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SCIENTIFIC OPINION

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Flavouring group evaluation 418 (FGE. 418): 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester

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The declarations of interest of all scientific experts active in EFSA's work are available at https://open.efsa.europa.eu/experts

Abstract

The EFSA Panel on Food Additives and Flavourings (FAF) was requested to evaluate the safety of 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] as a new flavouring substance, in accordance with Regulation (EC) No 1331/2008. The substance has not been reported to occur naturally and it is chemically synthesised. The information provided on the manufacturing process, the composition and the stability of [FL-no: 16.136] was considered sufficient. The chronic dietary exposure to [FL-no: 16.136] estimated using the added portions exposure technique (APET) is calculated to be 860 μg/person per day for a 60-kg adult and 540 μg/person per day for a 15-kg 3-year-old child. [FL-no: 16.136] did not show genotoxic effects in bacterial mutagenicity and mammalian cell micronucleus assays in vitro. No ADME studies on [FL-no: 16.136] were provided. In a prenatal developmental toxicity study, no maternal or fetal toxicity was observed in rats dosed up to 1000 mg/kg body weight (bw) per day. In a 90-day toxicity study in rats, no adverse effects were observed. In this study, the Panel considered that the NOAEL is 777 and 923 mg/kg bw per day (the highest dose tested) for male and female rats, respectively. Considering the lowest NOAEL of 777 mg/kg bw per day, as a reference point, adequate margins of exposure of 55×10^3 and 21×10^3 were calculated for adults and children, respectively, when considering the chronic APET dietary exposure estimates. The Panel concluded that the use of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] as a flavouring substance under the proposed conditions of use does not raise a safety concern at the dietary exposure estimates calculated using the APET approach.

KEYWORDS

3-[3-(2-Isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester, FGE.418, Flavouring, FL-no: 16.136

CONTENTS

1 | INTRODUCTION

The present scientific opinion, Flavouring Group Evaluation 418 (FGE.418), deals with the safety assessment of 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] proposed for use as a new flavouring substance in and on food.

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

1.1.1 | Background

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

Regulation (EC) No 1331/2008[2] established a common authorisation Procedure for food additives, food enzymes and food flavourings.

An application has been introduced for the authorisation of 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester as a new flavouring substance.

In order for the Commission to be able to consider its inclusion in the Union list of flavourings and source materials (Annex I of Regulation (EC) No 1334/2008), EFSA should carry out a safety assessment of this substance.

1.1.2 | Terms of reference

The European Commission requests the European Food Safety Authority to perform a safety assessment of 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester as a new flavouring substance in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation Procedure for food additives, food enzymes and food flavourings.

1.2 | Existing authorisations and evaluations

3-[3-(2-Isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester was considered by the expert Panel of the Flavor and Extract Manufacturers Association (FEMA) as 'Generally recognised as safe' (GRAS) and allocated the FEMA No. 4766.

The substance was evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in the group of miscellaneous nitrogen-containing substances, with the JECFA no. 2203 (JECFA, 2014, 2015). JECFA evaluated 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester via the B-side of the Procedure. JECFA reported that '*The no-observed-adverse-effect level (NOAEL) of 776.5 mg/kg body weight (bw) per day (the highest dose tested) in a 90-day study in rats (..) provides an MOE of 60 000 (SPET = 800 µg/day or 13 µg/kg bw per day) when No. 2203 is used as a flavouring agent. The Committee concluded that, on the basis of all of the available evidence, 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (No. 2203) would not pose a safety concern at current estimated dietary exposures*.' (JECFA, 2014, 2015).

The Panel noted that 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester is not registered in the ECHA chemical database.[3]

2 | DATA AND METHODOLOGIES

2.1 | Data

The present evaluation is based on data on 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] provided by the applicant in a dossier (Documentation provided to EFSA No. 1) to support its evaluation as a food flavouring substance. Additional information was provided by the applicant during the risk assessment process on 20 March 2024 (Documentation provided to EFSA No. 2) and on 17 July 2024 (Documentation provided to EFSA No. 3) in response to a request from EFSA sent on 11 May 2022 and on 16 April 2024 (with addendum letter sent on 7 June 2024), respectively.

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

[2]Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation Procedure for food additives, food enzymes and food flavourings. OJ L 354, 31.12.2008, p. 1–6.

[3]Accessed 2/12/2024: https://chem.echa.europa.eu/.

2.2 | Methodologies

This opinion was prepared following the principles described in the EFSA Guidance of the Scientific Committee on transparency with regard to scientific aspects of risk assessment (EFSA Scientific Committee, 2009) and following the relevant existing Guidance documents from the EFSA Scientific Committee.

The application on 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester was submitted to EFSA before the adoption and publication of the latest EFSA guidance on data required for the risk assessment of flavourings to be used in or on foods (EFSA FAF Panel, 2022). Therefore, the safety assessment of 3-[3-(2-isopropyl-5-methyl-cyclo hexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] was carried out in accordance with the Procedure as outlined in the EFSA scientific opinion '*Guidance on the data required for the risk assessment of flavourings to be used in or on foods*' (EFSA CEF Panel, 2010) and the EFSA technical report '*Proposed template to be used in drafting scientific opinions on flavouring substances (explanatory notes for guidance included)*' (EFSA, 2012).

3 | ASSESSMENT

3.1 | Technical data

3.1.1 | Identity of the substance

The chemical structure of the flavouring substance 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester and the specification data are shown in Table 1. The flavouring substance was allocated the FLAVIS number [FL-no: 16.136]. The SMILES code is O=C(NC1CC(C)CCC1C(C)C)NC(C)CC(=O)OCC.

The identity of the flavouring substance was investigated by gas chromatography (GC), high-performance liquid chromatography (HPLC), gas chromatography–mass spectrometry (GC–MS), liquid chromatography-mass spectrometry (LC–MS), Fourier transform infrared spectroscopy (FT-IR) and ^{1}H nuclear magnetic resonance (NMR) analyses. Details of the analytical techniques were provided to EFSA (Documentation provided to EFSA No. 1, 2).

Upon request by EFSA, the applicant provided compositional data on eight batches of the flavouring substance; quantifications were performed by GC using ethyl 3-(cyclohexylcarbamoylamino)propanoate as internal standard (Documentation provided to EFSA No. 2).

The flavouring substance has four asymmetric centres, resulting in 16 possible stereoisomers. According to the data provided by the applicant, the employed manufacturing process results in the generation of four of the stereoisomers in large excess: (3*R*)(1*S*,2*S*,5*R*), (3*S*)(1*S*,2*S*,5*R*), (3*R*)(1*R*,2*R*,5*S*) and (3*S*)(1*R*,2*R*,5*S*). The sum of these four stereoisomers in the investigated batches of the flavouring substance ranged from 93.5 wt% to 98.7 wt%; on average, these stereoisomers amounted to 95.7 ± 1.66 wt% ($n = 8$) (Documentation provided to EFSA No. 2).

The applicant also provided quantitative data on other stereoisomers that correspond to three minor peaks in the chromatogram. Their total amounts ranged from 0.9 wt% to 4.3 wt%; the average was 3.0 ± 0.9 wt% (Documentation provided to EFSA No. 2).

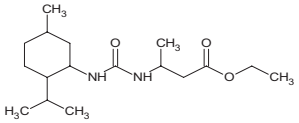
For one of the tested batches (batch no: 10300015), the applicant tentatively identified five minor impurities, and the sum of their peak areas amounted to 1.3% of the total peak area in the presented chromatogram (Documentation provided to EFSA No. 2). They are all structurally based on the menthylamine (CAS no: 21411-81-4) scaffold and their formation could be explained by side reactions occurring during the synthesis. The detection of the volatile impurities menthylamine (0.06% of total GC peak area) and 2-isocyanato-1-isopropyl-4-methylcyclohexane (0.02% of total GC peak area) was explained by the applicant due to the possible degradation of the flavouring substance during the chromatographic analysis. For three other minor impurities detected via GC (sum: 0.1% of total GC peak area), no mass spectrometric data could be obtained.

In the submitted batches, the total amounts of all 16 stereoisomers of the flavouring substance ranged from 98.1 wt% to 99.9 wt%; the average amount ($n = 8$) was 98.7 ± 1.08 wt% (Documentation provided to EFSA No. 2).

In addition, to demonstrate batch-to-batch variability/reproducibility, the applicant provided data on the amounts of all stereoisomers of the flavouring substance in 13 batches produced in the period from December 2010 to July 2022. The total amounts of all 16 stereoisomers ranged from 97.0% to 98.7%, with an average of 97.8%. The low relative standard deviation of 0.8% indicates the consistency of the production process.

Regarding the solvents applied in the manufacturing process, the applicant provided data for 13 batches of the flavouring substance in which the residual concentration of heptane ranged from 90 to 928 mg/kg, with an average of 446 mg/kg. In 10 batches, the content of methyl-*tert*-butyl-ether (MtBE) was reported to be below 10 mg/kg (Documentation provided to EFSA No. 2); the limit of detection (LOD) and limit of quantification (LOQ) for MtBE were not specified.

TABLE 1 Specification data for 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester (Documentation provided to EFSA No. 1–3). The table was compiled by the Panel according to the information provided in the technical dossier.

Chemical name	CAS no FL-no EC no CoE no JECFA no FEMA no	Chemical formula MW	Structural formula	Physical form	Solubility data	ID test	Purity	Impurities	Boiling point melting point specific gravity refractive index
3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester	1160112-20-8 16.136 – – 2203 4766	$C_{17}H_{32}N_2O_3$ 312.45 g/mol		Pearl-white powder	Water: insoluble Propan-1-ol: soluble[a] Ethanol: soluble[b] DMSO: soluble[b] Non-polar solvents: sparingly soluble[b]	GC HPLC GC–MS LC–MS ^{1}H-NMR FT-IR	Sum of all 16 stereoisomers: ≥ 98 wt%; Sum of the four main stereoisomers: (3*R*)(1S,2S,5R), (3*S*)(1S,2S,5R), (3R)(1R,2R,5S), (3S)(1R,2R,5S) ≥ 95 wt%	Not more than 2.2% of the total GC-FID peak area	N.A. 101–108°C N.D. N.D.

Abbreviations: FT-IR, Fourier-transform Infrared Spectroscopy; GC, Gas Chromatography; GC–MS, Gas Chromatography-Mass Spectrometry; HPLC, High-Pressure Liquid Chromatography; ID, Identity; LC–MS, Liquid Chromatography-Mass Spectrometry; MW, Molecular Weight; N.A., not applicable; N.D., not determined; NMR, Nuclear magnetic Resonance.

[a]At least 20% w/w (Documentation provided to EFSA No. 2).

[b]Based on visual testing only (Documentation provided to EFSA No. 2).

3.1.2 | Organoleptic characteristics

The applicant described the flavouring substance as a pearl-white powder to be used as a 'flavouring substance with cooked and roasted, meat, spice/herb, and vegetable notes'.

3.1.3 | Manufacturing process

In the first step of the manufacturing process, menthone/isomenthone is converted to menthylamine via a so-called Leuckart–Wallach reaction. The flavouring substance is obtained by reaction of this intermediate with ethyl 3-isocyanatobutanoate (Figure 1). The applicant provided adequate information on purity and specifications of the reagents and solvents used, the reaction conditions and the purification steps employed.

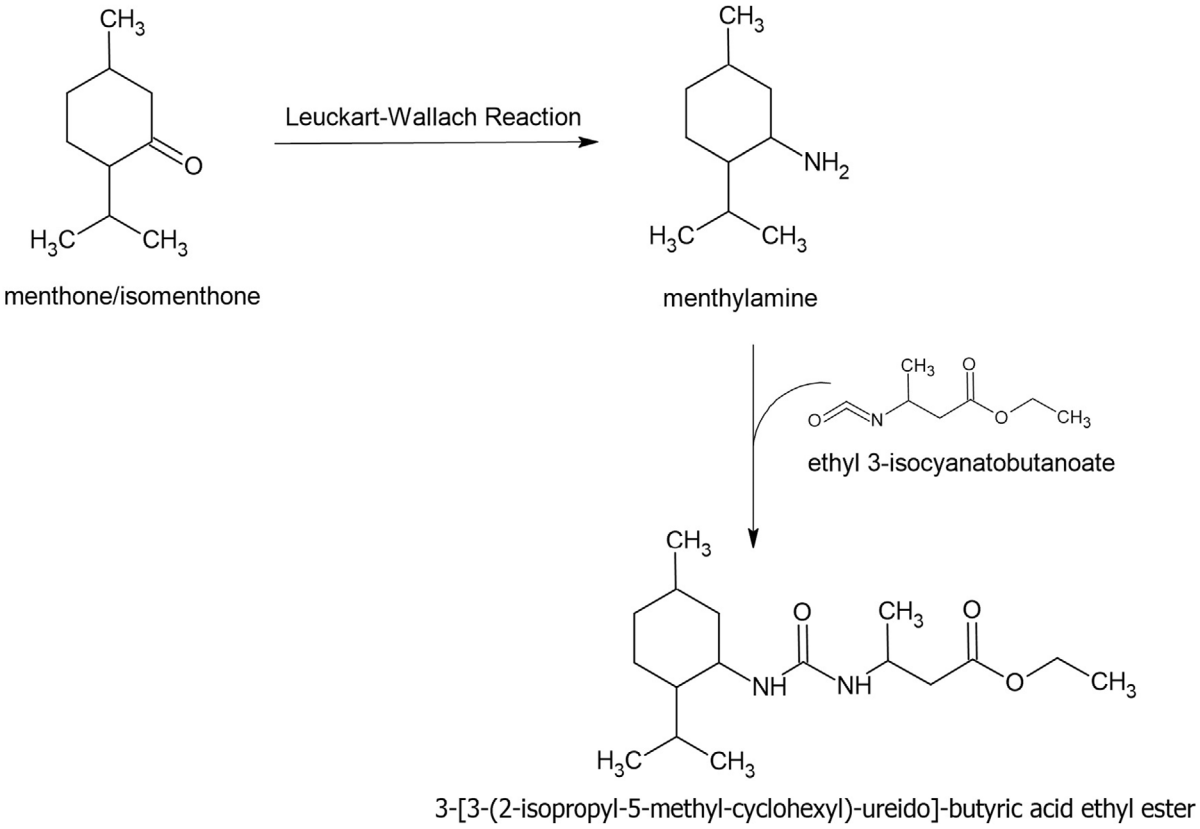


FIGURE 1 Production process of 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester.

3.1.4 | Particle size distribution and solubility

Particle size distribution

The applicant provided results from scanning electron microscopy (SEM) analysis coupled with energy-dispersive X-ray spectrometer (EDX) on dry samples of three batches of pristine 3-[3-(-2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester (Documentation provided to EFSA No. 2). The sample preparation and method of analysis, along with justification for choosing dry samples for quantitative analysis, were thoroughly described. The SEM images were taken with magnifications up to x120,000. The particle size distribution, shape and elemental composition of the material were analysed. Three independent number-based particle size distributions referring to length, width and thickness, along with their descriptive parameters, were provided. The applicant reported that the particles are irregular polyhedron-shaped with a smooth or slightly uneven surface. They are micrometric or submicrometric. Smaller particles can be detected in aggregates/agglomerates, mainly on top of large particles, but few isolated constituent particles are also detected. These smaller particles have a quite smooth surface and are mainly spheroids or irregular polyhedrons. The applicant reported that all samples are characterised by a broad particle size distribution for all three dimensions (i.e. length, width and thickness).

Quantitative analysis of SEM images revealed that, in the analysed batches of the flavouring substance, the d10 of particle size ranged from 240 to 347 nm, 147 to 203 nm and 122 to 143 nm in length, width and thickness, respectively. In the fraction containing only particles below 500 nm, the d10 particle size ranged from 177 to 201 nm, 129 to 163 nm and 90 to 116 nm in length, width and thickness, respectively.

The applicant concluded that the pristine flavouring substance contains a fraction of small particles, including nanoparticles, as defined in the EFSA Guidance on Particle-TR.

Solubility

The applicant stated that the flavouring substance is soluble in ethanol and dimethylsulfoxide (DMSO). The applicant provided further information on the solubility of the flavouring substance in water and other organic solvents. The tests were not performed according to the OECD 105 method (OECD, 1995). Saturated solutions of the flavouring substance in selected solvents were prepared and analysed by HPLC-UV. The details of the HPLC method employed were submitted. The solubility in water was reported as 85 mg/L after 1 day and 15 mg/L after 56 days in the solution (Documentation provided to EFSA No. 2).

The Panel considered that the method used in this study was insufficient to substantiate the reported solubility of the flavouring substance in water or organic solvents tested.

The applicant provided further information regarding the dissolution rate of 3-[3-(-2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester in water to investigate whether the particles would be solubilised under conditions simulating gastrointestinal digestion (EFSA Scientific Committee, 2021) (Documentation provided to EFSA No. 2). The applicant followed the main steps of the protocol of dissolution rate test as included in section 2.3.2.3 of the EFSA Guidance on Particle-TR (EFSA Scientific Committee, 2021). Test item concentrations were 25 mg/L (at pH 7) and 50 mg/L (at pH 3) in 0.5 L of medium (ultrapure water containing 5 mmol/L $NaHCO_3$ and 40 mmol/L NaCl). The concentration of dissolved flavouring substance was determined at six time points (0, 5, 10, 30, 60 and 120 min) by LC–MS following ultrafiltration using a 5-kDa membrane filter. The dissolution tests were done in triplicate (nEcoTox GmbH, 2024a).

The reported results indicated that, at pH 3, the dissolution rate was 1.01^{-4} mg /L s, and at pH 7, the dissolution rate was 2.81^{-4} mg /L s. The amount of flavouring substance dissolved after 30 min at both pH 3 and pH 7 was below the LOQ of the LC–MS method, which was 5 µg/L. The applicant reported that the concentration of dissolved test item increased linearly with time, but no equilibrium was reached. Additionally, visual observations indicated that the test item did not fully dissolve during the 120-min duration of the tests.

The applicant further stated that the ultrafiltration membranes adsorbed considerable amounts of the flavouring substance, suggesting the true dissolution rate exceeds 5.8 mg/L after 120 min, compared to the measured < 1 mg/L.

Based on the data provided, the Panel concluded that the dissolution rate of 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester in water at pH 3 and pH 7 does not meet the criterion outlined in the EFSA Guidance on Particle-TR, which specifies that at least 88% of the substance should dissolve within 30 min (EFSA Scientific Committee, 2021).

The applicant declared that 3-[3-(–2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester is intended to be used as a flavouring substance in food as a solution in propan-1-ol at up to 20% w/w, with an intended maximum use level of up to 20 mg/kg food. The Panel noted that, based on the evaluation by JECFA (JECFA, 1999), propan-1-ol [FL-no: 02.002] is an authorised flavouring substance in the European Union (EU) without restrictions according to Regulation (EC) No 1334/2008.[1]

The applicant provided a study on the solubility behaviour of 3-[3-(–2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester in propan-1-ol. Four concentrations of the flavouring substance 5, 10, 15 and 20% w/w in propan-1-ol were subjected to ultrafiltration applying a 10-kDa polyether sulfone membrane. Concentrations of 3-[3-(–2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester prior to and after ultrafiltration were compared. For all the solutions, the mean recovery rates were above 95%, and not dependent on the starting concentration of 3-[3-(–2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester (Documentation provided to EFSA No. 2; nEcoTox GmbH, 2024b). Following these results, the Panel considered that the solubility of 3-[3-(–2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester in propan-1-ol is at least 200 g/L.

Taking into account the data provided on the solubility of 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester in propan-1-ol (i.e. at least 200 g/L), and the proposed use of the flavouring substance in food only as a solution in propan-1-ol (up to 20% w/w), resulting in a maximum concentration of 20 mg/kg in the final product, the Panel considered that there is no concern with regard to the potential presence of small particles, including nanoparticles, in the flavouring substance under the proposed conditions of use. Consequently 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester can be assessed following the conventional risk assessment, i.e. Guidance on risk assessment of Flavourings (EFSA CEF Panel, 2010) should be applied.

3.1.5 | Proposed specifications

The chemical structure of the flavouring substance [FL-no: 16.136] and the specification data provided by the applicant, in the original dossier and in response to the EFSA requests for additional information, are summarised in Table 1 (Documentation provided to EFSA No. 1–3).

The Panel noted that, according to the proposed specifications, the flavouring substance is reported as 'soluble' in ethanol, DMSO and non-polar solvents, but no numerical data were provided by the applicant to support this proposal.

Additionally, the applicant specified the solubility of the candidate substance in propan-1-ol as 'soluble' (Table 1). However, the Panel noted that the data submitted by the applicant indicates a solubility of the proposed food flavouring

in propan-1-ol of at least 20% w/w (see Section 3.1.4). Furthermore, the Panel noted that 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester is intended to be added to food solely as a solution in propan-1-ol (max 20% w/w), and therefore, the solubility in propan-1-ol of at least 20% w/w should be captured in the specifications.

3.1.6 | Stability and fate in food

The storage stability of the flavouring substance was investigated for 18 months; the flavouring substance was kept in the applicant's warehouses at room temperature (10–30°C), packed in corrugated cardboard boxes lined with polyethylene bags (Documentation provided to EFSA No. 1, 2, 3). The analyses of the flavouring substance were performed on samples taken at seven time points (0, 1, 2, 10, 16, 18 and 18.5 months). The sum of all stereoisomers ranged from 97.7% to 99.4% with an average of 98.4%. The low standard deviation of 1.04% confirmed the stability of the flavouring substance under the employed storage conditions (Documentation provided to EFSA No. 1).

The applicant informed that the flavouring substance is dissolved in propan-1-ol prior to its addition to foods; therefore, two accelerated stability studies were performed in propan-1-ol to demonstrate its stability in solution (Documentation provided to EFSA No. 2). Tests were performed in duplicate. In test 1, samples were prepared at 5, 10, 15 and 20 wt% in propan-1-ol, following sonication for 30 min. Samples were stored for 4 weeks at 40°C and analysed at 2 and 4 weeks. In test 2, samples at 20 wt% were kept at 40°C under 5 bar of oxygen pressure, for 86 and 168 h. In test 1, samples showed a recovery rate ranging from 84.8% (at conc. of 20 wt%) to 100% (at conc. of 5 wt%). According to the applicant, the storage conditions applied in test 1 correspond to approximately 16 weeks of normal storage conditions. In test 2, samples showed a recovery rate ≥ 98% at 86 h and ≥ 96.5% at 168 h. According to the applicant, the storage conditions applied in test 2 correspond to approximately 6 and 12 months of normal storage conditions, respectively.

Following an additional data request from EFSA in which the applicant was requested to investigate the stability of the flavouring substance in aqueous solutions, the applicant provided data on the stability of the flavouring substance in a bouillon base (15 g of a model broth (42.5% maltodextrin, 20% salt, 14% yeast extract, 10% monosodium glutamate, 3.5% vegetable stock extract; pH 6.24) in 1 L water). The flavouring substance was added at a concentration of 10 mg/L and the mixture was stored at 40°C for 12 weeks. HPLC analysis of the main stereoisomers was performed after 1, 3, 6 and 12 weeks. After 6 and 12 weeks, slight decreases of the concentration of the flavouring substance compared to the start of the storage experiment (from 9.4 mg/L to 8.1 and 7.8 mg/L, respectively) were observed. At these time points, 0.8 mg/L and 1.1 mg/L, respectively, of the ester hydrolysis product 3-(3-(2-isopropyl-5-methyl-cyclohexyl)-ureido)-butyric acid were detected. The sum of the starting ethyl ester and the hydrolysis product remained constant, indicating that this hydrolysis was the only degradation process occurring under the test conditions (Documentations provided to EFSA No. 2, 3).

3.1.7 | Interactions with food components

No specific tests aimed to evaluate potential interactions of the flavouring substance with food components were provided by the applicant. Nevertheless, in the study on the stability of the substance in food (bouillon base; see Section 3.1.6), the unchanged concentration of the flavouring substance (determined after 3 weeks of storage) along with the constant combined concentration of the flavouring substance plus the free acid formed by hydrolysis (determined after 6 and 12 weeks of storage) indicated that there were no interactions with the constituents of the employed food matrix.

3.2 | Structural/metabolic similarity to flavouring substances in existing FGE

No flavouring substances with sufficient structural/metabolic similarity to 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] were identified in existing FGEs.

3.3 | Exposure assessment

3-[3-(2-Isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] is intended to be added to food solely as a solution in propan-1-ol (max 20% w/w). The use levels as proposed by the applicant (see Table B.1, Appendix B) refer to the concentration of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester in the final product (Documentation provided to EFSA No. 1, 2, 3).

3.3.1 | Natural occurrence in food

3-[3-(2-Isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester has not been reported to occur naturally in any food or food source (Volatile Compound in Food database (VCF, 2024)). Therefore, according to the information available, the only occurrence levels in food arise from its use as an added flavouring substance.

3.3.2 | Non-food sources of exposure

Non-food sources of exposure to 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester were not identified by the applicant (Documentation provided to EFSA no. 1). 3-[3-(2-Isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester is also not listed in the ECHA chemical database.[3]

3.3.3 | Chronic dietary exposure

In accordance with the applicable guidance, the exposure assessment to be used in the Procedure for the safety evaluation of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] is the chronic added portions exposure technique (APET) estimate (EFSA CEF Panel, 2010). The chronic APET was calculated for adults and children[4] (see Table 2). These values, expressed per kg body weight (bw) per day, will be used in the Procedure (see Appendices A and B). The chronic APET calculation is based on the proposed normal use levels and the standard portion sizes in both food and beverages (see Table B.1, Appendix B) (Documentation provided to EFSA No. 1).

Based on the information provided by the applicant, the Panel considered that [FL-no: 16.136] is not intended to be used in the food category 13.2 (foods for infants and young children).

TABLE 2 APET – Chronic dietary exposure to 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester as calculated by EFSA.

	Added as flavouring substance[a]		Other dietary sources[b]		Combined[c]	
Chronic APET	µg/kg bw per day	µg/person per day[f]	µg/kg bw per day	µg/person per day	µg/kg bw per day	µg/person per day
Adults[d]	14	860	0	0	14	860
Children[e]	36	540	0	0	36	540

Abbreviations: APET, added portions exposure technique; bw, body weight.

[a]APET added is calculated on the basis of the amount of flavouring added to a specific food category.

[b]APET other dietary sources are calculated based on the natural occurrence of the flavouring in a specified food category.

[c]APET combined is calculated based on the combined amount of added flavour and naturally occurring flavouring in a specified food category.

[d]For the adult APET calculation, a 60-kg person is considered representative.

[e]For the child APET calculation, a 3-year-old child with 15 kg bw is considered representative.

[f]Mathematical discrepancies derive from the degree of approximation used in the calculations.

3.3.4 | Acute dietary exposure

Based on the applicable guidance (EFSA CEF Panel, 2010), the acute APET calculation for 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] is based on the proposed maximum use levels (see Table B.1, Appendix B) and large portion size for both food and beverages (i.e. three times standard portion size). Acute exposure is reported in Table 3.

TABLE 3 APET – Acute dietary exposure to 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester as calculated by EFSA.

	Added as flavouring substance[a]		Other dietary sources[b]		Combined[c]	
Acute APET	µg/kg bw	µg/person	µg/kg bw	µg/person	µg/kg bw	µg/person
Adults[d]	100	6000	0	0	100	6000
Children[e]	252	3780	0	0	252	3780

Abbreviations: APET, Added portions exposure technique; bw, body weight.

[a]APET added is calculated on the basis of the maximum amount of flavouring added to a specific food category considering large portion sizes.

[b]APET other dietary sources are calculated based on the natural occurrence of the flavouring in a specified food category.

[c]APET combined is calculated based on the combined amount of added flavouring and naturally occurring flavouring in a specified food category.

[d]For the adult APET calculation, a 60-kg person is considered representative.

[e]For the child APET calculation, a 3-year-old child with 15 kg bw is considered representative.

3.3.5 | Cumulative dietary exposure

The Panel considered that there are no flavouring substances with close structural similarity to 3-[3-(2-isopropyl-5-methyl cyclohexyl)-ureido]-butyric acid ethyl ester. Therefore, calculation of the cumulative exposure is not applicable.

[4]A child of 3 years of age is considered in order to provide a conservative scenario for all children aged more than 3 years, since the consumption of foods and beverages per kg bw decreases with age. In the 3-year-old child, a 15 kg bw is considered (EFSA CEF Panel, 2010).

3.4 | Biological and toxicological data

3.4.1 | Absorption, distribution metabolism and excretion (ADME)

No experimental data on ADME were submitted for the flavouring substance [FL-no: 16.136].

3.4.2 | Metabolism

Metabolism studies have not been provided for the flavouring substance [FL-no: 16.136].

Based on general knowledge on biotransformation reactions, JECFA (JECFA, 2014, 2015) considered that the substance is likely to undergo ester hydrolysis followed by conjugation of the free acid. Additionally, oxidation by CYP450 could lead ultimately to the formation of menthone, urea and 3-oxo-butanoic acid. The Panel considered that the intermediates in this reaction are unknown, as is the rate at which such metabolism may occur. Therefore, the Panel considered that the substance should be evaluated through the B-side of the Procedure.

3.4.3 | Genotoxicity

Based on in silico analysis (OECD QSAR Toolbox[5] and ToxTree[6]), the substance 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester does not contain any structural alerts for genotoxicity, for genotoxic carcinogenicity or for non-genotoxic carcinogenicity.

The flavouring substance was tested in a bacterial reverse mutation assay (LPT, 2008) and an in vitro micronucleus (MN) assay (Covance, 2014), which are described below.

3.4.3.1 | *Bacterial reverse mutation assay*

A bacterial reverse mutation assay was conducted in Salmonella typhimurium strains TA98, TA100, TA102, TA1535 and TA1537 to assess the mutagenicity of 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester (purity of the batch used: 98.5% sum of 4 main stereoisomers; 99.4% sum of all stereoisomers), both in the absence and in the presence of metabolic activation by Aroclor 1254-induced rat liver S9 fraction (S9-mix). Two separate experiments were conducted, the first using the plate incorporation method and the second using the preincubation method (LPT, 2008). The study was performed according to OECD Test Guideline (TG) 471 (OECD, 1997) and in compliance with GLP. Positive control chemicals and vehicle control (dimethyl sulfoxide, DMSO) were evaluated concurrently. All tests were evaluated in triplicate plates. The highest concentration tested, based on a preliminary cytotoxicity test, was 3160 µg/plate.

In both experiments, 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester was tested at five concentrations from 31.6 to 3160 µg/plate, with and without S9-mix. Precipitate was observed at the concentration of 3160 µg/plate with and without S9-mix.

In the plate incorporation test with and without metabolic activation, cytotoxicity was observed at the concentration of 3160 µg/plate in test strains TA98, TA1535 and TA1537. In the preincubation test without metabolic activation, cytotoxicity was observed at the top concentration of 3160 µg/plate in test strain TA98.

All positive control chemicals both with and without S9-mix induced significant increases in revertant colony numbers. Mean revertant frequencies in vehicle controls were within the historical control ranges.

In both experiments, no increase in the mean number of revertant colonies was observed at any tested concentration in any tester strains in the absence or presence of metabolic activation (LPT, 2008).

The Panel considered the study to be reliable without restrictions and the relevance of its results as high. The Panel considered that 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester did not induce gene mutations in bacteria.

3.4.3.2 | *In vitro micronucleus assay*

Human peripheral blood lymphocytes from healthy donors were treated with 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester (purity of the batch used: 95.7% sum of 4 main stereoisomers; 99.3% sum of all stereoisomers). The in vitro MN assay was carried out according to OECD TG 487 (OECD, 2010) and in compliance with GLP. The cytokinesis block micronucleus assay protocol was applied. Positive controls were cyclophosphamide, mitomycin C and vinblastine. The vehicle DMSO was used as negative control. The highest concentration studied in the cytotoxicity range-finding experiment was 500 µg/mL, selected on the basis of solubility. No marked changes in osmolality or pH were observed at the

[5] https://www.oecd.org/chemicalsafety/risk-assessment/oecd-qsar-toolbox.htm.

[6] https://toxtree.sourceforge.net/.

highest concentration tested (500 µg/mL). Concentrations for the micronucleus experiments were selected based on the results of this cytotoxicity range-finding experiment (Covance, 2014).

For the MN experiment, lymphocytes were treated with 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester in three different test conditions: 3 h treatment followed by 21-h recovery period (3 h + 21 h) in the presence (3 + 21 h with S9-mix) or in the absence (3 + 21 h without S9-mix) of rat liver S9 metabolic activation (from rats treated with Aroclor 1254) and 24-h treatment in the absence of metabolic activation (24 h without S9-mix). Replication index (RI) cytotoxicity data were used to select the concentrations for the MN analysis.

In the treatment of 3 h + 21 h with S9-mix, 11 concentrations were tested ranging between 25 and 400 µg/mL and the following concentrations were chosen for MN analysis: 100, 180, 220 and 240 µg/mL (cytotoxicity of 0%, 19%, 32% and 54%, respectively).

In the treatment of 3 h + 21 h without S9-mix, 15 concentrations were tested ranging between 15 and 300 µg/mL and the following concentrations were chosen for MN analysis: 50, 75, 100 and 125 µg/mL (cytotoxicity of 0%, 14%, 41% and 54%, respectively).

In the treatment of 24 h without S9-mix, 12 concentrations were tested ranging between 10 and 100 µg/mL and the following concentrations were chosen for MN analysis: 30, 40 and 50 µg/mL (cytotoxicity of 6%, 27% and 50%, respectively).

The frequency of micronucleated cells in the vehicle controls was within the historical control 95% reference range in all culture conditions. The Panel noted that no historical positive control ranges were provided. However, the concurrent positive controls induced a statistically significant increase in the frequency of micronucleated cells as compared to the concurrent negative control and exceed the historical negative control range. 3-[3-(2-Isopropyl-5-methyl-cycloh exyl)-ureido]-butyric acid ethyl ester did not increase the frequency of micronucleated cells compared to the respective vehicle (DMSO) controls in any of the testing conditions.

The Panel considered the study to be reliable without restrictions and the relevance of its results as high.

The Panel considered that 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester did not induce the formation of MN in human lymphocytes in any experimental conditions of this study.

A summary of the genotoxicity studies is reported in Appendix C.

3.4.3.3 | *Information on the hydrolysis product 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid*

In addition, the applicant evaluated the genotoxic potential of the hydrolysis product 3-[3-(2-isopropyl-5-methyl-cyclohex yl)-ureido]-butyric acid that was detected in the course of the stability study (see Section 3.1.6). This evaluation was based on QSAR prediction (DEREK Nexus, V. 6.2.1 (Derek KB 2022 2.0); OECD QSAR Toolbox, V. 4.5 SP1) and read-across from the available in vitro genotoxicity studies provided for the parent compound 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester. No indications for genotoxicity were identified. The Panel agreed with this evaluation.

3.4.3.4 | *Conclusions on genotoxicity*

3-[3-(2-Isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] did not induce gene mutations in a bacterial reverse mutation assay and was neither clastogenic nor aneugenic in an in vitro micronucleus test. Therefore, there was no requirement to test the candidate substance for genotoxicity in vivo (EFSA Scientific Committee, 2011).

The Panel considered that the hydrolysis product 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid is closely structurally related to the proposed flavouring substance [FL-no: 16.136].

Therefore, based on the genotoxicity studies with the flavouring substance and based on QSAR analysis and read-across considerations on the hydrolysis product, the Panel concluded that the substance 3-[3-(2-isopropyl-5-methyl-cyclohexyl) -ureido]-butyric acid ethyl ester and its hydrolysis product 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid do not raise concern for genotoxicity.

3.4.4 | Toxicity data

3.4.4.1 | *Acute oral toxicity study*

The acute oral toxicity of 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester was determined according to OECD TG 423 (acute toxic class method; OECD, 2001a) and in compliance with GLP. The study authors reported that the test item (purity of the batch used: 98.5% sum of 4 main stereoisomers; 99.4% sum of all stereoisomers) was dissolved in a 4:6 mixture of ethanol and water for administration to female Wistar rats (stock CRl:WI of Charles River). Two groups of rats (each $n = 3$, fasted overnight) received a single dose of 2000 mg/kg bw by gavage followed by an observation period of up to 14 days. At 30 min and 2 h after application of the test item, all six rats showed a hunched posture and piloerection; after 4- and 6-h normal behaviour was observed. From day 1 to day 14, no abnormalities were observed; all rats had a normal body weight gain (at group mean and individual level). The gross necropsy on day 14 revealed no pathological abnormalities. In the absence of treatment-related death or severe signs of toxicity, the study authors concluded that the oral median lethal dose (LD50) in rats was above 2000 mg/kg bw (Frey-tox, 2009). The Panel agreed with this conclusion.

3.4.4.2 | *14-day dietary administration range-finding toxicity study in rats*

A 14-day dose range-finding study (Product Safety Labs, 2012) was performed with a batch of 3-[3-(-2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester with a purity of 95.8% (no further details provided).

Eight-week -old Hsd:SD® rats (5/sex/group) were fed for 14 days a diet containing 3-[3-(-2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester at concentrations that corresponded to doses of 0, 250, 500 and 1000 mg/kg bw per day (based on food consumption data, the actual doses were equal to 0, 256, 523 and 1050 mg/kg bw per day for male rats and 0, 271, 525 and 1096 mg/kg bw per day for female rats).

In the study report, it was reported that the study was conducted according to OECD TG 407 for a 28-day oral toxicity study. However, the Panel noted that this 14-day dose-range finding study was not fully consistent with OECD TG 407 (only a few parameters were evaluated, e.g. haematology and clinical chemistry were not evaluated).

No test substance-related mortality or other adverse effects were observed. It was concluded that the test substance was well tolerated at doses up to 1050 and 1096 mg/kg bw per day for male and female rats, respectively.

3.4.4.3 | *90-day oral toxicity study*

The test substance, 3-[3-(–2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester (purity of the batch used: 95.7% sum of the 4 main stereoisomers; 99.3% sum of all stereoisomers), was tested in a 90-day repeated dose toxicity study in rats (Product Safety Labs, 2013) according to OECD TG 408 (OECD, 1998) and in compliance with GLP.

Seven- to 8-week-old Crl: Sprague–Dawley® CD® IGS rats (10/sex/group) were fed a diet containing 3-[3-(-2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester at concentrations that corresponded to doses of 0, 250, 500 and 1000 mg/kg bw per day (based on food consumption data, the actual doses were equal to 0, 198, 388 and 777 mg/kg bw per day for male rats and 0, 228, 460 and 923 mg/kg bw per day for female rats).

The substance was added as a powder to the diet. The neat test substance was shown to be stable for the duration of the study. The test substance was shown to be evenly distributed in the feed and was demonstrated to be stable in the diet over 10 days. The feed was prepared fresh every week.

Ophthalmoscopy was performed at the start and at the end (Day 90) of the study. Cage-side observations were performed daily during the study period. Animals were inspected for clinical signs weekly in more detail. Animals were weighed twice during acclimation (including prior to test initiation (Day 0)) and approximately weekly thereafter, and prior to sacrifice. Food consumption and efficiency were measured and calculated weekly. Samples for blood biochemistry, haematology and urinalysis were collected at the end of the study. Full necropsy, collection of tissues and measurements of organ weights and histological examination were performed on all animals.

No test substance-related adverse effects were observed on survival, clinical signs, food consumption, body weight, body weight gain, ophthalmological changes, macroscopic findings and microscopic findings.

For male rats in the high-dose group, there was a statistically significant increase (6%) in red blood cells (RBC). In male rats, there were also a dose-dependent decreases in mean corpuscular volume (MCV) (1%–5%, statistically significant for the high dose) and mean corpuscular haemoglobin (MCH) levels (1%–5%, statistically significant for the high dose group).

For female rats, there were a small dose-dependent decrease in RBC (2%–6%) and haematocrit (HCT) (2%–5%), which reached statistically significance for the mid- and high-dose groups. Haemoglobin (HGB) was decreased in a dose-dependent manner for which the difference compared to controls reached statistical significance for the high-dose group. However, for the males, the observed changes were all less than 10%, and for the females, they were all less than 5%. Since all values remained within the range of historical control values and given the low magnitude of the observed changes, they were considered of no biological relevance.

Statistically significant, dose-dependent, increases in activated partial thromboplastin clotting time (APPT) were observed in all exposed male rats (12–32%, up to 22.10 s in the high-dose group). All values were within the range of historical control values (mean 18.5 s, range: 13.5–33.4 s). The increase in APPT was not considered of biological relevance.

For clinical chemistry parameters, a few statistically significant differences were observed. For both sexes, a dose-dependent decrease in bilirubin was observed (12%–35%). The mean bilirubin concentrations for the high-dose groups were at the lower limit of the historical control range, but in absence of major changes in the haematological parameters or in other organs, the Panel considered the decrease in bilirubin to be not adverse.

An increase in the number of animals with fine granular casts in urine, assessed semi-quantitatively, was seen in all male dose groups and in one female of the mid-dose group. The occurrence of these casts was reported as 'few' and was not clearly dose related.

There were no statistically significant differences in mean absolute organ weights. However, higher relative (to body weight) liver weight was observed for both sexes, reaching statistical significance for the high-dosed females and for males in the low- and high-dose groups. However, the differences compared to controls were < 15% and in the absence of histological changes, this increase in relative liver weight is not considered adverse.

Overall, the Panel considered the changes observed in the haematology, clinical chemistry and coagulation parameters to be non-adverse due to either the small magnitude of the effects and/or because the values are within the historical control range and for some of them without a clear dose response.

The Panel considered that the NOAEL is 777 and 923 mg/kg bw per day (the highest doses tested) for male and female rats, respectively, in this study.

3.4.4.4 | *Prenatal developmental toxicity study in rats*

3-[3-(2-Isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester (purity of the batch used: 94.3% sum of 4 main stereoisomers; purity 98.6%, sum of all stereoisomers) was tested in a dose range finding prenatal developmental toxicity study in timed-pregnant CRL Sprague–Dawley CD® IGS rats (Product Safety Labs, 2017) according to OECD TG 414 (OECD, 2001b). The study was not performed in full compliance with GLP, but it was conducted in a GLP-compliant facility.

Four groups of animals (6 per group) were administered the test substance at 0 (corn oil as vehicle), 250, 500 and 1000 mg/kg bw per day via oral gavage during gestational days 5–19, with sacrifice on day 20 of pregnancy. The Panel noted that the mid dose was not analysed for homogeneity and concentration verification.

No mortalities and no maternal or developmental effects were observed; therefore, the same dose levels were tested in the main study.

3-[3-(2-Isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester (purity of the batch used: 94.3% sum of 4 main stereoisomers; purity 98.6%, sum of all stereoisomers) was subsequently tested in a prenatal developmental toxicity study in CRL Sprague–Dawley CD® IGS rats (Product Safety Labs, 2018) according to OECD TG 414 (OECD, 2001b) and in compliance with GLP.

Four groups of animals (20 time-mated females per group) were administered the test substance at 0 (Group 1, vehicle control), 250 (Group 2), 500 (Group 3) or 1000 (Group 4) mg/kg bw per day in corn oil vehicle 5 mL/kg bw per day.

The test substance or vehicle control was administered daily (7 days/week) via oral gavage to each rat during gestation days (GD) 5–19. All animals survived until sacrifice at GD 20.

Number of pregnant animals was 17, 17, 18 and 18 in the 0, 250, 500 and 1000 mg/kg dose groups, respectively.

Incidental clinical signs noted in females included: anogenital staining in 1/20 Group 2 and 1/20 Group 4 animals; superficial eschar of the back in 1/20 Group 2 animals; moist rales in 1/20 Group 4; nasal discharge in 1/20 Group 3 animals.

No changes in body weight and body weight gain were observed compared to control group.

No effects were observed in uterine and reproductive parameters (including early and late resorptions). Litter sizes were between 12 and 14 for the four groups. For one female in group 3 detachment of placenta was observed, resulting in death of several fetuses. Since this was an observation in one animal only in the mid-dose group, this is not considered substance-related. Apart from resorptions, there were no dead fetuses in any litter in any dose group.

Approximately half of the total number of live fetuses (209, 242, 218 and 239 from Groups 1 to 4, respectively) were evaluated for visceral malformations and variations and the remaining fetuses were evaluated for skeletal malformations and variations. Several skeletal variations are reported in the study but considered by the study authors as not related to the test substance administration. No visceral or skeletal malformations were observed.

Overall, the Panel considered that no treatment-related adverse effects were identified in this study and that 1000 mg/kg bw per day (the highest dose tested) is the NOAEL in this study.

Summaries of the toxicity studies are reported in Appendix D.

3.5 | Application of the Procedure

No flavouring substances with sufficient structural/metabolic similarity to 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] were identified in existing FGEs. Since 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] does not raise a concern for genotoxicity, it is therefore appropriate to evaluate the use of [FL-no: 16.136] as a flavouring substance following the stepwise evaluation Procedure for individual substances as outlined in the 'Guidance on the data required for the risk assessment of flavourings to be used in or on foods' (EFSA CEF Panel, 2010) and Appendix A.

Step 1

The substance 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester is allocated to structural class III.[7]

Step 2

Since no data on metabolism are available to demonstrate that the substance can be expected to be metabolised to innocuous products, the substance is evaluated via the right (B-)side of the Procedure (see Appendix A, Figure A.1).

Step B3–B4

The conditions of use result in APET exposure estimates of 14 and 36 μg/kg bw per day (860 and 540 μg /person per day), for adults and children, respectively. These estimates are above the threshold of toxicological of concern (TTC) for Cramer Class III (90 μg/person per day), but below 10-fold this TTC (900 μg/person per day). Therefore, a 90-day toxicity study and a developmental toxicity study were submitted by the applicant and considered by the Panel. In both these studies, no

[7]Determined with OECD QSAR Toolbox (version 4.7 available at https://qsartoolbox.org/).

adverse effects were observed. In the prenatal developmental toxicity study, the Panel considered that the NOAEL is 1000 mg/kg bw per day (the highest dose tested). In the 90-day toxicity study, the Panel considered that the NOAEL is 777 and 923 mg/kg bw per day (the highest dose tested) for male and female rats, respectively. Using the lowest NOAEL of 777 mg/kg bw per day as a reference point, adequate margins of exposure (MOEs) of 55×10^3 and 21×10^3 were calculated for adults and children, respectively, when assessing the intake based on APET.

3.6 | Assessment of acute, combined and cumulative exposure

The estimates for acute exposure to 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] are 100 and 252 μg/kg bw corresponding to 6000 and 3780 μg/person for adults and children, respectively (see Table 3). No signs of acute toxicity were observed in an acute oral toxicity study in rats, from which an oral LD50 above 2000 mg/kg bw (single dose tested) was derived. Since this dose level is far above the potential acute exposure in humans, there is no concern for acute toxicity in humans.

Since 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester does not occur naturally in food and exposure to the substance from non-food sources is not expected (see Section 3.3), combined exposure estimates do not need to be addressed.

Because no flavouring substances with sufficient structural/metabolic similarity to 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] were identified in existing FGEs, cumulative exposure does not need to be addressed either.

4 | DISCUSSION

The European Commission requested EFSA to carry out the safety assessment of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester (CAS no. 1160112-20-8) as a new flavouring substance in accordance with Regulation (EC) No 1331/2008.[2]

EFSA evaluated 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] in the Flavouring Group Evaluation 418 (FGE.418) and used the Procedure as referred to in Regulation (EC) No 1334/2008.[1] No other substances with structural similarity to the flavouring substance have been identified in existing FGEs. The substance is obtained through chemical synthesis and is not known to occur naturally.

The specifications for the flavouring substance (as a powder) are considered adequate, including the information on purity (i.e. ≥ 95 wt% purity as sum of the four main stereoisomers; ≥ 98 wt% purity as sum of all 16 stereoisomers). The information provided on the manufacturing process (i.e. chemical synthesis) was considered sufficient. The flavouring substance was shown to be stable upon storage (i) in powder form for up to 18 months and (ii) dissolved in propan-1-ol (which, according to the applicant, is the solvent used prior to addition of the flavouring substance to foods) for up to 12 months.

Regarding particle size distribution, data submitted showed that 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester, as pristine material, contains a fraction of small particles, including nanoparticles, as defined in EFSA Guidance-TR (EFSA Scientific Committee, 2021).

The applicant provided results from dissolution rate tests in aqueous medium at pH 3 and pH 7, simulating gastrointestinal conditions, in line with main steps of the EFSA Guidance-TR protocol (EFSA Scientific Committee, 2021). After 60 min, the dissolution of the flavouring substance was below the method's LOQ, i.e. < 5 μg/L. Based on the data provided, the Panel concluded that the dissolution rate of 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester in aqueous medium at pH 3 and pH 7 does not meet the criterion outlined in the EFSA Guidance on Particle-TR, which specifies that 88% of the substance should dissolve within 30 min.

The applicant declared that 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester is intended to be added to food solely as a solution in propan-1-ol (max 20% w/w), resulting in a concentration of the flavouring substance of maximally 20 mg/kg in the final food. The Panel noted that, based on the evaluation by JECFA (JECFA, 1999), propan-1-ol [FL-no: 02.002] is an authorised flavouring substance in the European Union (EU) according to Regulation (EC) No 1334/2008.[1]

The applicant provided a solubility behaviour test of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester in propan-1-ol up to 20% w/w demonstrating that the flavouring substance is soluble in propan-1-ol at least at 200 g/L.

Taking into account the provided analytical data on the solubility of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester in propan-1-ol, as well as the proposed conditions of use, the Panel considered that there is no concern with regard to the presence of small particles, including nanoparticles, in the flavouring substance under the proposed conditions of use and that 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester can be assessed following the conventional risk assessment, i.e. by applying the guidance on risk assessment of flavourings (EFSA CEF Panel, 2010). This conclusion only applies when the flavouring substance is dissolved in propan-1-ol up to 20% w/w prior to its addition to food, and this condition of use should be reflected in the specifications.

For the use of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] as a flavouring substance, adequate information on uses and use levels has been provided, as specified in Appendix B. The substance is not

intended to be used in food for infants and young children. The chronic and acute dietary exposure to the proposed flavouring substance have been estimated using the APET method. The chronic APET exposure estimates are 14 and 36 µg/kg bw per day (860 and 540 µg /person per day) for adults (60 kg bw) and children (15-kg bw; 3-year-old), respectively. The acute APET exposure estimates are 100 and 252 µg /kg bw (6000 and 3780 µg/person, for adults and children, respectively).

The applicant submitted adequate studies to investigate the genotoxic potential of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester. Based on the available data, the Panel concluded that there is no concern with respect to genotoxicity.

No substance-specific information on absorption, distribution, metabolism and excretion (ADME) of [FL-no: 16.136] has been submitted. However, based on general knowledge on biotransformation reactions, the substance [FL-no: 16.136] is likely to undergo ester hydrolysis followed by conjugation of the free acid. Additionally, oxidation by CYP450 could lead ultimately to the formation of menthone, urea and 3-oxo-butanoic acid. The Panel considered that the intermediates in this reaction are unknown, as is the rate at which such metabolism may occur. Therefore, the Panel considered that the substance should be evaluated through the B-side of the Procedure (Appendix A).

The substance [FL-no: 16.136] is allocated to structural class III and the APET exposure estimates are between the TTC (90 µg/person per day) and 10 times the TTC applicable for this class. In accordance with the applicable guidance document (EFSA CEF Panel, 2010), the applicant submitted a 90-day subchronic toxicity study, and a developmental toxicity study carried out in the rat. No developmental toxicity was observed with dose levels up to 1000 mg/kg bw per day. In the 90-day study, the Panel considered the changes observed in the haematology, clinical chemistry and coagulation parameters to be non-adverse due to either the small magnitude of the effects and/or because the values are within the historical control range and for some of them without a clear dose response. The Panel noted that the study was performed in 2013 according to the OECD TG 408 in the version of 1998, which lacks some endpoints specified in the currently applicable version (OECD, 2018). Nevertheless, the Panel is of the view that the submitted study can be used. The Panel considered that the NOAEL is 777 and 923 mg/kg bw per day (the highest dose tested) for male and female rats, respectively. Using the lowest NOAEL of 777 mg/kg bw per day as a reference point, adequate MOEs of 55×10^3 and 21×10^3 were calculated for adults and children, respectively, based on the chronic APET dietary exposure estimates.

Based on the available acute toxicity study on 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester, the Panel concluded that there is no concern for acute toxicity.

Overall, the use of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester as food flavouring at the proposed uses and use levels, as specified in Appendix B, is of no safety concern.

5 | CONCLUSIONS

The Panel concluded that the use of 3-[3-(2-isopropyl-5-methylcyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] as a flavouring substance under the proposed conditions of use (to be dissolved in propan-1-ol up to 20% w/w prior to its addition to food) does not raise a safety concern at the dietary exposure estimates calculated using the APET approach.

6 | DOCUMENTATION AS PROVIDED TO EFSA

1. Technical Information Submission for a New Flavouring Substance by Symrise AG to the European Food Safety Authority (EFSA) according to the 'Common Authorisation Procedure for the application for evaluation of a new flavouring substance' (Regulation (EC) No 1334/2008, Regulation (EC) No 1331/2008, Regulation (EU) No 234/2011). January 2022. Submitted by Symrise AG.
2. Additional information received on 20 March 2024, submitted by Symrise AG in response to a request from EFSA (11 May 2022).
3. Additional information received on 17 July 2024, submitted by Symrise AG in response to a request from EFSA (16 April 2024 with addendum letter sent on 7 June 2024).
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ABBREVIATIONS

ADME	Absorption, Distribution, Metabolism and Excretion
APET	Added Portions Exposure Technique
APPT	Activated Partial Thromboplastin Clotting Time
BW	Body Weight
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
DMSO	Dimethyl Sulfoxide
ECHA	European Chemicals Agency
EDX	Energy-dispersive X-ray spectrometer
FAF	Panel on Food Additives and Flavourings
FEMA	Flavour and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS (FL)	Flavour Information System (database)
FL-no	FLAVIS number
FT-IR	Fourier Transform Infrared Spectroscopy
GC	Gas Chromatography
GC–MS	Gas Chromatography–Mass Spectrometry
GD	Gestation Day
GLP	Good Laboratory Practice
GRAS	Generally Recognised As Safe
HCT	Haematocrit
HGB	Haemoglobin
HPLC	High-Performance Liquid Chromatography
HPLC-UV	High-Performance Liquid Chromatography-Ultraviolet
ID	Identity
IR	Infrared Spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LC–MS	Liquid Chromatography-Mass Spectrometry
LD50	Mean Lethal Dose
LOD	Limit of Detection
LOQ	Limit of Quantification
MCH	Mean Corpuscular Haemoglobin
MCV	Mean Corpuscular Volume
MN	Micronucleus
MoE	Margin of Exposure
MtBE	methyl-*tert*-butyl-ether
NMR	Nuclear Magnetic Resonance
No	Number
NOAEL	No Observed Adverse Effect Level
OECD	Organization For Economic Co-operation And Development
RBC	Red Blood Cells
RI	Replication Index
SEM	Scanning Electron Microscopy
SPET	Single-Portion Exposure Technique
TG	Test Guideline
TTC	Threshold of Toxicological of Concern
VCF	Volatile Compounds in Food
WHO	World Health Organization

REQUESTOR

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APPENDIX A

Procedure for the safety evaluation of 'stand-alone' chemically defined flavouring substances

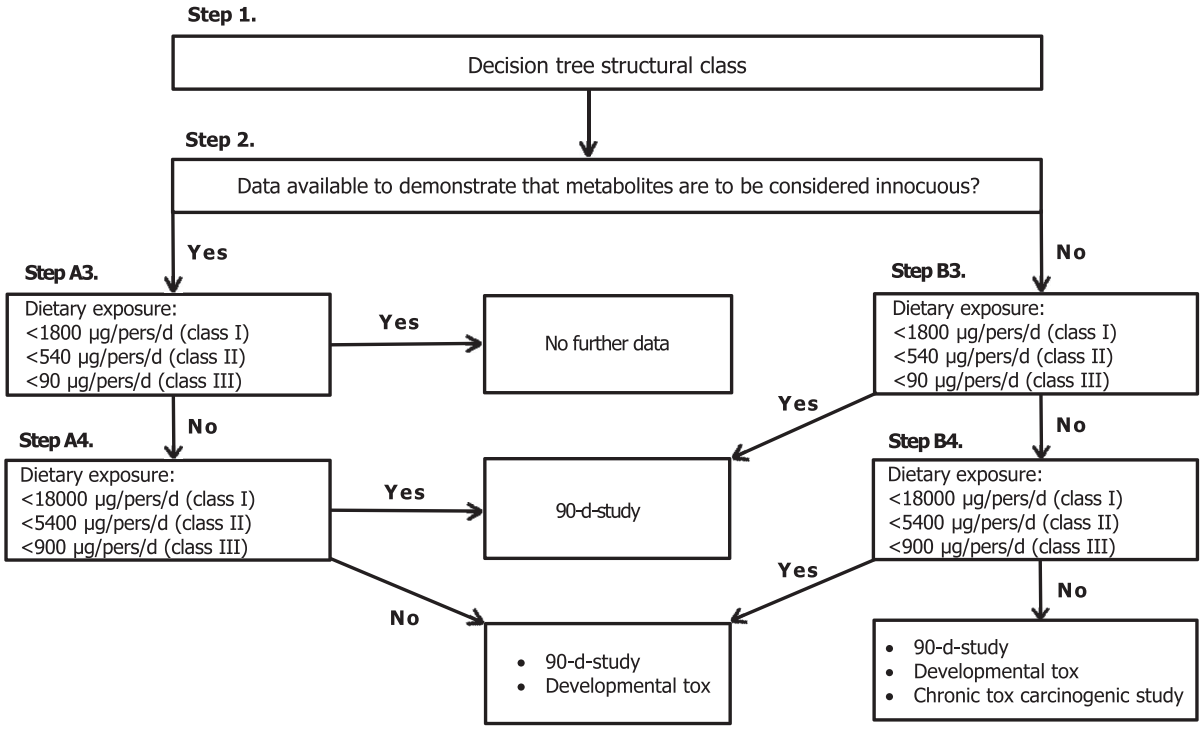

FIGURE A.1 Procedure applied for the safety evaluation of 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester according to the data requirements for the risk assessment of flavourings for which no structurally related flavouring substances in existing FGEs can be identified (EFSA CEF Panel, 2010). For the safety evaluation of 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester, the B side of the Procedure was applied (see Sections 3.4 and 3.5).

APPENDIX B

Food categories and use levels provided for 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester

TABLE B.1 Food categories and use levels for 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester (only these food categories are included for which use levels were provided). Portion sizes are according to the EFSA Guidance on the data required for the risk assessment of flavourings to be used in or on foods (EFSA CEF Panel, 2010) (Documentation provided to EFSA No. 1).

CODEX code	Food Categories[a]	Standard portions[b,c] (g)	Intended use level as flavouring substance (mg/kg)		Occurrence level from other sources (mg/kg)		Combined occurrence level from all sources (mg/kg)	
			Normal	Maximum	Normal	Maximum	Normal	Maximum
01.6	Cheese and analogues	40	2	6	–	–	2	6
02.1	Fats and oils essentially free from water	15	2	8	–	–	2	8
04.2.2	Processed vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes and aloe vera), seaweed and nuts and seeds	200	2	8	–	–	2	8
08.1	Fresh meat, poultry and game	200	2	8	–	–	2	8
08.2	Processed meat, poultry and game products in whole pieces or cuts	100	2	8	–	–	2	8
09.2	Processed fish and fish products, including molluscs, crustaceans and echinoderms	100	2	8	–	–	2	8
10.2	Egg products	100	4	10	–	–	4	10
12.2	Herbs, spices, seasonings and condiments (e.g. seasoning for instant noodles)	1	10	20	–	–	10	20
12.5	Soups and broths	200	4	10	–	–	4	10
12.6	Sauces and like products	30	4	10	–	–	4	10
14.1	Non-alcoholic ('soft') beverages	300	0.2	1.7	–	–	0.2	1.7
15.1	Snacks, potato-, cereal-, flour- or starch-based (from roots and tubers, pulses and legumes)	30	8	20	–	–	8	20

[a]Most of the categories reported are the subcategories of Codex GSFA (General Standard for Food Additives, available at https://www.fao.org/gsfaonline/foods/index.html) used by the JECFA (FAO/WHO, 2008).

[b]For adults. In case of foods marketed as powder or as concentrates, occurrence levels have been reported for the reconstituted product, considering the instructions reported on the product label or one of the standard dilution factors established by the JECFA (FAO/WHO, 2008):

- 1/25 for powder used to prepare water-based drinks such as coffee, containing no additional ingredients,
- 1/10 for powder used to prepare water-based drinks containing additional ingredients such as sugars (ice tea, squashes, etc.),
- 1/7 for powder used to prepare milk, soups and puddings,
- 1/3 for condensed milk.

[c]Portion sizes are according to the EFSA Guidance on the data required for the risk assessment of flavourings to be used in or on foods (EFSA CEF Panel, 2010). According to the guidance, standard portion sizes for children are obtained by multiplying the adult standard portion sizes by a factor of 0.63.

APPENDIX C

Genotoxicity studies

TABLE C.1 Summary of in vitro genotoxicity data on 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] (Documentation provided to EFSA No. 1).

Chemical name [FL-no]	Test system in vitro	Test object	Concentrations[a] and test conditions	Result	Reliability/comments	Relevance of test system/relevance of the result	Reference
3-[3-(2-Isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester [16.136]	Bacterial Reverse Mutation test	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	Experiment 1: 31.6, 100, 316, 1000, 3160 μg/plate (+/− S9, plate incorporation) Experiment 2: 31.6, 100, 316, 1000, 3160 μg/plate (+/− S9, pre-incubation)	Negative	Reliable without restrictions. Study performed according to OECD TG 471 and in compliance with GLP.	High/High	LPT (2008)
	Micronucleus assay	Human peripheral blood lymphocytes	100, 180, 220, 240 μg/mL (3 h + 21 h, +S9) 50, 75, 100, 125 μg/mL (3 h + 21 h, –S9) 30, 40, 50 μg/mL (24 h, –S9)	Negative	Reliable without restrictions. Study performed according to OECD TG 487 and in compliance with GLP.	High/High	Covance (2014)

Abbreviations: GLP, Good Laboratory Practice; OECD, Organisation for Economic Co-operation and Development.

[a]For the in vitro MN study, the given concentrations are those for the cultures that were scored for micronuclei.

APPENDIX D

Toxicity studies

TABLE D.1 Summary of toxicity studies for 3-[3-(2-isopropyl-5-methyl-cyclohexyl)-ureido]-butyric acid ethyl ester [FL-no: 16.136] (Documentation provided to EFSA No. 1, 2, 3).

Species; sex No/group	Route of administration	Dose levels (mg/kg bw per day)	Duration (days)	Results	Reference	Comments
Acute oral toxicity study						
Wistar Rat; F 3/group	Gavage	2000	Single dose followed by an observation period of up to 14 days	No toxicity observed	Frey-tox (2009)	Study performed in accordance with OECD TG 423 and in compliance with GLP. The oral LD50 in rats was above 2000 mg/kg bw.
Repeated dose toxicity studies						
Sprague–Dawley Rats; M, F 5/sex/group	Oral (feed)	0, 250, 500, 1000	14	No toxicity observed	Product Safety Labs (2012)	Dose range-finding study. Doses up to 1000 mg/kg bw per day were considered appropriate for testing in the 90-day toxicity study.
Sprague–Dawley Rats; M, F 10/sex/group	Oral (feed)	0, 250, 500, 1000	90	No toxicity observed	Product Safety Labs (2013)	Study performed in accordance with OECD TG 408 and in compliance with GLP. The highest dose tested (actual dose of 777 and 923 mg/kg bw per day for male and female rats, respectively) was considered as the NOAEL in this study.
Prenatal developmental toxicity study						
Sprague–Dawley Rats; F 6/group	Oral gavage	0, 250, 500, 1000	During GD 5–19	No maternal or fetal toxicity observed	Product Safety Labs (2017)	Dose range-finding study for maternal toxicity and developmental effects. Study performed in accordance with OECD TG 414 but not in full compliance with GLP.
Sprague–Dawley Rats; F 20/group	Oral gavage	0, 250, 500, 1000	During GD 5–19	No maternal or fetal toxicity observed	Product Safety Labs (2018)	Study performed in accordance with OECD TG 414 and in compliance with GLP. The highest dose tested (1000 mg/kg bw per day) was considered as the NOAEL in this study.

Abbreviations: F, female; GD, gestation day; GLP, Good Laboratory Practice; M, male; OECD, Organization for Economic Co-operation and Development.