimate of exposure is only a small fraction of their TWIs. For As, margin of exposure (MOE) values fall far below the target of 1000. For Pb, the MOE is only just above 1. For Hg and Cd, there are no safety concerns, and the choice of the exposure model would not

affect this outcome. For Pb, the magnitude of the MOE would increase by a factor of 2 or 3 when calculated on the basis of SMK-TAMDI and SMK-EPIC, respectively.

Overall, the resulting figures indicate that the potential exposure to As from the uses and use levels of Prosmoke at the specification limit (Table 8) would be expected to be of concern. For As, the estimates of exposure based on SMK-TAMDI and SMK-EPIC would overlap with the estimates obtained using the FAIM tool and would still indicate a safety concern. The panel considers lowering the specification limits for As and Pb to be both appropriate and technologically achievable, based on the analytical data provided for one batch of Prosmoke (Documentation provided to EFSA No. 1) (see Table 3).

3.3. Biological and toxicological data

3.3.1. Genotoxicity

Prosmoke BW 01 is a complex mixture in which the fraction of unidentified components amounts to 16 wt% (see Section 3.1.2.4.4). The Panel decided to apply the approach recommended by the EFSA Scientific Committee (EFSA Scientific Committee, 2019) for the genotoxicity assessment of mixtures containing a substantial fraction of unidentified components. This approach foresees that first the genotoxic potential of the chemically identified components in the smoke flavouring primary product should be assessed individually, using all available data. Genotoxicity data should be collected and evaluated based on the Scientific Committee guidance on genotoxicity (EFSA Scientific Committee, 2011, 2017, 2021). Conclusions on genotoxicity are required for all identified components. Structure–activity relations (SAR) information about the genotoxic potential of an identified component is considered when no information on genotoxicity from published or unpublished studies is available. When experimental data are available from structurally related substances, these were given prevalence over the results of the SAR analysis (see Appendix A – Table A.1).

3.3.1.1. Genotoxicity assessment of the individual components

The 48 identified constituents of Prosmoke BW 01 were evaluated individually for genotoxicity considering first the data available from the literature as provided by the applicant and then, in the absence of relevant information from the literature, with *in silico* information submitted by the applicant (see Appendix A – Table A.1).

The Panel noted that the investigation of volatile constituents has been performed using a sample of the primary product subjected to evaporation prior to GC analysis. This evaporation step is expected to result not only in the intended removal of water and acetic acid, but it might also lead to a partial loss particularly of highly volatile constituents. The Panel assumed that the application of this sample preparation step did not result in a complete loss of any of the volatile constituents and therefore does not invalidate the component-based genotoxicity assessment of the primary product.

The applicant submitted literature data on 44 identified components and performed *in silico* analysis for all the 48 identified components, mainly using VEGA QSAR platform (version 1.1.5)[12], applying the following models (Documentation provided to EFSA No. 3):

- Mutagenicity Classification Model version 2.1.13
- Mutagenicity SarPy/IRFMN Model version 1.0.7
- Mutagenicity ISS Model version 1.0.2
- Mutagenicity Read-Across version 1.0.0
- *In vitro* Micronucleus Activity (IRFMN/VERMEER) Version 1.0.0.

In case of equivocal or highly uncertain predictions with VEGA QSAR models, the OECD QSAR Toolbox (version 4.4.1)[13] was used by the applicant for read-across evaluations. If equivocal or uncertainty predictions could not be resolved, the Danish (Q)SAR database[14] was also used.

In addition to this, the Panel conducted a (Q)SAR analysis using the following six profilers contained in the OECD QSAR Toolbox v. 4.5:

- DNA alerts for AMES, Chromosomal Aberrations (CA) and Micronucleus (MN) by OASIS;
- DNA binding by OASIS;

[12] https://www.vegahub.eu/a-new-version-of-vega-qsar-available-with-more-than-70-models/
[13] https://www.oecd.org/chemicalsafety/oecd-qsar-toolbox.htm
[14] https://qsar.food.dtu.dk/

- DNA binding by OECD;
- Protein binding alerts for chromosomal aberration by OASIS;
- *In vitro* mutagenicity (Ames test) alerts by ISS;
- *In vivo* mutagenicity (Micronucleus) alerts by ISS.

When necessary, the application of profilers was followed by an expert review (e.g. check of close analogues/structurally related substances).

The Panel noted that genotoxicity data from literature were available for 31 constituents, either on the substance or on structurally related substances, that were assessed by EFSA or by JECFA as chemically defined flavouring substances.

Based on these (often limited) data, the Panel concluded for 30 substances that these data did not indicate a concern for genotoxicity. However, it should be noted that the expression used in Appendix A – Table A.1 *'limited experimental genotoxicity data on the substance and on structurally related substances did not indicate concern for genotoxicity'* should not be interpreted as being equivalent to 'no concern with respect to genotoxicity'. The approach taken here is therefore pragmatic rather than based on robust experimental data.

For one constituent (i.e. furan-2(5H)-one (formerly [FL-no: 10.066])[15], see Section 3.3.2.1 for further details), the Panel identified a concern for genotoxicity.

For the remaining 17 substances, the information from the literature on genotoxicity was not conclusive. For these 17 substances, the genotoxicity was assessed only based on (Q)SAR analysis and no indications for concerns on genotoxicity emerged (see Appendix A – Table A.2).

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

Furan-2(5H)-one (formerly [FL-no: 10.066]) has been identified among the chemical constituents of the mixture in all four analysed batches (batch #1: 0.35%[16], batch #2: 1.21%, batch #3: 0.88%, batch #4: 1.07%; average concentration: 0.88 wt %). This substance was evaluated by the FAF Panel as genotoxic *in vivo* (EFSA FAF Panel, 2019).

The applicant did not provide any new experimental data on (formerly [FL-no: 10.066]), but submitted a review by the Flavor and Extract Manufacturers Association of the United States (FEMA) Expert Panel (Gooderham et al., 2020). This review merely commented on the assessment of the genotoxicity data on furan-2(5H)-one (formerly [FL-no: 10.066]) made by the FAF Panel in FGE.217Rev2 (EFSA FAF Panel, 2019). The applicant quoted the FEMA Expert Panel's conclusion '*the criteria for a clear positive outcome were not met, and when considered in combination with the negative bacterial reverse mutation outcome, the negative in vivo micronucleus results, and the inconsistent results in the in vitro micronucleus studies, concluded that based upon weight of evidence that furan-2(5H)-one did not display genotoxic potential*'.

Regarding the evaluation of *in vitro* MN studies, the FEMA Expert Panel noted:

- '*in vitro micronucleus studies using TK6 cells are generally considered to be appropriately sensitive, if not more sensitive, than those conducted in human peripheral blood lymphocytes*'.

In response to the FEMA Expert Panel comments, the FAF Panel reiterated its view which was already expressed in FGE.217Rev2: '*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).*[17] *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(5H)-one [FL-no: 10.066] clearly increased the frequency of MN in the presence of metabolic activation'* (EFSA FAF Panel, 2019).

The FEMA Expert Panel further noted:

- '*the two in vitro micronucleus studies conducted in human peripheral blood lymphocytes displayed exceedingly steep cytotoxicity curves and shifts from trial to trial in the cytotoxicity measured at the same or very similar concentrations. The cytotoxicity curves for the in vitro*

[15] Removed from the Annex I of Regulation (EC) No 1334/2008 by Regulation (EU) No 2019/799.
[16] Calculated as follows: (reported furan-2(5H)-one concentration (g/L)*100/reported weight (g) of 1 L sample.
[17] Further division of cells would result in a lower frequency of MN. This would be prevented by the use of cytochalasin B.

micronucleus assay in TK6 cells were less steep and it was correspondingly easier, it appears, to choose concentrations for scoring of micronuclei. There is not a clear explanation as to why there were differing outcomes within these studies'.

In response to this, the FAF Panel emphasised that a steep increase in cytotoxicity was not observed in the MN study in TK6 cells (BioReliance, 2018) nor in the MN studies in human peripheral blood lymphocytes (Whitwell, 2012; Covance, 2013). The highest concentrations tested in the MN assays reached a level of cytotoxicity in agreement with OECD TG 487. Results from cytotoxicity tests and from MN analysis are consistent between the two studies in human peripheral blood lymphocytes (Whitwell, 2012, Covance, 2013). The inconsistency with the negative results observed in TK6 cells may be due to the different application of cytochalasin B as described above and in FGE.217Rev2 (EFSA FAF Panel, 2019). Scoring of MN in mononucleated cells could generate false-negative results (Fenech, 2000).

Regarding the evaluation of the *in vivo* comet assay, the FEMA Expert Panel noted:

- *'At the top dose of 250 mg/kg bw/day, small, less than two-fold increases in % tail DNA and tail moment were observed in the liver. The Panel notes that the increase in % tail DNA and tail moment at the top dose were within both the historical control range and the 95% reference range of the historical controls. Additionally, overlap between tail DNA values were reported for concurrent vehicle control animals and those in the top dose group'.*

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

- First criterion: At least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control;
- Second criterion: The increase is dose-related when evaluated with an appropriate trend test.

The Panel considered that the third criterion, i.e. '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 the very wide range for historical negative controls reported was overlapping with the range of historical positive control.

The FEMA Expert Panel considered that

- *the results from the in vivo MN assay in rats bone marrow were negative*.

However, as explained in FGE.217Rev2, the FAF Panel evaluated the study as inconclusive, because there was no evidence that bone marrow was exposed.

Based on the considerations above, the FAF Panel does not agree with the FEMA Expert Panel conclusion that *furan-2(5H)-one did not display genotoxic potential*.

For more details, see Appendix B and FGE.217Rev2 (EFSA FAF Panel, 2019).

3.3.1.1.2. Exposure to and risk assessment of furan-2(5H)-one

The FAF Panel calculated the exposure to furan-2(5H)-one (formerly [FL-no: 10.066]) by multiplying the estimated exposure to the primary product (see Section 3.2) using the average furan-2(5H)-one content of 0.88 wt % in the mixture. Using the SMK-TAMDI and SMK-EPIC techniques furan-2(5H)-one intakes of 81 μg/kg bw per day and 54.6 μg/kg bw per day, respectively, were obtained. In addition to that, the exposure to furan-2(5H)-one was calculated based on the exposure estimate for the primary product using the FAIM tool (Table 11).

Table 14: Exposure to furan-2(5H)-one based on FAIM (μg/kg bw per day)

	Infants (12 weeks to 11 months)	Toddlers (12–35 months)	Children (3–9 years)	Adolescents (10–17 years)	Adults (18–64 years)	The elderly (≥ 65 years)
• Mean	7.8–22.9	21.0–102.4	28.8–86.6	17.2–53.0	17.2–50.5	12.1–43.4
• 95th percentile	30.2–78.4	68.2–118.6	67.8–157.4	38.8–117.4	41.0–147.6	28.3–90.0

Considering that new data on the genotoxicity of furan-2(5H)-one (formerly [FL-no: 10.066]) are not available, the FAF Panel confirms the conclusion stated in FGE.217Rev2 that the substance is genotoxic *in vivo*. All exposure estimates for furan-2(5H)-one are above the threshold of toxicological

concern (TTC) value of 0.0025 μg/kg bw per day (or 0.15 μg/person per day) for DNA-reactive mutagens and/or carcinogens. Therefore, the concerns for genotoxicity could not be ruled out based on this TTC approach.

3.3.1.2. Genotoxicity assessment of the whole smoke flavouring mixture

The EFSA Scientific Committee recommends, as first option, to test the fraction of unidentified substances for genotoxicity separately from the rest of the mixture. Alternatively, if this is not feasible, the testing of the whole mixture should be undertaken (EFSA Scientific Committee, 2019). In the case of Prosmoke BW 01 mixture, the Panel considered that the separation and testing of the unidentified and/or of the tentatively identified part of the mixture would not be technically feasible. Therefore, the Panel considered not only the available information on individual constituents of the chemically characterised fraction but also the experimental data on the whole mixture for the genotoxicity assessment of the flavouring.

In vitro genotoxicity studies

3.3.1.2.1. Bacterial reverse mutation test

A bacterial reverse mutation assay was conducted in Salmonella Typhimurium strains TA98, TA100, TA1535, TA1537 and in *Escherichia coli* WP2 uvrA to assess the mutagenicity of the whole Prosmoke BW 01 mixture, both in the absence and in the presence of metabolic activation by phenobarbital/β-naphthoflavone-induced rat liver post-mitochondrial fraction (S9-mix) in two separate experiments using standard plate incorporation (in the first experiment) and modified pre-incubation treatments (in the second experiment) (TOXI-COOP ZRT, 2018a). The study was conducted in accordance with GLP (good laboratory practice) and OECD Test Guideline (TG) 471 (OECD, 1997).

An initial toxicity range-finding experiment was carried out using the plate incorporation method in the presence and absence of S9-mix, for *S*. Typhimurium strains TA98 and TA100 at concentrations of 5, 16, 50, 160, 500, 1,600 and 5,000 μg/plate, plus negative vehicle (DMSO) and positive controls. Inhibitory effect of the test item on bacterial growth was not observed and the colony and background lawn development were not affected at any concentration tested.

In the first and in the second experiments, *S*. Typhimurium TA98, TA1535, TA1537 and *E. coli* WP2 uvrA strains were incubated with 16, 50, 160, 500, 1,600 and 5,000 μg/plate of the whole Prosmoke BW 01 mixture in the absence and presence of S9-mix; *S*. Typhimurium TA100 strain was incubated with 16, 50, 160, 500, 1,600, 2,500, 4,000, 4,500 and 5,000 μg/plate in the absence and presence of S9-mix. No precipitation of the mixture was observed on the plates at any examined concentration level (±S9 mix) in any of the experiments conducted. The criteria considered to assess the biological relevance of results were the fold increase of revertant number over the solvent control values set at 2, for *S*. Typhimurium TA100 strain, and at 3, for the other *S*. Typhimurium strains and for *E. coli* WP2 uvrA.

In the first experiment, no biologically relevant increases in revertant colony numbers were observed.

In the second experiment, using the modified pre-incubation method, Prosmoke induced an increase of revertant colony numbers in *S*. Typhimurium TA100 both in the absence (up to 4.9-fold) and presence (up to 6.9-fold) of S9-mix at the concentration range of 1,600–5,000 μg/plate. These increases were more than twofold compared to the negative control and were therefore considered biologically relevant. Furthermore, the increase in revertant colony numbers was concentration-related. In strain TA98, a concentration-related increase in revertant colony numbers was observed both in the absence (up to 2.8-fold) and presence (up to 2.8-fold) of S9-mix, which was not considered biologically relevant. In strain TA1535, a concentration-related increase in revertant colony numbers (up to 2.6-fold) was observed (also not biologically relevant). However, in TA1537, a biologically relevant increase in revertant colony numbers (3.2-fold) was observed at 5,000 μg/plate in the presence of S9-mix). No increases in revertants were observed in *Escherichia coli* WP2 uvrA.

The Panel concluded that Prosmoke BW01 induced gene mutations in *Salmonella typhimurium*.

3.3.1.2.2. In vitro *mammalian cell gene mutation test*

An *in vitro* mammalian cell gene mutation test (*hprt* locus) was conducted in CHO-K1 cells to assess the mutagenicity of the whole Prosmoke BW 01 mixture, both in the absence and in the presence of metabolic activation by phenobarbital/β-naphthoflavone-induced rat liver post-mitochondrial fraction

(S9-mix) (TOXI-COOP ZRT, 2018b). The study was conducted in accordance with GLP and OECD TG 476 (OECD, 2016a).

CHO-K1 cells were treated for 5 h with 50, 100, 150, 200 and 250 μg/mL Prosmoke BW 01 mixture in the absence of S9-mix or with 150, 200, 250, 300 and 350 μg/mL in the presence of S9-mix. No precipitation of the mixture and no relevant changes in pH or osmolality of the medium were noted at the different concentrations tested. At the highest concentration applied, 250 μg/mL in the absence and 350 μg/mL in the presence of S9-mix, cytotoxicity (relative survival between 14% and 21%) was observed.

Mutation frequencies in negative and positive control samples were within the respective historical control ranges.

No increases in mutation frequency were observed when compared to the concurrent solvent control and the laboratory historical control data, at any concentration tested in the absence and presence of metabolic activation.

The Panel concluded that Prosmoke BW 01 was not mutagenic in this *in vitro* gene mutation test in CHO-K1 cells.

3.3.1.2.3. In vitro *mammalian chromosomal aberration test*

Prosmoke BW 01 was tested in an *in vitro* mammalian chromosomal aberration test in V79 cells (Chinese hamster lung cells) in the absence and in the presence of metabolic activation by phenobarbital/β-naphthoflavone-induced rat liver post-mitochondrial fraction (S9-mix) (TOXI-COOP ZRT, 2018c). The study was conducted in accordance with GLP and OECD TG 473 (OECD, 2016b).

A range-finding test was carried out applying the relative increase in cell counts (RICC) as a measure of cytotoxicity. On the basis of the results of this experiment, selected concentrations were tested (in duplicate cultures) in two main experiments (A and B).

In experiment A, cells were treated for 3 h and sampling was done at 20 h from the beginning of the treatment (approximately 1.5 cell cycles). The concentrations tested were 39.1, 78.2 and 156.3 μg/mL in the absence of S9-mix and 78.2, 156.3 and 312.5 μg/mL in the presence of S9-mix. In this experiment, cytotoxicity percentages at the highest concentrations tested were 51% and 53% in the absence and in the presence of S9-mix, respectively.

In experiment B, the following test conditions and concentrations were used:

- 20 h treatment (sampling time at the end of the treatment) in the absence of S9-mix, 39.1, 78.2 and 156.3 μg/mL (54% cytotoxicity);
- 20 h treatment/28 h sampling time (approximately two cell cycles) in the absence of S9-mix, 39.1, 78.2 and 156.3 μg/mL (51% cytotoxicity);
- 3 h treatment/28 h sampling time in the presence of S9-mix 78.2, 156.3 and 312.5 μg/mL (52% cytotoxicity).

No precipitation of the mixture and no relevant changes in pH or osmolality of the medium were noted at the different concentrations tested.

For each culture, 150 metaphases were scored (300 in total for each duplicate cultures).

A number of cells with structural chromosomal aberrations in negative and positive control samples were within the respective historical control ranges.

No significant increase of cells with structural chromosomal aberrations was observed in any of the experimental conditions when compared to concurrent control data. Only two values of mean aberrant cells were slightly above the upper confidence limit for the historical negative control range, but not statistically different with respect to the concurrent negative control. Furthermore, no concentration–response relationship was observed.

The Panel concluded that Prosmoke BW 01 did not induce structural chromosomal aberrations in Chinese hamster lung V79 cells, when tested up to cytotoxic concentrations in the absence and presence of metabolic activation.

3.3.1.2.4. In vitro *micronucleus test with FISH analysis*

Prosmoke BW 01 was tested in an *in vitro* micronucleus (MN) test in the mouse lymphoma L5178Y tk +/− 3.7.2c cell line (TOXI-COOP ZRT, 2021a), with the purpose of evaluating the aneugenic and clastogenic potential of the tested substance. The study was performed according to GLP and OECD TG 487 (OECD, 2016c).

A range-finding test was performed at different ranges of concentrations, based on the results of a previous experiment (data not shown) in which the test item had shown high cytotoxicity between concentrations of 1,000–5,000 μg/mL.

Based on the results of this cytotoxicity test, in a first micronucleus test, mouse lymphoma cells were treated for 4 h in the absence or presence of S9-mix (from phenobarbital/β-naphthoflavone induced rats) or for 24 h in the absence of S9-mix at the following concentrations:

- for the 4-h treatment in the absence of metabolic activation 12.5, 25, 50, 100, 200 μg/mL;
- for the 4-h treatment in the presence of metabolic activation 50, 100, 150, 200, 250 μg/mL;
- for the 24-h treatment in the absence of metabolic activation 12.5, 25, 50, 75, 100 μg/mL.

DMSO was used as the vehicle. Positive controls were cyclophosphamide (CP), mitomycin C (MMC) and colchicine (CL). Each concentration was tested in duplicate cultures. For each replicate, 10,000 cells were analysed through flow cytometry. Sampling was done at 24 h, approximately two normal cell cycles. Due to cytotoxicity, the highest concentrations evaluated for MN induction were 250 μg/mL (57% cytotoxicity) for the 4-h treatment in the presence of S9-mix, 200 μg/mL (50% cytotoxicity) for the 4-h treatment and 100 μg/mL (55% cytotoxicity) for the 24-h treatment in the absence of S9-mix.

After 4-h treatment, in the absence of S9-mix, at 200 μg/mL, a statistically significant increase in micronucleated cell frequency (1.6% vs. 0.7% in the negative control) was observed, which was above the upper confidence limit of the historical negative control.

After 4-h treatment, in the presence of S9-mix, at 250 μg/mL, a statistically significant increase in micronucleated cell frequency (0.9% vs. 0.6% in the negative control) was observed, which was above the upper confidence limit of the historical negative control. After 24-h treatment, in the absence of S9-mix, at 50, 75 and 100 μg/mL, a statistically significant increase in micronucleated cell frequencies (0.5, 0.6, 0.8%, respectively, vs. 0.3% in the negative control) was observed, which was below the upper confidence limit of the historical negative control.

The Panel noted that the micronucleated cell frequency in the 24-h solvent control was below the lower confidence limit of the historical negative controls. The micronucleated cell frequency after CL treatment was statistically significantly higher than the concurrent negative control, but below the lower confidence limit of the historical control for CL at 4-h and 24-h treatments in the absence of S9-mix. MMC and CP increased the micronucleated cell frequency above the upper confidence limit of the respective historical control range at 4 h in the absence of S9-mix and at 4 h in the presence of S9-mix, respectively.

A further *in vitro* MN test was performed in order to investigate the clastogenic and aneugenic potential of the mixture applying the fluorescence in situ hybridisation (FISH).

Based on the results from the first experiment, the following concentrations were tested in duplicate cultures:

- for the 4-h treatment in the absence of metabolic activation: 50, 100, 200 μg/mL;
- for the 4-h in the presence of metabolic activation: 150, 200, 250 μg/mL;
- for the 24-h treatment in the absence of metabolic activation: 25, 50, 100 μg/mL.

For each cell culture, 1,000 cells were analysed (2,000 cells/concentration) applying the visual scoring. At the highest concentrations, tested cytotoxicity was 56% at 200 μg/mL (4-h treatment in the absence of S9-mix), 51% at 250 μg/mL (4-h treatment in the presence of S9-mix) and 53% at 100 μg/mL (24-h treatment in the absence of S9-mix).

After the 4-h treatment in the absence of S9-mix, at 200 μg/mL, a statistically significant increase in micronucleated cell frequency (3.1% vs. 0.9% in the negative control) was observed. After the 4-h treatment, in the presence of S9-mix, at 250 μg/mL, a statistically significant increase in micronucleated cell frequency (3% vs. 0.7% in the negative control) was observed. After the 24-h treatment, in the absence of S9-mix, at concentrations of 50 and 100 μg/mL, a statistically significant increase in micronucleated cell frequency (2.1% and 2.6%, respectively, vs. 0.9% in the negative control) was observed.

No historical control data were provided for the visual scoring of MN to establish the proficiency of the laboratory and to evaluate and interpret the results.

The FISH analysis performed on a too low number of cells per slide did not provide robust data for negative and positive controls which are prerequisites to evaluate the clastogenicity and aneugenicity of Prosmoke BW 01. No historical control data were available to establish the proficiency of the laboratory for the FISH analysis.

Overall, the Panel concluded that these studies provided some indications for an increase of micronucleated cell frequency which in the light of the limitations of the study, would need to be further investigated.

In vivo genotoxicity studies

3.3.1.2.5. In vivo *mammalian erythrocyte micronucleus test*

The genotoxic potential of whole Prosmoke BW 01 mixture was assessed *in vivo* using the bone marrow micronucleus assay (TOXI-COOP ZRT, 2019a). The study was conducted in accordance with GLP and OECD TG 474 (OECD, 2016d).

A preliminary toxicity test was performed to identify the appropriate maximum dose level for the main test. Groups of two male and two female Hsd Win: NMRI mice were treated twice at 24-h intervals by oral gavage at 2,000 mg/kg body weight (bw) per day. No mortality and no adverse reactions to treatment were observed in male and female mice.

Based on this study, the highest dose level used for the main study was 2,000 mg/kg bw per day. As no gender-specific effects were seen, only male mice were used in the main study.

Groups of five mice per dose group were administered doses of Prosmoke BW 01 by gavage at 0 (Polyethylene glycol 400 (PEG 400)), 500, 1,000 or 2,000 mg/kg bw twice at 24-h intervals. Sampling time was at 24 h after the second treatment. A positive control group of five male mice was administered an i.p. dose of 60 mg CP/kg bw and sampling time was 24 h after administration.

No clinical signs of toxicity were observed in any animal following treatments with Prosmoke BW 01, vehicle or the positive control (CP).

A total of at least 500 polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) were scored to calculate the bone marrow toxicity by the relative decrease in PCE. For MN analysis 4,000 PCE per animal were scored for the presence of MN.

Mice treated with the whole Prosmoke BW 01 mixture exhibited group mean frequencies of micronucleated polychromatic erythrocytes (MNPCE) that were not statistically different from those observed in the concurrent vehicle control for all dose groups. Furthermore, they were within the historical negative control 95% confidence interval. A small reduction in PCE/total erythrocytes ratio was observed, which was not considered sufficient to demonstrate bone marrow exposure. No other signs of toxicity, which could be indicative of systemic exposure, were observed. Since target tissue exposure was not demonstrated, the Panel concluded that the results from the *in vivo* MN assay are inconclusive.

3.3.1.2.6. In vivo *alkaline comet assay*

The genotoxic potential of whole Prosmoke BW 01 mixture was assessed *in vivo* using the alkaline comet assay in rat stomach and liver (TOXI-COOP ZRT, 2019b). The study was conducted in accordance with GLP and OECD TG 489 (OECD, 2016e).

A preliminary toxicity test was performed to identify the appropriate maximum dose level for the main test. A group of two male Han Wistar rats was treated twice at 24-h intervals by oral gavage at 2,000 mg/kg bw. No mortality and no adverse reactions to treatment were observed. Therefore, 2,000 mg/kg bw per day was chosen as the highest dose level in the main study.

Groups of six male rats per dose group were administered doses of Prosmoke BW 01 by oral gavage at 0 (PEG 400), 500, 1000 or 2,000 mg/kg bw twice at 24-h intervals. A positive control group of four male rats was treated by oral gavage once with ethyl methanesulfonate (EMS) at the dose of 200 mg/kg bw. For all groups, sampling time was at 3–4 h after the last treatment.

No mortality and no clinical signs of toxicity were observed during the treatments and expression period in any dose group and controls. At tissue isolation, a normal appearance anatomy of examined organs (liver, stomach) was observed in all dose levels and control groups. Slight tympanites in the stomach were noticed at 1,000 and 2,000 mg/kg bw per day; furthermore, stomach hyperaemia and thin, faint structured gastric mucosa was noticed at 2000 mg/kg bw per day. No significant cytotoxicity was noticed in any dose group and controls (trypan blue dye exclusion method).

Comet analyses were performed on three slides per animal from five rats of all dose groups and negative control, and from three rats of positive control group. Olive tail moment (OTM), tail intensity (%TI) and tail length of a total of 150 cells per animal were analysed.

In both stomach and liver, there was no increase in the percentage of clouds following treatment with whole Prosmoke BW 01 mixture, thus demonstrating that treatment did not cause toxicity that could have interfered with comet analysis.

In stomach, no increase in any of the parameters analysed was observed in exposed groups with respect to the negative control group. Negative and positive controls of %TI values were within the lower and upper 95% confidence intervals of corresponding historical control values.

In liver, after the analysis of 150 cells per animal, a statistically significant increase of all parameters at the dose of 2,000 mg/kg bw per day was observed. The increase of OTM and tail length was statistically significant also at 1,000 mg/kg bw per day. The study authors indicated that the increases were above the upper 95% confidence limit of the historical control database (three studies only). However, results from this first analysis were not reported in detail in the study report. The study authors repeated the reading only of the slides of the highest dose group. In this second reading, one animal was added (six animals in total) and 50 additional cells were analysed from each animal (200 cells per animal and 1,200 cells in total). In the analysis of this second reading, the %TI values were not statistically significant different from the negative control (6.91 $\pm$ 0.87%TI at 2,000 mg/kg bw per day and 6.03 $\pm$ 0.34%TI for the negative control). However, a significant difference remained for the other two parameters, tail length and OTM.

The Panel considered that the additional analysis of cells only from the 2,000 mg/kg bw per day dose group may have introduced a bias in the study. Therefore, the applicant was requested to submit a second reading of all the four slides from all the six animals in each group, performed blinded and with the same number of cells in all treated and control groups, by an independent laboratory.

A new analysis was conducted on photos of slides from both stomach and liver (Toxi-Coop ZRT, 2021b). Two hundred cells per animal were analysed, five animals in each dose group (1000 cells per dose group) and three animals in the EMS group (600 cells for the positive control group).

Both in stomach and liver, no statistically significant increase in %TI was observed at any dose level. In liver, at the highest dose, %TI was 7.08 $\pm$ 0.59 and in the concurrent control %TI was 6.33 $\pm$ 0.73.

The Panel noted the inconsistencies among results obtained in the different scorings in liver. Moreover, the requested reanalysis (four slides per animal of six animals per group) was performed on photographs of the first scoring rather than on slides. It was not reported whether these photographs were taken and evaluated blinded and whether they covered the whole slide. These are limitations of the study. In addition, the Panel noted that the negative and positive historical control values database comprised only three and six studies, respectively, which was considered inadequate to establish the proficiency of the laboratory and to evaluate and interpret the results.

Due to all these limitations, the Panel concluded that the study could not rule out the concern for genotoxicity of the whole mixture, that emerged from the effects observed *in vitro*.

3.3.1.3. Conclusions on genotoxicity assessment

Overall, due to the presence of furan-2(5H)-one, a concern for genotoxicity was identified for Prosmoke BW 01. In addition, the data on the genotoxicity studies performed on the whole mixture were inconclusive and could not rule out a potential genotoxicity of the fraction of unidentified constituents.

3.3.2. Toxicity other than genotoxicity

3.3.2.1. Subchronic toxicity

Prosmoke BW 01 (batch #1 produced on 27/2/2017) was tested in a 90-day repeated dose toxicity study in rats with GLP compliance and according to OECD TG 408 (OECD, 2018). Prosmoke BW 01 was administered to four groups of male and female Han Wistar rats (10 animals per group) at doses of 0 (vehicle only), 500, 750 and 1000 mg/kg bw per day by oral gavage (TOXI-COOP ZRT, 2019c). The dose levels were not based on the results of a dose range finding study, but where chosen based on literature data as stated by the applicant. (see Appendix C – Table C.3).

According to OECD TG 408, all individual data for the control and high-dosed animals should be provided and the data should be summarised in a tabular form. Data should be reported for all dosage groups where there are treatment-related changes in the high-dose group. In this respect, the Panel noted that the reporting of the results of gross necropsy and histopathology are not in accordance with the OECD TG 408 as only deviant observations were reported.

The Panel noted that one female rat in the highest dose group died on day 78 due to a zootechnical mistake. Food consumption, mean body weight and body weight gains were not affected by the treatments. Statistically significant changes were observed in a range of haematological parameters including the numbers of monocytes, white blood cells and red blood cells and a reduction

of prothrombin time in male rats at all dose levels. There were also significant changes in the haematocrit value, mean corpuscular concentration, mean platelet volume, mean corpuscular haemoglobin concentration and in the percentage of reticulocytes in male rats. In females, the mean corpuscular volume was reduced at all dose levels and the mean red blood cell count and the mean corpuscular haemoglobin concentration were significantly but marginally increased and the percentage of reticulocytes was decreased at the top dose. However, many of these effects were small in degree, not clearly dose dependent and within the range of historical controls. This was not the case regarding the dose-dependent increase in the percentage of monocytes in male animals. In addition, basophil percentages were lower as the dose increased in males.

A number of changes were seen in both sexes with respect to clinical chemistry and urinalysis (e.g. a reduction of both serum AST and glucose at all dose levels in males and a decrease in cholesterol and LDL in females at 750 and 1,000 mg/kg bw per day, a small decrease in urine specific gravity at 500 and 1,000 mg/kg and an increased pH at the top dose). The Panel considered all these changes as not of toxicological significance.

According to the study authors, macroscopic or histopathological changes associated with these changes in clinical chemistry were not observed at any of the dose levels studied except for changes in the weight of liver and thyroid (i.e. statistically significant increase in the mean absolute and relative liver weight in male rats at all dose levels but not in females and a reduction of the mean weight of the thyroids in females compared to controls, which was statistically significant at 500 mg/kg bw per day only). Functional observations did not reveal an effect on behaviour or neurological function. Studies on oestrous cycle and sperm also did not raise a concern and ophthalmic examinations showed no alterations when tested at the high dose.

There was a statistically significant and dose-dependent increase in serum T3 observed in both female and male rats (significantly increased at 750 and 1,000 mg/kg bw per day in males and at all dose levels in females), with no significant alteration of serum T4 concentrations. The applicant stated that it was not possible to determine if TSH was altered since the concentrations were below the limit of quantification in all animals, including the control animals.

In view of the genotoxicity concerns for Prosmoke BW 01 as described in Section 3.3.1, the Panel considered that a further evaluation of the 90-day study, including the identification of a dose to be used as toxicological reference point for the derivation of a health-based guidance value, would not be appropriate. Should the genotoxicity concern be ruled out, further information would be needed to clarify the changes in serum T3. In addition, the Panel noted that the study was performed with a batch of Prosmoke BW 01 (i.e. batch #1) whose representativity for the material of commerce is uncertain (see Section 3.1.3).

4. Discussion

The Panel has been requested to evaluate the safety of a new smoke flavouring primary product, Prosmoke BW01, produced by pyrolysis of beechwood (Fagus sylvatica L.) sawdust and intended for use in several food categories.

The applicant provided compositional data for four batches of the primary product.

For all four investigated batches of the Primary Product, the identified and quantified proportion of the solvent-free fraction was higher than 50 wt%, and thus, it meets the legal requirement of Commission Regulation (EC) No 627/2006.

Regarding the identified and quantified proportion of the volatile fraction, two of the analysed batches (i.e. batches #1 and #2) comply with the legal limit of having at least 80 wt% of the volatile fraction identified and quantified, in line with Commission Regulation (EC) No 627/2006. For the other two batches (batches #3 and #4), the identified and quantified proportions of the volatile fraction are slightly below the legal limit (74.2% and 76.1%). Taking the limitations of the analytical procedure into account, the Panel considered that the deviations of these analytical values from the legal limits do not hamper the safety assessment of the primary product.

Data provided for three batches of the primary product demonstrated that their batch-to-batch-variability was sufficiently low. However, for the batch used for the toxicological studies, there were substantial deviations in the concentration of nearly all constituents compared to the other three batches. Therefore, the representativity of this batch for the material of commerce is uncertain.

Dietary exposure to Prosmoke BW 01 was estimated to be between 6.2 and 9.2 mg/kg bw per day, respectively, using SMK-EPIC and SMK-TAMDI. Using the FAIM tool, dietary exposure ranged from 0.9 mg/kg bw per day in infants to 11.6 mg/kg bw per day for toddlers at the mean. The 95th

percentile exposure estimates ranged from 3.2 mg/kg bw per day for the elderly to 17.9 mg/kg bw per day for children. Exposure estimated through FAIM tool is considered to be more accurate considering the food categories in which the smoke flavouring is requested to be used. In this case, the exposure estimate obtained using the FAIM tool is more refined than those obtained using with SMK-EPIC and SMK-TAMDI.

Furan-2(5H)-one (formerly [FL-no: 10.066]) is present in all batches of Prosmoke BW 01 at an average concentration of 0.88 wt%. This substance was evaluated by the FAF Panel as genotoxic in vivo after oral exposure (EFSA FAF Panel, 2019). Considering that new experimental data on the genotoxicity of furan-2(5H)-one (formerly [FL-no: 10.066]) are not available, the FAF Panel confirms the conclusion stated in its previous evaluation that the substance raises concern with respect to genotoxicity in vivo. In line with the Scientific Committee statement on the genotoxicity assessment of mixture (EFSA Scientific Committee, 2019) and considering that the exposure to furan-2(5H)-one that would result from the intended use of the primary product would be above the TTC of 0.0025 μg/kg bw per day for DNA-reactive mutagens and/or carcinogens, the Panel concluded that also the whole Prosmoke BW 01 mixture raises a concern with respect to genotoxicity.

The whole Prosmoke BW 01 mixture induced gene mutations in bacteria both in the presence and in the absence of metabolic activation, but not in mammalian cells in vitro.

Prosmoke BW 01 did not induce structural chromosomal aberrations in V79 cells.

In the *in vitro* micronucleus tests in the mouse lymphoma L5178Y tk +/− cell line, increases in micronucleated cell frequency were observed, which were further investigated using FISH analysis. However, due to limitations in the study, it was not possible to determine if this increase was due to clastogenicity or aneugenicity.

The concern raised by the increase in micronucleated cell frequency observed in vitro was not ruled out by the negative results from the in vivo MN test because there was no evidence of bone marrow exposure, and therefore, the results of this test are inconclusive. Even in case of toxicity indicating target tissue exposure, it would not be possible to attribute this to individual components, since these are not completely identified, and thus, their kinetic behaviour cannot be investigated.

In the in vivo comet assay in rats, the whole Prosmoke BW 01 mixture did not induce DNA single-strand breaks in the stomach. For liver, the test was inconclusive, due to shortcomings in the study. Therefore, this study could not rule out the concern for genotoxicity of the whole mixture, that emerged from the effects observed in vitro. Due to the presence of furan-2(5H)-one, a concern for genotoxicity was identified for Prosmoke BW 01. In addition, the data on the genotoxicity studies performed on the whole mixture were inconclusive and could not rule out a potential genotoxicity of the fraction of unidentified constituents.

The Panel considered the available subchronic oral toxicity study conducted with the primary product. Since this subchronic toxicity study was reported incompletely and, most importantly, taking the outcome of the genotoxicity assessment into account, the Panel considered that the available information does not enable the identification of a reference point (e.g. BMDL, NOAEL).

Furthermore, since all toxicity studies were performed with a batch of Prosmoke BW 01 that may not be representative of the material of commerce, the results of these studies would not be useful for the safety assessment of Prosmoke BW 01.

5. Conclusions

Prosmoke BW 01 contains furan-2(5H)-one (formerly [FL-no: 10.066]), for which a concern for genotoxicity was identified *in vivo* upon oral administration. Considering that the exposure estimates for this component are above the TTC of 0.0025 μg/kg bw per day (or 0.15 μg/person per day) for DNA-reactive mutagens and/or carcinogens, the Panel concluded that Prosmoke BW 01 raises concern with respect to genotoxicity.

The available toxicity studies are not adequate to support the safety assessment of the whole mixture, due to limitations in the studies and due to the fact that they were performed with a batch which may not be representative for the material of commerce.

6. Documentation as provided to EFSA

1) Dossier "Complete technical dossier for a new smoke flavouring". June 2019. Submitted by M PROFOOD ZRT.

2) Additional information received on 03/11/2020, submitted by M Profood Zrt in response to a request from EFSA (04/12/2019).
3) Additional information received on 17/12/2021, submitted by M Profood Zrt in response to a request from EFSA (18/12/2020).
4) BioReliance, 2018. Furan-2(5H)-one: in vitro mammalian cell micronucleus assay in TK6 cells. BioReliance Laboratories Study Number AE84GL. 361.BTL. 14 March 2018. Unpublished final report submitted by EFFA.
5) Covance, 2013. Furan-2(5H)-one. Induction of micronuclei in cultured human peripheral blood lymphocytes. Covance Laboratories LTD. Study no. 8272052. 25 October 2013. Unpublished report submitted by EFFA.
6) TOXI-COOP ZRT, 2018a. Bacterial reverse mutation assay with Prosmoke BW 01. TOXI-COOP ZRT study number 916-471-3569. 1 March 2018. Unpublished report submitted by M PROFOOD ZRT.
7) TOXI-COOP ZRT, 2018b. In Vitro Mammalian Cell Gene Mutation Test: HPRT Assay with Prosmoke BW 01. TOXI-COOP ZRT study number 916-476-3571. 26 June 2018. Unpublished report submitted by M PROFOOD ZRT.
8) TOXI-COOP ZRT, 2018c. Prosmoke BW 01 In Vitro Mammalian Chromosome Aberration Test. TOXI-COOP ZRT study number 916-473-3570. 31 May 2018. Unpublished report submitted by M PROFOOD ZRT.
9) TOXI-COOP ZRT, 2019a. Prosmoke BW 01 In Vivo Mouse Micronucleus Test. TOXI-COOP ZRT study number 916-474-3572. 14 February 2019. Unpublished report submitted by M PROFOOD ZRT.
10) TOXI-COOP ZRT, 2019b. In Vivo Alkaline Comet Assay On The Rat Stomach And Liver With Prosmoke BW 01. TOXI-COOP ZRT study number 916-489-3573. 2 April 2019. Unpublished report submitted by M PROFOOD ZRT.
11) TOXI-COOP ZRT, 2019c. 90-Day Repeated Dose Oral Gavage Toxicity Study of Prosmoke BW 01 in Rats. TOXI-COOP ZRT study number 916-408-3574. 13 June 2019. Unpublished report submitted by M PROFOOD ZRT.
12) TOXI-COOP ZRT, 2020. Validation of the Analytical Method for the Determination of Prosmoke BW 01. TOXI-COOP ZRT study number 916-100-5607. 26 October 2020. Unpublished report submitted by M PROFOOD ZRT.
13) TOXI-COOP ZRT, 2020. Accelerated Storage Stability Test of Prosmoke BW 01. TOXI-COOP ZRT study number 916-160-5608r. 26 October 2020. Unpublished report submitted by M PROFOOD ZRT.
14) TOXI-COOP ZRT, 2021a. In Vitro Mammalian Cell Micronucleus Test with Prosmoke BW 01. TOXI-COOP ZRT study number 916-487-6183. 15 November 2021. Unpublished report submitted by M PROFOOD ZRT.
15) TOXI-COOP ZRT, 2021b. In Vivo Alkaline Comet Assay On The Rat Stomach And Liver With Prosmoke BW 01. TOXI-COOP ZRT study number 916-489-3573. 28 September 2021. Unpublished report submitted by M PROFOOD ZRT
16) Whitwell J, 2012. Induction of micronuclei in cultured human peripheral blood lymphocytes. Furan-2(5H)-one. Covance Laboratories Ltd, England. Study no.8233100. February 2012. Unpublished report submitted by EFFA to FLAVIS Secretariat.

References

EFSA (European Food Safety Authority), 2011. Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment. EFSA Journal 2011;9(3):2097, 34 pp. https://doi.org/10.2903/j.efsa.2011.2097

EFSA (European Food Safety Authority), 2015. The food classification and description system FoodEx2 (revision 2). EFSA Supporting Publication 2015;12(5):EN-804, 90 pp. https://doi.org/10.2903/sp.efsa.2015.EN-804

EFSA AFC Panel (EFSA Panel on Food additives, Flavourings, Processing Aids and Materials in contact with Food), 2006. Flavouring Group Evaluation 20 (FGE.20): Benzyl alcohols, benzaldehydes, a related acetal, benzoic acids, and related esters from chemical group 23. EFSA Journal 2006;296, 117 pp. https://doi.org/10.2903/j.efsa.2006.296

EFSA AFC Panel (EFSA Panel on Food additives, Flavourings, Processing Aids and Materials in contact with Food), 2007. Flavouring Group Evaluation 22 (FGE.22): Ring-substituted phenolic substances from chemical groups 21 and 25 (Commission Regulation (EC) No 1565/2000 of 18 July 2000). EFSA Journal 2007;393, 78 pp. https://doi.org/10.2903/j.efsa.2007.393

EFSA AFC Panel (EFSA Panel on Food additives, Flavourings, Processing Aids and Materials in contact with Food), 2008a. Flavouring Group Evaluation 58 (FGE.58): consideration of phenol derivatives evaluated by JECFA (55th meeting) structurally related to ring substituted phenolic substances evaluated by EFSA in FGE.22 2006 Commission Regulation (EC) No 1565/2000 of 18 July 2000. EFSA Journal 2008;711, 50 pp. https://doi.org/10.2903/j.efsa.2008.711

EFSA AFC Panel (EFSA Panel on Food additives, Flavourings, Processing Aids and Materials in contact with Food), 2008b. Flavouring Group Evaluation 52 (FGE.52): Consideration of hydroxy- and alkoxy-substituted benzyl derivatives evaluated by JECFA (57th meeting) structurally related to benzyl alcohols, benzaldehydes, a related acetal, benzoic acids, and related esters evaluated by EFSA in FGE.20 2005 Commission Regulation (EC) No 1565/2000 of 18 July 2000. EFSA Journal 2008;6(3):637, 69 pp. https://doi.org/10.2903/j.efsa.2008.637

EFSA AFC Panel (EFSA Panel on Food additives, Flavourings, Processing Aids and Materials in contact with Food), 2009. Flavouring Group Evaluation 60 (FGE.60): Consideration of eugenol and related hydroxyallylbenzene derivatives evaluated by JECFA (65th meeting) structurally related to ring- substituted phenolic substances evaluated by EFSA in FGE.22 (2006). EFSA Journal 2009;ON-965, 53 pp. https://doi.org/10.2903/j.efsa.2009.965

EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources added to Food), 2017. Re-evaluation of fatty acids (E 570) as a food additive. EFSA Journal 2017;15(5):4785, 55 pp. https://doi.org/10.2903/j.efsa.2017.4785

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes Flavourings and Processing Aids), 2005. Guidance on submission of a dossier on a Smoke Flavouring Primary Product for evaluation by EFSA. https://doi.org/10.2903/j.efsa.2005.492

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes Flavourings and Processing Aids), 2009a. Dietary exposure assessment methods for smoke flavouring Primary Products. EFSA Journal 2009;RN-284, 30 pp. https://doi.org/10.2903/j.efsa.2009.248r

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes Flavourings and Processing Aids), 2009b. Flavouring Group Evaluation 213: alpha, beta-Unsaturated alicyclic ketones and precursors from chemical subgroup 2.7 of FGE.19. EFSA Journal 2009;ON-879, 27 pp. https://doi.org/10.2903/j.efsa.2009.879

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2011a. Scientific Opinion on Flavouring Group Evaluation 66, Revision 1 (FGE.66Rev1): Consideration of Furfuryl Alcohol and Related Flavouring Substances Evaluated by JECFA (55th meeting). EFSA Journal 2011;9(9):2314, 34 pp. https://doi.org/10.2903/j.efsa.2011.2314

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2011b. Scientific Opinion on Flavouring Group Evaluation 3, Revision 2 (FGE.03Rev2): acetals of branched- and straight-chain aliphatic saturated primary alcohols and branched- and straight-chain saturated or unsaturated aldehydes, an ester of a hemiacetal and an orthoester of formic acid, from chemical groups 1, 2 and 4. EFSA Journal 2011;9(10):2312, 12 pp. https://doi.org/10.2903/j.efsa.2011.2312

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes Flavourings and Processing Aids), 2012. Scientific Opinion on Flavouring Group Evaluation 20, Revision 4 (FGE.20Rev4): Benzyl alcohols, benzaldehydes, a related acetal, benzoic acids, and related esters from chemical groups 23 and 30. EFSA Journal 2012;10(12):2994, 32 pp. https://doi.org/10.2903/j.efsa.2012.2994

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes Flavourings and Processing Aids), 2014. Scientific Opinion on Flavouring Group Evaluation 11, Revision 3 (FGE.11Rev3): Aliphatic dialcohols, diketones, and hydroxyketones from chemical groups 8 and 10. EFSA Journal 2014;12(11):3888, 45 pp. https://doi.org/10.2903/j.efsa.2014.3888

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2015a. Scientific Opinion on Flavouring Group Evaluation 213, Revision 2 (FGE.213Rev2): Consideration of genotoxic potential for α,β-unsaturated alicyclic ketones and precursors from chemical subgroup 2.7 of FGE.19. EFSA Journal 2015;13(9):4244, 12 pp. https://doi.org/10.2903/j.efsa.2015.4244

EFSA CEF Panel (EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids), 2015b. Scientific Opinion on Flavouring Group Evaluation 212 Revision 3 (FGE.212Rev3): α,β-unsaturated alicyclic ketones and precursors from chemical subgroup 2.6 of FGE.19. EFSA Journal 2015;13(5):4116, 12 pp. https://doi.org/10.2903/j.efsa.2015.4116

EFSA CONTAM Panel (Panel on Contaminants in the Food Chain), 2009a. Scientific opinion on Arsenic in food. EFSA Journal, 2009;7(3):980, 9 pp. https://doi.org/10.2903/j.efsa.2009.980

EFSA CONTAM Panel (Panel on Contaminants in the Food Chain), 2009b. Scientific opinion on Cadmium in food. EFSA Journal 2009;7(10):1351, 45 pp. https://doi.org/10.2903/j.efsa.2009.1351

EFSA CONTAM Panel (Panel on Contaminants in the Food Chain), 2010. Scientific opinion on Lead in food. EFSA Journal 2010;8(4):1570, 12 pp. https://doi.org/10.2903/j.efsa.2010.1570

EFSA CONTAM Panel (Panel on Contaminants in the Food Chain), 2012. Scientific Opinion on the risk for public health related to the presence of mercury and methylmercury in food. EFSA Journal 2012;10(12):2985, 22 pp. https://doi.org/10.2903/j.efsa.2012.2985

EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings), 2019. Scientific Opinion on Flavouring Group Evaluation 217 Revision 2 (FGE.217Rev2), consideration of genotoxic potential for a,b-unsaturated ketones and

precursors from chemical subgroup 4.1 of FGE.19: lactones. EFSA Journal 2019;17(1):5568, 75 pp. https://doi.org/10.2903/j.efsa.2019.5568
EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings), 2021a. Scientific Guidance for the preparation of applications on smoke flavouring primary products. EFSA Journal 2021;19(3):6435, 45 pp. https://doi.org/10.2903/j.efsa.2021.6435
EFSA FAF Panel (EFSA Panel on Food Additives and Flavourings), 2021b. Scientific Opinion on Flavouring Group Evaluation 67, Revision 3 (FGE.67Rev3): consideration of 23 furan-substituted compounds evaluated by JECFA at the 55th, 65th, 69th and 86th meetings. EFSA Journal 2021;19(2):6362, 45 pp. https://doi.org/10.2903/j.efsa.2021.6362
EFSA Scientific Committee, 2011. Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment. EFSA Journal 2011;9(9):2379, 32 pp. https://doi.org/10.2903/j.efsa.2011.2379
EFSA Scientific Committee, 2012. Statement on the applicability of the Margin of Exposure approach for the safety assessment of impurities which are both genotoxic and carcinogenic in substances added to food/feed. EFSA Journal 2012;10(3):2578, 56 pp. https://doi.org/10.2903/j.efsa.2012.2578
EFSA Scientific Committee, 2017. Clarification of some aspects related to genotoxicity assessment. EFSA Journal 2017;15(12):5113, 32 pp. https://doi.org/10.2903/j.efsa.2017.5113
EFSA Scientific Committee, 2019. Genotoxicity assessment of chemical mixtures. EFSA Journal 2019;17(1):5519, 12 pp. https://doi.org/10.2903/j.efsa.2019.5519
EFSA Scientific Committee, 2021. Guidance on aneugenicity assessment. EFSA Journal 2021;19(8):6770, 45 pp. https://doi.org/10.2903/j.efsa.2021.6770
Fenech M, 2000. The in vitro micronucleus technique. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 455, 81–95. https://doi.org/10.1016/s0027-5107(00)00065-8
Gooderham NJ, Samuel MC, Eisenbrand G, Fukushima S, Guengerich FP, Hecht SS, Rietjens IMCM, Rosol TJ, Bastaki M, Linman MJ and Taylor SV, 2020. The safety evaluation of food flavoring substances: the role of genotoxicity studies. Critical Reviews in Toxicology, 50, 1–27. https://doi.org/10.1080/10408444.2020.1712589
JECFA (Joint FAO, WHO Expert Committee on Food Additives), 2011. Evaluation of certain food additives and contaminants. Seventy-third Meeting of the Joint FAO/WHO Expert Committee on Food Additives. WHO food additives series, no. 64, Geneva.
JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1997. Evaluation of certain food additives and contaminants. Forty-sixth Meeting of the Joint FAO/WHO Expert Committee on Food Additives. Geneva, 6–15 February 1996. WHO Technical Report Series, no. 868. Geneva.
JECFA (Joint FAO/WHO Expert Committee on Food Additives), 1999. Evaluation of certain food additives and contaminants. Forty-ninth Meeting of the Joint FAO/WHO Expert Committee on Food Additives. Rome, 17–26 June 1997. WHO Technical Report Series, no. 884. Geneva.
OECD (Organisation for Economic Co-operation and Development), 1997. Test Guideline No. 471: Bacterial Reverse Mutation Test. OECD Guidelines for the Testing of Chemicals, Section 4. https://doi.org/10.1787/9789264071247-en
OECD (Organisation for Economic Co-operation and Development), 2016a. Test Guideline No. 476: *In vitro* Mammalian Cell Gene Mutation Tests using the Hprt and xprt genes. OECD Guidelines for the Testing of Chemicals, Section 4. https://doi.org/10.1787/9789264071322-en
OECD (Organisation for Economic Co-operation and Development), 2016b. Test Guideline No. 473: *In vitro* Mammalian Chromosome Aberration Test. OECD Guidelines for the Testing of Chemicals, Section 4. https://doi.org/10.1787/9789264264649-en
OECD (Organisation for Economic Co-operation and Development), 2016c. Test Guideline no. 487: *In vitro* Mammalian Cell Micronucleus Test. OECD Guidelines for the Testing of Chemicals, Section 4. https://doi.org/10.1787/9789264264861-en
OECD (Organisation for Economic Co-operation and Development), 2016d. Test Guideline No. 474: Mammalian Erythrocyte Micronucleus Test. OECD Guidelines for the Testing of Chemicals, Section 4. https://doi.org/10.1787/9789264264762-en
OECD (Organisation for Economic Co-operation and Development), 2016e. Test Guideline No. 489: *In vivo* Mammalian Alkaline Comet Assay. OECD Guidelines for the Testing of Chemicals, Section 4. https://doi.org/10.1787/9789264264885-en
OECD (Organisation for Economic Co-operation and Development), 2018. Test Guideline No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents. OECD Guidelines for the Testing of Chemicals, Section 4. https://doi.org/10.1787/9789264304741-en

Abbreviations

AFC	Panel on Food additives, Flavourings, Processing Aids and Materials in contact with Food
ANS	Panel on Food Additives and Nutrient Sources added to Food
AST	Aspartate Aminotransferase
BW	body weight
CA	Chromosomal Aberration

CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CL	Colchicine
CP	Cyclophosphamide
ECHA	European Chemicals Agency
EPIC	European Prospective Investigation into Cancer and Nutrition
DMSO	dimethyl sulfoxide
EMS	Ethyl Methanesulfonate
FAF	Panel on Food Additives and Flavourings
FAIM	Food Additive Intake Model
FEMA	Flavor and Extract Manufacturers Association
FLAVIS	Flavour Information System database
FID	flame ionisation detector
FISH	Fluorescence in situ hybridisation
FSSC	Food Safety System Certification
GC	Gas Chromatography
GLP	good laboratory practices
HBGV	Health based guidance value
HPRT	hypoxanthine phosphorybosyltransferase
HS-GC	Headspace Gas Chromatography
IRFMN	Istituto Di Ricerche Farmacologiche Mario Negri
ISS	Istituto Superiore di Sanità
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LDL	Low Density Lipoprotein
MMC	Mitomycin C
MMS	Methyl methanesulfonate
MN	Micronucleus
MNPCE	Micronucleated Polychromatic Erythrocytes
MOE	margin of exposure
MOS	margin of safety
MS	Mass Spectrometry
NCE	Normochromatic Erythrocytes
OECD	Organisation for Economic Co-operation and Development
OASIS-LMC	OASIS-Laboratory of Mathematical Chemistry
OTM	Olive Tail Moment
PAHs	polycyclic aromatic hydrocarbons
PCE	Polychromatic Erythrocytes
PEG	Polyethylene Glycol
(Q)SAR	quantitative structure–activity relationship
SAR	structure–activity relationship
SMILES	simplified molecular input - line entry system
SMK-EPIC	Smoke Flavouring - Epic Model
SMK-TAMDI	Smoke Flavouring - Theoretical Added Maximum Daily Intake
TG	Test Guideline
TI	Tail Intensity
TIC	Total Ion Current
TSH	Thyroid Stimulating Hormone
TTC	Threshold of Toxicological Concern
TWI	Tolerable Weekly Intake
WT	Weight

Appendix A – Genotoxicity assessment of the identified components of Prosmoke BW 01

Table A.1: Compilation of the 48 fully identified volatile constituents in the primary product (Documentation provided to EFSA No. 2)

CAS number	Chemical name	Average[(a)] wt%
498-02-2	3-Methoxy-4-hydroxyphenylethanone	2.67
91-10-1	2,6-Dimethoxyphenol	1.36
134-96-3	4-Hydroxy-3,5-dimethoxybenzaldehyde	1.22
498-07-7	Levoglucosan (1,6-Anhydro-β-D-glucose)	1.01
497-23-4	2(5H)-Furanone	0.88
80-71-7	3-Methyl-1,2-cyclopentanedione	0.70
696-59-3	2,5-Dimethoxytetrahydrofuran[(b)] (II)	0.52
150-76-5	4-Methoxyphenol	0.51
1575-57-1	1-Acetoxy-2-butanone	0.51
696-59-3	2,5-Dimethoxytetrahydrofuran[(b)] (I)	0.42
121-33-5	Vanillin	0.40
90-05-1	2-Methoxyphenol	0.38
2478-38-8	4-Acetyl-2,6-dimethoxyphenol	0.34
2503-46-0	4-Hydroxy-3-methoxyphenyl-2-propanone	0.34
118-71-8	Maltol	0.31
2380-78-1	Homovanillyl alcohol	0.30
6627-88-9	2,6-Dimethoxy-4-(2-propenyl)phenol	0.25
2785-89-9	4-Ethyl-2-methoxyphenol	0.24
5932-68-3	(*E*)-2-Methoxy-4-(1-propenyl)phenol	0.20
93-51-6	2-Methoxy-4-methylphenol	0.20
934-00-9	2-Hydroxy-3-methoxyphenol	0.20
608-25-3	2-Methyl-1,3-benzenediol	0.16
98-00-0	2-Furanylmethanol	0.14
21835-01-8	3-Ethyl-2-hydroxy-2-cyclopenten-1-one	0.13
452-86-8	2-Hydroxy-4-methylphenol	0.12
97-53-0	2-Methoxy-4-(2-propenyl)phenol (eugenol)	0.11
513-85-9	(R,R)-Butane-2,3-diol	0.09
488-17-5	2-Hydroxy-3-methylphenol	0.08
930-30-3	2-Cyclopenten-1-one	0.08
1192-62-7	2-Acetylfuran	0.06
57-11-4	Octadecanoic acid (stearic acid)	0.06
624-45-3	Methyl levulinate	0.05
2758-18-1	3-Methyl-2-cyclopenten-1-one	0.05
7786-61-0	2-Methoxy-4-vinylphenol	0.04
2785-87-7	2-Methoxy-4-propyl-phenol	0.03
110-13-4	2,5-Hexanedione	0.03
1599-47-9	1,1-Dimethoxyhexane	0.03
3943-74-6	4-Hydroxy-3-methoxybenzoic acid methyl ester	0.024
1120-73-6	2-Methyl-2-cyclopenten-1-one	0.022
57-10-3	*n*-Hexadecanoic acid	0.021
611-13-2	5-(Methoxymethyl)-furoic acid	0.012
111-55-7	1,2-Diacetoxyethane	0.008
109-21-7	Butyl butanoate	0.008
930-68-7	2-Cyclohexen-1-one	0.006

CAS number	Chemical name	Average[(a)] wt%
930-60-9	4-Cyclopentene-1,3-dione	0.002
101-81-5	Diphenylmethane	0.00
105-54-4	Ethyl butanoate	0.00
547-64-8	Methyl 2-hydroxypropanoate	0.00
39151-19-4	3′,5′-Dimethoxyacetophenone	0.00

(a): From the analysis of the four batches presented in Table 1.
(b): Roman numbering according to the GC elution order of the diastereoisomers.

Table A.2: Conclusions on genotoxicity on the 48 identified components of Prosmoke BW 01

Molecule no.	CAS. no.	SMILES	FL-no	Compound name	Data from literature	QSAR analysis		EFSA's conclusions on genotoxicity based on available data[18]
						By the applicant	By EFSA	
1	498-07-7	C1C2C(C(C(C(O1)O2)O)O)O		Levoglucosan (1,6-Anhydro-β-D-glucose)	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis
2	91-10-1	COC1=C(C(=CC=C1)OC)O	04.036	2,6-Dimethoxyphenol	Limited experimental genotoxicity data on this substance and on structurally related substances did not indicate concern for genotoxicity in FGE.58 (EFSA AFC Panel, 2008a)	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement
3	134-96-3	COc1cc(C = O)cc(OC)c1O	05.153	4-Hydroxy-3,5-dimethoxybenzaldehyde	Limited experimental genotoxicity data on this substance and on structurally related substances did not indicate concern for genotoxicity in FGE.20 (EFSA AFC Panel, 2006)	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement
4	497-23-4	O=C1OCC=C1	10.066	2(5H)-Furanone	Experimental *in vivo* genotoxicity data on this substance indicated a concern for genotoxicity in FGE.217Rev2 (EFSA FAF Panel, 2019)	Predicted as positive	Predicted as negative	Concern for genotoxicity based on experimental data on this substance. See Section 3.3.1.1 of this opinion
5	80-71-7	CC1CCC(=O)C1=O		3-Methyl-1,2-cyclopentanedione	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis

[18] It should be noted that the following expression used in this table (under column 'Data from literature'): *'limited experimental genotoxicity data on the substance and on structurally related substances did not indicate concern for genotoxicity'* should not be interpreted as being equivalent to 'no concern with respect to genotoxicity'. The approach taken here is therefore pragmatic rather than based on robust experimental data.

Molecule no.	CAS. no.	SMILES	FL-no	Compound name	Data from literature	QSAR analysis		EFSA's conclusions on genotoxicity based on available data[18]
						By the applicant	By EFSA	
6	696-59-3	COC1CCC (O1)OC		2,5-Dimethoxytetrahydrofuran (peak 1 + 2)	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis
7	90-05-1	COC1=CC= CC=C1O	04.005	4-Methoxyphenol	Limited experimental genotoxicity data on this substance and on structurally related substances did not indicate concern for genotoxicity in FGE.58 (EFSA AFC Panel, 2008a)	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement
8	1575-57-1	CCC(=O)COC (C)=O		1-Acetoxy-2-butanone	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis
9	121-33-5	COC1=C (C=CC(=C1) C = O)O	05.018	Vanillin	Limited experimental genotoxicity data on this substance and on structurally related substances did not indicate concern for genotoxicity in FGE.52 (EFSA AFC Panel, 2008b)	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement
10	2478-38-8	CC(=O)C1=CC (=C(C(=C1) OC)O)OC	07.164	4-Acetyl-2,6-dimethoxyphenol	Limited experimental genotoxicity data on this substance and on structurally related substances did not indicate concern for genotoxicity in FGE.22 (EFSA AFC Panel, 2007)	Predicted as positive	Predicted as positive	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement

Molecule no.	CAS. no.	SMILES	FL-no	Compound name	Data from literature	QSAR analysis		EFSA's conclusions on genotoxicity based on available data[18]
						By the applicant	By EFSA	
11	2503-46-0	CC(=O) CC1=CC(=C (C=C1)O)OC		4-Hydroxy-3-methoxyphenyl-2-propanone	No relevant information on genotoxicity available on this substance. Limited experimental data on structurally related substances, i.e. 2-methoxyphenol [FL-no: 04.005] and eugenol [FL-no: 04.003], evaluated by EFSA as chemically defined flavouring substances in FGE.58 (EFSA AFC Panel, 2008a) and FGE.60 (EFSA AFC Panel, 2009) respectively, did not indicate concern for genotoxicity.	Predicted as positive	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement.
12	2380-78-1	COc1cc(CCO) ccc1O		Homovanillyl alcohol	No relevant information on genotoxicity available on this substance. Limited experimental data on structurally related substances, i.e. 2-methoxyphenol [FL-no: 04.005] and eugenol [FL-no: 04.003], evaluated by EFSA as chemically defined flavouring substances in FGE.58 (EFSA AFC Panel, 2008a) and FGE.60 (EFSA AFC Panel, 2009) respectively, did not indicate concern for genotoxicity.	Predicted as positive	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement.

Molecule no.	CAS. no.	SMILES	FL-no	Compound name	Data from literature	QSAR analysis		EFSA's conclusions on genotoxicity based on available data[18]
						By the applicant	By EFSA	
13	93-51-6	CC1=CC(=C(C=C1)O)OC	04.007	2-Methoxy-4-methylphenol	Limited experimental genotoxicity data on this substance and on structurally related substances did not indicate concern for genotoxicity in FGE.58 (EFSA AFC Panel, 2008a)	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement
14	118-71-8	CC1=C(O)C(=O)C=CO1	07.014	Maltol	Experimental *in vivo* genotoxicity data on this substance did not indicate concern for genotoxicity in FGE.213Rev2 (EFSA CEF Panel, 2015a)	Predicted as positive	Predicted as negative	No concern for genotoxicity based on available experimental data
15	6627-88-9	COc1cc(CC=C)cc(OC)c1O	04.051	2,6-Dimethoxy-4-(2-propenyl)phenol	No relevant information on genotoxicity available on this substance. Limited experimental genotoxicity data on structurally related substances within the same FGE did not indicate concern for genotoxicity in FGE.58 (EFSA AFC Panel, 2008a)	Predicted as positive	Predicted as positive	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement
16	498-02-2	CC(=O)C1=CC(=C(C=C1)O)OC	07.142	3-Methoxy-4-hydroxyphenylethanone	Limited experimental genotoxicity data on this substance and on structurally related substances did not indicate concern for genotoxicity in FGE.22 (EFSA AFC Panel, 2007)	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement

Molecule no.	CAS. no.	SMILES	FL-no	Compound name	Data from literature	QSAR analysis		EFSA's conclusions on genotoxicity based on available data[18]
						By the applicant	By EFSA	
17	2785-89-9	CCC1=CC(=C(C=C1)O)OC	04.008	4-Ethyl-2-methoxyphenol	Limited experimental genotoxicity data on this substance and on structurally related substances did not indicate concern for genotoxicity in FGE.58 (EFSA AFC Panel, 2008a)	Predicted as positive	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement
18	21835-01-8	CCC1=C(C(=O)CC1)O	07.057	3-ethyl-2-hydroxy-2-cyclopenten-1-one	Evaluated by EFSA as chemically defined flavouring substance in FGE.213 (EFSA CEF Panel, 2009b). No experimental genotoxicity data on this substance nor on structurally related substances in FGE.213.	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis
19	513-85-9	CC(C(C)O)O		(R,R)-Butane-2,3-diol	No relevant information on genotoxicity available on this substance. Limited experimental data on a structurally related substance, i.e. butan-2,3-diol [FL-no: 02.133], evaluated by EFSA as chemically defined flavouring substances in FGE.11Rev3, (EFSA CEF Panel, 2014) did not indicate concern for genotoxicity	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement

Molecule no.	CAS. no.	SMILES	FL-no	Compound name	Data from literature	QSAR analysis		EFSA's conclusions on genotoxicity based on available data[18]
						By the applicant	By EFSA	
20	97-53-0	COC1=C(C=CC(=C1)CC=C)O	04.003	2-Methoxy-4-(2-propenyl) phenol (eugenol)	Experimental genotoxicity data on this substance did not indicate concern for genotoxicity in FGE.60 (EFSA AFC Panel, 2009)	Predicted as positive	Predicted as negative	No indications for concern on genotoxicity based on available experimental data on this substance, based on expert judgement
21	488-17-5	Oc1cccc(c1(O))C		2-Hydroxy-3-methylphenol	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis
22	934-00-9	COC1=CC=CC(=C1O)O		2-Hydroxy-3-methoxyphenol	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis
23	57-11-4	CCCCCCCCCCCCCCCC(=O)O	08.015	Octadecanoic acid (stearic acid)	Limited experimental genotoxicity data on this substance did not indicate concern for genotoxicity (EFSA ANS Panel, 2017)	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on available experimental data on this substance, based on expert judgement
24	624-45-3	COC(=O)CCC(C)=O		Methyl levulinate	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis
25	930-30-3	C1CC(=O)C=C1		2-Cyclopenten-1-one	No relevant information on genotoxicity available on this substance. Experimental genotoxicity data on a structurally related substance, i.e. [FL-no: 07.112] 3-methyl-2-cyclopenten-1-one, evaluated by EFSA as chemically defined flavouring	Predicted as positive	Predicted as negative	No indications for concern on genotoxicity based on read-across from a structurally related substance, based on expert judgement.

Molecule no.	CAS. no.	SMILES	FL-no	Compound name	Data from literature	QSAR analysis		EFSA's conclusions on genotoxicity based on available data[18]
						By the applicant	By EFSA	
					substances in FGE212Rev3 (EFSA CEF Panel, 2015b), did not indicate concern for genotoxicity.			
26	452-86-8	CC1=CC(=C(C=C1)O)O		2-Hydroxy-4-methylphenol	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis.
27	1192-62-7	CC(=O)C1=CC=CO1	13.054	2-Acetylfuran	Experimental genotoxicity data on this substance ruled out the concern for genotoxicity in FGE.67Rev3 (EFSA FAF Panel, 2021)	Predicted as positive	Predicted as negative	No concern for genotoxicity based on available experimental data
28	2758-18-1	CC1=CC(=O)CC1	07.112	3-Methyl-2-cyclopenten-1-one	Experimental genotoxicity data on this substance ruled out the concern for genotoxicity in FGE.212Rev3 (EFSA CEF Panel, 2015b)	Predicted as positive	Predicted as negative	No concern for genotoxicity based on available experimental data
29	5932-68-3	CC=CC1=CC(=C(C=C1)OC(=O)C)OC		(E)-2-Methoxy-4-(1-propenyl)phenol	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis
30	110-13-4	CC(=O)CCC(=O)C		2,5-Hexanedione	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis
31	611-13-2	COC(=O)C1=CC=CO1	13.002	5-(Methoxymethyl)-furoic acid	Limited experimental genotoxicity data on this substance and on structurally related substances did not indicate concern for genotoxicity in FGE.66Rev1 (EFSA CEF Panel, 2011a)	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement

Molecule no.	CAS. no.	SMILES	FL-no	Compound name	Data from literature	QSAR analysis		EFSA's conclusions on genotoxicity based on available data[18]
						By the applicant	By EFSA	
32	3943-74-6	COC1=C(C=CC(=C1)C(=O)OC)O	09.799	4-Hydroxy-3-methoxybenzoic acid methyl ester	No relevant information on genotoxicity available on this substance. Limited experimental genotoxicity data on a structurally related substances within the same FGE did not indicate concern for genotoxicity in FGE.20Rev4 (EFSA CEF Panel 2012)	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement
33	57-10-3	CCCCCCCCCCCCCCCC(=O)O	08.014	*n*-Hexadecanoic acid	Experimental genotoxicity data available on ECHA website[19] on structurally related substances, i.e. docosanoic acid and other fatty acids, did not indicate concern for genotoxicity. This substance was evaluated by JECFA before 2000 as of no safety concern (JECFA 1999).	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances
34	2785-87-7	CCCC1=C(C=CC(=C1)C)O		2-Methoxy-4-propyl-phenol	No relevant information on genotoxicity	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis
35	7786-61-0	COC1=C(C=CC(=C1)C = C)O	04.009	2-Methoxy-4-vinylphenol	Limited experimental genotoxicity data on this substance and on structurally related substances did not indicate	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related

[19] https://echa.europa.eu/it/registration-dossier/-/registered-dossier/15218/7/7/2

Molecule no.	CAS. no.	SMILES	FL-no	Compound name	Data from literature	QSAR analysis		EFSA's conclusions on genotoxicity based on available data[18]
						By the applicant	By EFSA	
					concern for genotoxicity in FGE.58 (EFSA AFC Panel, 2008a)			substances, based on expert judgement
36	1120-73-6	CC1=CCCC1=O		2-Methyl-2-cyclopenten-1-one	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis
37	98-00-0	C1=COC(=C1)CO	13.019	2-Furanylmethanol	Limited experimental genotoxicity data on this substance and on structurally related substances did not indicate concern for genotoxicity in FGE.66Rev1 (EFSA CEF Panel, 2011a)	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement
38	111-55-7	CC(=O)OCCOC(=O)C		1,2-Diacetoxyethane	Experimental genotoxicity data available on ECHA website on this substance did not indicate concern for genotoxicity.[20]	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on available experimental data
39	1599-47-9	CCCCCC(OC)OC	06.073	1,1-Dimethoxyhexane	No relevant information on genotoxicity available on this substance. Limited experimental genotoxicity data on structurally related substances within the same FGE did not indicate concern for genotoxicity in FGE.03Rev2 (EFSA CEF Panel, 2011b)	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement

[20] https://echa.europa.eu/hu/registration-dossier/-/registered-dossier/14437/7/7/1

Molecule no.	CAS. no.	SMILES	FL-no	Compound name	Data from literature	QSAR analysis		EFSA's conclusions on genotoxicity based on available data[18]
						By the applicant	By EFSA	
40	930-68-7	C1CC=CC(=O)C1		2-Cyclohexen-1-one	Limited experimental genotoxicity data with inconsistent results on this substance (JECFA 2011). Experimental genotoxicity data on a structurally related substance, i.e. [FL-no: 07.112] 3-methyl-2-cyclopenten-1-one, evaluated by EFSA as chemically defined flavouring substances in FGE212Rev3 (EFSA CEF Panel, 2015b), did not indicate concern for genotoxicity.	Predicted as positive	Predicted as negative	No indications for concern on genotoxicity based on read-across from a structurally related substance, based on expert judgement
41	109-21-7	CCCCOC(=O)CCC	09.042	Butyl butanoate	Experimental genotoxicity data available on ECHA website on this substance did not indicate concern for mutagenicity.[21] This substance was evaluated by JECFA before 2000 as of no safety concern. Limited experimental genotoxicity data on structurally related substances did not indicate concern for genotoxicity (JECFA 1999).	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on available experimental data on this substance and on read-across from structurally related substances

[21] https://echa.europa.eu/it/registration-dossier/-/registered-dossier/20635/7/7/2

Molecule no.	CAS. no.	SMILES	FL-no	Compound name	Data from literature	QSAR analysis		EFSA's conclusions on genotoxicity based on available data[18]
						By the applicant	By EFSA	
42	105-54-4	CCCC(=O)OCC	09.039	Ethyl butanoate	Experimental genotoxicity data available on ECHA website on this substance did not indicate concern for genotoxicity.[22] This substance was evaluated by JECFA before 2000 as of no safety concern (JECFA 1997).	Predicted as negative	Predicted as negative	No indications for concern for genotoxicity based on available experimental data
43	930-60-9	C1C(=O)C=CC1=O		2-Cyclohexen-1-one/ 4-Cyclopentene-1,3-dione	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis.
44	101-81-5	C1=CC=C(C=C1)CC2=CC=CC=C2	01.036	Diphenylmethane	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concerns on genotoxicity based on (Q)SAR analysis
45	608-25-3	CC1=C(C=CC=C1O)O		2-Methyl-1,3-benzenediol	No relevant information on genotoxicity available on this substance. Limited experimental genotoxicity data on a structurally related substance, i.e. [FL-no: 04.047] benzene-1,3-diol, evaluated by EFSA as chemically defined flavouring substances in FGE.58, (EFSA AFC Panel, 2008a) did not indicate concern for genotoxicity.	Predicted as positive	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement

[22] https://echa.europa.eu/hu/registration-dossier/-/registered-dossier/17594/7/7/1

Molecule no.	CAS. no.	SMILES	FL-no	Compound name	Data from literature	QSAR analysis		EFSA's conclusions on genotoxicity based on available data[18]
						By the applicant	By EFSA	
46	39151-19-4	CC(=O)C1=CC(=CC(=C1)OC)OC		3',5'-Dimethoxyacetophenone	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on (Q)SAR analysis
47	150-76-5	COC1=CC=C(C=C1)O	04.077	4-Methoxyphenol	Limited experimental genotoxicity data on this substance and on structurally related substances did not indicate concern for genotoxicity in FGE.22 (EFSA AFC Panel, 2007)	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on read-across from structurally related substances, based on expert judgement
48	547-64-8	CC(C(=O)OC)O		Methyl 2-hydroxypropanoate	No relevant information on genotoxicity available	Predicted as negative	Predicted as negative	No indications for concern on genotoxicity based on (Q)SAR analysis

Appendix B – Publication on the evaluation of genotoxicity studies on furan-2(5H)-one (formerly [FL-no: 10.066]) (Gooderham et al., 2020)

As mentioned in Section 3.3.2.1, furan-2(5H)-one (formerly [FL-no: 10.066]) has been identified among the chemical constituents of Prosmoke BW 01 in all four analysed batches. This substance was evaluated by the FAF Panel as genotoxic *in vivo* (EFSA FAF Panel, 2019).

The applicant did not provide any new experimental data on (formerly [FL-no: 10.066]), but submitted a review by the Flavor and Extract Manufacturers Association of the United States (FEMA) Expert Panel (Gooderham et al., 2020). This review merely commented on the assessment of the genotoxicity data on furan-2(5H)-one (formerly [FL-no: 10.066]) made by the FAF Panel in FGE.217Rev2 (EFSA FAF Panel, 2019). The applicant quoted the FEMA Expert Panel's conclusion '*the criteria for a clear positive outcome were not met, and when considered in combination with the negative bacterial reverse mutation outcome, the negative in vivo micronucleus results, and the inconsistent results in the in vitro micronucleus studies, concluded that based upon weight of evidence that furan-2(5H)-one did not display genotoxic potential*'.

Regarding the evaluation of *in vitro* MN studies, the FEMA Expert Panel noted:

- '*in vitro micronucleus studies using TK6 cells are generally considered to be appropriately sensitive, if not more sensitive, than those conducted in human peripheral blood lymphocytes'.*

In response to the FEMA Expert Panel comments, the FAF Panel reiterated what was already explained in FGE.217Rev2: '*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).*[23] *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(5H)-one [FL-no: 10.066] clearly increased the frequency of MN in the presence of metabolic activation' (EFSA FAF Panel, 2019).*

The FEMA Expert Panel further noted:

- '*the two in vitro micronucleus studies conducted in human peripheral blood lymphocytes displayed exceedingly steep cytotoxicity curves and shifts from trial to trial in the cytotoxicity measured at the same or very similar concentrations. The cytotoxicity curves for the in vitro micronucleus assay in TK6 cells were less steep and it was correspondingly easier, it appears, to choose concentrations for scoring of micronuclei. There is not a clear explanation as to why there were differing outcomes within these studies'.*

In response to this, the FAF Panel emphasised that a steep increase in cytotoxicity was not observed in the MN study in TK6 cells (BioReliance, 2018) nor in the MN studies in human peripheral blood lymphocytes (Whitwell, 2012; Covance, 2013). The highest concentrations tested in the MN assays reached a level of cytotoxicity in agreement with OECD TG 487. Results from cytotoxicity tests and from MN analysis are consistent between the 2 studies in human peripheral blood lymphocytes (Whitwell, 2012; Covance, 2013). The inconsistency with the negative results observed in TK6 cells may be due to the different application of cytochalasin B as described above and in FGE.217Rev2 (EFSA FAF Panel, 2019). Scoring of MN in mononucleated cells could generate false negative results (Fenech, 2000).

In detail, the same range of concentrations (from 3 to 840 μg/mL) was tested in the cytotoxicity range finder experiments performed in the 2 *in vitro* MN studies in human peripheral blood lymphocytes (Whitwell, 2012; Covance, 2013).

At the concentrations of 109, 181, 302, 504 μg/mL, tested in the 3 h +21 h treatment in the presence of S9-mix, cytotoxicity was, respectively, 3, 9, 22, 57%, in the study by Whitwell (2012) and 5, 11, 26, 61% in the study by Covance (2013).

Based on the cytotoxicity range finder experiment, the concentrations tested in the *in vitro* MN test by Whitwell (2012), at 3h +21h in the presence of S9-mix, were 100, 250, 425, 450, 475 μg/mL resulting in a cytotoxicity of 16, 28, 40, 50, 51%, respectively. Statistically significant increase in micronucleated cell frequency was observed at 425 μg/mL and higher concentrations.

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

The concentrations tested in the *in vitro* MN test by Covance (2013), at 3 h +21 h in the presence of S9-mix, were 100, 300, 350, 400 μg/mL resulting in a cytotoxicity of 7, 35, 43, 53%, respectively. Statistically significant increase in micronucleated cell frequency was observed at 300 μg/mL and higher concentrations.

In the *in vitro* micronucleus assay in the human lymphoblastoid cell line TK6 cells, concentrations between 0.0841 and 840 μg/mL were tested in the cytotoxicity range finder experiments. In the 4 h + 23 h treatment, in the presence of metabolic activation, cytotoxicity observed at 8, 25, 84, 252, 840 μg/mL were 8, 12, 24, 79 and 97%, respectively. Based on these results the following concentrations were chosen for MN analysis: 25, 75, 150 μg/mL with a cytotoxicity of 11, 23 and 58%, respectively.

Regarding the evaluation of the *in vivo* comet assay, the FEMA Expert Panel noted:

- *'At the top dose of 250 mg/kg bw per day, small, less than two-fold increases in % tail DNA and tail moment were observed in the liver. The Panel notes that the increase in % tail DNA and tail moment at the top dose were within both the historical control range and the 95% reference range of the historical controls. Additionally, overlap between tail DNA values were reported for concurrent vehicle control animals and those in the top dose group'.*

The FAF Panel reiterated that on the basis of OECD TG 489, the evaluation should be based on a comparison between treatment-induced values and the concurrent vehicle control, the consideration of a potential dose-response relationship and on a comparison of treatment-induced values with (appropriate) historical negative control data, not primarily on a comparison between vehicle/positive control experimental data and historical controls.

As explained in FGE.217Rev2 (EFSA FAF Panel, 2019), the Panel reiterated that the following two criteria for evaluation and interpretation of results as positive (OECD TG 489) were fulfilled:

- First criterion: At least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control;
- Second criterion: 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 the very wide range for historical negative controls reported was overlapping with the range of historical positive control (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).

Regarding the overlap between tail DNA values for concurrent vehicle control animals and those in the top dose group, it is noted that the median tail intensity in one animal of the concurrent control group was 4.23% and in the other 5 animals the median was between 1.5% and 2.59%.

The lowest median tail intensity values reported in two animals of the highest dose group were 3.93% and 4.04%. For the other 4 animals in the highest dose group the median was between 4.34% and 5.27%.

The FAF Panel noted the statistically significant increase in % tail intensity at the highest dose ($p < 0.001$) and agreed with the authors of the study report that '*The response in the comet assay was consistent across all animals within the 250 mg/kg bw per day group'*.

The FEMA Expert Panel considered that

- *the results from the in vivo MN assay in rats bone marrow were negative.*

However, as explained in FGE.217Rev2, the FAF Panel evaluated the study as inconclusive, because there was no evidence that bone marrow was exposed.

Based on the considerations above, the FAF Panel does not agree with the FEMA Expert Panel conclusion that *furan-2(5H)-one did not display genotoxic potential*.

Appendix C – Toxicological studies for Prosmoke BW 01

Table C.1: Summary of the *in vitro* genotoxicity data for Prosmoke BW 01

Name	Test system	Test object	Concentrations of substance and test conditions	Result	Reference	Comments
Prosmoke BW 01	Bacterial Reverse Mutation test	*S.* Typhimurium TA98, TA1535,TA1537 and *Escherichia coli* WP2 uvrA	16, 50, 160, 500, 1,600 and 5,000 µg/plate[(a),(f),(g)]	Positive	TOXI-COOP ZRT (2018a)	Reliable without restrictions. Study performed in compliance with GLP and OECD TG 471.
		S. Typhimurium TA100	16, 50, 160, 500, 1,600, 2,500, 4,000, 4,500 and 5,000 µg/plate[(a),(f),(g)]			
	Mammalian Cell Gene Mutation test (HPRT)	CHO-K1 cells	50, 100, 150, 200, and 250 µg/mL[(b)]	Negative	TOXI-COOP ZRT (2018b)	Reliable without restrictions. Study performed in compliance with GLP and OECD TG 476.
			150, 200, 250, 300 and 350 µg/mL[(c)]			
	Chromosomal aberration test	Chinese Hamster lung V79 cells	39.1, 78.2 and 156.3 µg/mL[(b),(h)]	Negative	TOXI-COOP ZRT (2018c)	Reliable without restrictions. Study performed in compliance with GLP and OECD TG 473.
			39.1, 78.2 and 156.3 µg/mL[(b),(i)]			
			78.2, 156.3 and 312.5 µg/mL[(c),(h)]			
			39.1, 78.2 and 156.3 µg/mL[(b),(j)]			
			78.2, 156.3 and 312.5 µg/mL[(c),(k)]			
	Micronucleus test	Mouse lymphoma L5178Y tk +/− cells	12.5, 25, 50, 100, 200 µg/mL[(b),(d)]	Positive	TOXI-COOP ZRT (2021)	Reliable with restrictions. Study performed in compliance with GLP and mainly in line with OECD TG 487. MN scoring through flow cytometry.
			50, 100, 150, 200, 250 µg/mL[(c),(d)]			
			12.5, 25, 50, 75, 100 µg/mL[(b),(e)]			
	Micronucleus test with FISH analysis		50, 100, 200 µg/mL[(b),(d)]	Inconclusive		Reliable with restrictions. Study performed in compliance with GLP and mainly in line with OECD TG 487. Repeated experiment with FISH analysis.
			150, 200, 250 µg/mL[(c),(d)]			
			25, 50, 100 µg/mL[(b),(e)]			

(a): With and without S9 metabolic activation.
(b): Without S9 metabolic activation.
(c): With S9 metabolic activation.
(d): 4-h incubation, sampling at 24 h.
(e): 24-h incubation with no recovery period.
(f): Plate incorporation method.
(g): Pre-incubation method.
(h): 3-h treatment, sampling at 20 h.
(i): 20-h treatment with no recovery period.
(j): 20-h treatment, sampling at 28 h.
(k): 3-h treatment, sampling at 28 h.

Table C.2: Summary of *in vivo* genotoxicity data for Prosmoke BW 01

Name	Test system	Species; Sex	Route of administration	Dose levels (mg/kg bw per day)	Results	Reference	Comments
Prosmoke BW 01	Micronucleus assay (bone marrow)	NMRI Mice; M	Gavage	500, 1,000 or 2,000[(a)]	Inconclusive	TOXI-COOP ZRT (2019a)	Reliable with restrictions. Study performed in compliance with GLP and mainly in compliance with OECD TG 474. No clear evidence of bone marrow exposure.
	Comet assay (liver, stomach)	Han Wistar Rat; M	Gavage	500, 1,000 or 2,000[(a)]	Negative (stomach) Inconclusive (liver)	TOXI-COOP ZRT (2019b)	Reliable with restrictions. Study performed in compliance with GLP and mainly in compliance with OECD TG 489.

M: males.
(a): Administered via gavage twice at 24-h intervals.

Table C.3: Summary of toxicity study for Prosmoke BW 01

Name	Species; Sex No./Group	Route of administration	Dose levels (mg/kg bw per day)	Duration (days)	Results	Reference	Comments
Prosmoke BW 01	Han Wistar rats; M and F 10/group	Gavage	500, 750, 1,000	90	No reference point derived	TOXI-COOP ZRT (2019c)	Study GLP compliant. Study performed in compliance with OECD TG 408, but limited reporting of histopathology and gross necropsy.

F: females; M: males.

Appendix D – EFSA Comprehensive Database

EFSA Comprehensive European Food Consumption Database

Since 2010, the EFSA Comprehensive European Food Consumption Database (Comprehensive Database) has been populated with national data on food consumption at a detailed level. Competent authorities in European countries provide EFSA with data on the level of food consumption by the individual consumer from the most recent national dietary survey in their country (cf. Guidance of EFSA on the 'Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment' (EFSA, 2011). The version of the Comprehensive database taken into account in the exposure assessment was published in July 2021.[24] Data from EU Member States were considered for the estimations.

The food consumption data gathered by EFSA were collected by different methodologies and thus direct country-to-country comparisons may not be appropriate. Depending on the food category and the level of detail used for the exposure calculations, uncertainties could be introduced owing to possible subjects' underreporting and/or misreporting of the consumption amounts. Nevertheless, the EFSA Comprehensive Database includes the currently best available food consumption data across EU.

Food consumption data from infants, toddlers, children, adolescents, adults and the elderly were used in the exposure assessment. For the present assessment, food consumption data were available from 41 different dietary surveys carried out in 22 European countries (Table D.1). Not all countries provided consumption information for all population groups, and in some cases the same country provided food consumption data from more than one consumption survey.

Table D.1: Population groups considered in the exposure assessment of Prosmoke BW 01

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From more than 12 weeks up to and including 11 months of age	Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia
Toddlers[(a)]	From 12 months up to and including 35 months of age	Belgium, Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Latvia, Netherlands, Portugal, Slovenia, Spain
Children[(b)]	From 36 months up to and including 9 years of age	Austria, Belgium, Bulgaria, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Spain, Sweden
Adolescents	From 10 years up to and including 17 years of age	Austria, Belgium, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
Adults	From 18 years up to and including 64 years of age	Austria, Belgium, Croatia, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden
The elderly[(b)]	From 65 years of age and older	Austria, Belgium, Cyprus, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Netherlands, Portugal, Romania, Slovenia, Spain, Sweden

(a): The term 'toddlers' in the Comprehensive Database (EFSA, 2011) corresponds to 'young children' in Regulations (EC) No 1333/2008 and (EU) No 609/2013.[25]

(b): The terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in the Comprehensive Database (EFSA, 2011).

Since 2018, all consumption records in the Comprehensive Database are codified according to the FoodEx2 classification system (EFSA, 2015). Nomenclature from the FoodEx2 classification system has been linked to the food categorisation system of Annex II of Regulation (EC) No 1333/2008, part D, to perform the exposure assessments. In practice, the FoodEx2 food codes were matched to the food categories.

[24] https://www.efsa.europa.eu/en/data-report/food-consumption-data

[25] Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009. OJ L 181, 29.6.2013, p. 35–56.