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Scientific opinion on the renewal of the authorisation of proFagus Smoke R709 (SF-008) as a smoke flavouring Primary Product

EFSA Panel name 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 the smoke flavouring Primary Product proFagus Smoke R709 (SF-008), for which a renewal application was submitted in accordance with Article 12(1) of Regulation (EC) No 2065/2003. This opinion refers to the assessment of data submitted on chemical characterisation, dietary exposure and genotoxicity of the Primary Product. ProFagus Smoke R709 is obtained by pyrolysis of beech and oak wood as main source materials. The panel concluded that the compositional data provided on the Primary Product are adequate. At the maximum proposed use levels, dietary exposure estimates calculated with DietEx ranged from 0.8 to 12.2 mg/kg body weight (bw) per day at the mean and from 2.3 to 51.4 mg/kg bw per day at the 95th percentile. The Panel concluded that three components in the Primary Product raise a potential concern for genotoxicity. In addition, a potential concern for genotoxicity was identified for the unidentified part of the mixture. The Primary Product contains furan-2(5*H*)-one, 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 for DNA-reactive mutagens and/or carcinogens, the panel concluded that the Primary Product raises concern with respect to genotoxicity.

Keywords: proFagus Smoke R709, SF-008, smoke flavouring Primary Product, genotoxicity, furan-2(5*H*)-one

Requestor: European Commission

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1. Introduction

1.1. Background and terms of reference as provided by the requestor

1.1.1. Background

Regulation (EC) No 2065/2003[1] establishes a procedure for the safety assessment and the authorisation of smoke flavouring primary products with a view to ensuring a high level of protection of human health and the effective functioning of the internal market. No smoking flavouring or any food where such a smoking flavouring is present (in or on) can be placed on the market if the smoke flavouring is not an authorised primary product or is not derived therefrom and if the conditions of use laid down in the authorisation in accordance with this Regulation are not adhered to (Article 4 (2) of Regulation (EC) No 2065/2003).

Commission Implementing Regulation (EU) No 1321/2013[2] authorised 10 smoke flavouring primary products for a 10-year period, due to expire on 31 December 2023.

The European Commission has received an application for the renewal of the authorisation of the smoke flavouring primary product proFagus Smoke R709 (SF-008) for a 10-year period, in accordance with Article 12 of Regulation (EC) No 2065/2003.

1.1.2. Terms of reference

The European Commission requests the European Food Safety Authority (EFSA) to evaluate the safety of the smoke flavouring primary product proFagus Smoke R709 (SF-008), for which a renewal application has been submitted, in accordance with Article 12 of Regulation (EC) No 2065/2003.

The safety assessment shall be carried-out in two steps. Firstly, EFSA shall give a scientific opinion on the data included in the renewal application dossier related to the chemical characterisation, the genotoxicity and the dietary exposure of proFagus Smoke R709 (SF-008).

Secondly, provided that the genotoxic concern can be ruled out in the first part of the evaluation, EFSA shall complete the rest of the safety assessment without delay upon submission of the relevant pending data from the applicant.

1.2. Interpretation of the terms of reference

In line with the terms of reference (see Section 1.1.2), the safety of the Primary Product will be assessed in two steps.

The current (first) opinion addresses the chemical characterisation, genotoxicity and dietary exposure to the smoke flavouring Primary Product.

If in the first opinion, no concern for genotoxicity is raised, EFSA will issue a second opinion assessing the toxicity other than genotoxicity data, as required by the EFSA guidance for the preparation of applications on smoke flavouring Primary Products (EFSA FAF Panel, 2021).

1.3. Additional information

EFSA issued a previous opinion on the safety of this smoke flavouring Primary Product proFagus Smoke R709 (formerly Scansmoke R909) in 2010 (EFSA CEF Panel, 2010).[3]

Following the safety assessment from EFSA, proFagus Smoke R709 was authorised in the European Union and assigned the unique code 'SF-008', according to Commission Implementing Regulation (EU) No 1321/2013, establishing the Union list of authorised smoke flavouring Primary Products, for a 10-year period with effect from 1 January 2014.

[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] Commission Implementing Regulation (EU) No 1321/2013 of 10 December 2013 establishing the Union list of authorised smoke flavouring primary products for use as such in or on foods and/or for the production of derived smoke flavourings. OJ L 333, 12.12.2013, p. 54–67.

[3] In the first safety assessment (EFSA CEF Panel, 2010), the name of the Primary Product was Scansmoke R909. However, as reported in recital (12) of Commission Implementing Regulation (EU) No 1321/2013 of 10 December 2013, the applicant notified to the Commission on 26 November 2012 that the name of the primary product had been changed to proFagus Smoke R709.

The present opinion refers to an assessment of the data submitted by the authorisation holder for the renewal of the authorisation of proFagus Smoke R709 (SF-008) as a smoke flavouring Primary Product, in line with Article 12(1) of Regulation (EC) No 2065/2003.

2. Data and methodologies

2.1. Data

The present evaluation is based on the data provided by the applicant in the form of a technical dossier, submitted according to Article 12(1) of Regulation (EC) No 2065/2003 for the renewal of the authorisation of the smoke flavouring Primary Product proFagus Smoke R709 (SF-008).

In accordance with Article 38 of the Regulation (EC) No 178/2002[4] and taking into account the protection of confidential information and of personal data in accordance with Articles 39 to 39e of the same Regulation and of the Decision of the EFSA's Executive Director laying down practical arrangements concerning transparency and confidentiality,[5] the non-confidential version of the dossier is published on Open.EFSA.[6]

According to Art. 32c(2) of Regulation (EC) No 178/2002 and to the Decision of EFSA's Executive Director laying down the practical arrangements on pre-submission phase and public consultations, EFSA carried out a public consultation on the non-confidential version of the application from 28 October to 19 November 2022, for which no comments were received.

Additional information was sought from the applicant during the assessment process by requests from EFSA sent on 21 December 2022 and was subsequently provided (see Documentation provided to EFSA No. 2).

The Panel acknowledged the submission of data on toxicity other than genotoxicity by the applicant in the technical dossier (see Documentation provided to EFSA No. 1 and 3). As indicated in Section 1.2, the assessment of these data is outside the scope of the present opinion.

2.2. Methodologies

The safety assessment of the Primary Product proFagus Smoke R709 (SF-008) was conducted in line with the requirements laid down in Regulation (EC) No 2065/2003 and following the principles of the EFSA guidance for the preparation of applications on smoke flavouring Primary Products (EFSA FAF Panel, 2021).

The principles described in the EFSA Guidance on transparency with regard to scientific aspects of risk assessment (EFSA Scientific Committee, 2009) as well as the relevant cross-cutting guidance documents from the EFSA Scientific Committee published after the adoption of the guidance on smoke flavourings (EFSA FAF Panel, 2021), in particular the 'Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles' (EFSA Scientific Committee, 2021a), were also considered during the risk assessment.

The uncertainty analysis was performed by checking whether standard or non-standard sources of uncertainties are present, as outlined in the standard procedure described in section 4.2 of the EFSA guidance on smoke flavouring and listed in Table G.1 therein (EFSA FAF Panel, 2021). Standard uncertainties are not discussed in detail in the present assessment. In case of the presence of non-standard uncertainties, these are reported in the relevant sections of the opinion and their combined impact on the assessment was evaluated by the Panel (see Section 4).

[4] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–48.

[5] Decision available online: https://www.efsa.europa.eu/en/corporate-pubs/transparency-regulation-practical-arrangements

[6] The non-confidential version of the dossier, following EFSA's assessment of the applicant's confidentiality requests, is published on Open.EFSA and is available at the following link: https://open.efsa.europa.eu/dossier/SFL-2021-2373

3. Assessment

3.1. Technical data

3.1.1. Manufacturing process

3.1.1.1. Source materials for the Primary Product

The source materials of proFagus Smoke R709 are beech (*Fagus sylvatica* > 90%) and oak (*Quercus robur* < 10%); other wood species might be present at levels < 1%. The wood used for manufacturing the Primary Product is obtained in equal parts from industrial dried cuts (approximately 33.3%), industrial fresh cuts (approximately 33.3%) and untreated remaining material (slabs) from the wood industry (approximately 33.3%). Upon its arrival at the manufacturing site, the wood is inspected for the presence of impurities. The applicant submitted a certificate of quality stating that the wood used to manufacture the Primary Product is not treated with pesticides or subjected to chemical treatments prior to felling or afterwards (Documentation provided to EFSA No. 1).

3.1.1.2. Method of manufacture of the Primary Product

As described by the applicant (Documentation provided to EFSA No. 1), the manufacturing process comprises pyrolysis of the dried wood pieces and condensation of the generated wood gas. The obtained smoke condensate is further concentrated by evaporation; after adjustment of the total acid content, the remaining 'pyroligneous acid' forms the smoke flavouring Primary Product proFagus Smoke R709.

The applicant submitted a description of the manufacturing process, with information on the drying step, the range of temperatures during the pyrolysis and the distillation conditions.

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 proFagus Smoke R709 (formerly named as Scansmoke R909).

3.1.2.2. Information on existing evaluations from other regulatory bodies and authorisations in non-EU countries

The applicant indicated that the smoke flavouring proFagus Smoke R709 has not been evaluated by regulatory bodies other than EFSA (Documentation provided to EFSA No. 1).

Regarding the existing authorisations in non-EU countries, the applicant stated that proFagus Smoke R709 is currently authorised in the United Kingdom (Documentation provided to EFSA No. 3).

3.1.2.3. Description of the physical state and sensory characteristics

The applicant described the smoke flavouring Primary Product as a '*viscous liquid of brown colour with a characteristic odour of smoke*' (Documentation provided to EFSA No. 1). The Primary Product has an average density of approximately 1,020 g/L, a refraction index (at 20 °C) ranging from 1.340 to 1.355, a pH ranging from 2.0 to 2.5, a staining index (at 440 nm) ranging from 11 to 17 and a viscosity (at 20 °C) of 6 cP (n = 5) (Documentation provided to EFSA No. 1 and 2). The Primary Product was reported to have a residue on evaporation of 1–2% and to be '*fully miscible with water; fully miscible with polar solvents such as ethanol, acetone, isopropanol; immiscible with non-polar solvents such as toluene or benzene*.'

3.1.2.4. Chemical composition of the Primary Products

The compositional data provided by the applicant for seven 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 and 2).

Table 1: Overview on the compositional data provided for seven batches of the Primary Product (Documentation provided to EFSA No. 1 and 2)

Batch no.	Density (g/L)	Total volatiles (wt%)	Identified volatiles (wt%)	Unidentified volatiles (wt%)	Total non-volatiles (wt%)(1)	Identified non-volatiles (wt%)	Unidentified non-volatiles (wt%)	Water (wt%)	Solvent-free fraction (wt %)	Ident./quant. proportion of solvent-free fraction (wt%)(2),(a)	Ident./quant. proportion of volatile fraction (wt%)(3),(b)
400348021	1,030	10.8	10.5	0.3	4.8		4.8	84.4	15.6	67.3	97.2
401918021	1,023	11.3	11.1	0.2	6.6		6.6	82.1	17.9	62.0	98.2
404338021	1,022	10.6	10.3	0.3	5.8		5.8	83.6	16.4	62.8	97.2
406538021	1,021	11.2	11.0	0.2	4.0		4.0	84.8	15.2	72.4	98.2
407318021	1,021	10.7	10.4	0.3	–		–	89.1*	–	–	97.2
402578022#	1,023	10.9	10.5	0.4	4.5		4.5	84.6	15.4	68.2	96.3
400918021#	1,023	10.0	9.8	0.2	5.2		5.2	84.8	15.2	64.5	98.0
Average	1,023	10.8	10.5	0.3	5.2		5.2	84.1	16.0	66.2	97.5
SD	3.1	0.4	0.4	0.1	0.9		0.9	1.1	2.1	3.9	0.7
RSD (%)	0.3	4.0	4.2	27.9	18.2		18.2	1.3	13.4	5.9	0.7

(1): Calculated as 100 – water (wt%) – total volatiles (wt%).
(2): Calculated as: (identified volatiles + identified non-volatiles)/solvent free fraction) × 100.
(3): Calculated as: (identified volatiles/total volatiles) × 100.
#: Batches tested in toxicological studies.
*: The applicant considered this value as an outliner and consequently was not considered by the Panel in the calculations.
(a): Regulatory quality criterion for the applied method according to Regulation (EC) No 627/2006[7]: ≥ 50 (wt%).
(b): Regulatory quality criterion for the applied method according to Regulation (EC) No 627/2006: ≥ 80 (wt%).

[7] Commission 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.

3.1.2.4.1. Chemical characterisation

The applicant provided data on the content of the major chemical classes, i.e. acids, phenols and carbonyls (Table 2). The analyses were performed on seven production batches (Documentation provided to EFSA No. 1 and 2).

Table 2: Chemical composition reported for seven batches of the Primary Product

	Batch no.							Average	SD
	400348021	401918021	404338021	406538021	407318021	402578022#	400918021#		
Acids (wt%) (as acetic acid)	9.7	9.5	9.7	9.6	9.2	9.3	9.4	9.5	0.20
Phenols (g/kg) (as syringol)	6.2	6.3	6.3	5.8	5.8	6.2	6.2	6.1	0.22
Carbonyls (wt%) (as 2-butanone)	4.8	5.2	5.1	5.2	4.9	5.3	4.8	5.0	0.21
Water (wt%)	84.4	82.1	83.6	84.8	89.1*	84.6	84.8	84.1	1.1

#: Batches tested in toxicological studies.
*: The applicant considered this value as an outliner, and consequently was not considered by the Panel in the calculations.

Water is the solvent of the Primary Product and was determined by the Karl-Fischer titration method. The panel noted that in the current renewal application, the water content of the Primary Product amounted to 84 ± 1.1 wt% (n = 6), whereas, in the previous opinion issued by EFSA (EFSA CEF Panel, 2010), the water content amounted to 80.1 ± 1.7 wt% (n = 7). The Panel did not consider this inconsistency of relevance for the safety assessment.

Concentrations of arsenic, cadmium, lead and mercury were determined by ICP-MS (inductively coupled plasma-mass spectrometry) and were submitted to EFSA (Table 3) (Documentation provided to EFSA No. 1).

Table 3: Toxic elements reported for seven batches of the Primary Product

	Batch no. (mg/kg)							Average (mg/kg)	SD
	400348021	401918021	404338021	406538021	407318021	402578022#	400918021#		
Arsenic (As)	< 0.1(a)	< 0.1(a)	< 0.1(a)	< 0.1(a)	< 0.1(a)	< 0.1(a)	< 0.1(a)	< 0.1(a)	–
Cadmium (Cd)	0.02	0.02	0.03	0.02	0.02	0.03	0.03	0.02	0.005
Lead (Pb)	< 0.05(a)	< 0.05(a)	< 0.05(a)	< 0.05(a)	< 0.05(a)	< 0.05(a)	< 0.05(a)	< 0.05(a)	–
Mercury (Hg)	< 0.005(a)	< 0.005(a)	< 0.005(a)	< 0.005(a)	< 0.005(a)	< 0.005(a)	< 0.005(a)	< 0.005(a)	–

#: Batches tested in toxicological studies.
(a): Value below the corresponding limit of quantification (LOQ).

3.1.2.4.2. Identification and quantification of the volatile fraction

GC-MS (gas chromatography–mass spectrometry) and GC-FID (gas chromatography-flame ionisation detection) were applied for identification and quantification of the constituents of the volatile fraction of the Primary Product. Individual volatile constituents were considered as identified if their chromatographic (i.e. retention times) and mass spectral data were in agreement with those of reference standards. Quantifications of the volatile constituents were based on compound specific response factors and the use of anthracene d-10 as internal standard. The analytical methods employed to identify and quantify the volatile constituents were submitted to EFSA together with the validation parameters (Documentation provided to EFSA No. 1).

Overall, using this approach, 39 constituents were identified and quantified in seven batches of the Primary Product (for batch numbers, see Table 1) (Appendix A, Table A.1). The lowest concentration reported by the applicant was 0.01 wt% for 2,3-dimethyl-2-cyclopenten-1-one (CAS no.: 1121-05-7). In addition, the applicant reported that the constituent 3-methyl-2(5*H*)-furanone (CAS no.: 22122–36-7) was identified and quantified only in batch no. 406538021. However, the panel noted that the concentrations reported for this constituent were not consistent across the dossier (i.e. 0.01 and 0.08 wt%). The 20 principal volatile constituents are presented in Table 4.

The applicant reported about 45 tentatively identified volatile constituents (Documentation provided to EFSA No. 1). The identification was considered as tentative when it was (solely) based on structural similarities to identified components or when the mass spectral data were only compared to a fragmentation mass spectral library rather than to those of a reference standard. In accordance with the EFSA Scientific Guidance on Smoke Flavourings (EFSA FAF Panel, 2021), EFSA considered these tentatively identified constituents as part of the unidentified fraction.

According to the information provided by the applicant (Documentation submitted to EFSA No. 2), the total volatile fraction of proFagus Smoke R709 accounted on average for approximately 11 wt% of the Primary Product. The proportion of identified and quantified volatiles amounted to approximately 98 wt% of the total volatile fraction; thus, the applied methods meet the legal quality criterion that at least 80% by mass of the volatile fraction shall be identified and quantified (Regulation (EC) No 627/2006).

Following an additional data request from EFSA, the applicant commented on the fact that the current list of identified volatile constituents does not fully match the list of identified volatile constituents provided at the time of the previous EFSA assessment of proFagus Smoke R709 (EFSA CEF Panel, 2010). The applicant emphasised that there were no changes in the manufacturing process and explained that the observed differences are mainly due to the fact that in contrast to the previous application, volatiles were only considered as identified if their chromatographic and mass spectrometric data matched those of reference standards (Documentation provided to EFSA No. 2). The Panel acknowledges this explanation. The Panel further noted that the applicant reported differences in the conditions of the GC-based quantification approaches, e.g. limitation of the upper GC column temperature to 250 °C in the analysis performed for the present application. It is very likely that the use of more recent analytical techniques allowed the applicant to perform a more accurate characterisation of the volatile fraction. Although, in the current application, the portion of identified and quantified volatile components is lower than in the former application, the newly developed GC–MS method allowed better peak separation and shape, and enhanced the retention of components with high boiling point and polarity. For this reason, the characterisation performed here is more reliable than the characterisation performed in the previous application (EFSA CEF Panel, 2010), and the product evaluated in the present assessment does not fundamentally deviate from the product evaluated formerly (EFSA CEF Panel, 2010).

Table 4: Twenty principal volatile constituents of the Primary Product (Documentation provided to EFSA No. 1)

CAS no	FL-no	Chemical name[a]	Average concentration (wt%)	
			Current application[b]	Former application[c]
64–19-7	08.002	Acetic acid	6.9	8.4
116–09-6	07.169	1-hydroxypropan-2-one (1-hydroxy-2-propanone)	1.0	1.4
79–09-4	08.003	Propionic acid (propanoic acid)	0.4	1.4
23147–58-2	–	Glycolaldehyde dimer	0.3	0.1
98–01-1	13.018	Furfural	0.2	0.5
5077-67-8	07.090	1-hydroxybutan-2-one (1-hydroxy-2-butanone)	0.2	0.4
118–71-8	07.014	Maltol	0.1	0.0
765–70-8	07.056[d]	3-methylcyclopentan-1,2-dione (3-methyl-1,2-cyclopentanedione)	0.1	
90–05-1	04.005	2-methoxyphenol	0.1	0.1
91–10-1	04.036	2,6-dimethoxyphenol	0.1	0.4

CAS no	FL-no	Chemical name[a]	Average concentration (wt%)	
			Current application[b]	Former application[c]
96–48-0	10.006	butyro-1,4-lactone (butyrolactone)	0.1	0.1
123–76-2	08.023	4-oxovaleric acid (4-oxo-pentanoic acid)	0.1	
930–30-3		2-cyclopenten-1-one	0.1	0.0
497–23-4	former 10.066[e]	furan-2(5*H*)-one (2(5*H*)-furanone)	0.1	0.1
22122–36-7		3-methyl-2(5*H*)-furanone[f]	0.08	
107–92-6	08.005	Butyric acid (butanoic acid)	0.07	
107–21-1		1,2-ethandiol	0.05	
592–20-1	09.185	2-oxopropyl acetate (acetoxyacetone)[g]	0.04	
79–31-2	08.006	2-methylpropionic acid (2-methyl-propanoic acid)	0.04	
10493–98-8		2-hydroxy-2-cyclopenten-1-one	0.04	0.0
93–51-6	04.007	2-methoxy-4-methylphenol (creosol/methylguaiacol)	0.04	0.1

CAS: Chemical Abstract Service; FL-no: FLAVIS number; wt: weight.
(a): In case a constituent of the Primary Product is an authorised flavouring substance (FL-no), the assigned chemical name corresponds to the respective entry in the EU Union List of flavourings. Deviating chemical names reported by the applicant in the dossier are given in brackets, if applicable.
(b): From the analysis of the batches presented in Table 1.
(c): Calculated from the data presented in EFSA CEF Panel (2010) for the dry matter of the Primary Product, taking into account a water content of 80.1%.
(d): [FL-no: 07.056] refers to the mixture of the tautomeric forms of 3-methylcyclopentan-1,2-dione.
(e): 'Former FL-number' refers to substances that were initially included in the evaluation programme for chemically defined flavouring substances but were not included or were removed/withdrawn from the Union List.
(f): Identified only in batch 406538021; values (wt%) were not consistent across the technical dossier; in this table the highest reported value is included.
(g): Not found in all the investigated batches of the Primary Product.

3.1.2.4.3. Characterisation of the non-volatile fraction

In order to characterise the non-volatile fraction of the Primary Product, the applicant employed the following approaches (Documentation provided to EFSA No. 1):

a) Heating the Primary Product at 350°C for determination of the residual non-volatile fraction. The residue remaining amounted on average to ~ 0.7 wt%.
b) Size exclusion chromatography was performed for an untreated sample of the Primary Product and for a sample obtained after evaporation of the Primary Product at 80°C. Using a calibration curve for a polystyrene standard, the average molecular mass was estimated to be 1.3 kDa for both samples.
c) A sample of the Primary Product, obtained after evaporation at 80 °C, was subjected to alkaline oxidation using H_2O_2/NaOH followed by GC–MS analysis to identify volatile products resulting from degradation of the non-volatile material. After this oxidation step, 37 volatile constituents (Documentation provided to EFSA No. 1) were tentatively identified by GC–MS analysis, based on comparisons of their mass spectra to those from an MS-library. Full identifications and quantifications of the constituents were not performed. The range of detected volatile degradation products encompassed compound classes as described for the oxidative cracking of precipitated hardwood lignin by hydrogen peroxide (Xiang and Lee, 2000). Based on these data, the applicant suggested that the non-volatile fraction mainly consists of 'pyrolytic lignin' which has been described as a reaction product of wood pyrolysis (Figueiredo et al., 2022). However, the Panel noted that this investigation of the non-volatile fraction was performed following a treatment with H_2O_2 under alkaline conditions. Owing to the chemical changes expected under

these oxidative conditions, the detected degradation products do not necessarily represent monomers of the non-volatile material. Thus, the data provided contribute to a characterisation of the non-volatile fraction, but this part of the Primary Product cannot be considered as identified.

The Panel considered that the applicant could have used a more direct method to identify and quantify the constituents in the non-volatile fraction, e.g. high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS), as suggested in the EFSA guidance on smoke flavourings (EFSA FAF Panel, 2021).

3.1.2.4.4. Unidentified fraction

The unidentified fraction of the Primary Product amounts to approximately 6 wt% and comprises the unidentified volatile constituents and the unidentified non-volatile fraction (i.e. the total non-volatile fraction); for the individual values, see Table 1.

3.1.2.4.5. Overall composition of the Primary Product

Based on the chemical analyses performed on the production batches of the Primary Product (Table 1), the overall composition of proFagus Smoke R709 (wt% of Primary Product) is shown in Figure 1, whereas the composition (wt%) of the solvent-free fraction is shown in Figure 2.

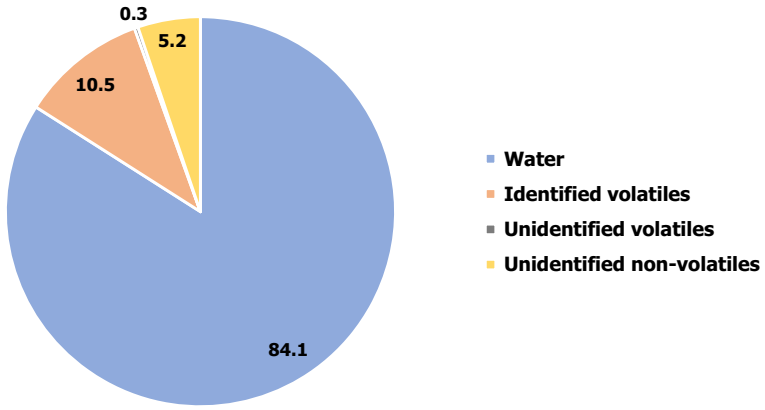

Figure 1: Overall composition of proFagus Smoke R709 (wt% of primary product)

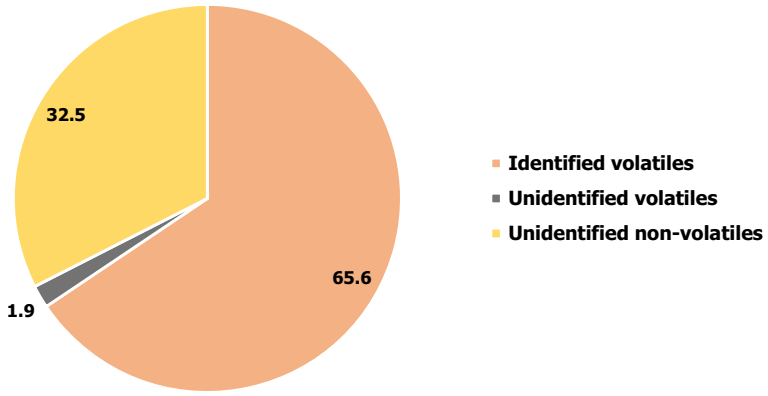

Figure 2: Composition (wt%) of the solvent-free fraction of proFagus Smoke R709

Regarding the identified and quantified proportion of the volatile fraction, the applied methods comply with the legal requirement that at least 80 wt% of the volatile fraction shall be identified and quantified (Regulation (EC) No 627/2006). The Panel noted that for the investigated batches of the Primary Product, the identified and quantified proportion of the solvent-free fraction is on the average approximately 66 wt%; thus, the applied methods meet the legal quality criterion that at least 50% by mass (wt%) of the solvent-free fraction shall be identified and quantified (Regulation (EC) No 627/2006).

3.1.2.5. Polycyclic aromatic hydrocarbons (PAHs)

Analytical data on the content of 16 PAHs were provided for seven batches of the Primary Product. The analysis meets the performance criteria as set in Regulation (EC) No 627/2006. The levels reported for the individual PAHs (Table 5) are consistently below the minimum required limits of quantification according to 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 5: Concentrations of PAHs in the Primary Product, average from seven batches (for batch numbers, see Table 1) (Documentation provided to EFSA No. 1). Averages and standard deviations were not included as the PAHs were reported at concentrations below their respective LOQs

PAH	Conc. (μg/kg)
benzo[a]anthracene[(a)]	< 0.5[(b)]
chrysene[(a)]	< 0.5[(b)]
benzo[b]fluoranthene[(a)]	< 0.5[(b)]
benzo[k]fluoranthene	< 0.5[(b)]
benzo[j]fluoranthene	< 0.5[(b)]
benzo[a]pyrene[(a)]	< 0.5[(b)]
indeno[123-cd]pyrene	< 0.5[(b)]
dibenzo[a,h]pyrene	< 1[(b)]
benzo[g,h,i]perylene	< 0.5[(b)]
dibenzo[a,l]pyrene	< 1[(b)]
dibenzo[a,i]pyrene	< 1[(b)]
dibenzo[a,h]anthracene	< 0.5[(b)]
dibenzo[a,e]pyrene	< 1[(b)]
cyclopenta[cd]pyrene	< 1[(b)]
5-methylchrysene	< 1[(b)]
benzo[c]fluorene	< 1[(b)]
PAH4	< 2.0[(c)]

(a): PAHs printed in bold are included in the calculation of 'PAH4', which is used for the evaluation of the exposure to these contaminants (see Section 3.3.3.2).
(b): Value below the corresponding limit of quantification (LOQ).
(c): Value below the sum of the LOQs for PAH4 in the individual batches.

3.1.2.6. Batch-to-batch variability

The batch-to-batch variability of the 20 principal volatile constituents of the batches presented in Table 1 was investigated by GC–MS and GC-FID. The Panel considered that the batch-to-batch-variability of the investigated production batches (Table 6), with production dates spanning 15 months, was acceptable, also taking into account information on the other measured parameters evaluated in this opinion. Information on the criteria underlying the selection of these batches was not provided.

Table 6: Batch-to-batch variability of the Primary Product

CAS-no	Chemical name	Batch no. (production date)							Average (wt%)	SD	RSD (%)
		400348021 (19-01-2021)	401918021 (30-03-2021)	404338021 (19-07-2021)	406538021 (12-11-2021)	407318021 (13-12-2021)	402578022# (20-02-2021)	400918021# (22-04-2022)			
64-19-7	acetic acid	7.0	7.2	6.8	7.2	6.8	6.7	6.7	6.9	0.2	3.0
116-09-6	1-hydroxy-2-propanone	1.0	1.1	1.0	1.0	1.0	1.0	0.9	1.0	0.1	5.9
79-09-4	propanoic acid	0.38	0.41	0.37	0.41	0.33	0.4	0.31	0.37	0.04	10.6
23147-58-2	glycolaldehyde dimer	0.26	0.28	0.29	0.29	0.29	0.28	0.24	0.28	0.02	6.90
98-01-1	furfural	0.22	0.25	0.2	0.27	0.25	0.26	0.15	0.23	0.04	18.5
5077-67-8	1-hydroxy-2-butanone	0.23	0.25	0.22	0.22	0.22	0.22	0.2	0.22	0.01	6.71
118-71-8	maltol	0.12	0.13	0.13	0.12	0.12	0.18	0.11	0.13	0.02	17.8
765-70-8	3-methyl-1,2-cyclopentanedione	0.12	0.13	0.11	0.11	0.11	0.11	0.11	0.11	0.01	6.88
90-05-1	2-methoxyphenol	0.11	0.12	0.1	0.11	0.11	0.12	0.09	0.11	0.01	9.85
91-10-1	2,6-dimethoxyphenol	0.1	0.12	0.1	0.1	0.1	0.09	0.1	0.10	0.01	8.87
96-48-0	butyrolactone	0.08	0.08	0.08	0.08	0.11	0.1	0.08	0.09	0.01	14.4
123-76-2	4-oxo-pentanoic acid	0.1	0.1	0.09	0.09	0.09	0.08	0.09	0.09	0.01	7.55
930-30-3	2-cyclopenten-1-one	0.08	0.09	0.08	0.08	0.08	0.09	0.07	0.08	0.01	8.47
497-23-4	2(5*H*)-furanone	0.08	0.08	0.08	0.08	0.08	0.09	0.07	0.08	0.01	7.22
107-92-6	butanoic acid	0.07	0.07	0.07	0.07	0.07	0.08	0.06	0.07	0.01	8.25
22122-36-7	3-methyl-2(5*H*)-furanone	–	–	–	0.01*	–	–	–			
107-21-1	1,2-ethandiol	0.04	0.05	0.04	0.05	0.04	0.04	0.05	0.04	0.01	12.1
592-20-1	acetoxyacetone	–	–	–	0.04	0.04	0.05	–	0.04	0.01	13.3
79-31-2	2-methylpropanoic acid	0.04	0.04	0.04	0.04	0.03	0.05	0.03	0.04	0.01	17.9
10493-98-8	2-hydroxy-2-cyclopenten-1- one	0.04	0.05	0.04	0.04	0.05	0.05	0.03	0.04	0.01	17.6
93-51-6	creosol/methylguaiacol	0.04	0.04	0.04	0.04	0.04	0.05	0.03	0.04	0.01	14.4

SD: standard deviation; RSD: relative standard deviation.
#: Batches used in the toxicological studies.
*: The Panel noted that this value is not in agreement with the concentration reported in Table 4.

3.1.2.7. Solubility and particle size

According to the applicant, the Primary Product has 1–2% of residue after evaporation (see Section 3.1.2.3); therefore, EFSA requested to clarify the potential presence of small particles including nanoparticles in line with the EFSA 'Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles' (EFSA Scientific Committee, 2021a).

The applicant submitted a study report based on DLS (dynamic light scattering) analysis (Documentation provided to EFSA No 2). The DLS profiles of the Primary Product and of the Primary Product in three selected solvents (i.e. water, propylene glycol and diethyl ether) were submitted. The justification for selecting these solvents was not provided, although it can be noted that they span a polarity range and could be used as carriers of the Primary Product in foods. In addition, the DLS profile of the Primary Product with stevia glycoside, used as a surfactant, was provided. As a positive control, the applicant added platinum nanoparticle clusters (Pt-NP) to the pure solvents at a concentration of 15 μg/mL (Documentation provided to EFSA No. 2). The applicant stated that '*mixing the Primary product with diethyl ether led to separation in two phases*'. Therefore, only the mixtures of the Primary Product with water and propylene glycol were investigated using DLS. Based on the analyses performed, the applicant concluded that the Primary Product is an unstable emulsion of liquids having different polarities.

The Panel noted that:

i) The DLS profiles of the pure Primary Product exhibit peaks up to 1,000 nm.
ii) When the Primary Product is mixed with water and propylene glycol, the DLS profiles show peaks up to 10,000 nm, which do not correspond to the peaks observed in the DLS profiles of the pure Primary Product and the pure solvents.
iii) After the addition of stevia glycoside, the DLS profiles of the Primary Product show changes in the distribution of the observed peaks. The fact that the peaks do not disappear may indicate the presence of particulate matter.

The Panel noted that the evidence provided is not sufficient to prove the absence of a fraction of small particles in the Primary Product since:

i) The presence of peaks in the DLS profiles of the pure Primary Product and the Primary Product mixed with propylene glycol and water is not explained by the applicant, and may indicate the presence of particulate matter, e.g. the DLS profile of the Primary Product in propylene glycol (from 1:1 to 1:10) shows various peaks from 10 to 10,000 nm;
ii) The platinum nanoparticle clusters (Pt-NP) do not represent an appropriate positive control, as they have different light scattering properties compared to the organic particles potentially present (if any) in the Primary Product. Moreover, since large nanoparticles produce very high DLS signal, this could have covered the signals belonging to smaller particles, thereby reducing the sensitivity of the testing. Since the size of the Pt-NP was not specified, it is not possible to judge if the Pt-NP positive control would have introduced DLS signalling bias.

Based on the above, the Panel concluded that more evidence is needed to exclude the presence of nanoparticles in the Primary Product. This creates a non-standard uncertainty with respect to the solubility and particle size of the Primary Product (see Section 2.2 of this opinion and Table G.1 of the EFSA guidance document on smoke flavouring [EFSA FAF Panel, 2021]).

3.1.3. Specifications

The applicant provided the required product specification data and reported that the Primary Product proFagus Smoke R709 is manufactured within its proposed specifications (Documentation provided to EFSA No. 1). Information on parameters relevant for the specifications has been compiled by the panel in Table 7.

Table 7: Relevant information for specifications of the Primary Product

	Specifications for proFagus Smoke R709 as proposed by the applicant	Specifications as reported in (EFSA CEF Panel, 2010)	Specifications as laid down in Regulation (EU) No 1321/2013
Description	Smoke flavouring Primary Product obtained from beech (*Fagus sylvatica* > 90%), oak (*Quercus robur* < 10%), other wood species < 1%		
Source materials			
• Woods	beech (*Fagus sylvatica* > 90%), oak (*Quercus robur* < 10%), other wood species < 1%.		90% beech (*Fagus sylvatica*), 10% oak (*Quercus alba*)
Identity parameters:			
• Physico-chemical parameters			
– pH	2.0–2.5	2.0–2.5	2.0–2.5
– Density	1.016–1.026 g/mL (at 20°C)	1.02–1.04 g/mL (as specific gravity at 20°C)	
– Refraction index	1.340–1.355 (at 20°C)		
– Staining index	11–17		
Chemical composition:			
• Chemical classes:			
– Acids	9.0–12.5 wt% (as acetic acid)	10.5–12.5% (as acetic acid)	10.5–12.5 mEq/g (as acetic acid)
– Carbonyls	3–10 wt% (as 2-butanone)		5–10 wt%
– Phenols	5.0–10.0 g/kg (as syringol)	5–10 mg/g*	5–10 wt%
– water	76.7–85.0 wt%		76.7–83.5%
20 principal constituents of the volatile fraction	See Table 4		
Purity:			
• Benzo[a]pyrene	< 10 μg/kg	< 10 μg/kg	
• Benz[a]anthracene	< 20 μg/kg	< 20 μg/kg	
• Toxic elements			
– Arsenic	< 3 mg/kg	< 0.1 mg/kg	< 3 mg/kg
– Cadmium	< 1 mg/kg	< 0.1 mg/kg	< 1 mg/kg
– Lead	< 5 mg/kg	< 0.1 mg/kg	< 5 mg/kg
– Mercury	< 1 mg/kg	< 0.1 mg/kg	< 1 mg/kg

*: Named as 'Smoke flavour compounds'.

The Panel noted that the analytical data for the batches analysed indicated that actual concentrations of toxic elements and PAHs, reported in Tables 3 and 5, respectively, are substantially lower than the currently proposed limits (Table 7), being the same as the limits laid down in the respective Regulations (i.e. Regulation (EU) No 1321/2013 for toxic elements, and Regulation (EC) No 2065/2003 for benzo[a]pyrene and benzo[a]anthracene).

With regard to the raw materials, the panel noted that according to the current application, wood from *Quercus robur* is used to manufacture the Primary Product. This differs from the wood species reported in the Union List (Regulation (EU) No 1321/2013), which is *Quercus alba*. The Panel further noted that also in the former assessment of the Primary Product (EFSA CEF Panel, 2010), *Quercus robur* had been reported as the raw material used in the manufacturing process.

With regard to the content of phenols and acids, the panel noted that the units used to express the numerical values in the current application differ from the units used in the Union List (Regulation (EU)

No 1321/2013). Following an additional data request, the applicant informed that the values expressed in the units used in the current application are correct, whereas the values expressed in the units used in the Annex of Regulation (EU) No 1321/2013 are not correct (Documentation provided to EFSA No. 2). In addition, the applicant informed that the difference between the concentration range of carbonyls in the current specifications and the specifications provided in the Annex of Regulation (EU) No 1321/2013 is due to the different reference compound used to calculate the concentration of carbonyls, i.e. 2-butanone (here, Table 7) rather than furfural in Regulation (EU) No 1321/2013 (Documentation provided to EFSA No. 2).

With regard to the contents of water and acids, the proposed values are based on the newly provided analytical data; therefore, the Panel considered that the proposed extensions of the range of water and acid content (see Table 7) are justified.

In general, the applicant stated that minor modifications were included in the currently proposed specifications compared to the specifications from the former opinion (EFSA CEF Panel, 2010) and from Regulation (EC) No 1321/2013, with regard to the chemical composition of the Primary Product. These modifications are not due to changes in the manufacturing process but reflect the results from the most recent (improved) analytical measurements (Documentation provided to EFSA No. 1). The Panel did not consider these modifications as of relevance for the safety assessment.

3.1.4. Stability and fate in food

Stability tests were performed for one of the batches of the Primary Product (batch no. 406538021) listed in Table 1.

A stability test was performed with the batch stored for 6 months under ambient conditions. In addition, the batch was subjected to an accelerated stability test (forced ageing) at 40 °C for 5.5 months. The storage stability of proFagus Smoke R709 was assessed by monitoring the volatile constituents of the Primary Product; based on the observed relative standard deviations (on average below 15%), their concentrations were sufficiently stable under both storage conditions. On the basis of these data, the Panel considered the stability of the Primary Product upon storage under the intended conditions not to be of concern. No data on the stability of the Primary Product in commercial formulations or in the proposed food categories were provided.

3.2. Proposed uses and use levels

The applicant applied for a renewal of authorisation of the Primary Product proFagus Smoke R709 for use in foods at the proposed maximum and expected typical use levels as presented in Table 8.

The proposed maximum and expected typical use levels were used to assess the dietary exposure to this Primary Product (see Section 3.3.2).

Table 8: Proposed maximum and expected typical use levels of Primary Product (mg/kg) in food categories according to Annex II of Regulation (EC) No 1333/2008[8]

Food category number	Food category Name	Restrictions/exceptions	Proposed maximum use levels (mg/kg)[(a)]	Expected typical use levels (mg/kg)[(a)]
1.7	Cheese and cheese products		1,000	20
3	Edible ices	Only dairy ice creams and similar	5	5
4.2.4.1	Fruit and vegetable preparations excluding compote	Only processed tomato products, vegetable puree or paste, extracts of plant origin and vegetables-based cooked sauce	20	5
4.2.5.4	Nut butters and nut spreads		20	5
5.1	Cocoa and chocolate products as covered by Directive 2000/36/EC	Only pralines and filled chocolate	20	1

[8] 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.

Food category number	Food category Name	Restrictions/exceptions	Proposed maximum use levels (mg/kg)[(a)]	Expected typical use levels (mg/kg)[(a)]
5.3	Chewing gum		20	1
5.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	Only flavoured sugar	20	1
6.4.5	Fillings of stuffed pasta (ravioli and similar)		160	10
6.6	Batters		80	10
8.2	Meat preparations as defined by Regulation (EC) No 853/2004		400	30
8.3	Meat products	Except preserved/processed fat tissues	1,000	30
9.2	Processed fish and fishery products including molluscs and crustaceans		1,000	30
9.3	Fish roe		200	12
12.2.1	Herbs and spices	Only seasoning mixes	1,300	50
12.2.2	Seasonings and condiments	Only taste enhancing RPC extracts	1,300	50
12.4	Mustard		100	50
12.5	Soups and broths		280	70
12.6	Sauces		750	600
12.7	Salads and savoury based sandwich spreads	Only prepared mixed egg/meat/ fish/vegetable salad and prepared meat salad	200	80
12.9	Protein products, excluding products covered in category 1.8	Only meat imitates	1,000	50
14.1.4	Flavoured drinks	Only flavoured milks, whey, soft drinks and functional drinks	20	10
14.1.5	Coffee, tea, herbal and fruit infusions, chicory; tea, herbal and fruit infusions and chicory extracts; tea, plant, fruit and cereal preparations for infusions, as well as mixes and instant mixes of these products		20	10
14.2	Alcoholic beverages, including alcohol-free and low-alcohol counterparts		20	10
15.1	Potato-, cereal-, flour- or starch-based snacks		1,000	50
15.2	Processed nuts		500	100

(a): Use levels are provided for the foods as consumed.

The applicant also proposed maximum and expected typical use levels for the Primary Product in composite dishes so to cover foods which could contain 'meat, fish and sauce ingredients containing smoke flavourings' (e.g. meat based dishes, legumes based dishes, sandwich and sandwich-like dishes, pizza and pizza-like dishes, finger food, pasta based dishes (cooked)). These foods and their proposed maximum and expected typical use levels are not listed in Table 8 because, in line with article 18(1) (a) and (c) of Regulation (EC) No 1333/2008, authorisations in prepared or composite dishes are covered by the authorisations in the relevant respective food categories. The composite dishes as mentioned by the applicant were considered in the exposure assessment, as described below.

3.3. Exposure

3.3.1. Food consumption data used for the exposure assessment

The food consumption data used for the exposure assessment are from the EFSA Comprehensive European Food Consumption Database (Comprehensive Database).[10] This database contains food consumption data at the level of the individual consumer from the most recent national dietary surveys carried out in EU countries and includes the currently best available food consumption data across the EU. These data cover infants (from 0 weeks of age), toddlers (1–2 years), children (3–9 years), adolescents (10–17 years), adults (18–64 years) and the elderly (65 years and older). As these data were collected by different methodologies, direct country-to-country comparisons of exposure estimates based on these data may not be appropriate.

The dietary exposure to the Primary Product was calculated by the applicant and EFSA using the FAIM (food additive intake model, version 2.1) and DietEx tools. The food consumption data in both tools are based on the version of the Comprehensive Database that was published in July 2021. These data cover 42 dietary surveys carried out in 22 EU countries (Table 9).

Table 9: Population groups and countries considered for the exposure estimates of the Primary Product with FAIM and DietEx

Population	Age range	Countries with food consumption surveys covering more than 1 day
Infants	From 0–12 weeks[(a)] up to and including 11 months of age	Bulgaria, Cyprus, Denmark, Estonia, Finland, France, Germany, Italy, Latvia, Portugal, Slovenia, Spain
Toddlers[(b)]	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[(c)]	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[(c)]	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): FAIM includes infants from 12 weeks of age and DietEx infants from 0 weeks of age.
(b): The term 'toddlers' in the Comprehensive Database (EFSA, 2011) corresponds to 'young children' (from 12 months up to and including 35 months of age) in Regulations (EC) No 1333/2008 and (EU) No 609/2013.[9]
(c): In FAIM, the terms 'children' and 'the elderly' correspond, respectively, to 'other children' and the merge of 'elderly' and 'very elderly' in Comprehensive Database (EFSA, 2011).

The food consumption data from the Comprehensive Database in FAIM are codified according to the food categories as presented in Annex II, Part D, of Regulation (EC) No 1333/2008, which is the relevant regulation for the food categories of the smoke flavourings. In DietEx, these consumption records are codified according to the FoodEx2 food classification and description system. As FoodEx2 includes more information on the foods coded in the food consumption data, this tool will potentially result in less conservative estimates of dietary exposure compared to FAIM.

[9] 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. 35–54.
[10] https://www.efsa.europa.eu/en/data-report/food-consumption-data

3.3.2. Exposure assessment of the Primary Product

Using both FAIM and DietEx, dietary exposure to the Primary Product was calculated by multiplying the relevant use level for each food category or FoodEx2 code with its respective consumption amount for each individual. This was done for all individuals in the surveys (i.e. the estimates are not based on consumers only). The exposures per food category or FoodEx2 code were subsequently added and divided by the individual body weight (as registered in the consumption survey) to derive an individual total exposure per day expressed per kilogram body weight. These exposure estimates were averaged over the number of survey days in the survey, resulting in an individual average exposure per day. Dietary surveys with only 1 day per subject were excluded as they are not considered adequate to assess repeated exposure. The calculations resulted in distributions of individual exposure per survey and population group. Based on these distributions, the mean and the 95th percentile of exposure were calculated per survey and population group. The 95th percentile of exposure was only calculated for those population groups with a sufficiently large sample size to obtain a reliable estimate (EFSA, 2011).

In FAIM, the infant population considers infants from 12 weeks up to and including 11 months of age. In DietEx, the infant population considers the infants from 0 weeks up to and including 11 months of age.

3.3.2.1. Exposure assessment using FAIM

The applicant provided estimates of dietary exposure to the Primary Product using FAIM, based on the proposed maximum (proposed maximum use level exposure assessment scenario) and expected typical use levels (expected typical use level exposure assessment scenario) (Documentation provided to EFSA n.1). These estimates were re-calculated by EFSA, following a submission of updated uses and use levels from the applicant (Documentation provided to EFSA n.2).

In FAIM, use levels were linked to the corresponding food categories according to the instructions provided for its use.[11] Furthermore, all foods belonging to the food categories (FC) were included in the assessment without applying the restrictions/exceptions as indicated in Table 8. This tool does not allow to include or exclude specific foods from the exposure assessment.

The composite foods for which the applicant provided proposed maximum and expected typical use levels were allocated to the relevant food categories based on their main ingredient at the use levels provided for these food categories. See Annex A1 for the food categories and use levels considered in FAIM.

Exposure estimates using FAIM

In Table 10, the dietary exposure estimates of the Primary Product with FAIM are presented.

Table 10: Summary of dietary exposure to the Primary Product from its proposed maximum and expected typical use levels as a smoke flavouring in six population groups and estimated with FAIM (minimum-maximum across the dietary surveys in mg/kg bw per day)

	Infants (12 weeks–11 months) (n = 11/9)	Toddlers (12–35 months) (n = 15/13)	Children (3–9 years) (n = 19/19)	Adolescents (10–17 years) (n = 21/20)	Adults (18–64 years) (n = 22/22)	The elderly (≥ 65 years) (n = 22/21)
Proposed maximum use level exposure assessment scenario						
Mean	0.3–7.0	2.0–11.6	2.5–11.8	1.5–6.2	1.1–4.0	0.8–3.0
95th percentile	2.0–25.8	5.4–42.2	5.5–36.5	3.4–19.7	2.6–13.3	2.1–10.0
Expected typical use level exposure assessment scenario						
Mean	0.02–1.1	0.1–2.1	0.2–2.3	0.1–1.2	0.1–0.7	0.1–0.5
95th percentile	0.1–4.0	0.4–10.0	0.4–8.5	0.3–4.4	0.2–3.0	0.2–2.1

n: number of surveys from which a mean/P95 could be calculated.

[11] Link to FAIM instructions: https://www.efsa.europa.eu/sites/default/files/applications/FAIM-instructions.pdf

At the proposed maximum use levels, the mean exposure to the Primary Product from its use as a smoke flavouring ranged from 0.3 mg/kg bw per day in infants to 11.8 mg/kg bw per day in children. The 95th percentile of exposure to the Primary Product ranged from 2.0 mg/kg bw per day in infants to 42.2 mg/kg bw per day in toddlers.

At the expected typical use levels, the mean exposure ranged from 0.02 mg/kg bw per day in infants to 2.3 mg/kg bw per day in children, and the 95th percentile of exposure from 0.1 mg/kg bw per day in infants to 10.0 mg/kg bw per day in toddlers.

The Primary Product is requested for renewal of authorisation in 25 food categories (Table 8). For all these 25 food categories considered, it was assumed that 100% of the foods belonging to these food categories will contain the Primary Product at the proposed maximum or expected typical use levels. As it is unlikely that the Primary Product will be added to all foods and given the restrictions/ exceptions for 10 food categories (Table 8), the Panel considered that the calculated exposure to the Primary Product using FAIM is an overestimation of the expected exposure in EU countries if this Primary Product is used at the proposed maximum or expected typical use levels.

Additionally, overall sources of standard uncertainties (Annex A9) also contributed to an overestimation of the exposure.

Detailed results per population group and survey are presented in Annexes A2 (proposed maximum use level exposure assessment scenario) and A3 (expected typical use level exposure assessment scenario).

3.3.2.2. Exposure assessment using DietEx

The applicant provided also estimates of dietary exposure to the Primary Product using DietEx, based on the proposed maximum and expected typical use levels (Documentation provided to EFSA n.1). These estimates were re-calculated by EFSA, following a submission of updated uses and use levels from the applicant (Documentation provided to EFSA No.2).

To assess the exposure using DietEx, the applicant provided a list of FoodEx2 codes per food category (Documentation provided to EFSA No.2). Using FoodEx2 codes, the applicant selected the foods to which the Primary Product could be added per food category, considering the restrictions/ exceptions (Table 8).

An examination of the FoodEx2 codes showed that some FoodEx2 codes belonging to a particular food category were missing based on the restrictions/exceptions provided by the applicant. Also, the applicant had selected FoodEx2 codes that were not available in DietEx. In the first case, for instance, the applicant did not include all FoodEx2 codes covering FC 15.2 Processed nuts, such as the FoodEx2 codes for tree nuts. In the second case, for instance, for FC 8.2 Meat preparations, the applicant selected the FoodEx2 code 'Pig minced meat'; however, this code is not available in DietEx and therefore the parent code 'Pig fresh meat' should have been selected.

The corrections applied by EFSA to the food categories considered for the exposure assessment using DietEx are:

- FC 8.2 Meat preparations as defined by Regulation (EC) No 853/2004 of the European Parliament and of the Council: all FoodEx2 codes belonging to this food category were considered. In addition, when FoodEx2 codes were not available in DietEx, their parent food code was selected. This is the case for minced meat of several species which are not available in DietEx. This will lead to an overestimation of the exposure.
- FC 15.2 Processed nuts: All FoodEx2 codes that belong to this food category were considered.
- The applicant also proposed to use the Primary Product in composite dishes restricted to 'Only foods containing meat, fish and sauces ingredients'; therefore, all composite dishes containing meat and/or fish and/or sauce as ingredient were considered in this assessment. When a FoodEx2 code for a dish with such ingredient(s) was not available in DietEx, its parent code was considered if this did not result in an unrealistically large overestimation of the exposure, based on expert judgement (e.g. 'Omelette with Bacon' is not available in DietEx and considering its parent food group 'Egg-based dishes' would grossly overestimate the dietary exposure). The use levels proposed by the applicant were used in the assessment and adjusted by a factor (see Annex A4), based on the percentage of meat and sauce in the dishes as proposed by the applicant.

For the other food categories, the FoodEx2 codes selected by the applicant could be included in DietEx. See Annex A4 for the list of FoodEx2 codes per food category that were used in the exposure assessment using DietEx (see Section 3.3.2.1).

Exposure estimates using DietEx

In Table 11, the dietary exposure estimates of the Primary Product with DietEx are presented.

Table 11: Summary of dietary exposure to the Primary Product from its proposed maximum and expected typical use levels as a smoke flavouring in six population groups, estimated with DietEx (minimum-maximum across the dietary surveys in mg/kg bw per day)

	Infants (0 weeks-11 months) (n = 12/11)[(a)]	Toddlers (12–35 months) (n = 15/13)	Children (3–9 years) (n = 19/19)	Adolescents (10–17 years) (n = 21/20)	Adults (18–64 years) (n = 22/22)	The elderly (≥ 65 years) (n = 33/29)[(b)]
Proposed maximum use level exposure assessment scenario						
Mean	0.8–8.8	2.2–12.0	3.0–12.2	1.8–6.4	1.4–4.3	1.0–3.2
95th percentile	2.7–51.4	5.6–41.8	5.9–36.8	3.5–19.9	2.8–13.6	2.3–10.1
Expected typical use level exposure assessment scenario						
Mean	0.04–1.4	0.1–2.1	0.2–2.4	0.1–1.2	0.1–0.7	0.1–0.5
95th percentile	0.1–12.6	0.4–10.0	0.4–8.5	0.3–4.4	0.2–3.0	0.1–2.1

n: number of surveys for which a mean/P95 could be calculated.
(a): The number of surveys for infants is different from FAIM as the age range for this population group differs between the two tools.
(b): DietEx provides exposure estimates for the elderly and the very elderly population groups. To ease the reading, and for consistency with FAIM, exposure results are reported as the range of these two population groups (i.e. the min being the minimum between both populations and max being the maximum between both populations).

At the proposed maximum use levels, the mean exposure to the Primary Product from its use as a smoke flavouring ranged from 0.8 mg/kg bw per day in infants to 12.2 mg/kg bw per day in children. The 95th percentile of exposure to the Primary Product ranged from 2.3 mg/kg bw per day in the elderly to 51.4 mg/kg bw per day in infants.

At the expected typical use levels, the mean exposure ranged from 0.04 mg/kg bw per day in infants to 2.4 mg/kg bw per day in children, and the 95th percentile of exposure from 0.1 mg/kg bw per day in infants and the elderly to 12.6 mg/kg bw per day in infants.

As for FAIM, the panel considered that the calculated exposure to the Primary Product using DietEx is an overestimation of the expected exposure in EU countries at the proposed maximum or expected typical use levels. In fact, it is assumed that the Primary Product is used in all foods within food categories without restrictions/exceptions, as well as in all foods within a food category with restrictions/exceptions that meet these restrictions/exceptions. Also, considering the parent food for foods not available in DietEx, unless this would have resulted in an unrealistically large overestimation of the exposure, contributed to an overestimation of the exposure with DietEx.

Additionally, overall sources of standard uncertainties (Annex A9) also contributed to an overestimation of the exposure.

Detailed results per population group and survey are presented in Annexes A5 (proposed maximum use level exposure assessment scenario) and A6 (expected typical use level exposure assessment scenario).

Main FoodEx2 codes contributing to exposure to the Primary Product using DietEx

Under the conservative assumptions mentioned above, the main FoodEx2 codes contributing to the total mean exposure to the Primary Product for both exposure scenarios contributing to at least 30% to the total mean exposure in at least one population group in one survey, listed in order of the number of the FCs, are:

- Cheese belonging to FC 1.7.
- Chicken fresh meat belonging to FC 8.2.
- Raw cured (or seasoned) meat belonging to FC 8.3.1.
- Sausages belonging to FC 8.3.1.
- Soups (ready-to-eat) belonging to FC 12.5.
- Stock cubes or granulate (bouillon base) belonging to FC 12.5.

- Savoury Sauces belonging to FC 12.6.
- Coffee, cocoa, tea and infusions belonging to FC 14.1.5.

Considering the conservative nature of the underlying assumption that 100% of the foods within the FoodEx2 codes (with the restrictions/exceptions, Table 8) contain the Priary Product, the Panel emphasises that the FoodEx2 codes listed here may not reflect the FoodEx2 codes that contribute most to the exposure in real life.

Detailed results of the contributing FoodEx2 codes per population group and dietary survey are presented in Annexes A7 and A8.

3.3.2.3. Comparison FAIM versus DietEx

The use of DietEx has the potential to calculate less conservative estimates of exposure to the Primary Product compared to FAIM by selecting foods, via FoodEx2 codes, within a food category to which the Primary Product may be added. Despite this, the exposure to the Primary Product calculated with both tools was similar for most population groups, except for infants (Tables 10 and 11). The exposure calculated with FAIM was lower for this age group than calculated with DietEx, because DietEx includes infants from 0 to 12 weeks of age, which are expected to consume more food per kg body weight than older infants.

For the other population groups, the exposure estimates were comparable. This was very likely due to the absence of restrictions/exceptions for FC 1.7 Cheese and cheese products which contributed largely to the exposure to the Primary Product using DietEx. Another important contributing food category in DietEx was 'Chicken fresh meat'. The applicant only requested authorisation for FC 8.2 to which 'minced meat' belongs. Because corresponding FoodEx2 codes for minced meat were not available in DietEx, their parent food codes (fresh meat) was used, resulting in an overestimation of the exposure.

The DietEx exposure estimates (see Table 11) will be used for the risk assessment of the Primary Product, because in general these estimates are considered more refined than the FAIM exposure estimates.

3.3.3. Anticipated exposure to impurities in the Primary Product

The potential exposure to impurities arsenic, lead, cadmium, mercury and PAHs (as PAH4) from the use of the Primary Product can be calculated by assuming that they are present in the Primary Product up to a limit value and then by calculating pro-rata to the estimates of exposure to the Primary Product itself.

With regard to the dietary exposure to the Primary Product, the Panel considered the highest mean and the highest 95th percentile exposure estimates resulting from the exposure assessment using DietEx among the different population groups, i.e. 12.2 mg/kg bw per day for children and 51.4 mg/kg bw per day for infants, respectively (Table 11).

The level of the impurities in the Primary Product combined with the estimated exposure to the Primary Product (Table 11) can be used to estimate the exposure to these impurities. This exposure can then be compared with reference points (RP, i.e. lower limit of the benchmark dose (BMDL) for arsenic, lead and PAH4) or health-based guidance values (HBGV, i.e. tolerable weekly intake (TWI) for cadmium and mercury) for the undesirable impurities present in the Primary Product (Table 12).

Table 12: Reference points/health-based guidance values for the impurities potentially present in the Primary Product

Impurity/ constituent/HBGV/ RP	Basis/Reference
Arsenic (As)/ 0.3–8 μg/kg bw per day ($BMDL_{01}$)	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. 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, i.e. a MOE of 1,000 would be sufficient (EFSA CONTAM Panel, 2009a; EFSA Scientific Committee, 2012)
Cadmium (Cd)/2.5 μg/ kg bw per week (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 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)
Lead (Pb)/0.5 μg/kg bw per day ($BMDL_{01}$)	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 $BMDL_{01}$ (MOE lower than 1) (EFSA CONTAM Panel, 2010).
Mercury (Hg)/4 μg/kg bw per week (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 per week, expressed as mercury was established (EFSA CONTAM Panel, 2012).
PAH4/340 μg/kg bw per day ($BMDL_{10}$)	Polycyclic aromatic hydrocarbons (PAHs) are considered genotoxic and carcinogenic. The reference point is based on a carcinogenicity study by Culp et al. (1998), as reported by the EFSA CONTAM Panel (2008), who concluded that PAH4 (i.e. the sum of benzo[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene and chrysene) is a suitable indicator for the occurrence and toxicity of PAHs in food. The MOE should be at least 10,000 (EFSA CONTAM Panel, 2008).

HBGV: Health-based guidance value; RP: Reference point; $BMDL_{01}$: lower confidence limit of the benchmark dose associated with a 1% extra risk for tumours (EFSA Scientific Committee, 2017a); $BMDL_{10}$: lower confidence limit of the benchmark dose associated with a 10% extra risk for tumours (EFSA Scientific Committee, 2017a); TWI: Tolerable Weekly Intake; MOE: margin of exposure.

The risk assessment of the undesirable impurities helps to determine whether there could be a possible health concern if these impurities were present at their limit values in the Primary Product. The assessment is performed by calculating the MOE (margin of exposure) by dividing the reference point (i.e. BMDL, Table 12) by the exposure estimate for an impurity (Table 11), or by estimating the contribution of the exposure to an impurity due to the use of Primary Product to the health-based guidance value (HBGV) (expressed as percentage of the HBGV).

3.3.3.1. Toxic elements

The results of the analysis of arsenic, cadmium, lead and mercury in seven batches of the Primary Product were reported (Table 3).

The applicant proposed maximum limits for these toxic elements, which are the same as the limits in the current EU specifications (Table 7). The Panel noted that the actual measured levels of the toxic elements in commercial samples of the Primary Product were substantially lower than these limits.

The Panel assessed the risk that would result if these toxic elements were present in the Primary Product according to two concentration scenarios: (i) at the current limits in the EU specifications, and (ii) at the highest measured value for Cd multiplied by a factor of 5 by the Panel, and the LOQs multiplied by a factor of 10 by the Panel for As, Hg and Pb, for which the measured values were below the LOQs; this to account for variability with respect to representativeness, homogeneity and analytical measurement.

The outcome of the risk assessment for the two concentration scenarios and based on the highest mean and the highest 95th percentile exposure estimates among the different population groups (see Section 3.3.2) is presented in Table 13.

Table 13: Risk assessment for four toxic elements present in the Primary Product according to two concentration scenarios, using the reference points/health-based guidance values as provided in Table 12

Exposure to proFagus Smoke R709 (mg/kg bw/day)	(i) Considering the presence of toxic elements at the current EU specification limits for proFagus SMOKE R709			
	MOE for As at 3 mg/kg	% of the TWI for Cd at 1 mg/kg	MOE for Pb at 5 mg/kg	% of the TWI for Hg at 1 mg/kg
12.2[(a)]	8.2–219	3.4	8.2	2.1
51.4[(b)]	1.9–51.9	14.4	1.95	9.0
	(ii) Considering the presence of toxic elements at the highest measured value for Cd multiplied by a factor of 5, and the LOQs multiplied by a factor of 10 by the Panel for As, Hg and Pb			
	MOE for As at 1 mg/kg	% of the TWI for Cd at 0.15 mg/kg	MOE for Pb at 0.5 mg/kg	% of the TWI for Hg at 0.05 mg/kg
12.2[(a)]	24.6–656	0.5	82	0.1
51.4[(b)]	5.8–156	2.2	19.5	0.4

(a): Highest mean exposure level among the different population groups (proposed maximum use level exposure assessment scenario – children [Table 11]).
(b): Highest 95th percentile exposure level among the different population groups (proposed maximum use level exposure assessment scenario – infants [Table 11]).

When considering the current limits of the EU specifications (scenario (i) in Table 13), the Panel concluded that for arsenic, the ranges of the calculated MOE values were insufficient, i.e. below the target value of 1,000 (Table 12). For the other three toxic elements (cadmium, lead and mercury), the EU current specification limit values do not give rise to safety concerns.

When considering the highest measured value for Cd multiplied by a factor of 5 and the LOQs multiplied by a factor of 10 for As, Hg and Pb (scenario (ii) in Table 13), the Panel concluded that for arsenic, the ranges of the calculated MOE values were insufficient, i.e. below the target value of 1,000 (Table 12). The presence of the other toxic elements in the Primary Product does not give rise to concern.

Overall, the Panel considered that the limits in the EU specifications for arsenic, cadmium, lead and mercury should be established based on actual levels in the commercial Primary Product. If the European Commission decides to revise the current limits in the EU specifications, the estimated exposure to the toxic elements as described above could be considered.

3.3.3.2. Polycyclic aromatic hydrocarbons (PAHs)

The results of the analysis of 16 PAHs were reported by the applicant for seven batches of the Primary Product (Table 5).

The proposed limits for two of these PAHs (i.e. benzo[a]pyrene and benzo[a]anthracene) are below their respective limits of 10 and 20 μg/kg as laid down in Regulation (EC) No 2065/2003. However, the Panel noted that the actual measured levels for benzo[a]pyrene and benzo[a]anthracene in the Primary Product (Table 5) were substantially lower than the current limits in Regulation (EC) No 2065/2003.

According to the data submitted by the applicant, the Panel considered the maximum reported level of PAH4 (i.e. the sum of their LOQs) in the Primary Product, i.e. 2.0 μg/kg (Table 5). Based on this level, the Panel assessed the risk that would result if PAH4 were present in the Primary Product: (i) at

the specifications limits for the sum of benzo[a]pyrene and benzo[a]anthracene in the Primary Product, i.e. 30 μg/kg, as proposed by the applicant (Table 7) and setting the concentration of the other two members of PAH4 (chrysene and benzo[b]fluoranthene) at zero for the purpose of this concentration scenario, and also (ii) at the maximum reported level of PAH4 in seven batches of the Primary Product (Table 5). The outcome of the risk assessment for the two concentration scenarios and based on the highest mean and the highest 95th percentile exposure estimates among the different population groups (see Section 3.3.2) is presented in Table 14.

Table 14: Risk assessment for PAH4, i.e. benzo[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene and chrysene in the Primary Product according to two concentration scenarios, using the reference points/health-based guidance values as provided in Table 12

Exposure to proFagus Smoke R709 (mg/kg bw per day)	MOE for PAH4
	(i) Considering the presence of PAH4 at the sum of the specifications limits for benzo[a]pyrene and benzo[a]anthracene in proFagus Smoke R709 (30 μg/kg)
12.2[(a)]	9.29×10^5
51.4[(b)]	2.20×10^5
	(ii) Considering the presence of PAH4 at their maximum reported level in proFagus SMOKE R709 (2.0 μg/kg)
12.2[(a)]	1.39×10^7
51.4[(b)]	3.31×10^6

(a): Highest mean exposure level among the different population groups (proposed maximum use level exposure assessment scenario – children [Table 11]).
(b): Highest 95th percentile exposure level among the different population groups (proposed maximum use level exposure assessment scenario – infants [Table 11]).

The Panel concluded that the resulting MOEs for PAH4 were far above the target value of 10,000 for both concentration scenarios and both exposure estimates of the Primary Product (EFSA Scientific Committee, 2012) (Table 12).

Furthermore, the Panel noted that at the highest proposed maximum use level of the Primary Product in any of the food categories, i.e. 1,300 mg/kg food (Table 8), and the maximum reported level of PAH4 (i.e. the sum of their LOQs) in the Primary Product, i.e. 2.0 μg/kg, the concentration of PAH4 in food would be 2.6×10^{-3} μg/kg food, which is far below the lowest maximum level (ML) of these contaminants in any of the foods listed in Regulation (EU) 2023/915[12] (i.e. 1 μg PAH4/kg food).

3.4. Genotoxicity data

The present evaluation is conducted in line with the new EFSA guidance on smoke flavourings (EFSA FAF Panel, 2021) which encompasses all the EFSA guidance documents on genotoxicity (EFSA Scientific Committee, 2011, 2017b, 2019, 2021b). These documents were not available at the time when the smoke flavourings were evaluated previously by the CEF Panel. In addition, for the assessment of the renewal applications, the reliability and relevance of all submitted genotoxicity studies were evaluate by the FAF Panel (see Sections 3.4.1 and 3.4.2) based on the criteria, described in Appendix C.

3.4.1. Genotoxicity assessment of the individual components

The 40 identified and quantified components of proFagus Smoke R709 (SF-008) were evaluated individually for potential concern of 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, considering the *in silico* information/data first submitted by the applicant and then generated by EFSA (see Annex B).

Out of the 40 identified and quantified components, the applicant reported that 27 have already been evaluated by EFSA and/or JECFA/CoE and were concluded not to represent genotoxicity concern.

[12] Commission Regulation (EU) 2023/915 of 25 April 2023 on maximum levels for certain contaminants in food and repealing Regulation (EC) No 1881/2006. OJ L 119, 5.5.2023, p. 103–157.

For those components, the applicant relied on EFSA's conclusion on the genotoxic potential as set out in the respective Scientific Opinions of EFSA.

For one component, i.e. furan-2(5*H*)-one (CAS No. 497–23-4; formerly [FL-no. 10.066]), EFSA previously concluded that based on the available data, the substance is genotoxic *in vivo* (EFSA FAF Panel, 2019). Therefore, for this substance, further experimental studies were performed by the applicant (see Appendices B and D).

For the remaining 12 substances, applicant's conclusions were based on literature search as well as *in silico* prediction of genotoxicity endpoints using a combination of independent and scientifically valid quantitative structure–activity relationship (QSAR) models.

In silico data were generated by the applicant using toxicity prediction tool Derek Nexus (version 6.1.0)[13], applying the following models:

- Mutagenicity *in vitro* and *in vivo*
- Chromosome damage *in vitro* and *in vivo*

The OECD QSAR Toolbox v. 4.5[14] was also used by the applicant to complement the *in silico* analysis, applying the following profilers:

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

The *in silico* analysis was also complemented by read-across (grouping) considerations with metabolically and structurally related substances.

A short summary of the data available from the literature as submitted by the applicant and of the overall conclusions from the applicant on the genotoxicity of the individual components, including the *in silico* analysis, when available, is reported in Annex B of this opinion (see columns 'G' and 'I'). The complete set of information from the applicant is available under the section 'Genotoxicity' of the technical dossier (see Documentation provided to EFSA No.1).

In line with the EFSA guidance on smoke flavourings (EFSA FAF Panel, 2021), the Panel conducted a (Q)SAR analysis for all the 40 identified and quantified components of the Primary Product using the six profilers as available in the OECD QSAR Toolbox v. 4.5, as listed above.

As described in column 'K' of Annex B, reporting the EFSA's conclusions on the genotoxicity of the components of the Primary Product based on the available data, the individual structural alerts identified by the six profilers may have different positive predictivity (i.e. rate of positives to the total number of substances with the alert) for the genotoxicity of the target substance. The concepts of the alerts are described by the European Chemicals Agency (ECHA, 2008) and the predictivities of the individual alerts are documented by Benigni et al. (2008, 2009). When necessary, the application of profilers was followed by an expert review (e.g. check of close analogues/structurally related substances).

Overall, regarding the genotoxicity assessment of the individual components of the Primary Product, the Panel noted that:

i) For 36 identified components, based on the (often limited) genotoxicity data available from the literature either on the substance or on structurally related substances, the Panel concluded that the data did not indicate a concern for genotoxicity (see Annex B).
ii) for one component, i.e. furan-2(5*H*)-one (CAS No. 497-23-4, former [FL-no: 10.066]), the panel identified a concern for genotoxicity (see Annex B and Appendices B and D).
iii) for three components, i.e. glycoaldehyde dimer (CAS No. 23147–58-2), 3-methyl-2(5*H*)-furanone (CAS No. 22122-36-7) and 2,5-hexanedione (CAS No. 110–13-4) the Panel identified potential concern for genotoxicity for which additional data would be needed to reach a final conclusion on the genotoxic potential of these substances (see Annex B and Appendix B).

[13] https://www.lhasalimited.org/products/derek-nexus.htm
[14] https://www.oecd.org/chemicalsafety/oecd-qsar-toolbox.htm

The Panel investigated if the concern for genotoxicity for furan-2(5*H*)-one and the potential concern for genotoxicity for the three components listed in (iii) could be ruled out by application of the threshold of toxicological concern (TTC) approach for DNA-reactive mutagens and/or carcinogens (EFSA Scientific Committee, 2019). For this purpose, the Panel calculated the exposure to each of these components by multiplying the estimated exposure to the Primary Product (proposed maximum use level exposure assessment scenario, estimated with DietEx – Table 11) by the average content of these components in the Primary Product (see Appendix A).

The obtained exposure estimates were compared with the TTC value of 0.0025 μg/kg bw per day for DNA-reactive mutagens and/or carcinogens. All exposure estimates were at least a factor of 4120 above this TTC value (see Table 15), and therefore, the application of the TTC approach could not rule out the (potential) concern for genotoxicity for these components.

The lack of robust experimental data on genotoxicity for the three components listed in (iii) for which a potential concern for genotoxicity was identified is a non-standard uncertainty with respect to the genotoxicity assessment of the individual components (see section 2.2 of this opinion and Table G.1 of the EFSA guidance document on smoke flavourings (EFSA FAF Panel, 2021)). This uncertainty can only be addressed by filling the data-gaps with genotoxicity studies, as described in Appendix B.

Table 15: Dietary exposure in μg/kg bw per day to the four individual components for which a (potential) concern for genotoxicity has been identified (see Appendix B), based on the proposed maximum use level exposure assessment scenario using DietEx (Table 11)

CAS No.	Chemical Name	Average content in the Primary Product (wt%)	Exposure	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)	Ratio between the highest exposure estimate and TTC
Component of concern for genotoxicity										
497-23-4	furan-2(5*H*)-one (2(5*H*)furanone)	0.1	Mean	0.8–8.8	2.2–12.0	3.0–12.2	1.8–6.4	1.4–4.3	1.0–3.2	2.056×10^4
			95th percentile	2.7–51.4	5.6–41-8	5.9–36.8	3.5–19.9	2.8–13.6	2.3–10.1	
Components for which a potential concern for genotoxicity is identified										
23147-58-2	glycoaldehyde dimer	0.3	Mean	2.4–26.4	6.6–36.0	9.0–36.6	5.4–19.2	4.2–12.9	3.0–9.6	6.168×10^4
			95th percentile	8.1–154.2	16.8–125.4	17.7–110.4	10.5–59.7	8.4–40.8	6.9–30.3	
22122-36-7	3-methyl-2(5*H*)-furanone	0.08	Mean	0.6–7.0	1.8–9.6	2.4–9.8	1.4–5.1	1.1–3.4	0.8–2.6	1.644×10^4
			95th percentile	2.2–41.1	4.5–33.4	4.7–29.4	2.8–15.9	2.2–10.9	1.8–8.1	
110-13-4	2,5-hexanedione	0.02	Mean	0.1–1.8	0.4–2.4	0.6–2.4	0.4–1.3	0.3–0.9	0.2–0.6	4.12×10^3
			95th percentile	0.5–10.3	1.1–8.4	1.2–7.4	0.7–4.0	0.6–2.7	0.5–2.0	

3.4.2. Genotoxicity assessment of the Primary Product (whole mixture)

The applicant resubmitted the genotoxicity studies on the whole smoke flavouring mixture that were already evaluated by the CEF Panel in 2010, to investigate the genotoxicity of the unidentified fraction of the Primary Product, in line with the EFSA Scientific Committee statement on genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019): a bacterial reverse mutation test (Lab International Research Centre, 2005a), an *in vitro* mammalian cell gene mutation assay in mouse lymphoma cells (Lab International Research Centre, 2005b), an *in vitro* mammalian chromosomal aberration test (Lab International Research Centre, 2005c), an *in vivo* micronucleus assay in mouse bone marrow (Lab International Research Centre, 2005d) and an *in vivo* unscheduled DNA synthesis (UDS) (Research Laboratory, 2007).

The evaluation of these studies as described in the scientific opinion 'Safety of smoke flavour Primary Product – Scansmoke R909[15]' (EFSA CEF Panel, 2010) is reported in Section 3.4.2.1. For each study, comments and evaluation by the FAF Panel are reported. These studies are summarised in Tables E.1 and E.2 (Appendix E), where the evaluation of reliability and relevance are reported (according to the approach described in Appendix C).

The Panel noted that the general compositional data of the product evaluated in 2010 do not fundamentally deviate from the product assessed in the current opinion. In addition, as stated by the applicant, the manufacturing process has not changed and the batch-to-batch variability was low both in the previous evaluation (EFSA CEF Panel, 2010) and in the current opinion (see Table 6 in Section 3.1.2.6). Therefore, the Panel considered the Primary Product that was evaluated in 2010 similar to the Primary Product evaluated in this opinion and that the batch used for the genotoxicity testing in the past can still be considered representative for the current product.

In addition, new *in vivo* genotoxicity studies were provided, which are described in Section 3.4.2.2 and summarised in Appendix F.

The batch used in these newly submitted genotoxicity studies (no. 400918021) fell within the reported range of batch-to-batch variability and could be considered representative (see Section 3.1.3).

The Panel noted that information provided to confirm the absence of a fraction of small particles is not sufficient (see Section 3.1.2.7). Therefore, the conclusions reached for each of the genotoxicity studies described below is applicable only under the assumption that the material is covered by the conventional risk assessment and does not require a separate assessment regarding nanoscale properties.

3.4.2.1. Studies evaluated in EFSA opinion (EFSA CEF Panel, 2010)

3.4.2.1.1. Bacterial reverse mutation test (Lab International Research Centre, 2005a)

*'The Primary Product did not induce gene mutations in a bacterial assay which was performed using Salmonella typhimurium strains TA98, TA100, TA1535 and TA1537 and the Escherichia coli strain WP2 uvrA (..). The experiments have been carried out in the presence and absence of a metabolic activation system prepared from enzyme-induced rat liver. Phenobarbitone and ß-naphthoflavone were used for enzyme-induction. The assay was in accordance with OECD guideline 471 (*1997*).'* (EFSA CEF Panel, 2010).

The FAF Panel agreed with this evaluation and considered the study to be reliable without restrictions and its result of high relevance.

3.4.2.1.2. In vitro *mammalian cell gene mutation assay in mouse lymphoma cells (Lab International Research Centre, 2005b)*

'Scansmoke R909 induced statistically significant and dose-related increases in the mutant frequency in mouse lymphoma cells in the MLTK assay (performed in accordance with OECD guideline 476 (1997)) both in the absence (up to 4.9-fold at 0.85 mg/ml resulting in a Relative Survival of 26%) and presence of metabolic activation (up to 4.3-fold at 1.4 mg/ml resulting in a Relative Survival of 24%) (..). Both large and small colonies were induced at similar rates with and without metabolic activation (with small colonies somewhat predominating) suggesting that the genotoxic effects

[15] In the first safety assessment (EFSA CEF Panel, 2010) the name of the Primary Product was Scansmoke R909. However, as reported in recital (12) of Commission Implementing Regulation (EU) No 1321/2013 of 10 December 2013, the applicant notified to the Commission on 26 November 2012 that the name of the Primary Product had been changed to proFagus Smoke R709.

observed may be due to both clastogenicity and the induction of gene mutations in this assay. The metabolic activation system was prepared from rat liver.' (EFSA CEF Panel, 2010).

The FAF Panel agreed with the previous evaluation of the CEF Panel that the Primary Product gave clearly positive results in all testing conditions.

The increase was to be considered positive also applying the global evaluation factor proposed in the successive OECD TG 490 (OECD, 2016a), starting from the concentrations of 1,200 μg/mL for 3 h exposure with S9-mix, 650 μg/mL for 3 h exposure without S9-mix and 550 μg/mL for 24 h exposure without S9-mix. Historical controls were not reported.

Therefore, the Panel considered the study to be reliable with restrictions and its positive results of limited relevance.

3.4.2.1.3. In vitro *mammalian chromosomal aberration test (Lab International Research Centre, 2005c)*

'The Primary Product did not induce chromosomal aberrations in Chinese Hamster Ovary cells in vitro in an assay performed in accordance with OECD guideline 473 (1997) (..). The experiments have been carried out in the presence and absence of a metabolic activation system. The type of S9-fraction was not reported, however, since the assay has been performed in the same laboratory as the bacterial assay it could be assumed that the metabolic activation system used was prepared from enzyme-induced rat liver likewise.' (EFSA CEF Panel, 2010).

The FAF Panel agreed that the Primary Product did not show evidence of chromosomal damage in this study. However, the study has some limitations: only 200 metaphases/concentration instead of 300 were scored as recommended in the most recent version of the OECD TG 473 (OECD, 2016b) and the method applied to assess the cytotoxicity was not in accordance with OECD TG 473 (OECD, 2016b).

The study was considered reliable with restrictions and the relevance of the negative result was evaluated as limited.

3.4.2.1.4. In vivo *bone marrow mouse micronucleus test (Lab International Research Centre, 2005d)*

'In vivo, the Primary Product was tested in a mouse micronucleus assay performed in accordance with OECD guideline 474 (1997) (..). It was administered orally to male and female mice at dose levels of 500, 1000 and 2000 mg/kg. After exposure to the Primary Product a statistically significant increase in the percentage of micronucleated polychromatic erythrocytes (MNPCE) in bone marrow of female mice at the lowest dose at 24 hours (1.75-fold compared to control) was reported. However, a dose-related increase was not observed as at 1000 mg/kg the increase was 1.4-fold and not significant and at 2000 mg/kg there was no increase at all. In addition, there was no change in MNPCE in the males at this time point at any dose. At 48 hours, a dose-related slight increase in male mice (up to 1.6-fold compared to control at the highest dose level) was seen, but this increase did not reach statistical significance. At 48 hours in females, there was a slight but statistically non-significant increase, but only at the mid-dose. The Panel noted that the data were not consistent between dose levels, time points and sexes and that the magnitude of these changes was very small. In addition, increases in MNPCE at later time points (i.e. after 48 hrs) are normally associated with indications of cytotoxicity but no such toxicity (recorded as changes in PCE/NCE ratios) was observed. Based on this variability and the lack of consistence between changes in MNPCE and (absence of) changes in PCE/NCE ratios, the Panel concluded that this study does not provide evidence for genotoxicity of the Primary Product in vivo.' (EFSA CEF Panel, 2010).

Considering the current version of the OECD TG 474 (OECD, 2016c) according to which the exposure of the bone marrow to the test substance needs to be demonstrated, the FAF Panel noted that there was no indication of bone marrow toxicity that could be considered as evidence of bone marrow exposure. Since only minor effects were observed in clinical investigations and since there were no other lines of evidence for systemic bioavailability that could be indicative of bone marrow exposure, the FAF Panel concluded that the result of this study was inconclusive. Moreover, no historical controls were reported.

It should also be noted that, according to the statement on genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019), even if there were demonstration of bone marrow exposure, the assessment of genotoxicity of mixtures in the bone marrow is limited by the fact that target tissue exposure to all potential genotoxic components cannot be demonstrated unequivocally.

Therefore, the Panel considered the study as reliable with restrictions and the study result of low relevance.

3.4.2.1.5. In vivo *rat liver UDS assay (Research Laboratory, 2007)*

'Scansmoke R909 was tested in vivo in an unscheduled DNA synthesis (UDS) assay which was performed in compliance with GLP (Research Laboratory, 2007). UDS was assessed in hepatocytes of Sprague Dawley rats following oral gavage administration of Scansmoke R909 on two separate occasions (the second dose being administered 14 hours after the first dose and 2 hours before perfusion). This study design deviated slightly from the OECD guideline 486 (1997) with respect to dosing and sampling. However, the protocol was considered acceptable. Scansmoke R909 was administered at dosages of 600 and 2000 mg/kg bw. Under the conditions of this study, Scansmoke R909 did not induce UDS in vivo.' (EFSA CEF Panel, 2010).

The study report on the *in vivo* UDS assay was included in the new dossier. However, the applicant considered that the negative results observed in this assay do not contribute to the overall assessment of genotoxicity. The FAF Panel agreed with this consideration and confirmed that the results of a negative UDS study are of low relevance, based on the EFSA Scientific Committee opinion on the adequacy of the UDS assay to follow-up positive results in *in vitro* gene mutation tests (EFSA Scientific Committee, 2017b).

3.4.2.2. New genotoxicity studies

Based on the available data and on the requirements of the EFSA guidance on smoke flavouring Primary Products (EFSA FAF Panel, 2021), new genotoxicity studies were submitted: an *in vitro* micronucleus test (Labcorp, 2022), an *in vivo* micronucleus test (BSRC, 2022a) and an *in vivo* gene mutation test in transgenic rodents (BSRC, 2022b).

3.4.2.2.1. In vitro *genotoxicity studies*

3.4.2.2.1.1. *In vitro* mammalian cell micronucleus test with FISH analysis

An *in vitro* micronucleus assay, with cytokinesis block protocol, was carried out based on the general provisions in OECD TG 487 (OECD, 2016d) and in compliance with good laboratory practices (GLP). Duplicate cultures of human peripheral blood lymphocytes from healthy donors were treated with proFagus Smoke R709 (batch no. 400918021) (Labcorp, 2022).

Treatments started after a 44–48 h stimulation period with phytohaemagglutinin. A single experiment tested the following exposure conditions: 4 h exposures with and without metabolic activation by phenobarbital–5,6-benzoflavone-induced rat liver S9 fraction (S9-mix), followed by a 24-h recovery period in the presence of cytochalasin B and a 24 h exposure without S9-mix and without cytochalasin B followed by a 24-h recovery period in the presence of cytochalasin B. The Panel noted that the extended treatment exposure conditions differed from the suggested cell treatment schedule in OECD TG 487 (OECD, 2016d). However, the Panel considered that the protocol applied for the extended treatment could potentially enhance the sensitivity of the micronucleus test (Whitwell et al., 2019); therefore, the Panel did not consider this aspect as a limitation.

Positive controls were cyclophosphamide, mitomycin C (MMC) and demecolcine. Sterile distilled water was used as negative control.

The concentrations tested in the micronucleus experiment were selected based on the cytokinesis block proliferation index (CBPI) cytotoxicity data detected in a cytotoxicity range-finding experiment carried out at a range of concentrations up to 5 mg/mL.

Lymphocytes were treated with proFagus Smoke R709 at eight and ten concentrations ranging from 117.2 to 2,500 μg/mL in the 4 h treatments in the absence and in the presence of S9-mix, respectively, and at eight concentrations from 117.2 to 1,250 μg/mL in the 24 h treatment in the absence of metabolic activation. No precipitate of the test item was noted in any of the exposure conditions.

In the treatment of 4 h + 24 h in the presence of S9-mix, the following concentrations were chosen for MN analysis: 625, 1,250 and 1,562.5 μg/mL (cytotoxicity of 26%, 34% and 48%, respectively). An additional 1,000 binucleated cells were scored to help clarify the result and increase the statistical power of the data. A statistically significant increase in the bi-nucleated cells with micronuclei (MNBN) was observed at 1,250 and 1,562.5 μg/mL (0.90% and 1.25%, respectively) compared to the concurrent vehicle control (0.21%), and these values were also outside the range of the 95% control limits of the historical negative control (0–0.81%).

In the treatment of 4 h + 24 h in the absence of S9-mix, the following concentrations were chosen for MN analysis: 625, 1,250 and 1,406.2 μg/mL (cytotoxicity of 28%, 36% and 51%, respectively). An additional 1,000 binucleated cells were scored to help clarify the result and increase the statistical

power of the data. A statistically significant increase in the frequency of MNBN was observed at 1,250 and 1,406.2 μg/mL (0.63% and 1.18%, respectively) compared to the concurrent negative control (0.18%), and the MNBN frequency for the top concentration was also outside the range of the 95% control limits of the historical negative control (0.08–0.72%).

In the treatment of 24 h + 24 h in the absence of S9-mix, the following concentrations were chosen for MN analysis: 234.4, 625 and 937.5 μg/mL (cytotoxicity of 24% and 44% for the mid and top concentrations, respectively). A statistically significant increase in the frequency of MNBN was observed at 937.5 μg/mL (1.2%) compared to the concurrent negative control (0.28%), which was also outside the range of the 95% control limits of the historical negative control (0.01–0.69%).

In all three test conditions, proFagus Smoke R709 induced statistically significant increases in the frequency of MNBN compared to vehicle controls, and concentration-dependent trends. Due to the clear positive response observed in all exposure groups, fluorescence *in situ* hybridisation (FISH) analysis was performed.

The results of the FISH analysis showed that 91%, 91% and 87% of the micronuclei induced by the corresponding top concentrations of proFagus Smoke R709 analysed for the 4 h exposure in the absence of S9-mix, for the 4 h exposure in the presence of S9-mix and for the 24 h exposure in the absence of S9-mix, respectively, were centromere-negative. The negative controls showed 86–88% micronuclei negative for centromere labelling. The clastogenic positive control, MMC, had a mean of 96% micronuclei negative for centromere labelling. The aneugenic positive control, demecolcine, had 35% micronuclei negative for centromere labelling. Therefore, the authors of this study considered that proFagus Smoke R709 induced micronuclei in human peripheral blood lymphocytes, under all exposure conditions, via predominant clastogenic mechanism.

The Panel agreed with this conclusion and considered the study reliable without restrictions and the positive results of high relevance.

Results of *in vitro* studies are summarised in Appendix F, Table F.1.

3.4.2.2.2. In vivo *genotoxicity studies*

The concentrations of the tested Primary Product in the formulations were confirmed by the analysis of 2(5*H*)-furanone as a typical component of the Primary Product. The applicant submitted a validated analytical method for the determination of 2(5*H*)-furanone in water for injection using high performance liquid chromatography (HPLC) (BSRC, 2022c).

The stability of test article formulations (1 and 100 mg/mL in water for injection) was confirmed in a separate study using the validated analytical method for the determination of 2(5*H*)-furanone (BSRC, 2022d). The Panel noted that the stability of the Primary Product in the test article formulations (within 8 days) was also confirmed by visual comparison of the HPLC chromatograms provided in the study report.

Additionally, the stability of test article formulations at a concentration of 300 mg/mL was confirmed in the study report for the *in vivo* gene mutation assay in Muta™Mouse (BSRC, 2022b).

The applicant provided the study report on the validation of an analytical method (LC–MS/MS) for determination of proFagus Smoke R709 in mouse plasma, using 2,6-dimethoxyphenol as analytical standard. However, a toxicokinetic study was not conducted, because the highest dose tested in the *in vivo* MN study and in the *in vivo* gene mutation study in transgenic rodents was considered by the applicant sufficiently high to maximise exposure of the target tissues as reported in the sections below.

3.4.2.2.3. In vivo *micronucleus assay*

ProFagus Smoke R709 (batch no. 400918021) was tested in a bone marrow MN assay in mice which was performed in compliance with GLP and according to OECD TG 474 (OECD, 2016c) (BSRC, 2022a).

A preliminary toxicity test was performed to identify the appropriate maximum dose level for the main test. Groups of three B6D2F1/Slc [SPF] male mice were treated twice at 24 h intervals by oral gavage at 2,500, 5,000, 7,500 and 10,250 mg/kg bw per day. In the top dose group, two mice died and another was humanely euthanised, in the 7,500 mg/kg bw per day group and the 5000 mg/kg bw per day group, three and two animals died, respectively. No mortality and no adverse reactions to treatment were observed in the low dose group, and therefore, a dose of 3,000 mg/kg bw per day was considered the maximum tolerated dose (MTD).

Groups of six B6D2F1/Slc [SPF] male mice were treated via oral gavage with proFagus Smoke R709 at doses of 0 (vehicle control, water), 750, 1,500 and 3,000 mg/kg bw per day. A single administration of cyclophosphamide administered orally at the concentration of 25 mg/kg bw was used as positive

control. Animals were dosed at 0 and 24 h, except the positive control group that was dosed only at 24 h. There were no clinical signs and no apparent suppression of body weight gain observed in any of the treatment groups.

Twenty-four hours after the final administration, animals were sacrificed, and femoral bone marrow was harvested and prepared for the MN analysis (three bone smears per animal) for five animals per group. A total of at least 500 polychromatic erythrocytes (PCE) and normochromatic erythrocytes (NCE) were scored to assess potential bone marrow toxicity by the relative decrease in PCE. For the MN analysis, 4000 PCE per animal were scored for the presence of MN.

The vehicle control data were comparable with the laboratory's historical vehicle control data. Positive controls resulted in a statistically significant increase in MNPCE (over the concurrent vehicle control), which was comparable to the laboratory's historical positive control data.

In all three dose groups of mice treated with proFagus Smoke R709, there were no statistically significant increases in MNPCE frequency compared to the vehicle controls. Individual frequencies of MNPCE for all treated animals were consistent with historical vehicle control data.

The PCE/NCE ratio was not affected by treatment with proFagus Smoke R709: 56.6 ± 2.3%, 55.8 ± 2.6%, 57.4 ± 3.2%, and 55.6 ± 4.1% at 0, 750, 1,500 and 3,000 mg/kg bw per day, respectively. The PCE/NCE ratio for the positive control was 58.0 ± 2.8%.

The study authors concluded that proFagus SMOKE R709 did not induce micronucleated erythrocytes in mouse bone marrow cells under the conditions of this study (BSRC, 2022a).

The applicant considered that the highest dose tested of 3,000 mg/kg bw per day, being higher than the limit dose of 2,000 mg/kg/day (OECD TG 474, 2016c), allows to maximise the exposure of bone marrow to the components of the mixture.

However, there was no evidence of bone marrow toxicity based on the PCE/NCE ratio and there appears to be insufficient evidence of systemic exposure based on the absence of toxicity observed in the treatment groups.

It should also be noted that, according to the statement on genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019), even if there were demonstration of bone marrow exposure, the assessment of genotoxicity of mixtures in the bone marrow is limited by the fact that target tissue exposure to all potential genotoxic components cannot be demonstrated unequivocally.

The Panel considered the study as reliable with restrictions (due to insufficient evidence of bone marrow exposure) and the inconclusive result of low relevance.

3.4.2.2.4. In vivo *gene mutation assay in Muta™Mouse transgenic mice*

ProFagus Smoke R709 (batch no. 400918021) was tested in a 14-day dose range finding (non-GLP) study in CD2F1/Slc mice (i.e. wild-type Muta™Mouse), in order to determine the MTD and dose levels for the transgenic rodent (TGR) gene mutation assay using the same rodent strain (BSRC, 2022e).

ProFagus Smoke R709 was initially administered via oral gavage (water for injection used as vehicle) to groups of CD2F1/Slc mice (three animals per sex per group) corresponding to the following dose levels: 0, 250, 500 and 1,000 mg/kg bw per day. As no signs of toxicity were observed, an additional study of 7 days of administration using doses of 3,000 and 10,250 mg/kg bw per day (i.e. the maximum dose that could practically be administered as undiluted test liquid) was conducted (two animals per sex per group). In both studies, animals were observed daily for clinical signs.

In the 10,250 mg/kg bw per day group, one male and one female were sacrificed moribund, and another female died. Both increases and decreases in locomotor activity, piloerection, emaciation, irregular respiration and incomplete eyelid opening were observed in the moribund male, and a decrease in locomotor activity, hypothermia and irregular respiration were observed in the moribund female. At scheduled necropsy, thickening of the forestomach was observed in one male in the 10,250 mg/kg bw per day group. There were no signs of toxicity observed in the 3,000 mg/kg bw per day group. No gender-specific differences were observed.

There were no obvious body weight changes, clinical signs of toxicity nor macroscopic changes at necropsy observed with proFagus Smoke R709 treatment at 3,000 mg/kg bw per day. Based on these results in which 3,000 mg/kg bw per day was considered to be the MTD, the study authors selected doses of 250, 500, 1,000 and 3,000 mg/kg bw per day for the *in vivo* gene mutation assay with Muta™Mouse (BSRC, 2022e).

In the *in vivo* gene mutation assay in Muta™Mouse (BSRC, 2022b), proFagus Smoke R709 (batch no. 400918021) was administered via oral gavage (water for injection used as vehicle) to four groups of male transgenic CD2-LacZ80/HazfBR mice (Muta™Mouse) (six animals per group) at dose levels of 0, 250, 500, 1,000 and 3,000 mg/kg bw per day for 28 consecutive days. The study was performed

according to OECD TG 488 (OECD, 2020a) and in compliance with GLP. The treatment period was followed by a 3-day manifestation period and then animals were sacrificed, and the liver, stomach and duodenum removed. N-Ethyl-N-nitrosourea (ENU) administered intraperitoneally at a dose of 100 mg/kg bw per day for two consecutive days followed by a 10-day manifestation period was used as concurrent positive control. Test item formulations were prepared on a weekly basis, 1–2 days prior to first use. The positive control was prepared just before use.

There were no clinical signs and no differences in body weight gain or food consumption observed for any group. At necropsy, in the top dose group, a white patch was observed in the forestomach of all animals and a thickening of the duodenum was observed in two animals. There were no other macroscopic findings related to treatment in the other groups.

Mutation frequency was determined for all but the low dose group. Liver, duodenum and stomach samples from five animals per group (including controls) were processed for DNA isolation. For each DNA sample, the number of plaques from 1 packaging was greater than 300,000 (i.e. more than the OECD recommended minimum of 125,000 plaques).

Treatment with proFagus Smoke R709 did not significantly increase the mutation frequency at the *lacZ* gene in liver, stomach or duodenum of Muta™Mouse mice compared to the concurrent negative control and the historical negative control data.

The applicant considered that the highest dose tested of 3,000 mg/kg bw per day, being higher than the limit dose of 1,000 mg/kg per day (OECD TG 488, 2020a), allows to maximise the exposure of liver to the components of the mixture.

The Panel acknowledged that the top dose of 3,000 mg/kg bw per day was higher than the limit dose recommended in the OECD TG 488 (OECD, 2020a), as per EFSA recommendations.[16] Although, no clinical signs of toxicity were observed at 3,000 mg/kg bw per day, some signs of tissue damage were observed in the stomach and duodenum at this dose, demonstrating that the exposure of these tissues could not have been very much higher. The study authors concluded that in this *in vivo* gene mutation assay in Muta™Mouse, proFagus Smoke R709 did not induce gene mutations in the liver, stomach or duodenum. The Panel agreed with this conclusion. The Panel considered the study reliable without restrictions and the negative result of high relevance.

Results of *in vivo* studies are summarised in Appendix F, Table F.2.

4. Discussion

The European Commission has requested the European Food Safety Authority (EFSA) to evaluate the safety of the smoke flavouring Primary Product proFagus Smoke R709 (SF-008) (formerly named and authorised as Scansmoke R909), for which a renewal application has been submitted, in accordance with Article 12(1) of Regulation (EC) No 2065/2003.

The Primary Product is produced from a mixture of beech (*Fagus sylvatica* > 90%) and oak (*Quercus robur* < 10%); other wood species might be present at < 1%.

The production of the Primary Product begins with the pyrolysis of the dried wood pieces and the condensation of the generated wood gas. The obtained smoke condensate is further concentrated by evaporation; after adjustment of the total acid content, the resulting 'pyroligneous acid' forms the smoke flavouring Primary Product proFagus Smoke R709. The Panel considered the information provided on the manufacturing process as sufficient. The data demonstrated that the Primary Product is produced in the same way as the product evaluated formerly (EFSA CEF Panel, 2010).

The applicant provided compositional data for seven batches of the Primary Product. The Panel noted that the applied method meets the legal quality criterion that at least 80% by mass of the volatile fraction shall be identified and quantified (Regulation (EC) 627/2006).

For the investigated batches, the identified and quantified proportion of the solvent-free fraction was on average approximatively 66 wt% (range from 62.0 to 72.4 wt%). Thus, the applied methods meet the legal quality criterion that at least 50% of the solvent-free fraction shall be identified and quantified (Regulation (EC) No 627/2006).

Data provided for the investigated batches of the Primary Product demonstrated that their batch-to-batch variability was sufficiently low (i.e. the observed relative standard deviations for the individual constituents was on average below 11%), based on the analytical data for the 20 principal volatile constituents and the chemical classes. The Panel noted that the applicant has adequate control over

[16] https://www.efsa.europa.eu/sites/default/files/2021-10/24th-plenary-meeting-faf-panel-proposed-be-open-observers-minutes.pdf

the relevant steps of the production process (pyrolysis and purifications) and concluded that the data provided in the selected batches are representative of the Primary Product.

Based on the data provided, the Panel considered that the combined evidence is not conclusive to exclude the possible presence of small particles including nanoparticles for the Primary Product. If, based on additional evidence, the presence of small particles including nanoparticles cannot be eventually excluded in the Primary Product, a specific assessment at the nanoscale would be required, in line with the EFSA Scientific Committee Guidance on risk assessment of nanomaterials (EFSA Scientific Committee, 2021a,b).

The applicant proposed limits for four toxic elements (arsenic, cadmium, lead and mercury), which are the same as in the current EU specifications (Table 7). The panel noted that the actual measured levels for these elements in seven batches of the Primary Product (Table 3) were substantially lower than these limits.

The Panel performed a risk assessment on the presence of these toxic elements in the Primary Product and concluded that, when considering the current limits of the EU specifications (scenario (i) in Table 13), the ranges of the calculated MOE values for arsenic were insufficient, i.e. below the target value of 1,000. For the other three toxic elements (cadmium, lead and mercury), their presence in the Primary Product up to the current limits in the EU specifications does not give rise to a safety concern. When considering the highest measured value for Cd multiplied by a factor of 5 and the LOQs multiplied by a factor of 10 for As, Hg, and Pb (scenario (ii) in Table 13), the Panel concluded that for arsenic the ranges of the calculated MOE values were still insufficient, i.e. below the target value of 1,000. In this scenario, the presence of the other toxic elements in the Primary Product does not give rise to concern.

The analytical procedure for the determination of 16 PAHs meets the performance criteria as set in Regulation (EC) No 627/2006. The levels of benzo[a]pyrene and benzo[a]anthracene were below the current limits in Regulation (EC) No 2065/2003. Based on the estimated exposure to the Primary Product and the maximum reported level of the PAH4 in the Primary Product (i.e. 2.0 μg/kg), an MOE of at least 3.31×10^6 could be calculated for the exposure to PAHs, which would be of low concern from a public health point of view and might be reasonably considered as a low priority for risk management actions (see EFSA Scientific Committee, 2012). The Panel noted that including a limit for PAH4 in the EU specifications would take better account of the presence of other PAHs than only the two PAHs benzo[a]pyrene and benzo[a]anthracene.

Overall, the Panel considered that limits in the EU specifications for the four toxic elements and PAH4 should be established based on actual levels in the commercial Primary Product. If the European Commission decides to revise the limits already present and to include a limit for PAH4, the estimated exposure to the four toxic elements and PAH4 as presented in Sections 3.3.3.1 and 3.3.3.2 could be considered.

The Primary Product is requested to be authorised for use in 25 food categories. The Panel performed an exposure assessment for this product based on proposed maximum and expected typical use levels in these food categories, using both FAIM and DietEx. In general, the use of FAIM or DietEx results in an overestimation of the exposure. However, since DietEx allows a better selection of the actual foods to which the Primary Product may be added, the DietEx exposure estimates have been used for the risk assessment of the Primary Product.

At the maximum proposed use levels, mean DietEx exposure estimates to the Primary Product from its use as a smoke flavouring ranged from 0.8 mg/kg bw per day in infants to 12.2 mg/kg bw per day in children (Table 11). The 95th percentiles DietEx exposure estimates ranged from 2.3 mg/kg bw per day in the elderly to 51.4 mg/kg bw per day in infants. At the expected typical use levels, the mean DietEx dietary exposure estimates ranged from 0.04 mg/kg bw per day in infants to 2.4 mg/kg bw per day in children, and the 95th percentile DietEx exposure estimates ranged from 0.1 mg/kg bw per day in infants and the elderly to 12.6 mg/kg bw per day in infants (Table 11).

Regarding the genotoxicity data, the Panel conducted the evaluation in line with the currently applicable EFSA guidance on smoke flavourings (EFSA FAF Panel, 2021) which encompasses all the EFSA guidance documents on genotoxicity (EFSA Scientific Committee, 2011, 2017b, 2019, 2021b).

From the analysis of the available information on genotoxicity of the 40 individual components of the Primary Product, the Panel considered that:

i) for 36 individual components no concern for genotoxicity is identified (see Annex B);
ii) a concern for genotoxicity is identified for furan-2(5*H*)-one which is present in the Primary Product at average concentration of 0.1 wt%;
iii) for three components a potential concern for genotoxicity is identified, for which additional data would be to reach a conclusion on the genotoxic potential of these substances.

The details of the genotoxicity data available on the four components listed in (ii) and (iii) are given and discussed in Appendix B.

Regarding furan-2(5*H*)-one, the available data raise a concern for genotoxicity. As described in detail in Appendix B, furan-2(5*H*)-one induced MN in liver of rats. Based on the positive results observed in the *in vivo* MN study, the applicant submitted a study to investigate the pro-oxidative potential of furan-2(5*H*)-one and an *in vitro* Multiflow® screen with the aim of clarifying the mode of action (MOA) and to determine whether the genotoxic effect observed is threshold mediated. The Panel considered that there is inadequate evidence to suggest that the genotoxicity of furan-2(5*H*)-one is mediated through reactive oxygen species (ROS) production. An association of reactive oxygen species (ROS) production with DNA strand breaks and toxicity is not evidence of a causative role. Results from the *in vitro* MultiFlow® test on TK6 cells support a clastogenic MOA for furan-2(5*H*)-one. Overall, these new studies confirm the genotoxicity of furan-2(5*H*)-one, for which the Panel already expressed a concern in flavouring group evaluation (FGE) 217Rev2 (EFSA FAF Panel, 2019).

The Panel investigated if the concern for genotoxicity for furan-2(5*H*)-one and the potential concern for genotoxicity for the 3 components mentioned above in (iii) could be ruled out by application of the Threshold of Toxicological Concern (TTC) approach for DNA-reactive mutagens and/or carcinogens (EFSA Scientific Committee, 2019). The obtained exposure estimates were compared with the TTC value of 0.0025 μg/kg bw per day for DNA-reactive mutagens and/or carcinogens. For all the 4 substances, the exposure estimates were well above this TTC value (see Table 15) and therefore the application of the TTC approach could not rule out the (potential) concern for genotoxicity for these components.

The Panel considered whether refined exposure estimates for the Primary Product (in line with the principles described in the guidance on smoke flavourings (EFSA FAF Panel, 2021)) could mitigate the concern for the (potential) genotoxicity of each of these 4 components. However, taking into account:

- the magnitude of the calculated ratios between the exposure estimates and the above mentioned TTC value (see Table 15);
- the uses of the Primary Product and the nature of the restrictions/exceptions indicated by the applicant for the different food categories (see Table 8),

the Panel considered that a more refined exposure assessment will not reduce the exposure estimates for these components to such an extent that they will be below the TTC value of 0.0025 μg/kg bw per day.

The Primary Product (whole mixture) was tested in *in vitro* and *in vivo* genotoxicity studies to investigate the genotoxicity of the unidentified fraction of the Primary Product, in line with the EFSA Scientific Committee statement on genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019).

The Primary Product, *in vitro*, did not induce gene mutations in a bacterial reverse mutation test, but it induced gene mutations in mammalian cells. However, *in vivo*, it did not induce gene mutations in liver, stomach and duodenum of transgenic mice.

The Primary Product did not show evidence of chromosomal damage in an *in vitro* chromosomal aberration assay in CHO-KI cells (study results of limited relevance), but it showed clastogenic effects in an *in vitro* micronucleus test in human peripheral blood lymphocytes. The Primary Product was tested in two *in vivo* MN studies in bone marrow. In both studies no increase in micronucleated cells was observed, but studies results were considered of low relevance due to insufficient demonstration of bone marrow exposure.

Since the results of the *in vivo* MN studies are of low relevance, they are not sufficient to rule out the concern for genotoxicity for the whole mixture that is raised by the observation of chromosomal aberrations in the *in vitro* MN assay.

In principle, based on the EFSA Scientific Committee statement on genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019) as well as on the EFSA guidance on smoke flavourings (EFSA FAF Panel, 2021), if aneugenicity can be excluded, an *in vivo* Comet assay (OECD TG 489 (2016e)) at the site of contact and in the liver might also be considered appropriate to follow up the chromosomal aberrations observed *in vitro*. The studies at the site of contact allow investigation of genotoxic effects at the site where the exposure to the components is expected to be maximal. However, in this case, the concern for genotoxicity for the Primary Product cannot be ruled out by an additional *in vivo* Comet assay performed on the whole mixture, since the exposure estimate for the genotoxic component furan-2(5*H*)-one is above the TTC value of 0.0025 μg/kg bw per day for DNA-reactive mutagens and/or carcinogens. In fact, as outlined in the Scientific Committee statement on genotoxicity assessment on chemical mixtures (EFSA Scientific Committee, 2019), 'if the mixture contains one or more chemical substances that are evaluated to be genotoxic *in vivo* via a relevant route of administration, the whole mixture raises concern about genotoxicity.'

For the same reason, the Panel noted that, filling of the data-gaps for the three components that raise a potential concern for genotoxicity, as pointed out in Appendix B, will not remove the safety concern for the Primary Product.

5. Conclusions

In line with the ToR as provided by the European Commission, in the current opinion EFSA assessed the chemical characterisation, the genotoxicity, and the dietary exposure to proFagus Smoke R709 (SF-008).

From all data available on characterisation, the Panel concluded that the Primary Product considered in this opinion is representative for the one authorised in Commission Implementing Regulation (EU) No 1321/2013 under the code name SF-008. The Panel concluded that the compositional data provided on the Primary Product were adequate. The Panel concluded that the applicant has adequate control over the production process and that the Primary Product is sufficiently stable upon storage.

Based on the data submitted by the applicant, the Panel could not exclude the presence of small particles including nanoparticles and hence could not conclude if conventional risk assessment is sufficient or whether it needs to be complemented with nano-specific considerations.

The Panel identified a potential concern for genotoxicity for three components in the Primary Product as well as for the unidentified fraction of the mixture. More importantly, the Primary Product contains furan-2(5*H*)-one, a known *in vivo* genotoxic substance via the oral route. Considering that the exposure estimates for furan-2(5*H*)-one 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 proFagus Smoke R709 (SF-008) raises concern with respect to genotoxicity.

6. Documentation as provided to EFSA

1) Dossier 'Application for renewal of an already authorised smoke flavouring – proFagus Smoke R709'. Dossier number: SFL-2021-2373. June 2022. Submitted by proFagus GmbH.[6]
2) Additional data received on 10 February 2023, submitted by proFagus GmbH in response to additional data request from EFSA sent on 21 December 2022.
3) Additional data received on 9 and 10 May 2023, submitted by proFagus GmbH as spontaneous submissions.
4) BSRC, 2022a. Micronucleus Test of proFagus SMOKE R709 in Mice. BioSafety Research Center Inc., Japan. Experiment No. K433 (820-018). May 2022. Unpublished study report submitted by proFagus GmbH.
5) BSRC, 2022b. In Vivo Gene Mutation Assay of proFagus SMOKE R709 in MutaMouse. BioSafety Research Center Inc., Japan. Experiment No. K033 (820-004). April 2022. Unpublished study report submitted by proFagus GmbH.
6) BSRC, 2022c. Validation of Analytical Method for Determination of proFagus SMOKE R709 in Water for Injection [Non-GLP]. BioSafety Research Center Inc., Japan. Experiment No. K030 (820-001). September 2021. Unpublished study report submitted by proFagus GmbH.
7) BSRC, 2022d. Stability Study of proFagus SMOKE R709 in Water for Injection. BioSafety Research Center Inc., Japan. Experiment No. K031 (820-002). October 2021. Unpublished study report submitted by proFagus GmbH.
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16) LSIM, 2021. Analytical Method Validation for Determination of Furan-2(5*H*)-one in Dosing Formulations. LSIM Safety Institute Corporation, Japan. Study No. B210495. December 2021. Unpublished study report submitted by proFagus GmbH.
17) LSIM, 2022a. A Repeated Dose Liver Micronucleus Assay of Furan-2(5*H*)-one in Rats. LSIM Safety Institute Corporation, Japan. Study No. B210475. June 2022. Unpublished study report submitted by proFagus GmbH.
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Abbreviations

BMDL	benchmark dose lower limit
BSRC	Bioscience Research center
BW	body weight
CA	chromosomal aberration
CAS	Chemical Abstract Service
CBPI	cytokinesis-block proliferation index
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CHO	Chinese hamster ovary
CM-H2DCF	chloromethyl-2′,7′-dichloro-dihydro-fluorescein
CONTAM	Panel on Contaminants in the Food Chain
DCFH	2′,7′-dichloro-dihydro-fluorescein
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
ECHA	European Chemicals Agency

EMS	ethyl methanesulfonate
ENU	N-ethyl-N-nitrosourea
FAF	Panel on Food Additives and Flavourings
FAIM	Food Additive Intake Model
FC	food category
FGE	flavouring group evaluation
FISH	fluorescence *in situ* hybridisation
FL-no	FLAVIS number
GC-FID	gas chromatography-flame ionization detection
GC-MS	gas chromatography-mass spectrometry
GEF	global evaluation factor
GLP	good laboratory practices
GSH	reduced glutathione
H3P	H3 phosphorylation
HBGV	health-based guidance values
HPLC	high performance liquid chromatography
ICP-MS	inductively coupled plasma-mass spectrometry
IQ	intelligence quotient
ISS	Istituto Superiore di Sanità
ITEM	Institute for Toxicology and Experimental Medicine
IWGT	international workshops on genotoxicity testing
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC-MS	liquid chromatography-mass spectroscopy
LDH	lactate dehydrogenases
ML	maximum level
MMC	mitomycin C
MN	micronucleus
MNBN	bi-nucleated cells with micronuclei
MNHEP	micronucleated hepatocytes
MNPCE	micronucleated polychromatic erythrocytes
MOE	margin of exposure
MS	mass spectrometry
NCE	normochromatic erythrocytes
OECD	Organisation for Economic Co-operation and Development
P95	95th percentile
PAHs	polycyclic aromatic hydrocarbons
PCE	polychromatic erythrocytes
PCLS	precision cut liver slices
Pt-NP	platinum nanoparticle clusters
QSAR	quantitative structure–activity relationship
RFU	random fluorescence units
ROS	reactive oxygen species
RP	reference points
RSD	relative standard deviation
SD	standard deviation
SOPs	Standard Operating Procedures
TG	test guideline
TI	tail intensity
TK	thymidine kinase
TR	technical requirements
TTC	threshold of toxicological concern
TWI	tolerable weekly intake
UDS	unscheduled DNA synthesis
WT	weight

Appendix A – Full list of identified and quantified constituents of smoke flavouring Primary Product SF-008

Table A.1: Compilation of the 40 identified and quantified volatile constituents in the Primary Product (Documentation provided to EFSA No. 1)

CAS no	FL-no	Chemical name[a]	Average[b] (wt%)
64-19-7	08.002	Acetic acid	6.9
116-09-6	07.169	1-hydroxypropan-2-one (1-hydroxy-2-propanone)	1.0
79-09-4	08.003	Propionic acid (propanoic acid)	0.4
23147-58-2		Glycolaldehyde dimer	0.3
98-01-1	13.018	Furfural	0.2
5077-67-8	07.090	1- hydroxybutan-2-one (1-hydroxy-2-butanone)	0.2
118-71-8	07.014	maltol	0.1
765-70-8	07.056[c]	3-methylcyclopentan-1,2-dione (3-methyl-1,2-cyclopentanedione)	0.1
90-05-1	04.005	2-methoxyphenol	0.1
91-10-1	04.036	2,6-dimethoxyphenol	0.1
96-48-0	10.006	butyro-1,4-lactone (butyrolactone)	0.1
123-76-2	08.023	4-oxovaleric acid (4-oxo-pentanoic acid)	0.1
930-30-3		2-cyclopenten-1-one	0.1
497-23-4	former 10.066[d]	furan-2(5*H*)-one (2(5*H*)-furanone)	0.1
22122-36-7		3-methyl-2(5*H*)-furanone[e]	0.08
107-92-6	08.005	butyric acid (butanoic acid)	0.07
107-21-1		1,2-ethandiol	0.05
592-20-1	09.185	2-oxopropyl acetate (acetoxyacetone)[f]	0.04
79-31-2	08.006	2- methylpropionic acid (2-methyl-propanoic acid)	0.04
10493-98-8		2-hydroxy-2-cyclopenten-1-one	0.04
93-51-6	04.007	2-methoxy-4-methylphenol (creosol/methylguaiacol)	0.04
620-17-7	04.021	3-ethylphenol	0.04
134-96-3	05.153	4-hydroxy-3,5-dimethoxy- benzaldehyde	0.04
576-26-1	04.042	2,6-dimethylphenol	0.03
2785-87-7	04.049	2-methoxy-4-propylphenol	0.03
6638-05-7	04.053	4-methyl-2,6-dimethoxyphenol (2,6-dimethoxy-4-methylphenol)	0.03
19037-58-2		syringylacetone	0.03
1120-73-6		2-methyl-2-cyclopenten-1-one	0.03
95-48-7	04.027	2-methylphenol	0.03
14059-92-8	04.052	4-ethyl-2,6-dimethoxyphenol	0.03
110-13-4		2,5-hexanedione	0.02
108-39-4	04.026	3-methylphenol	0.02
19444-84-9		2-hydroxy-γ-butyrolactone	0.02
20736-25-8		dihydrosyringenin	0.02
2758-18-1	07.112	3-methyl-2-cyclopenten-1-one[f]	0.01

CAS no	FL-no	Chemical name[a]	Average[b] (wt%)
108-95-2	04.041	phenol[f]	0.01
2785-89-9	04.008	4-ethylguaiacol (4-ethyl-2-methoxyphenol)[f]	0.01
97-53-0	04.003	eugenol	0.01
10374-51-3		5-(hydroxymethyl) dihydrofuran- 2(3*H*)-one[f]	0.01
1121-05-7		2,3-dimethyl-2-cyclopenten-1-one[f]	0.01

(a): In case a constituent of the Primary Product is an authorised flavouring substance (FL-no), the assigned chemical name corresponds to the respective entry in the EU Union List of flavourings. Deviating chemical names reported by the applicant in the dossier are given in brackets, if applicable.
(b): From the analysis of the batches presented in Table 1.
(c): [FL-no: 07.056] refers to the mixture of the tautomeric forms of 3-methylcyclopentan-1,2-dione.
(d): 'Former FL-number' refers to substances that were initially included in the evaluation programme but were not included or were removed/withdrawn from the Union List.
(e): Identified only in batch 406538021; values (wt%) were not consistent across the technical dossier; in this table the highest reported value is included.
(f): Not found in all the investigated batches of the Primary Product.

Appendix B – Genotoxicity data available on 4 individual components for which a (potential) concern for genotoxicity is identified

The data on the four substances discussed in this appendix relate to:

i) furan-2(5*H*)-one, for which a concern for genotoxicity has been identified; and
ii) three substances described in Section 3.4.1 for which a potential concern for genotoxicity has been identified, i.e. glycoaldehyde dimer (CAS No. 23147-58-2), 3-methyl-2(5*H*)-furanone (CAS No. 22122-36-7) and 2,5-hexanedione (CAS No. 110-13-4).

B.1. Furan-2(5*H*)-one (CAS No. 497-23-4, former [FL-no: 10.066])

Furan-2(5*H*)-one (former [FL-no: 10.066]) was evaluated as genotoxic *in vivo* (EFSA FAF Panel, 2019). To further assess the *in vivo* genotoxicity, furan-2(5*H*)-one was tested in an *in vivo* MN assay in liver of rats (summary of study results reported in Appendix D). In addition, the systemic exposure to furan-2(5*H*)-one was investigated in a satellite group of animals.

Three additional studies were performed by the applicant which were assessed by the Panel as described below.

In vivo micronucleus assay in the liver

Furan-2(5*H*)-one (batch: NEWJF, purity: 94.3%) was tested in an *in vivo* liver micronucleus assay in rats (LSIM, 2022a) which was performed in compliance with GLP and following a validated protocol recommended by the international workshops on genotoxicity testing (IWGT) (Hamada et al., 2015; Uno et al., 2015; Kirkland et al., 2019). An OECD test guideline for the *in vivo* MN study in liver is not yet available. The stability of test article formulations (5 and 20 mg/mL) for up to 8 days was confirmed through a validated analytical method (HPLC) for the determination of furan-2(5*H*)-one in corn oil (0.5–20 mg/mL) (LSIM, 2021).

A dose range-finding study was performed to identify the appropriate maximum dose level for the *in vivo* liver micronucleus assay. Groups of three Crl:CD(SD) male rats (6 weeks old at the beginning of dosing) were administered furan-2(5*H*)-one (batch: NEWJF) via oral gavage at 50, 100 and 200 mg/kg bw per day (vehicle control: corn oil) for 14 consecutive days. There were no abnormal clinical signs in any of the groups, but in the top dose group suppression of body weight gain was observed. Therefore, 200 mg/kg bw per day was considered the maximum tolerated dose (MTD) (LSIM, 2022b).

In the *in vivo* micronucleus assay, groups of five Crl:CD(SD) male rats (6 weeks old at the beginning of dosing) were administered furan-2(5*H*)-one via oral gavage at doses of 0 (vehicle control: corn oil), 50, 100 and 200 mg/kg bw per day for 28 consecutive days. A group of three animals dosed with 12.5 mg/kg bw per day diethyl nitrosamine, administered as per the test item, was used as the positive control (LSIM, 2022a).

Twenty-four hours after the final administration, liver cells were sampled and prepared for the micronuclei analysis for all animals. For the micronuclei analysis, 4000 hepatocytes per animal (excluding cells in M-Phase) were scored for the presence of micronucleated hepatocytes (MNHEPs). The number of hepatocytes in M-phase was recorded separately and used to calculate the mitotic index.

In order to confirm the systemic exposure of the test item, this study also included a toxicokinetic (TK) analysis of furan-2(5*H*)-one (batch: NEWJF) in plasma for a satellite group of rats (LSIM, 2022a). Groups of three Crl:CD(SD) male rats were treated via oral gavage with furan-2(5*H*)-one at doses of 0 (vehicle control: corn oil) or 200 mg/kg bw per day for 28 consecutive days. Furan-2(5*H*)-one concentrations in rat plasma were analysed via LC-MS/MS using a validated method (LSIM, 2021). The lower limit of quantification was 100 ng/mL and the upper limit of quantification was 10,000 ng/mL; the calibration curve showed linearity between 100 ng/mL and 10,000 ng/mL (LSIM, 2022c).

In the top dose group of the main study, one animal showed slight salivation 1 h after dosing on Day 23 and Day 24. No clinical signs were observed in any other animals of any group. Also in the top dose group, there were significant decreases in body weight from Day 15 onwards and significant increases in relative liver weight compared to the negative control.

The acceptance criteria of the study were fulfilled. A statistically significant increase in micronucleated hepatocytes (MNHEP) with respect to the concurrent vehicle control was reported in positive control group, which was within the range of laboratory's historical positive control data (based only on 16 animals, number of experiments not reported). The vehicle control data were within

the range of laboratory's historical vehicle control data (based on 68 animals, number of experiments not reported).

A statistically significant and dose-dependent increase in MNHEPs, compared with the concurrent vehicle control group was detected in 100 and 200 mg/kg bw per day test compound groups. At 200 mg/kg bw per day, the increase (9-fold the values in the control animals) in MNHEPs exceeded the range of historical negative control data (min–max, 0.00–0.25%).

The mitotic index in the liver was not affected by the treatment with furan-2(5*H*)-one at any dose level.

In the TK analysis, Tmax occurred at 0.5 h after both the initial and final administrations. The Cmax and AUC0-24 h values at the final dosing were lower than at the initial dosing, but systemic exposure was confirmed throughout the dosing period. Salivation was noted in all furan-2(5*H*)-one-treated animals on various days and body weight gain was lower compared to the vehicle control group.

According to the study authors, satisfactory linearity, recovery and repeatability were found for furan-2(5*H*)-one when the substance was spiked and analysed in rat plasma samples. However, the Panel noted that linearity in plasma extracts was in the range of 100–10,000 ng/mL, but the concentration reported for furan-2(5*H*)-one in rat plasma samples was above this range after the initial dosing, i.e. Cmax 15,060 ng/mL. Moreover, the recovery and accuracy of the method were only determined for the ranges 200–8,000 ng/mL and 100–8,000 ng/mL, respectively.

The study authors concluded that the study is valid and that furan-2(5*H*)-one has the potential to induce micronuclei in rat liver hepatocytes *in vivo* under the conditions of this study. The Panel concurred with this finding. The panel considered the study as reliable without restrictions and the results of high relevance (see Appendix D).

Based on the positive results observed in the *in vivo* MN study, the applicant submitted a study to investigate the pro-oxidative potential of furan-2(5*H*)-one and an *in vitro* Multiflow® screen with the aim of clarifying the mode of action and to determine whether the genotoxic effect observed is threshold mediated.

Pro-oxidative potential of furan-2(5*H*)-one

An exploratory study was conducted to investigate the potential of furan-2(5*H*)-one to elevate concentrations of reactive oxygen species (ROS) in two different *in vitro* liver models: human hepatoblastoma (HepG2) cells and precision cut liver slices (PCLS) obtained from healthy (nulliparous and non-pregnant) Wistar rats (strain Crl:VVI (Han)) (Fraunhofer ITEM, 2023). The PCLS model consists of different cell types, including immune cells, and therefore can include assessment of ROS production resulting from inflammatory processes.

Furan-2(5*H*)-one (batch: BCCD2607; purity: 98.6%) was tested according to the relevant Standard Operating Procedures (SOPs) of the Fraunhofer ITEM and, for the two *in vitro* comet assay pilot experiments, also in line with the principles suggested in OECD TG 489 (OECD, 2016e) regarding the most appropriate measure for DNA damage following analyses of single cells. According to the study report, the study was conducted in the spirit of the basic requirements of GLP.

Prior to ROS measurements, a cytotoxicity screen was performed in HepG2 cells over a concentration range of 15.6–500 μg/mL furan-2(5*H*)-one for 3 h in the absence and presence of S9-mix and for 24 h without S9-mix. Cytotoxicity was determined using cell morphology and cell density for 24 h in the absence of S9-mix only (i.e. light microscopy), cell proliferation/cell loss (i.e. automatic cell counting) for all treatments, membrane damage (i.e. lactate dehydrogenase (LDH) release assay) 3 h and 24 h in the absence of S9-mix only, and metabolic activity (i.e. WST-1 assay) for all treatments. Triton™ X-100 (0.1% [v/v]) served as positive control and dimethyl sulfoxide (DMSO) (0.8% [v/v]) as vehicle control. Three separate experiments each in triplicate were performed. Significant furan-2(5*H*)-one cytotoxicity was only evident after 24 h exposures and mainly at concentrations higher than 62.5 μg/mL, with steep increases observed between 62.5 and 125 μg/mL in the WST-1 and LDH release assays. The S9-mix had no considerable impact on the induction of cytotoxicity by furan-2(5*H*)-one as measured using the WST-1 assay. Subsequent studies to assess ROS were performed at higher concentrations than applied in this cytotoxicity study in which the authors attempted to determine if ROS could be a potential MOA in these cytotoxicity screening assays.

For the ROS studies, HepG2 cells were exposed to five different concentrations (0, 125, 250, 500 and 1,000 μg/mL) of furan-2(5*H*)-one for 3 h or 24 h exposures with or without reduced glutathione (GSH, 5 mM, approximately 1537 μg/mL). Luperox® tert-butyl hydroperoxide (TBH70X) was used as a positive control (1 mM, approximately 242 μg/mL) and 0.8% DMSO was used as negative/vehicle

control. ROS-dependent intracellular 2′,7′-dichloro-dihydro-fluorescein (DCFH) fluorescence was measured at 60, 120 and 180 min. Velocity of ROS generation was subsequently calculated considering the linear increase in random fluorescence units (RFU)/time between 60 min and 180 min. Three separate experiments each in triplicate were performed. In other studies, rat PCLS were exposed to four different concentrations (0, 250, 375 and 500 μg/mL) of furan-2(5*H*)-one for 4 h exposures with or without GSH (5 mM, ~ 1,537 μg/mL). TBH7OX (10 mM, ~ 2,422 μg/mL) was used as positive control and 0.4% DMSO was used as negative/vehicle control. ROS-dependent fluorescence of intracellular chloromethyl-2′,7′-dichloro-dihydro-fluorescein (CM-H2DCF) was measured after 240 min. Three separate experiments each in duplicate were performed. A sample of PCLS-treated media was also obtained for the determination of LDH release.

In HepG2 cells, furan-2(5*H*)-one induced a slight increase of ROS at 500 and 1,000 μg/mL following 3 h exposures, reaching statistical significance only at the highest concentration. This significant increase was partially, but statistically significantly, counteracted by GSH. Following a 24 h co-exposure with furan-2(5*H*)-one and GSH, relative cell counts were decreased and LDH release increased for the +GSH control compared to the -GSH control. At 250 and 500 μg/mL (and also at 1,000 μg/mL in the case of LDH release), the effects of furan-2(5*H*)-one were partially and significantly counteracted by GSH ($p \leq 0.01 - p \leq 0.001$).

In rat PCLS, 4 h furan-2(5*H*)-one exposures induced a slight increase of ROS at 250 μg/mL, which was increased at 375 μg/mL and at both concentrations completely counteracted by GSH when compared to the negative control +GSH. At the highest concentration (500 μg/mL of furan-2(5*H*)-one), no significant increase in ROS was observed. LDH release showed similar patterns as for ROS production, but GSH treatment had a lesser counteractive effect.

To determine the clastogenic potential of furan-2(5*H*)-one in HepG2 cells, pilot alkaline comet assays in HepG2 cells were performed after 3 h exposures with furan-2(5*H*)-one at concentrations of 0, 62.5, 125, 250 and 500 μg/mL with or without concomitant addition of GSH (5 mM, ~ 1,537 μg/mL). Ethyl methanesulfonate (EMS, 0.75 μL/mL, 1 h) was used as positive control for induction of DNA strand breaks and DMSO (0.4% [v/v]) was used as vehicle control. Three biological replicates were analysed. A slight (but statistically significant) increase in mean tail intensity (TI) was evident at all concentrations that was maximal at 125 μg/mL (mean TI 1.82 ± 0.31% vs. 0.45 ± 0.04% for negative control without GSH), but it was not concentration-dependent. GSH treatment completely (62.5–250 μg/mL) or partially (500 μg/mL; mean TI without GSH: 1.79 ± 0.55% vs. mean TI with GSH: 1.14 ± 0.37%) inhibited effects as compared to the negative control +GSH (mean TI: 0.69 ± 0.160%).

Additionally, an acellular comet assay was performed using lysed L5178Y/TK$^{+/-}$ mouse lymphoma cells as a DNA source as no cell-type specific functions were needed for the assay. This assay was performed to determine the direct DNA-damaging potential of furan-2(5*H*)-one without cellular enzymes such as those for metabolic activation or DNA repair. Furan-2(5*H*)-one was tested at concentrations of 0, 125, 250 and 500 μg/mL for a 1-h exposure. With EMS (1 μL/mL, 1 h) single strand breaks were observed. DMSO (0.4% [v/v]) was used as vehicle control. Four biological replicates were analysed. Tail intensity (TI) was used as a measure of DNA strand breakage. In this acellular comet assay, furan-2(5*H*)-one had no effect at any concentration.

Overall, furan-2(5*H*)-one was able to induce oxidative stress in the two liver cell models, and as the slight clastogenic potential of furan-2(5*H*)-one in HepG2 cells was effectively counteracted by GSH, induction of ROS might, therefore, be at least partly responsible for the slight clastogenic activity. However, the exact mechanism(s) and types of ROS generated, as well as cell type specificity, remain to be specified. From this information, the study author considered that clastogenicity in the presence of oxidative stress may be consistent with a non-DNA reactive MOA, which is expected to exhibit a threshold, i.e. at concentrations below which physiological stress/significant cytotoxicity is caused, no genotoxic effects will occur.

The Panel concluded that, whilst it is possible that ROS contributed to the responses seen at relatively high concentrations in the comet assays with HepG2 cells, it is not possible to confirm from the available evidence that this mode of action contributes to the genotoxicity of furan-2(5*H*)-one. In particular, the potential of ROS production to be a cause or a consequence of cytotoxicity is not clear and the mode(s) of action of the inhibitory effect of GSH on reducing ROS concentrations, comet responses and cell toxicity has not been demonstrated. The fact that GSH can protect against the effect of a direct acting genotoxicant as well as a pro-oxidant is evident from the result of the positive control agent. Further to these considerations, it should also be noted that in HepG2, a clastogenic effect was observed at concentrations well below those inducing a significant production of ROS

(62.5 vs. 1,000 μg/mL). There is no specific evidence of ROS-induced DNA lesions or on absence of direct reaction of furan-2(5*H*)-one with DNA.

In vitro MultiFlow® Screen

To evaluate genotoxic potential, with a focus on mode of action (MOA; i.e. clastogenicity and aneugenicity), furan-2(5*H*)-one (batch: BCCD2607, purity 98.6%) was tested in an *in vitro* MultiFlow® Assay using TK6 human lymphoblasts, both in the absence and in the presence of induced rat liver S9 fraction (S9-mix) (Charles River, 2023). A panel of nuclear biomarkers that have been shown to distinguish between aneugenicity and clastogenicity mechanisms was used: γH2AX, H3 phosphorylation (H3P), polyploidisation and p53 translocation (Bryce et al., 2018). Cleaved-PARP was also tested as an indicator of the health of the cultures. As there is no OECD TG for this method, the assay was carried out in accordance with the MultiFlow® DNA Damage Kit's manual (Litron Laboratories; Rochester, US). This study is not GLP. The study report only provided a protocol with limited and sometimes contradictory summary, but results were adequately reported.

There were four positive controls tested at four concentrations each: methyl methanesulfonate (12.5, 25, 50 and 100 μM), carbendazim (12.5, 25, 50 and 100 μM), benzo(a)pyrene (1.25, 2.5, 5 and 10 μM) and cyclophosphamide (2.5, 5, 10 and 20 μM). DMSO was used as vehicle control. In the absence of metabolic activation, all test item concentrations and positive controls were evaluated after 4 h or 24 h of exposure. In the presence of metabolic activation, the exposure lasted for 4 h followed or not by a 20-h expression period and for 24 h continuous exposure with and without S9-mix. The test item and positive controls were evaluated in single replicate cultures while the vehicle control was evaluated in eight replicate cultures.

The highest concentration for all exposure conditions was 842 μg/mL (~ 10 mM) furan-2(5*H*)-one, which was reported to be freely soluble at the end of both exposure periods. At the end of the 4 h and 24 h exposure periods, an aliquot from each culture was taken, placed in lysis solution and analysed using flow cytometry with FACSDiva software. Cytotoxicity was calculated based on relative nuclei counts. The highest furan-2(5*H*)-one concentrations evaluated for genotoxicity were those inducing ≤ 80% cytotoxicity. In the absence of S9-mix, furan-2(5*H*)-one was tested at 11 concentrations ranging from 12.1 to 113 μg/mL (74% cytotoxicity at 24 h) and in the presence of S9-mix was tested at 12 concentrations ranging 12.1–141 μg/mL (64% cytotoxicity at 24 h).

Clastogenicity and aneugenicity were assessed using independent machine learning models developed using JMP Pro statistical software (v12.2.0). Clastogenicity signatures were demonstrated by either two consecutive concentrations with clastogenic probability scores ≥ 80% or one concentration with a probability score ≥ 90% and conversely, aneugenic signatures were demonstrated by either two consecutive concentrations with aneugenic probability scores ≥ 80% or one concentration with a probability score ≥ 90%. For any test concentration to be considered as having clastogenic or aneugenic signatures two of the three models needed to be in agreement. In addition, global evaluation factors (GEFs) were created using JMP statistical software (v12.2.0) and applied. Clastogenicity signatures were demonstrated by fold increases in two consecutive concentrations that met or exceeded cut-offs for at least two of the following clastogenic responses: ≥ 1.51-fold 4 h γH2AX, ≥ 2.11-fold 24 h γH2AX, ≥ 1.40-fold 4 h nuclear p53 and ≥ 1.45-fold 24 h nuclear p53. Conversely, aneugenic signatures for cultures without S9-mix were demonstrated by fold increases in two consecutive concentrations that met or exceeded cut-offs for at least two of the following aneugenic responses: ≥ 1.71-fold 4 h H3P+ nuclei, ≥ 1.52-fold 24 h H3P+ nuclei, ≥ 5.86-fold 24 h polyploidy and ≥ 1.45-fold 24 h nuclear p53.

For with S9-mix cultures, only the clastogen MOA was investigated. Clastogenicity MOA probability scores were assessed using independent machine learning models as described above. In addition, GEFs were created as described above. Clastogenicity signatures were demonstrated by fold increases in two consecutive concentrations that met or exceeded cut-offs for at least two of the following clastogenic responses (at least one being γH2AX): ≥ 1.44-fold 4 h γH2AX, ≥ 1.31-fold 24 h γH2AX, ≥ 1.23-fold 4 h nuclear p53 and ≥ 1.12-fold 24 h nuclear p53.

In the absence of S9-mix, from the machine learning models, there was a prediction of a clastogenic but not aneugenic signature at test concentrations ≥ 90.4 μg/mL. Also, at the same concentration, statistically significant increases in γH2AX and p53 translocation were observed at 24 h, exceeding the respective GEFs. In the presence of S9-mix, there were no statistically significant increases in markers for clastogenicity observed at any concentration evaluated based on the machine learning models. Nevertheless, statistically significant increases, exceeding their respective GEFs, were

observed for γH2AX at 4 h and p53 translocation at 4 h and 24 h at concentrations ≥ 113 μg/mL, which would indicate a clastogenic potential.

The study author concludes that the results indicate that furan-2(5*H*)-one was not aneugenic in the absence of metabolic activation and that it was clastogenic, both in the absence and in the presence of S9-mix, under the conditions of the study.

Despite the fact that the study methodology is not validated, the Panel recognises that the study results provide further support for the clastogenic properties of furan-2(5*H*)-one.

Conclusion: The positive results from previously available studies (see EFSA FAF Panel, 2019) as well as from the newly submitted *in vivo* MN assay in liver and the positive results obtained by the newly submitted *in vitro* MultiFlow® test in TK6 cells support a clastogenic MOA for furan-2(5*H*)-one. Evidence to suggest that the genotoxicity of furan-2(5*H*)-one is mediated through ROS production is inadequate. Therefore, a safety concern emerges for this component, since the exposure to furan-2 (5*H*)-one exceeds the TTC for DNA-reactive mutagens and/or carcinogens (see Table 15).

B.2. Glycoaldehyde dimer (CAS No. 23147-58-2)

The applicant reported that in the body under physiological conditions, the target substance hydrolyses to glycolaldehyde (i.e. hydroxyacetaldehyde), which is expected to be completely metabolised to endogenous metabolites carbon dioxide or glycolic acid and oxalic acid (WHO, 2002). According to the applicant, these considerations may support the absence of concern for genotoxicity *in vivo* for the target substance, also considering read-across with substances in FGE.03Rev2 (EFSA CEF Panel, 2011) and FGE.10Rev3 (EFSA CEF Panel, 2012).

On the other hand, EFSA noted that the hydrolysis product of the target substance, glycolaldehyde, shows reactivity towards DNA and gave equivocal results in a bacterial gene mutation assay (Denkel et al., 1986). Therefore, EFSA considered that the suggestion from the applicant to waive a concern for potential genotoxicity of the target substance solely based on the metabolic considerations or on read-across with substances evaluated by EFSA in FGE.03Rev2 (EFSA CEF Panel, 2011) and FGE.10Rev3 (EFSA CEF Panel, 2012) is not acceptable, since the similarity with the substances in these FGEs is not sufficiently robust. In addition, based on the (Q)SAR analysis performed by EFSA, a weak indication for potential genotoxicity was also identified for glycoaldehyde dimer (see Annex B).

Conclusion: The Panel concluded that the (Q)SAR analysis provides a weak indication for potential genotoxicity of this constituent. In addition, taking into account experimental data on the hydrolysis product glycolaldehyde, appropriate *in vitro* studies addressing gene mutations as well as structural and numerical chromosomal aberrations would be needed to evaluate the genotoxic potential of the substance, since exposure to glycoaldehyde dimer, exceeds the TTC for DNA-reactive mutagens and/or carcinogens (see Table 15).

B.3. 3-Methyl-2(5*H*)-furanone (CAS No. 22122-36-7)

The Panel noted that this component is structurally related to furan-2(5*H*)-one [former FL-no: 10.066] and to the 2(5*H*)-furanone-derivative 3,4-dimethyl-5-pentylidenefuran-2(5*H*)-one [FL-no: 10.042]. For 2(5*H*)-furanone, a concern for genotoxicity was identified and for [FL-no: 10.042] only a concern for structural and numerical chromosomal aberrations could not be ruled out in FGE.217Rev2 (EFSA FAF Panel, 2019).

Conclusion: A potential concern for genotoxicity is identified for this derivative of 2(5*H*)-furanone considering the read-across from the above-mentioned structurally related substances. Considering the data presented in FGE.217Rev2 for 3-methyl-2(5*H*)-furanone, information on clastogenic and aneugenic potential would be needed to evaluate the genotoxic potential of the substance, since the exposure to this component exceeds the TTC for DNA-reactive mutagens and/or carcinogens (see Table 15).

B.4. 2,5-Hexanedione (CAS No. 110-13-4)

Four papers on 2,5-hexanedione, retrieved from the literature, were submitted by the applicant.

Aeschbacher et al. (1989) tested the substance in a group of about 40 coffee aroma constituents in a bacterial reverse mutation assay on three Salmonella Typhimurium (TA98, TA100 and TA102) with and without metabolic activation, with negative results. The test conduct was overall in line with the current standard; however, the set of the bacterial strains used was not complete compared to the recommendations of OECD TG 471 (OECD, 2020b). The study is of limited reliability and relevance.

Zimmermann et al. (1989) and Mayer and Goin (1994) tested the substance for the induction of mitotic chromosome loss in the D61.M strain of the yeast *Saccharomyces cerevisiae*, an obsolete assay not validated for regulatory purposes. The first study found the 2,5-hexanedione weakly positive, with an apparent synergic effect with propionitrile. The second study reported a clearly positive outcome. These two studies indicate a possible aneugenic effect. Both studies are of limited reliability and relevance.

Muhammad et al. (2018) reported induction of 'DNA fragmentation in the blood' micronuclei in the bone marrow of rats after oral administration. The technical procedure used to investigate DNA fragmentation is not clearly described. In addition, this endpoint could reflect cytotoxic effect rather than genotoxicity. The micronucleus assay is considered inconclusive, because of several limitations in the description of the experimental procedure and in data reporting. The study is of limited reliability and relevance.

Overall, the data set for 2,5-hexanedione is incomplete: The Ames test was not conducted in the complete set of bacterial strains recommended by OECD and the other available studies are insufficient to conclude on the possible induction of structural and numerical chromosomal aberrations. Two non-guideline studies indicated a possible aneugenic activity. It should be noted that this indication is supported by mechanistic studies that reported formation of covalently crosslinked tubulin dimer in an acellular *in vitro* system (Boekelheide 1987a) and alterations in microtubule assembly induced by the substance in experimental animals after oral exposure (Boekelheide 1987b).

Conclusion: Given the limited relevance of the available data set and the indications for structural and numerical chromosomal aberrations, appropriate *in vitro* studies addressing gene mutations as well as structural and numerical chromosomal aberrations would be needed to evaluate the genotoxic potential of the substance, since the exposure to 2,5-hexanedione exceeds the TTC for DNA-reactive mutagens and/or carcinogens (see Table 15).

Appendix C – Approach for assessing reliability and relevance of genotoxicity studies

Evaluation of data quality for hazard/risk assessment includes evaluation of reliability of studies and relevance of study results (Klimisch et al., 1997; ECHA, 2011; EFSA Scientific Committee, 2011, 2017b, 2021b). Reliability is assessed using a scoring system based on published criteria (Klimisch et al., 1997) described in the following section. In a second step, the relevance (high, limited or low) of study results is assessed based on several aspects (genetic endpoint, route of administration, status of validation of the assay, etc.) discussed in Section C.2, and also taking into account the assessment of the reliability of the study.

Only studies with acceptable relevance (high or limited) are considered in the weight of evidence approach (WoE). Genotoxicity studies evaluated as of low relevance are not further considered in the WoE.

C.1. Evaluation of reliability of results of genotoxicity studies – general considerations

The scoring system for reliability is based on the scoring system of Klimisch et al. (1997). Reliability is defined by Klimisch as 'evaluating the inherent quality of a test report or publication relating to preferably standardised methodology and the way that the experimental procedure and results are described to give evidence of the clarity and plausibility of the findings'. In assigning the reliability score, the compliance with the OECD test guidelines (TGs) or standardised methodology and the completeness of the reporting should be considered.

The reliability scores are:

1) Reliable without restriction
2) Reliable with restrictions
3) Reliability insufficient
4) Reliability cannot be evaluated.

1. Reliable without Restriction 'This includes studies or data from the literature or reports which were carried out or generated according to generally valid and/or internationally accepted testing guidelines (preferably performed according to GLP) or in which the test parameters documented are based on a specific (national) testing guideline (preferably performed according to GLP) or in which all parameters described are closely related/comparable to a guideline method.'

2. Reliable with Restrictions 'This includes studies or data from the literature, reports (mostly not performed according to GLP), in which the test parameters documented do not totally comply with the specific testing guideline, but are sufficient to accept the data or in which investigations are described which cannot be subsumed under a testing guideline, but which are nevertheless well documented and scientifically acceptable.'

3. Reliability Insufficient[17] 'This includes studies or data from the literature/reports in which there are interferences between the measuring system and the test substance or in which organisms/test systems were used which are not relevant in relation to the exposure (...) or which were carried out or generated according to a method which is not acceptable, the documentation of which is not sufficient for an assessment and which is not convincing for an expert judgment.'

4. Reliability cannot be evaluated[18] 'This includes studies or data from the literature, which do not give sufficient experimental details, and which are only listed in short abstracts or secondary literature (books, reviews, etc.).'

[17] Klimisch et al. (1997) used the term "Not reliable" however, "Reliability Insufficient" was considered more appropriate.
[18] Klimisch et al. (1997) used the term "Not assignable" however, "Reliability cannot be evaluated" was considered more appropriate.

C.2. Evaluation of relevance of results of individual genotoxicity studies – general considerations

The relevance of the test system and test results are reported separately.
The relevance of the test systems (high, limited, low) is principally based on the following criteria:

- Genetic endpoint: higher relevance is given to studies providing information on apical endpoints, i.e. gene mutations, structural and numerical chromosomal alterations. Supporting information may be obtained from indicator assays; exception is the *in vivo* Comet assay that is considered with high relevance when applied as follow-up to a positive *in vitro* result (as recommended by the EFSA Scientific Committee (2011)).
- Status of validation of the test system (e.g. (in order of decreasing relevance) availability of an OECD TG consolidated or in the course of development or internationally recommended protocol, validation at national level only).

The relevance of the study results (high, limited, low) are principally based on the following criteria:

- Reliability of studies: the results of studies with reliability that are insufficient or which cannot be evaluated (see points 3–4 in Section C.1) are considered of low relevance.
- Relevance of the test system.
- Route of administration: higher relevance is given to oral vs. intravenous or subcutaneous injection and inhalation exposure in case of *in vivo* studies. Lower relevance is given to studies using the intraperitoneal route, which is not physiological and not recommended by OECD TGs.
- Biological relevance of the test results, considering: purity of the test substance; the metabolic capabilities of the test system; the bioavailability of the test substance, with particular consideration of the evidence of target tissue exposure in tests *in vivo*: negative results without evidence of target tissue exposure are considered as inconclusive and their relevance low; the interference of high cytotoxicity; the reproducibility of test results.

Appendix D – Genotoxicity studies on individual components

Table D.1: Summary of *in vivo* genotoxicity data on furan-2(5*H*)-one

Chemical Name CAS No.	**Test System *in vivo***	**Test Object Route**	**Doses (mg/kg bw per day)**	**Result**	**Reliability/Comments**	**Relevance of test system/ Relevance of the result**	**Reference**
Furan-2(5*H*)-one 497–23-4	Micronucleus assay in liver	Crl:CD(SD) male rats gavage	50, 100 and 200[(a)]	Positive	Reliable without restrictions Study performed in compliance with GLP. An OECD test guideline is not yet available; however, the study was performed following a validated protocol recommended by the international workshops on genotoxicity testing (IWGT) (Hamada et al., 2015; Uno et al., 2015; Kirkland et al., 2019).	High/High	LSIM (2022a)

(a): The test substance was administered once daily for 28 consecutive days; sampling 24 h after the final administration.

Appendix E – Genotoxicity studies on the Primary Product (whole mixture) evaluated by the CEF Panel (EFSA CEF Panel, 2010)

Table E.1: Summary of *in vitro* genotoxicity studies on proFagus Smoke R709 (SF-008) including re-evaluation of reliability and relevance by the FAF Panel (approach described in Appendix C)

Name	Test System *in vitro*	Test Object	Concentrations and Test Conditions	Result	Reliability/Comments	Relevance of test system/ Relevance of the result	Reference
proFagus Smoke R709	Bacterial Reverse Mutation test	S. typhimurium TA98, TA100, TA1535, TA1537 *E. coli* WP2 uvrA	Experiment 1: 51.2–5,000 μg/plate (+/-S9, plate incorporation) Experiment 2: 51.2–5,000 μg/plate (+/-S9, pre-incubation)	Negative	Reliable without restrictions. Study performed according to OECD TG 471 and in compliance with GLP.	High/High	LAB International Research Centre (2005a)
	In vitro mammalian cell gene mutation test in mouse lymphoma cells	L5178Y TK $^{+/-}$ mouse lymphoma cells	Experiment 1: 500–1,400 μg/mL (3 + 21 h, +S9); 250–850 μg/mL (3 + 21 h, -S9) Experiment 2: 500–1,400 μg/mL (3 + 21 h, +S9); 250–650 μg/mL (24 h -S9)	Positive	Reliable with restrictions (historical controls not provided). Study performed according to OECD TG 476 (applicable at that time, now OECD TG 490) and in compliance with GLP.	High/Limited	LAB International Research Centre (2005b)
	In vitro mammalian chromosomal aberrations test	Chinese hamster ovary cells (CHO-KI cell line)	Experiment 1: 20, 100, 200 μg/mL (4 + 20 h, +S9) 20, 100, 200 μg/mL (4 + 20 h, -S9) Experiment 2: 20, 100, 200 μg/mL (20 + 20 h, -S9) 20, 100, 200 μg/mL (4 + 20 h, +S9)	Negative	Reliable with restrictions (200 metaphases/ concentration instead of 300 were scored; a method for cytotoxicity not recommended in the OECD TG 473 was applied). Study performed according to OECD TG 473 and in compliance with GLP.	High/Limited	LAB International Research Centre (2005c)

Table E.2: Summary of *in vivo* genotoxicity studies on proFagus Smoke R709 (SF-008) including re-evaluation of reliability and relevance by the FAF Panel (approach described in Appendix C)

Name	Test System *in vivo*	Test Object Route	Doses (mg/kg bw per day)	Result	Reliability/Comments	Relevance of test system/ Relevance of the result	Reference
proFagus Smoke R709	Micronucleus assay in bone marrow	NMRI BR mice; M and F Oral	500, 1,000 and 2,000[(a)]	Inconclusive (negative, but without demonstration of bone marrow exposure)	Reliable with restrictions (no demonstration of bone marrow exposure; historical control data not reported). Study performed according to OECD TG 474 and in compliance with GLP	High/Low	LAB International Research Centre (2005d)

M: males; F: females.
(a): One administration with sampling at: 24 and 48 h.

Appendix F – New genotoxicity studies on the Primary Product (whole mixture)

Table F.1: Summary of *in vitro* genotoxicity study on proFagus Smoke R709

Name	Test system *in vitro*	Test object	Concentrations[(a)] and test conditions	Result	Reliability/Comments	Relevance of test system/ Relevance of the result	Reference
proFagus Smoke R709	Micronucleus assay with FISH analysis	Human peripheral blood lymphocytes	625, 1250, 1,562.5 µg/mL (4 + 24 h, +S9) 625, 1,250, 1,406.2 µg/mL (4 + 24 h, -S9) 234.4, 625, 937.5 µg/mL (24 + 24 h, -S9)	Positive FISH analysis indicates that proFagus Smoke R709 induced MN by a clastogenic mechanism.	Reliable without restrictions. Study performed according to OECD TG 487 and in compliance with GLP.	High/High	Labcorp (2022)

(a): The given concentrations are those for the cultures that were scored for micronuclei.

Table F.2: Summary of *in vivo* genotoxicity studies on proFagus Smoke R709

Name	Test System *in vivo*	Test Object Route	Doses (mg/kg bw per day)	Result	Reliability/ Comments	Relevance of test system/ Relevance of the result	Reference
proFagus Smoke R709	Micronucleus assay in bone marrow	B6D2F1/Slc [SPF] mice; M Gavage	750, 1,500, 3,000[(a)]	Inconclusive (negative, but without demonstration of bone marrow exposure)	Reliable with restrictions (no demonstration of bone marrow exposure). Study performed according to OECD TG 474 and in compliance with GLP.	High/Low	BSRC (2022a)
	Gene mutation assay in liver, stomach and duodenum	Muta™Mouse CD_2-LacZ80/HazfBR SPF transgenic mice; M Gavage	250, 500, 1,000 and 3,000	Negative	Reliable without restrictions. Study performed according to OECD TG 488 and in compliance with GLP.	High/High	BSRC (2022b)

M: males.
(a): The primary product was administered once daily on two consecutive days; sampling 24 h after the last administration.

Annex A – Exposure assessment results

- Annex A1 Occurrence data per food category considered in FAIM (mg/kg).
- Annex A2 Total estimated exposure of proFagus Smoke R709 (SF-008) for the proposed maximum use level exposure assessment scenario using FAIM, per population group and survey: mean and 95th percentile (mg/kg bw per day).
- Annex A3 Total estimated exposure of proFagus Smoke R709 (SF-008) for the expected typical use level exposure assessment scenario using FAIM, per population group and survey: mean and 95th percentile (mg/kg bw per day).
- Annex A4 Proposed food categories and use levels linked to FoodEx2 foods, considered within DietEx, and their dilution factors (mg/kg or mg/L).
- Annex A5 Total estimated exposure of proFagus Smoke R709 (SF-008) for the proposed maximum use level exposure assessment scenario using DietEx, per population group and survey: mean and 95th percentile (mg/kg bw per day).
- Annex A6 Total estimated exposure of proFagus Smoke R709 (SF-008) for the expected typical use level exposure assessment scenario using DietEx, per population group and survey: mean and 95th percentile (mg/kg bw per day).
- Annex A7 Main food categories contributing to exposure to proFagus Smoke R709 (SF-008) at the proposed maximum use level exposure assessment scenario using DietEx (> 5% to the total mean exposure).
- Annex A8 Main food categories contributing to exposure to proFagus Smoke R709 (SF-008) at the expected typical use level exposure assessment scenario using DietEx (> 5% to the total mean exposure).
- Annex A9 Qualitative evaluation of the influence of standard uncertainties on the dietary exposure estimates of the Primary Product.

Annex A can be found in the online version of this output, in the 'Supporting information' section.

Annex B – Genotoxicity assessment of the identified components in the Primary Product

Annex B can be found in the online version of this output (in the 'Supporting information' section).