SCIENTIFIC OPINION

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Scientific Opinion of Flavouring Group Evaluation 406 (FGE.406): (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one

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Abstract

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) of EFSA was requested to deliver a scientific opinion on the safety of the use of the substance (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one [FL-no: 16.129], as a flavouring substance. The substance is intended to be used in the form of its sodium salt as a flavour modifier in beverages. The Panel concluded that [FL-no: 16.129] would not raise a concern with respect to genotoxicity under conditions where it remains stable and does not undergo photodegradation. However, the data provided do not rule out genotoxicity for the degradation products. A 90-day toxicity study with [FL-no: 16.129] in rats showed no adverse effects at exposure up to 100 mg/kg body weight (bw) per day. No developmental toxicity was observed in rats at dose levels up to 1,000 mg/kg bw per day. An adequate margin of safety was calculated for [FL-no: 16.129]. The Panel concluded that [FL-no: 16.129] and its sodium salt are not expected to be of safety concern at the estimated levels of intake. This conclusion applies only to the use of the substance as a flavour modifier at levels up to those specified in beverages, but not to the degradation products that may be formed upon exposure to ultraviolet-A (UV-A) light. The conditions protecting [FL-no: 16.129] from photodegradation have not been adequately investigated. It is also unclear if degradation occurs in the absence of UV light. Based on the data provided, the Panel cannot conclude on the safety of [FL-no: 16.129] when used as a flavour modifier.

Keywords: (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one, FGE.406, FL-no: 16.129, CAS no 1469426-64-9, CAS no 1479020-92-2, S617

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Ulla Beckman Sundh was a Member of the Standing Working Group on Flavourings of the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) until 16 June 2017.

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1. Introduction

1.1. Background and Terms of Reference as provided by the European Commission

The use of flavouring in food 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] applies for the evaluation and approval of new flavouring substances.

The applicant has submitted an application for authorisation as a new flavouring substance of the substance: (S)-1-(3-(((4-amino-2,2-dioxido-1H-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one.

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.

Terms of reference

The European Commission requests the European Food Safety Authority to carry out a safety assessment on: (S)-1-(3-(((4-amino-2,2-dioxido-1H-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one as a new flavouring substance in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and flavourings.

1.2. Interpretation of the Terms of Reference

The present scientific opinion Flavouring Group Evaluation 406 (FGE.406) covers the safety assessment of the (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl) piperidin-1-yl)-3-methylbutan-1-one [FL-no: 16.129]. This substance will be evaluated as a flavouring substance with modifying properties[3] in line with Regulation (EC) No 1334/2008. The Panel noted that the substance is intended to be used as the sodium salt. The Union List would also allow use of the sodium salt under the condition that the free acid is not of concern.

2. Data and methodologies

The present evaluation is based on data on (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6] thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one [FL-no: 16.129] provided in a dossier submitted to support the authorisation of the substance in accordance with Regulation (EC) No 1334/2008.

The estimation of the dietary intake of [FL-no: 16.129] will be based on use and use levels submitted by the applicant for the following categories of beverages: 14.1c, 14.2.1, 14.2.3 and 14.2.5 (see Appendix C).

The safety assessment of [FL-no: 16.129] has been carried out by the Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) Panel in accordance with the procedure as lined out 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 technical report of EFSA 'Proposed template to be used in drafting scientific opinions on flavouring substances (explanatory notes for guidance included)' (EFSA, 2012).

The Procedure for the safety evaluation of the flavouring substance is given in Appendix A.

[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] https://ec.europa.eu/food/sites/food/files/safety/docs/fs_food-improvement-agents_flavourings-guidance_modifying_properties.pdf

3. Assessment

3.1. Identity of the substance

(*S*)-1-(3-(((4-Amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one has been allocated the FLAVIS number: [FL-no: 16.129].

The flavouring substance is intended to be used in beverages. It is the sodium salt and not the parent compound that is intended to be added to foodstuffs.

3.2. Organoleptic characteristics

The substance [FL-no: 16.129] is intended to be used as a flavouring substance with modifying properties. According to the applicant, it helps to restore the flavour balance in foods with less added sweetener. At or below 5 mg/kg, it is not inherently sweet as demonstrated in a two-alternative forced choice testing vs 1% sucrose solution. As specified in the 'Guidance notes on the classification of a flavouring substance with modifying properties and a flavour enhancer',[4] it intensifies substantially some and/or reduces some other flavour characteristics. At the specified use levels, the change in sweetness was not significantly greater than other flavour changes, i.e. flavour modification.

The Panel has not further evaluated these statements, but noted that the intended maximum use levels reported in Table C.1 are above 5 mg/kg food, which suggests that the substance might also be used as a sweetener.

3.3. Existing authorisations and evaluations

The Panel is not aware of any official evaluations of (*S*)-1-(3-(((4-amino-2,2-dioxido-1H-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one [FL-no: 16.129] performed by national or international authorities. In November 2014, [FL-no: 16.129] was allocated the status 'Generally recognised as safe' (GRAS) by the Flavor and Extract Manufactures Associations (FEMAs) expert Panel (FEMA GRAS 4802).

3.4. Technical data

The specifications of the flavouring substance [FL-no: 16.129] and its sodium salt are summarised in Table 1.

3.4.1. Information on the configuration of the flavouring substance

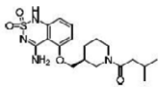
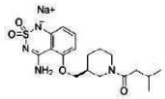
The flavouring substance [FL-no: 16.129] possesses a chiral centre at the three-position of the piperidine ring. Synthesis from a pure chiral precursor ((*S*)-tert-butyl 3-(hydroxymethyl)piperidine-1-carboxylate) precludes generation of the *R* isomer. This was verified for the GMP (good manufacturing practice) batch of compound used in the safety studies.

3.4.2. Manufacturing process

The compound was chemically synthesised from (S)-tert-butyl 3-(hydroxymethyl)piperidine-1-carboxylate, isovaleryl chloride, 2-amino-6-fluorobenzencarbonitrile and chlorosulfonyl isocyanate.

The synthesis is schematically outlined in Figure 1.

Table 1: Specifications of the candidate substance and its sodium salt

FL-no	Chemical name	Structural formula	JECFA no FEMA no CoE no CAS no EINECS no	Phys.form Mol.formula Mol.weight	Impurities	Solubility[a] Solubility in ethanol[b] Others	Boiling point, °C[c] Melting point, °C ID test Assay minimum	Refrac. Index[d] Spec. gravity[e]	Comments
16.129	(*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)3-methylbutan-1-one		– 4802 – 1469426-64-9 – –	Off-white solid $C_{18}H_{26}N_4O_4S$ 394.49	< 0.1% (by HPLC)	Not determined 1.23 mM 0.14 mM[f] 62.5 mM[g] 0.24 mM[h]	n.a. 233–238 IR NMR MS 99.0 min (by HPLC)	n.a. n.a.	pKa = 6.31
	Sodium (*S*)-4-amino-5-((1-(3-methylbutanoyl)piperidin-3-yl)methoxy)benzo[*c*][1,2,6]thiadiazin-1-ide 2,2-dioxide		– – – 1479020-92-2 – –	White to off-white solid $C_{18}H_{25}N_4NaO_4S$	< 0.1% (by HPLC)	235 mM 217 mM 3.17 mM[f] 227 mM[g]	n.a. – IR NMR MS 99.0 min (by HPLC)	n.a. n.a.	Name to be included in Union List:(*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)3-methylbutan-1-one sodium salt

(a): Solubility in water, if not otherwise stated.
(b): Solubility in 95% ethanol, if not otherwise stated.
(c): At P = 1 atm, if not otherwise stated.
(d): At 20°C, if not otherwise stated.
(e): At 25°C, if not otherwise stated.
(f): Solubility in triacetin.
(g): Solubility in DMSO.
(h): Solubility in phosphate buffer, pH = 7.1.

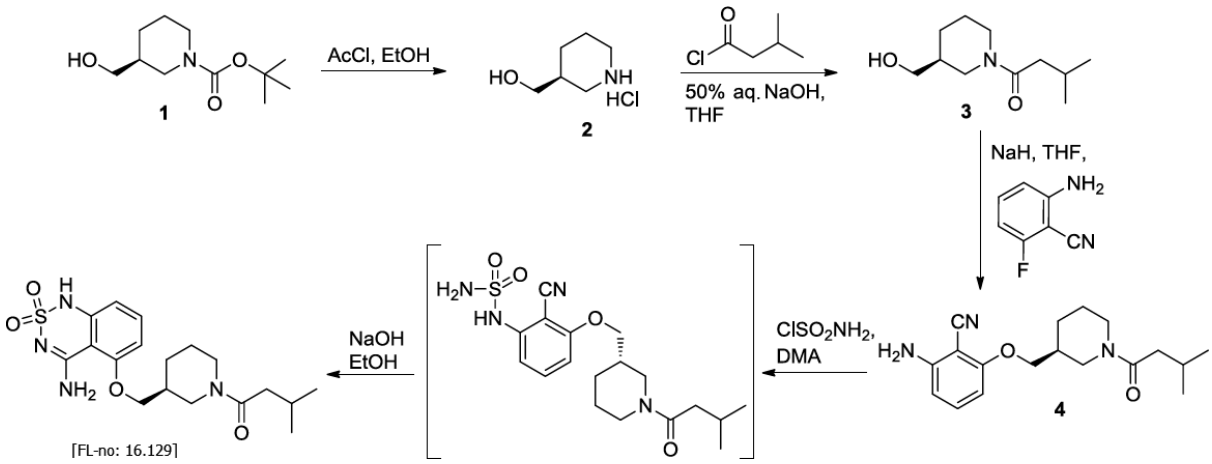


Figure 1: Synthesis of (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl) piperidin-1-yl)3-methylbutan-1-one [FL-no: 16.129]

3.4.3. Stability and degradation products

Thermal stability

The thermal stability as a dry powder was evaluated for both the flavouring substance and its sodium salt. Both forms were found to be stable up to at least 24 h at 110°C (see Table 2).

Table 2: Dry powder stability of [FL-no: 16.129] and its sodium salt

Form tested	Temperature (°C)	Time (h)	Percent remaining (%) ± standard deviation
[FL-no: 16.129]	110	24	98.7 ± 0.2
[FL-no: 16.129], sodium salt			98.7 ± 0.2

Hydrolytic stability

The stability of 50 μM of the flavouring substance [FL-no: 16.129] was determined in aqueous buffer solutions of pH 2.8, 4.0 and 7.1 at 100°C (Table 3).

Table 3: Hydrolytic stability of [FL-no: 16.129] (parent compound)

pH	Temperature (°C)	Time (h)	Percent remaining (%) ± standard deviation
2.8	100	24	99.3 ± 2.7
4.0			97.7 ± 0.4
7.1			100.1 ± 2.9

Photostability

The flavouring substance [FL-no: 16.129] was tested in photostability studies at concentrations 5–10 mg/kg (12.5–25 μM) in pH 2.8 buffer or in 'prototype beverage' products using the Q-Sun set as a light source at room temperature (25 °C) and irradiance of 0.35 W/m^2 at 340 nm. The applicant reported that 24-h exposure to Q-Sun is equivalent to 12.4 days exposure to average sunlight in the southwestern US.

The studies showed that the substance can be degraded in beverages in the presence of light when stored in clear ultraviolet-A (UV-A) transparent containers. The percent of the remaining flavouring substance after 24 h of exposure was as follows: ready to drink iced tea (104.7%), root beer carbonated soft drink (96.5%), cola carbonated soft drink (95.3%), fruit punch sports drink (86.4%), citrus carbonated soft drink (56.5%), lemon-lime sports drink (30.2%). In lemon-lime carbonated soft drinks, only 8.2% of the flavouring substance remained after 24 h, i.e. ~ 92% of [FL-no: 16.129] was degraded under the conditions of the test (see Figure B.1, Appendix B).

The photodegradation products of [FL-no: 16.129] have been identified and are shown in Figure 2.

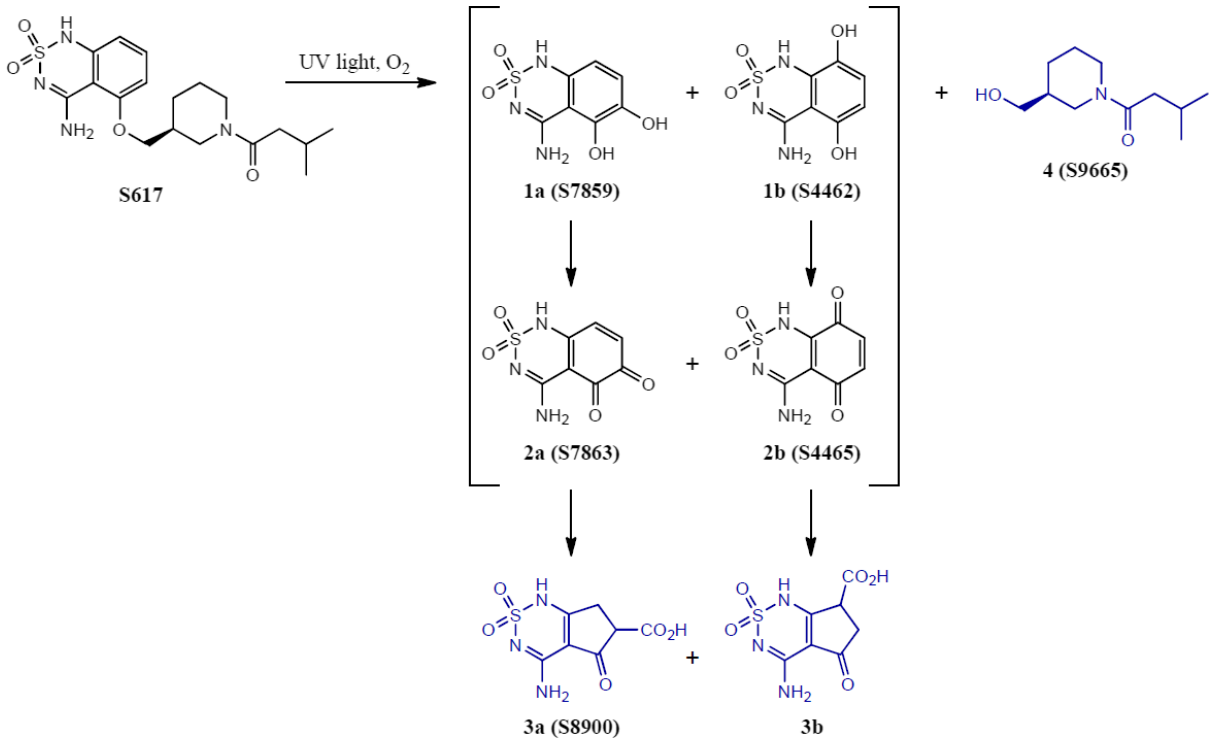


Figure 2: Photodegradation pathway of [FL-no: 16.129] (S617)

In a sucrose solution or in various beverage prototypes after addition of chlorogenic acid (CGA) or enzyme-modified isoquercetin (natural antioxidants in coffee or tea, respectively), the photodegradation was prevented (CGA) or strongly reduced (enzyme-modified isoquercetin). However, these antioxidants cannot be added to foodstuffs as none of them is included in the Union list[4] of food additives approved for use in foods. To investigate the efficiency of permitted antioxidants in beverages, the photostability of [FL-no: 16.129] in the presence of tocopherols (E306-E309) and ascorbic acid (E300) was examined. However, none of these authorised food additives (antioxidants) was effective in protecting the substance from photodegradation. The Panel noted that the absence of photodegradation in the sucrose solution and in several prototype drinks after addition of chlorogenic acid or enzyme-modified isoquercetin may be the result of high UV absorbance by these two substances.

To address possible concerns on the photodegradation of [FL-no: 16.129], the stability of the substance in 'prototypes beverage' was examined under UV irradiation and when packed in bottles containing a UV barrier or no UV barrier. Even after the use of protective packaging/UV barrier, the degradation of [FL-no: 16.129] was still up to 5% (see Figures B.2, Appendix B).

[FL-no: 16.129] was further tested for photodegradation in flavoured beer, wine cooler, cordial and energy drink in product packaging. The percentage of [FL-no: 16.129] remaining after 24 h (0.35 W/m^2 at 340 nm, 25°C) was as follows: flavoured beer (99.21%), a wine cooler (90.03%), a cordial (101.81%) and an energy drink (99.49%) (see Appendix B). No study details were provided and the type of packaging (UV transparent or opaque) was not specified.

The Panel noted that [FL-no: 16.126] evaluated in FGE.312 (EFSA CEF Panel, 2013) is based on the same 4-amino-5-alkoxy-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazine molecular scaffold. Photodegradation also occurs with [FL-no: 16.126], but the degradaton products were not identified in FGE.312. However, it is stated in the dossier for [FL-no: 16.129] that [FL-no: 16.126] will form the same photo-breakdown products 1a/b, 2a/b and 3a/b as [FL-no: 16.129].

3.4.4. Interaction with food components

No data are available.

[4] Annex II of Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on Food Additives. OJ L354/16, 31.12.2008.

3.4.5. Particle size

The particle size distribution of the flavouring substance [FL-no: 16.129] gave a mean particle size of 30 μm, with a density of 0.36 g/cm^3 and a surface area of 0.6 m^2/g.

3.4.6. Conclusion

The information provided on specifications (Table 1), the manufacturing process and the stability of the substance in beverages was considered adequate by the Panel. The Panel took note of the limited stability of the substance in clear beverages in UV-A-transparent packagings. With the use of protective packaging/UV barrier, the photodegradation can be limited to 5% (or less) under conditions that reflect approximately 12 days exposure to southwestern USA sunlight intensity. For some beverages, it is not clear if [FL-no: 16.129] may also degrade by 10%, even in UV-opaque packaging.

3.5. Structural/metabolic similarity to substances in an existing FGE

The flavouring substance (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy) methyl)piperidin-1-yl)-3-methylbutan-1-one [FL-no: 16.129] contains the 4-amino-2,2-dioxido-1*H*-benzo [c][1,2,6]thiadiazin-5-yl)oxy)methyl group as a common structural element with 3-[(4-amino-2,2-dioxido-1*H*-2,1,3-benzothiadiazin-5-yl)oxy]-2,2-dimethyl-*N*-propylpropanamide [FL-no: 16.126] from FGE.312. However, the other part of the molecule, [(piperidin-1-yl)-3-methylbutan-1-one], is different from the corresponding part in [FL-no: 16.126]. Therefore, these two flavouring substances are not sufficiently structurally related to justify a read-across with respect to toxicity data. The Panel decided to evaluate [FL-no: 16.129] as an individual substance.

3.6. Exposure assessment

All data necessary for the calculation of normal and maximum occurrence levels for refined subcategories of foods and beverages are reported in Appendix C.

3.6.1. Concentration in processed and non-processed foods from natural occurrence

The flavouring substance [FL-no: 16.129] is chemically synthesised. It has not been identified in unprocessed or processed natural vegetable or animal source materials. Based on the chemical structure of [FL-no: 16.129], its formation during food processing is not anticipated.

3.6.2. Non-food sources of exposure

For [FL-no: 16.129], there is no known non-food source of exposure to humans.

3.6.3. Chronic dietary exposure

The exposure assessment to be used in the Procedure for the safety evaluation of [FL-no: 16.129] is the chronic added portions exposure technique (APET) estimate (EFSA CEF Panel, 2010a). The chronic APET for [FL-no: 16.129] has been calculated for adults and children (see Table 4 and Appendix C). The chronic APET calculation is based on the combined normal occurrence level and the standard portion size (see Appendix C).

Table 4: Chronic dietary exposure estimate

Chronic APET	Added as flavouring substance[a]		Other dietary sources[b],[f]		Combined[c]	
	µg/kg bw per day	µg/person per day	µg/kg bw per day	µg/person per day	µg/kg bw per day	µg/person per day
Adults[d]	14.5	870	n.a.	n.a.	14.5	870
Children[e]	36.5	548	n.a.	n.a.	36.5	548

APET: added portions exposure technique; bw: body weight; n.a.: not applicable, the chronic APET calculation is based on the combined normal occurrence level.
(a): APET calculated on the basis of the normal amount of flavouring added to a specific food category.
(b): APET other dietary sources is 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 a 15 kg bw is considered representative.
(f): Other dietary sources refer to the substance as such.

3.6.4. Acute dietary exposure

The acute APET calculation for [FL-no: 16.129] (Table 5) is based on the combined maximum occurrence level and large portion size, i.e. three times standard portion size (see Appendix C).

Table 5: Acute dietary exposure estimate

Acute APET	Added as flavouring substance[a]		Other dietary sources[b]		Combined[c]	
	µg/kg bw per day	µg/person per day	µg/kg bw per day	µg/person per day	µg/kg bw per day	µg/person per day
Adults[d]	90	5,400	n.a.	n.a.	90	5,400
Children[e]	227	3,402	n.a.	n.a.	227	3,402

APET: added portions exposure technique; bw: body weight; n.a.: not applicable, the acute APET calculation is based on the combined maximum occurrence level.
(a): APET is calculated on the basis of the maximum amount of flavouring added to a specific food category.
(b): APET other dietary sources is 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 a 15 kg bw is considered representative.

3.6.5. Cumulative dietary exposure

Since no structurally related substances have been identified to justify a supporting read-across with respect to toxicity data, the Panel concluded that cumulative dietary exposure does not need to be calculated.

3.7. Genotoxicity (Appendix E)

In all studies, the substance administered was the free acid of [FL-no: 16.129]. However, the Panel noted that it can be anticipated that the free acid will dissociate under the conditions (pH ~ 7) of the genotoxicity tests.

The genotoxic potential of [FL-no: 16.129] was studied *in vitro* in a battery of three genotoxicity assays: a gene mutation assay in bacteria, a chromosomal aberration assay in cultured human peripheral blood lymphocytes and an *in vitro* micronucleus assay in Chinese hamster ovary (CHO) cells. All tests were performed according to OECD guidelines and good laboratory practice (GLP).

[FL-no: 16.129] induced no significant increases in revertant colonies in *Salmonella* Typhimurium strains TA1535, TA1537, TA98 and TA100 and *Escherichia coli* strain WP2uvrA with and without metabolic activation using the plate incorporation and preincubation methods (WIL Research, 2012; Nucro-Technics, 2012a).

[FL-no: 16.129] was tested in cultured human peripheral blood lymphocytes in a chromosome aberration test (Nucro-Technics, 2012b) and produced no statistically significant increases in the frequency of cells with aberrations in the absence and presence of metabolic activation.

[FL-no: 16.129] did not increase the number of CHO cells with micronuclei in any test conditions applied (Nucro-Technics, 2012c).

This testing strategy is consistent with the more recent recommendations of the 'Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment' (EFSA Scientific Committee, 2011).

Taken together, the results of this battery of tests (described in Appendix E) allow the conclusion that [FL-no: 16.129] by itself does not raise a concern with respect to genotoxicity if it remains stable and does not undergo photodegradation. However, the Panel noted that there is evidence for photodegradation.

An experimental photodegradation study (Appendix B) demonstrates the possibility of photodegradation of the substance [FL-no: 16.129] into seven photo-breakdown products. These photo-breakdown products were not identified as metabolites of the substance [FL-no: 16.129]. Genotoxicity studies were performed on five of them: S4462 (photoproduct 1b), S4465 (photoproduct 2b), S7859 (photoproduct 1a), S7863 (photoproduct 2a) and S8900 (photoproduct 3a). The studies provided are incomplete and do not allow to assess the *in vivo* genotoxic potential of these substances. The results are summarised in Appendix E, Tables E.2 and E.3. No genotoxicity studies were performed on two photo-products: photoproduct 3b (that was not identified) and S9665.

All the photo-breakdown products tested demonstrated genotoxic potential *in vitro*, the genotoxicity potential of S8900 (photo-breakdown product 3a) was poorly studied.

The Panel concluded that the data provided are insufficient to rule out the concern resulting from positive *in vitro* data of most of the degradation products and lacking genotoxicity data on other degradation products. It is unclear if degradation may also occur in the absence of UV light (e.g. in wine cooler, see Appendix B).

Genotoxicity studies are described in more detail in Appendix E.

3.8. Absorption, distribution, metabolism and elimination (Appendix D)

The Panel noted that of the two forms of [FL-no: 16.129], it is the soluble sodium salt and not the free acid that is intended to be added to beverages. When they are administered by gavage in 1% methyl cellulose (MC) in water, the soluble salt is more bioavailable than the insoluble acid (which is administered as a suspension). However, when the acid is administered in a food ad-mix, its bioavailability is comparable to (if not higher than) that of the sodium salt administered in 1% MC by oral gavage (Senomyx, 2013b, 2016a). This may be explained by a longer residence time of the substance in the gastrointestinal (GI) tract, when given via the food. In addition, the presence of food will increase the pH within the GI tract, and then the solubility of the acid will also increase, possibly resulting in a higher absorption. The Panel notes the lack of information on the bioavailability of the sodium salt of [FL-no: 16.129] given via food. However, for the current submission, this is of limited relevance since the substance (as sodium salt) is only intended to be added in beverages. From the data, it appears that the bioavailability of the sodium salt in solution (5–10% of the dose) is comparable to or somewhat less than the absorption of the free acid from food (up to 13.5% of the dose).

The bioavailability of the compound as free acid is no more than 13.5% of the orally administered dose if it is mixed with the food. Without food, the bioavailability of [FL-no: 16.129] is less than 1.6% (MPI Research, 2013; Senomyx, 2016a).

Analysis of urine and faeces from Sprague–Dawley rats, after oral gavage administration with a single dose of 30 mg/kg of [FL no: 16.129] sodium salt in 1% MC, showed that within 24 h the substance is excreted unchanged for approximately 93% of the dose in faeces and for 0.7% of the dose in urine (Senomyx, 2016b).

Toxicokinetics of [FL-no: 16.29] have also been studied as side study of the 90-day oral toxicity study. As in the single dose toxicokinetics study, a low oral bioavailability was observed. No sex difference in maximum plasma concentration (Cmax) or area under curve (AUCs) was observed. Systemic exposure increased with repeated dosing. Dose normalised accumulation ratios were 1.1, 2.2 and 2.9 for the 10, 30 and 100 mg/kg body weight (bw) per day dose groups, respectively.

In *in vitro* studies with rat and human liver microsomal systems, two possible monohydroxylated metabolites were identified (Senomyx, 2013a); however, no metabolites were detected in plasma after

intravenous injection in rats (see Appendix D). Based on the available information, it is not possible to conclude that the metabolites formed or the parent substance may be considered as innocuous substances. The evaluation should therefore proceed via the B-side of the Procedure (see Appendix A).

More detailed information is reported in Appendix D.

3.9. Phototoxicity test

[FL-no: 16.129] was tested in 3T3 Neutral Red Uptake (NRU) assay (CiToxLAB, 2016). The study was compliant with OECD test guideline 432 (OECD, 2004) and GLP.

The mouse fibroblast cell line Balb/c 3T3 was tested for cytotoxicity induced by [FL-no: 16.129], in the presence or absence of non-cytotoxic levels of UV-A light. Cytotoxicity is measured as the inhibition of cellular uptake of the vital dye Neutral Red (NR). A concentration range of 6.7–100 μg/ml [FL-no: 16.129] was tested.

In the non-irradiated plate, no change in cell morphology and no decrease in NR uptake were observed.

In the irradiated plate, a change in cell morphology was observed at concentrations higher than 31.5 μg/ml and a decrease in NR uptake was noted at concentrations higher than 68 μg/ml. However, no IC_{50} was calculated as the individual viabilities at 100 μg/mL were closely distributed above and below the cut-off of 50%. Based on a Mean Photo Effect (MPE) of 0.019, the substance [FL-no: 16.129] was classified as not phototoxic, according to the interpretation of results defined by OECD test guideline 432 (MPE < 0.1 predicts: 'no phototoxicity').

The authors of the study concluded that [FL-no: 16.129] is not phototoxic for concentrations up to 100 μg/mL, under the experimental conditions of this study, according to the classifications presented in the OECD test guideline 432 (OECD, 2004).

3.10. Subchronic and developmental toxicity studies (Appendix F)

In all studies, the substance administered was the free acid of [FL-no: 16.129].

A 28-day dose-range toxicity study with dietary administration of the candidate substance [FL-no: 16.129] was performed in CD® [Crl:CD®(SD)] rats (MPI Research, 2012). Three treatment groups of rats (eight animals/ sex per group) were administered a diet with [FL-no: 16.129] to provide doses of 10, 30 and 100 mg/kg bw per day. A vehicle control group was included. There were no test article-related effects among haematology or urine parameters, or clinical chemistry analytes in either sex at termination. There were no macroscopic or microscopic findings or changes in organ weights. As a result, the no-observed-adverse- effect-level (NOAEL) of [FL-no: 16.129] was 100 mg/kg bw per day, the highest dose level tested.

A 90-day feeding study was carried out in CD® [Crl:CD®(SD)] rats with the candidate substance (MPI Research, 2013). [FL-no: 16.129] was administered in the diet to rats (20 animals per sex per group) at dose levels of 0, 10, 30 and 100 mg/kg bw per day. Additionally, one control group and three treated groups (three animals per sex per group) dosed at 0, 10, 30 and 100 mg/kg bw per day were used for toxicokinetic investigation (see Appendix F). The vehicle or test article diet was available *ad libitum* for 13 weeks. Average daily compound consumption for the 10 mg/kg bw per day group was 10.2 and 10.4 mg/kg bw per day; for the 30 mg/kg per day group, 30.7 and 30.8 mg/kg bw per day and for the 100 mg/kg bw per day group, 102.5 and 103.9 mg/kg bw per day, for males and females, respectively. There were no test article-related deaths. There were no effects among haematology parameters, coagulation times or clinical chemistry analytes in either gender at any dose level. There were no alterations observed among urinalysis parameters. No changes in organ weight, macroscopic or microscopic examination were observed at any dose level. There were no test-article related effects on Functional Observation Battery (FOB) and Motor Activity (MA) parameters in males or females. The NOAEL following 13 weeks of dietary administration was 100 mg/kg bw per day in male and female rats.

In a dose-range finding, developmental toxicity study (WIL Research, 2013a), the candidate substance [FL-no: 16.129] was administered by gavage to 4 groups of eight female Crl:CD®(SD) rats once daily from gestation days 6 through 20, at dose levels of 0, 125, 250, 500 and 1,000 mg/kg bw per day. No toxicity on any of the measured paramethers was observed at any dose levels tested. Based on this lack of toxicity, doses of 250, 500 and 1,000 mg/kg bw per day were selected for a definitive embryo/foetal development study on [FL-no: 16.129].

A developmental toxicity study (WIL Research, 2013b) was performed with maternal exposure from implantation to one day prior to expected parturition to study maternal and developmental toxicity. A suspension of the candidate substance [FL-no: 16.129] was administered orally by gavage in 1% MC to Crl:CD®(SD) rats (25 bred females per group) once daily from gestation days 6 through 20, at dosage levels 0, 250, 500 and 1,000 mg/kg bw per day. All females survived to the scheduled necropsy on gestation day 21. No test article-related clinical or macroscopic findings were noted at any dosage level. Mean maternal body weights, body weight gains, gravid uterine weights and food consumption were unaffected by administration of the candidate substance at all dosage levels. No treatment-related findings were noted on intrauterine growth and survival and foetal morphology at any dose level. Mean numbers of corpora lutea and implantation sites and the mean litter proportions of preimplantation loss were similar across all groups. There were no test article-related soft tissue or skeletal malformations noted for foetuses at any dose levels. Based on the lack of adverse maternal toxicity or effects on intrauterine growth and survival and foetal morphology at any dose levels, 1,000 mg/kg bw per day was considered to be the NOAEL for maternal toxicity and embryo/foetal development.

The toxicity studies are described in Appendix F.

3.11. Exposure compared to TTC

As shown in Table 6, the APET exposure estimate is higher than the threshold of toxicological concern (TTC) of structural class III (90 μg/person per day), but lower than the TTC × 10 of structural class III (Cramer et al., 1978). Therefore, for the evaluation of the candidate flavouring substance, a 90-day oral toxicity study and a developmental toxicity study are required (see Appendix A). These studies have been submitted by the applicant.

Table 6: Summary Table on Calculated Chronic APET and Threshold of Concern

FL-no	Structural class	Add APET as a flavouring substance	Add APET as a flavouring substance[a]	Threshold of Concern	Threshold of Concern × 10
16.129		μg/kg bw per day	μg/person per day		
Adult[b]	III	14.5	870	90	900
Child[c]		36.5	548		

APET: Added portions exposure technique.
(a): The APET figure to be used in the Procedure is based on exposure per person per day.
(b): For the adult APET calculation, a 60 kg person is considered representative.
(c): For the child APET calculation, a 3-year old child with a 15 kg bw is considered representative.

3.11.1. Procedure for the safety assessment

The principles of the evaluation and the applied Procedure are described in Appendix A.

3.11.2. Safety assessment for acute exposure

Estimates of maximum acute dietary exposure indicate that this would be about 0.3 mg/kg bw for a 3-year-old child. Doses of 100 mg/kg bw issued from the 90-day toxicity study and probably higher are well tolerated in rats without adverse effects. This results in a margin of exposure of more than 300 for children and adults, which indicate no concern for acute effects. In a developmental toxicity study with an exposure period of 14 days at dose levels up to 1,000 mg/kg bw per day, no overt maternal toxicity was observed which supports this conclusion.

3.11.3. Safety assessment for long-term exposure

Since no clear structural/metabolic similarity of the candidate substance to flavouring substances in an existing FGE was identified, the Panel proceeded with the individual evaluation of the candidate substance [FL-no: 16.129], according to the EFSA Guidance (EFSA CEF Panel, 2010).

Procedure steps

Does the candidate give rise to concern with respect to genotoxicity?

The candidate substance [FL-no: 16.129] is not considered to be of concern with respect to genotoxicity (see Section 3.7). Therefore, the unchanged substance can be evaluated using the Procedure. However, there are indications for genotoxicity of the photodegradation products, and therefore, these are not covered by the Procedure. It is not clear if the substance may degrade in absence of UV light.

Step 1. Decision on structural class

On the basis of its chemical structure, the candidate substance [FL-no: 16.129] is classified in structural class III. The toxicological threshold of concern for a structural class III substance is 90 μg/person per day.

Step 2. Are there data available to demonstrate that metabolites are to be considered innocuous?

Based on the pharmacokinetic and metabolism studies carried out on the candidate substance, it cannot be concluded that the potential metabolites are innocuous, and therefore, the substance proceeds via the B-side of the Procedure.

Step B3. Is the dietary exposure below the respective structural class threshold?

Based on the APET calculation, the chronic exposure to the candidate substance is 870 μg/adult person per day and 548 μg/child per day.

There are no contributions from structurally related flavouring substances to these values.

The chronic APET for both adults and children exceeds the threshold of 90 μg/person per day and accordingly the Candidate substance proceeds to Step B4.

Step B4. Is the dietary exposure below 10 × the respective structural class threshold?

The daily exposure based on the chronic APET is located between the threshold and 10 times the threshold (see Table 6). Following the Procedure (see Appendix A), a 90-day study and a developmental study are required to finalise the safety evaluation of the candidate substance.

3.11.4. Margin of safety

A 90-day study (MPI Research, 2013) and a developmental study (WIL Research, 2013b) have been performed with the flavouring substance [FL-no: 16.129] and provide NOAELs of 100 and 1,000 mg/kg bw per day, respectively (see Section 3.10).

The NOAEL of the 90-day oral toxicity study (MPI Research, 2013) (100 mg/kg bw per day) was considered in the risk assessment of the flavouring substance (see Section 3.10).

Based on the higher APET (36.5 μg/kg bw per day) and the NOAEL of 100 mg/kg bw per day, a lowest margin of safety of 2,740 could be derived for long-term exposure in children (Table 7).

Table 7: Summary table of calculated margins of safety

FL-no: 16.129	Study type	NOAEL mg/kg bw per day	Add APET μg/kg bw per day	Margin of Safety
Adult	90-day oral toxicity study in the rat[(a)]	100	14.5	6,900
Child			36.5	2,740

NOAEL: No Observed Adverse Effect Level; APET: Added portions exposure technique.
(a): MPI Research, 2013

Based on the Procedure, the Panel concluded that there is no safety concern for the use of [FL-no: 16.129] as a flavouring substance with modifying properties at the estimated level of dietary exposure calculated using the APET approach and based on the use levels in food as specified in Appendix C. This conclusion, reached for the free acid and the sodium salt of [FL-no: 16.129], does not apply to the degradation products that may be formed upon exposure to UV-A light.

4. Conclusions

The flavouring substance (*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy) methyl)piperidin-1-yl)-3-methylbutan-1-one [FL-no: 16.129] contains the 4-amino-2,2-dioxido-1*H*-benzo

[c][1,2,6]thiadiazin-5-yl)oxy)methyl group as a common structural element with 3-[(4-amino-2,2-dioxido-1*H*-2,1,3-benzothiadiazin-5-yl)oxy]-2,2-dimethyl-*N*-propylpropanamide [FL-no: 16.126] from FGE.312. However, as the other part of the molecule [(piperidin-1-yl)-3-methylbutan-1-one] is different from the corresponding part in [FL-no: 16.126], these two flavouring substances are not sufficiently structurally related to justify a supporting read-across with respect to toxicity data.

[FL-no: 16.129] has not been reported to occur in natural source materials of botanical or animal origin. There are no reports of its detection in processed foods.

Specifications

The Panel noted that it is the soluble sodium salt of [FL-no: 16.129] and not the (acid) parent compound that is intended to be added to foodstuffs.

Specifications including complete purity criteria and identity (including specification of stereoisomeric composition) for the materials of commerce for both [FL-no: 16.129] and its salt have been provided and considered adequate.

The Panel notes that the substance is unstable when exposed to sunlight.

Use and exposure

(*S*)-1-(3-(((4-amino-2,2-dioxido-1*H*-benzo[c][1,2,6]thiadiazin-5-yl)oxy)methyl)piperidin-1-yl)-3-methylbutan-1-one [FL-no: 16.129] is intended to be used as a flavouring substance with modifying properties[4] in alcoholic (category 14.2) and non-alcoholic (category 14.1) beverages. However, the Panel noted that the intended maximum use levels reported in Table C.1 are above 5 mg/kg food, which suggests that the substance might also be used as a sweetener.

The chronic dietary exposure to the flavouring substance has been estimated using the APET. It is calculated to be 870 μg/person per day (14.5 μg/kg bw per day for a 60-kg adult) and 548 μg/person per day (36.5 μg/kg bw per day for a 15-kg 3-year-old child).

The highest acute intake of the flavouring substance results from the consumption of non-alcoholic beverages containing 6.0 mg/kg of the candidate substance consumed by a 15-kg 3-year-old child. This results in an intake of 3.4 mg/person per day (or 227 μg/kg bw per day for a 15-kg 3-year-old child).

Absorption, distribution, metabolism and elimination

Absorption, distribution, metabolism and elimination (ADME) studies in rats available for the free acid of [FL-no: 16.129] indicate that the bioavailability does not exceed 13.5% of the orally administered dose, when administered as food ad-mixture. The Panel notes the lack of information on the bioavailability of the sodium salt of [FL-no: 16.129] when given via food. Studies in both rat and human liver microsomes showed that the flavouring substance is expected to be a poor substrate for oxidative metabolism. Analysis of urine and faeces from Sprague–Dawley rats, where a single dose of 30 mg/kg sodium salt as a suspension solution in 1% MC was administered by gavage, showed that within 24 h, 93% and 0.5% of the substance were excreted unchanged in the faeces and the urine, respectively.

Genotoxicity

In tests carried out *in vitro*, [FL-no: 16.129] showed no potential to cause gene mutations, structural or numerical chromosomal aberrations. The Panel concluded that the substance would not cause concern with respect to genotoxicity under conditions where it remains stable and does not undergo photodegradation.

The Panel concluded that the data provided are insufficient to rule out the concern resulting from positive *in vitro* data of most of the degradation products and lacking genotoxicity data on other degradation products. It is unclear if degradation may also occur in the absence of UV light (e.g. in wine cooler, see Appendix B).

Systemic toxicity

A 90-day systemic toxicity study in the rat has been performed. Dietary administration of [FL-no: 16.129] to CD rats for 13 weeks at doses up to 100 mg/kg bw per day was well tolerated. Based on the findings in this study, the NOAEL was considered to be 100 mg/kg bw per day in both sexes.

Developmental toxicity

In a developmental toxicity study, rats were administered oral doses of 250–1,000 mg/kg bw per day of the candidate substance. There were no statistically significant differences between the treated

and control groups. Therefore, there is no concern for developmental toxicity of [FL-no: 16.129] in rats at dose levels up to 1,000 mg/kg bw per day.

Safety assessment for acute exposure

Estimates of maximum acute dietary exposure indicate that this would be about 0.3 mg/kg bw for a 3-year-old child. Doses of 100 mg/kg bw issued from the 90-day toxicity study and probably higher are well tolerated in rats without adverse effects. This results in a margin of exposure of more than 300 for children and adults, which indicates no concern for acute effects. In a developmental toxicity study with an exposure period of 14 days, no overt maternal toxicity was observed which supports this conclusion.

Safety assessment for long-term exposure

For [FL-no: 16.129], there is no concern in relation to genotoxicity, and therefore, the safety from the long-term exposure was evaluated according to the Procedure for the evaluation of chemically defined flavouring substances.

Since no clear structural/metabolic similarity of the candidate substance to flavouring substances in an existing FGE was identified, the Panel proceeded with the individual evaluation of the candidate substance [FL-no: 16.129], according to the EFSA Guidance (EFSA CEF Panel, 2010).

Based on its chemical structure, the substance has been assigned to structural class III. The results of studies on metabolism and pharmacokinetics do not allow to conclude that its metabolites are innocuous. Accordingly, the candidate substance is evaluated via the B-side of the Procedure scheme. Based on the comparison of APET with the structural class III threshold, a 90-day study and a developmental toxicity study were required and carried out for this substance.

Overall, the Panel concluded that using the NOAEL obtained from a 90-day dietary study in rats, there is no safety concern for [FL-no: 16.129], when used as a flavour modifier at the estimated level of dietary exposure calculated using the APET approach, based on the use levels in beverages as specified in Appendix C. Adequate margins of safety of 6,900 for adults and 2,740 for 3-year-old children have been calculated.

The conclusion reached for the free acid and the sodium salt of [FL-no: 16.129] does not apply to the degradation products that may be formed. The degradation products have not been adequately studied with respect to their toxicological properties, including genotoxicity. In addition, the conditions under which (photo)degradation does not occur have not been adequately investigated.

Based on the incomplete information on photostability and genotoxic potential of (photo) degradation products, the safety of [FL-no: 16.129] when used as a flavouring substance with modifing properties cannot be established.

Documentation provided to EFSA

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2) BioReliance, 2013b. Bacterial Reverse Mutation Assay, test article S4462 (BioReliance Study No. AD77KV.501.BTL).
3) BioReliance, 2013c. *In Vitro* Mammalian Cell Gene Mutation Test (L5178Y/TK$^{+/-}$ Mouse Lymphoma Assay), test article S4462 (BioReliance Study No. AD77KV.704.BTL).
4) BioReliance, 2013d. *In Vivo* Micronucleus and Comet Assay in Rats, test article S4462 (BioReliance Study No. AD77KV.433.BTL).
5) BioReliance, 2013e. *In Vivo* Micronucleus and Comet Assay in Rats (S7859) (BioReliance Study No. AD80DN.433.BTL).
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9) Huntingdon, 2009c. S7863, Bacterial Reverse Mutation Screening Test (2 strains), HLS study number: TEI0011.
10) Huntingdon, 2009d. S4465, Bacterial Reverse Mutation Screening Test (2 strains), HLS study number: TEI0008.
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19) Senomyx, 2013c. Metabolic Profiling of S52617 in Plasma Following a Single Oral Dose to Sprague-Dawley Rats. Study No. S52617-PK2.
20) Senomyx, 2016a. Relative bioavailability study of S617 and S9701 by oral administration in Sprague-Dawley rats, study No. S617-S9701BE.
21) Senomyx, 2016b. Excretion of S9701 in feces and urine following a single oral administration in Sprague-Dawley rats, study No. S617-EXC.
22) WIL Research, 2012. Salmonella/Mammalian Microsome Assay, S52617 (summary results for Study No. MBR12-277).
23) WIL Research, 2013a. An Oral (Gavage) Dose Range-finding Developmental Toxicity Study of S617 in Rats. (Study No. WIL-884016).
24) WIL Research, 2013b. An Oral (Gavage) Developmental Toxicity Study of S617 in Rats. Study No. WIL-884017.
25) WIL Research, 2013c. Salmonella-E.Coli/Mammalian Microsome Reverse Mutation Assay (S4462). Study No. MBR12-461.
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27) WIL Research, 2013e. Salmonella-E.Coli/Mammalian Microsome Reverse Mutation Assay (S4465). Study No. MBR12-465.
28) WIL Research, 2013f. Salmonella-E.Coli/Mammalian Microsome Reverse Mutation Assay (S7863) Study No. MBR12-467.
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Abbreviations

APET	Added Portions Exposure Technique
BW	Body Weight
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CGA	ChloroGenic Acid
CHO	Chinese hamster ovary
CoE	Council of Europe
CSDs	Carbonated soft drinks
EC	European Commission
EINECS	European Inventory of Existing Commercial chemical Substances
EMIQ	Enzyme Modified Isoquercetin
EMS	Ethyl methanesulfonate
FAO	Food and Agriculture Organization of the United Nations
FOB	Functional Observation Battery
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FLAVIS	Flavour Information System database
GC	Gas Chromatography
GEMS	Global Environment Monitoring System
GI	gastrointestinal tract
GLP	Good Laboratory Practice
GMP	good manufacturing practice
GRAS	Generally recognised as safe
GSFA	General Standard for Food Additives
HPBL	human peripheral blood lymphocytes
IR	Infra Red
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LC	Liquid Chromatography
LC/MS	Liquid chromatography-mass spectrometry
LC/MS/MS	Liquid chromatography-tandem mass spectrometry
MA	Motor Activity
MS	Mass Spectrometry
MN	Micronuclei
MPE	Mean Photo Effect
NCE	Normochromatic erythrocytes
NOAEL	No Observed Adverse Effect Level
NRU	Neutral Red Uptake
OECD	Organisation for Economic Co-operation and Development

PCE	polychromatic erythrocytes
PET	polyethylene terephthalate
RCG	Relative Cell Growth
RSD	relative standard deviation
RMI	Relative Mitotic Index
RTD	ready to drink
SPET	Single Portion Exposure Technique
TTC	threshold of toxicological concern
WHO	World Health Organisation

Appendix A – Procedure for evaluation of a new flavouring substance

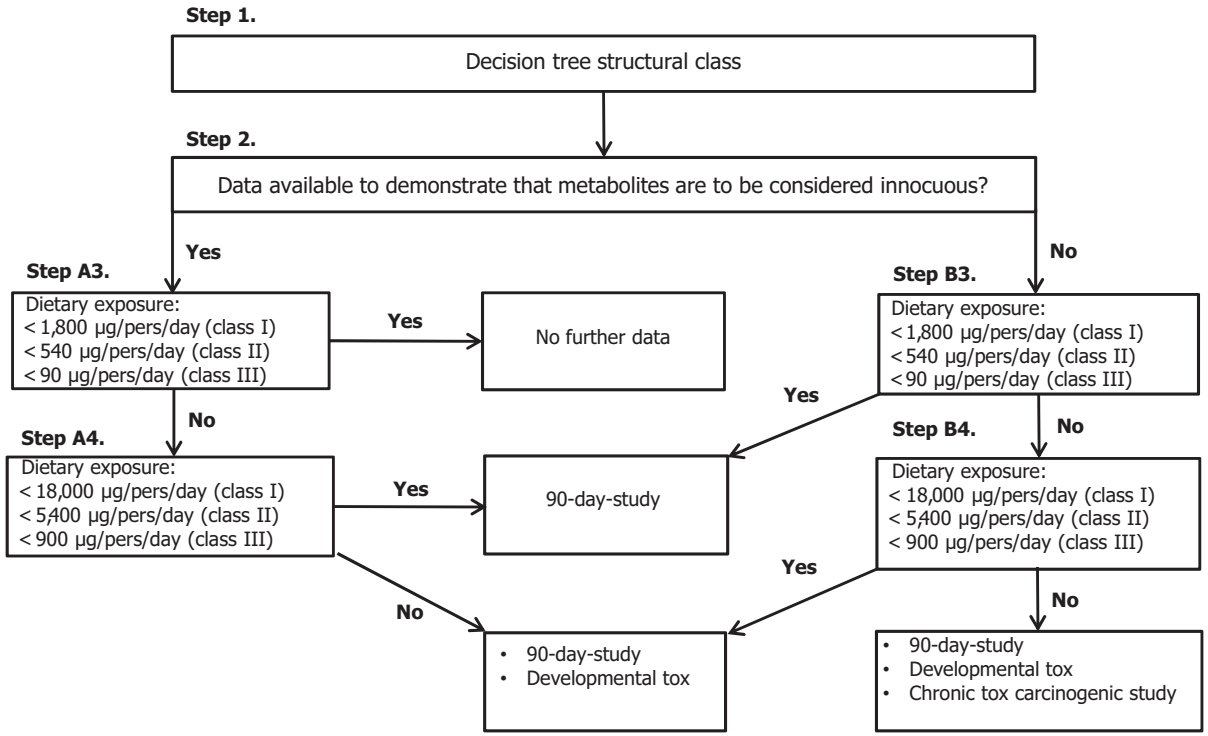

Figure A.1: The stepwise procedure for evaluation of a new flavouring substances according to Commission Regulation 1334/2008

Appendix B – Stability of the flavouring substance in the presence of light

Conditions of direct sunlight exposure were simulated using a Q-Sun Xenon Test Chamber, which reproduces the entire spectrum of natural sunlight. This test system is used to evaluate the photostability of ingredients or products under conditions of accelerated photodegradation controlling the duration, temperature and intensity of the light. According to the applicant, it is estimated that 24-h exposure to Q-Sun at a constant temperature of 25 °C and irradiance of 0.35 W/m^2 at 340 nm is comparable to approximately 12.4 days of average sunlight in the southwestern US.

According to the applicant, products containing natural antioxidants (e.g. chlorogenic acid (CGA) enzyme modified isoquercetin (EMIQ)) could decrease the photodegradation rate. Packaging and the presence of natural or artificial colors can also reduce the photodegradation rate. The applicant showed that ultraviolet(UV)-opaque packaging can prevent the photodegradation of [FL-no: 16.129] nearly completely. Indeed, CGA and EMIQ can also prevent photodegradation, but it was not demonstrated that this is because of their antioxidant properties or because of their UV absorption characteristic. In addition, no evidence was submitted to show that natural or artificial food colours can prevent photodegradation of [FL-no: 16.129].

Photostability studies of the flavouring substance [FL-no: 16.129] were performed at 5–10 mg/kg (12.5–25 μM) in pH 2.8 buffer or in prototype beverage products using the Q-Sun Test at room temperature (25 °C) and irradiance of 0.35 W/m^2 at 340 nm. Methods were developed to determine the concentrations of [FL-no: 16.129] as well as the photo-breakdown products as a function of time under conditions of continuous irradiation. Figure 2 in Section 3.4.3 shows the photodegradation pathway for [FL-no: 16.129]. The photo-breakdown products were identified using high pressure liquid chromatography-ultraviolet (HPLC-UV) and Liquid Chromatography Mass Spectrometry (LC/MS/MS) and, with the exception of compound 3b, all of these structures have been confirmed by direct comparison to synthesized materials. The structure of 3b was proposed on the basis of its exact mass and mass spectrometry fragmentation pattern which is nearly identical to that of keto-acid 3a (S8900). Under conditions of continuous UV irradiation, compounds 1a/b and 2a/b are transient intermediates that are further degraded to photostable end products 3a/b. The relative rates of decomposition of [FL-no: 16.129] and intermediates 1a/b and 2a/b are pH dependent. At higher pHs, the turnover of 1a/b and 2a/b is more rapid than that of the parent substance [FL-no: 16.129].

The percent of [FL-no: 16.129] remaining after 24 h in different beverages exposed in the Q-Sun Xenon Test Chamber under the conditions described above was: ready to drink iced tea (104.7%), root beer-carbonated soft drink (96.5%), cola-carbonated soft drink (95.3%), fruit punch sports drink (86.4%), citrus-carbonated soft drink (56.5%), lemon-lime sports drink (30.2%) and lemon-lime-carbonated soft drink (8.2%) (see Figure B.1). The packaging used was not UV opaque.

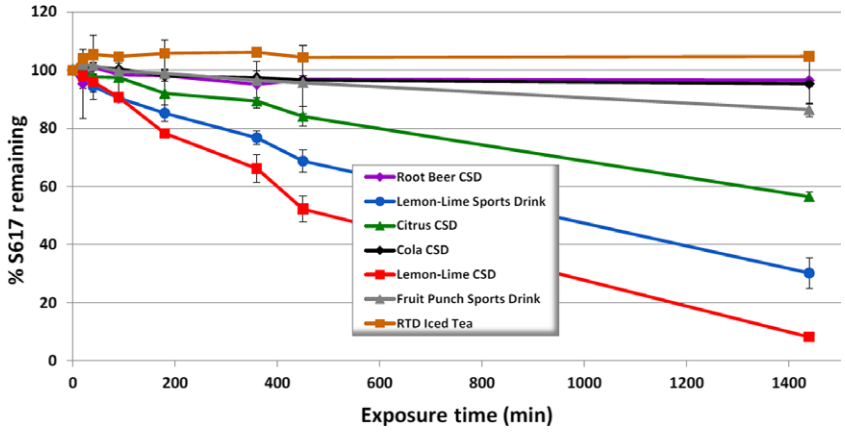

Figure B.1: Photostability of 5 mg/kg of [FL-no: 16.129] in beverage product prototypes in standard (not UV opaque) packaging

To address possible concerns on the degradation of [FL-no: 16.129] in the presence of light, the applicant examined the stability of the substance when stored in bottles containing a UV barrier. Prototype beverage products containing 10 mg/kg of [FL-no: 16.129] in 20 fl oz (591 mL) polyethylene terephthalate (PET) bottles with/without Clearshield 390 UV barrier were irradiated in the Q-Sun Xenon Test Chamber (0.35 W/m^2 at 340 nm, 25°C). The percent candidate substance remaining after 24 h

was greater than 95% in products with UV barrier compared to less than or equal to 15% in products without UV barrier (see Figure B.2).

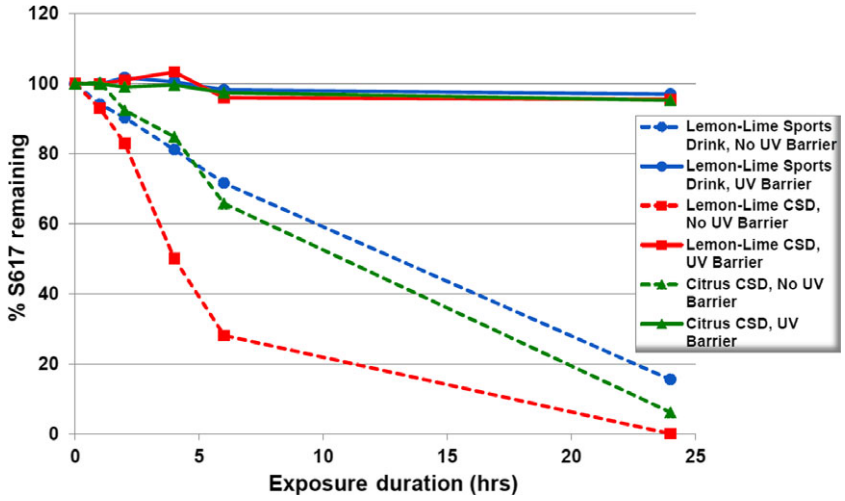

Figure B.2: Photostability of [FL-no: 16.129] in beverage product prototypes in bottles with and without UV barrier

The applicant analysed also a flavoured beer (355 mL), a wine cooler (187 mL), a cordial (750 mL) and an energy drink (100 mL) in product packaging (unclear if this is a UV-transparent or UV-opaque). The percent of [FL-no: 16.129] remaining after 24 h (0.35 W/m^2 at 340 nm, 25°C) was as follows: flavoured beer (99.21%), a wine cooler (90.03%), a cordial (101.81%) and an energy drink (99.49%) as shown in Figure B.3.

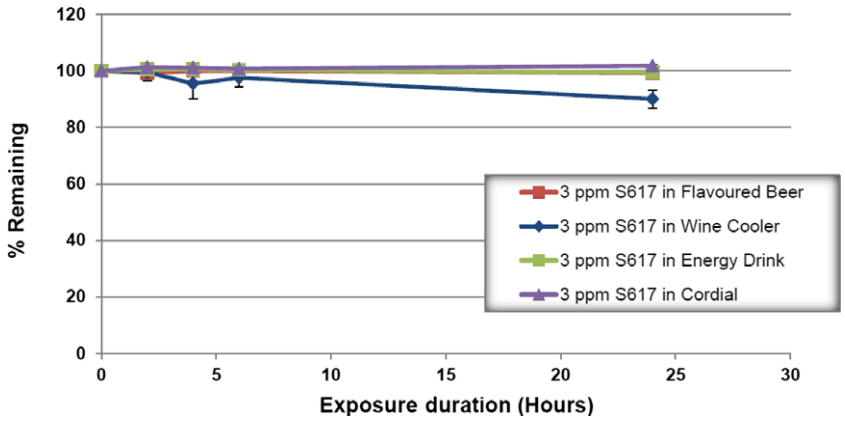

Figure B.3: Photostability of [FL-no: 16.129] in additional beverage product prototypes

In summary, UV-blocking packaging can provide considerable protection of the candidate substance [FL-no: 16.129] from photodegradation in product prototypes. There is no evidence that antioxidants can also prevent photodegradation. The candidate substance appears to be inherently photostable in several types of beverage applications (e.g. cola and root beer carbonated soft drinks (CSDs), ready to drink (RTD) tea, select sports drinks). In several product applications where the candidate substance is not inherently photostable, the photostability of the compound can be improved by the addition of CGA or EMIQ (no authorised food additives in the EU) or prevented in UV-opaque packing. For one beverage application (wine cooler-type drinks), some degradation was observed, but testing conditions were not properly reported. It is unclear if, in this beverage type, degradation also occurs in UV-opaque packaging.

Appendix C – Use Levels and Exposure Calculations

Table C.1: Normal and Maximum Occurrence Levels for Refined Categories of Foods and Beverages

Food categories[a]		Standard portions[b] (g)	Occurrence level as added flavouring substance (mg/kg)		Occurrence level from other sources[c] (mg/kg)		Combined occurrence level from all sources[e] (mg/kg)	
			Normal	Maximum	Average[d]	Maximum	Normal	Maximum
01.1	Milk- and dairy-based drinks	200						
01.2	Fermented and renneted milk products (plain), excluding food category 01.1.2 (dairy-based drinks)	200						
01.3	Condensed milk and analogues (plain)	70						
01.4	Cream (plain) and the like	15						
01.5	Milk powder and cream powder and powder analogues (plain)	30						
01.6	Cheese and analogues	40						
01.7	Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	125						
01.8	Whey and whey products, excluding whey cheeses	200						
02.1	Fats and oils essentially free from water	15						
02.2	Fat emulsions mainly of type water-in-oil	15						
02.3	Fat emulsions mainly of type water-in-oil, including mixed and/or flavoured products based on fat emulsions	15						
02.4	Fat-based desserts excluding dairy-based dessert products of category 1.7	50						
03.0	Edible ices, including sherbet and sorbet	50						
04.1.1	Fresh fruit	140						
04.1.2	Processed fruit	125						
04.1.2.5	Jams, jellies, marmalades	30						
04.2.1	Fresh vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes and aloe vera), seaweed and nut and seed	200						
04.2.2	Processed vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes and aloe vera), seaweed and nut and seed purees and spreads (e.g. peanut butter) and nuts and seeds	200						

Food categories[a]		Standard portions[b] (g)	Occurrence level as added flavouring substance (mg/kg)		Occurrence level from other sources[c] (mg/kg)		Combined occurrence level from all sources[e] (mg/kg)	
			Normal	Maximum	Average[d]	Maximum	Normal	Maximum
04.2.2.5	Vegetables (including mushrooms and fungi, roots and tubers, pulses and legumes and aloe vera), seaweed and nut and seed purees and spreads (e.g. peanut butter)	30						
05.1	Cocoa products and chocolate products, including imitations and chocolate substitutes	40						
05.1.3	Cocoa-based spreads, including fillings	30						
05.2	Confectionery, including hard and soft candy, nougats, etc. other than 05.1, 05.3 and 05.4	30						
05.3	Chewing gum	3						
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	35						
06.1	Whole, broken or flaked grain, including rice	200						
06.2	Flours and starches (including soya bean powder)	30						
06.3	Breakfast cereals, including rolled oats	30						
06.4	Pastas and noodles and like products (e.g. rice paper, rice vermicelli, soya bean pastas and noodles)	200						
06.5	Cereal- and starch-based desserts (e.g. rice pudding, tapioca pudding)	200						
06.6	Batters (e.g. for breading or batters for fish or poultry)	30						
06.7	Precooked or processed rice products, including rice cakes (Oriental type only)	200						
06.8	Soya bean products (excluding soya bean products of food category 12.9 and fermented soya bean products of food category 12.10)	100						
07.1	Bread and ordinary bakery wares	50						
07.2	Fine bakery wares (sweet, salty, savoury) and mixes	80						
08.1	Fresh meat, poultry and game	200						
08.2	Processed meat, poultry and game products in whole pieces or cuts	100						
08.3	Processed comminute meat, poultry and game products	100						

Food categories[a]		Standard portions[b] (g)	Occurrence level as added flavouring substance (mg/kg)		Occurrence level from other sources[c] (mg/kg)		Combined occurrence level from all sources[e] (mg/kg)	
			Normal	Maximum	Average[d]	Maximum	Normal	Maximum
08.4	Edible casings (e.g. sausage casings)	1						
09.1.1	Fresh fish	200						
09.1.2	Fresh molluscs, crustaceans and echinoderms	200						
09.2	Processed fish and fish products, including molluscs, crustaceans and echinoderms	100						
09.3	Semipreserved fish and fish products, including molluscs, crustaceans and echinoderms	100						
09.4	Fully preserved, including canned or fermented, fish and fish products, including molluscs, crustaceans and echinoderms	100						
10.1	Fresh eggs	100						
10.2	Egg products	100						
10.3	Preserved eggs, including alkaline, salted and canned eggs	100						
10.4	Egg-based desserts (e.g. custard)	125						
11.1	Refined and raw sugar	10						
11.2	Brown sugar excluding products of food category 11.1	10						
11.3	Sugar solutions and syrups, and (partially) inverted sugars, including molasses and treacle, excluding products of food category 11.1.3 (soft white sugar, soft brown sugar, glucose syrup, dried glucose syrup, raw cane sugar)	30						
11.4	Other sugars and syrups (e.g. xylose, maple syrup, sugar toppings)	30						
11.5	Honey	15						
11.6	Table-top sweeteners, including those containing high-intensity sweeteners	1						
12.1	Salt and salt substitutes	1						
12.10	Protein products other than from soybeans	15						
12.2	Herbs, spices, seasonings and condiments (e.g. seasoning for instant noodles)	1						

Food categories[a]		Standard portions[b] (g)	Occurrence level as added flavouring substance (mg/kg)		Occurrence level from other sources[c] (mg/kg)		Combined occurrence level from all sources[e] (mg/kg)	
			Normal	Maximum	Average[d]	Maximum	Normal	Maximum
12.3	Vinegars	15						
12.4	Mustards	15						
12.5	Soups and broths	200						
12.6	Sauces and like products	30						
12.7.a	Salads 120 g (e.g. macaroni salad, potato salad) excluding cocoa- and nut-based spreads of food categories	120						
12.7.b	Sandwich spreads (20 g), excluding cocoa- and nut-based spreads of food categories	20						
12.8	Yeast and like products	1						
12.9	Soybean-based seasonings and condiments	15						
12.9.1	Fermented soya bean products (e.g. miso)	40						
12.9.2	Soybean sauce	15						
12.9.3	Fermented soybean sauce	15						
13.2. a	Complementary foods for infants and young children: Dry instant cereals (with or without milk), including pasta	110						
13.2. b	Complementary foods for infants and young children: Meat-based or fish-based dinner	170						
13.2. c	Complementary foods for infants and young children: Dairy-based dessert	110						
13.2. d	Complementary foods for infants and young children: Vegetables, potatoes, broth, soups, pulses	170						
13.2. e	Complementary foods for infants and young children: Biscuits and cookies	20						
13.2. f	Complementary foods for infants and young children: Fruit purée	110						
13.2. g	Complementary foods for infants and young children: Fruit juice	120						
13.2. h	Milk for young children	200						

Food categories[a]		Standard portions[b] (g)	Occurrence level as added flavouring substance (mg/kg)		Occurrence level from other sources[c] (mg/kg)		Combined occurrence level from all sources[e] (mg/kg)	
			Normal	Maximum	Average[d]	Maximum	Normal	Maximum
13.3	Dietetic foods intended for special medical purposes (excluding food products of category 13.1 'Infant formulae, follow-up formulae and other formulae for special medical purposes for infants')	200						
13.4	Dietetic formulae for slimming purposes and weight reduction	200						
13.5	Dietetic foods (e.g. supplementary foods for dietary use), excluding products of food categories 13.1 (Infant formulae, follow-up formulae and other formulae for special medical purposes for infants), 13.2–13.4 and 13.6	200						
13.6	Food supplements	5						
14.1a	Coffee powder	12						
14.1b	Drinks mix powders	30						
14.1c	Other non-alcoholic ('soft') beverages (expressed as liquid)	300	2.9	6			2.9	6
14.2.1	Beer and malt beverages	300	2.9	6			2.9	6
14.2.2	Cider and perry	300						
14.2.3	Grape wines	150	3	6			3	6
14.2.4	Wines (other than grape)	150						
14.2.5	Mead	150	3	6			3	6
14.2.6	Distilled spirituous beverages containing more than 15% alcohol	30						
14.2.7	Aromatised alcoholic beverages (e.g. beer, wine and spirituous cooler type beverages, low alcoholic refreshers)	300						
15.1	Snacks, potato-, cereal-, flour- or starch-based (from roots and tubers, pulses and legumes)	30						
15.2	Processed nuts, including coated nuts and nut mixtures (with e.g. dried fruit)	30						
15.3	Snacks – fish based	30						

Food categories[a]		Standard portions[b] (g)	Occurrence level as added flavouring substance (mg/kg)		Occurrence level from other sources[c] (mg/kg)		Combined occurrence level from all sources[e] (mg/kg)	
			Normal	Maximum	Average[d]	Maximum	Normal	Maximum
16.0	Composite foods (e.g. casseroles, meat pies, mincemeat) – foods that could not be placed in categories 01–15	300						

(a): Most of the categories reported are the subcategories of Codex GSFA (General Standard for Food Additives) used by the JECFA in the SPET technique (FAO/WHO, 2008). In the case of category 13.2 (complementary foods for infants and young children), further refined categories have been created so that a specific assessment of dietary exposure can be performed in young children.

(b): For Adults. In case of foods marketed as powder or as concentrates, occurrence levels must be 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): As natural constituent and/or developed during the processing and/or as carry over resulting from their use in animal feed.

(d): In order to estimate normal values in each category, only foods and beverages in which the substance is present in significant amount will be considered (e.g. for the category 'Fresh fruit' 04.1.1., the normal concentration will be the median concentration observed in all kinds of fruit where the flavouring substance is known to occur).

(e): As added flavouring or from other sources. The normal and maximum combined occurrence levels of the substance will be assessed by the applicant either by adding up occurrence levels from added use to that from other sources or by expert judgment based on the likelihood of their concomitant presence. This will be done both for normal use levels and for maximum use levels.

Calculation of the Dietary Exposure – 'Added Portions Exposure Technique' (APET)

Chronic Dietary Exposure[5]

The chronic Added Portions Exposure Technique (APET) calculations for adults and children are based on the normal combined occurrence level by adding the highest contributing portion of food and highest contributing portion of beverages (either among soft drinks or alcoholic beverages). In the APET calculation, for children, the portion sizes listed in Table C.1 are adjusted by a factor 0.63 to take into account the smaller portion sizes consumed by children.

Adults

On the Basis of Normal Occurrence Level from Added Flavourings

The APET is calculated by adding the highest contribution from one portion of food and one portion of beverages:

Solid Food: the flavouring substance is not used in solid foods.

Beverage: the maximum intake will be from category 14.1 (Other non-alcoholic ('soft') beverages (expressed as liquid) with the normal combined occurrence level of 870 μg/adult per day.

The total APET will be 870 μg/adult per day corresponding to 14.5 μg/kg bw per day for a 60-kg person.

Children (3-year-old child of 15 kg body weight)

Solid Food: the flavouring substance is not used in solid foods.

Beverage: the maximum intake will be from category 14.1 (Other non-alcoholic ('soft') beverages (expressed as liquid)) with the normal combined occurrence level of 870 × 0.63 = 548 μg/child per day.

The total APET will be 548 μg/child per day corresponding to 36.5 μg/kg bw per day for a 15-kg child.

Conclusion

The higher of the two values among adults and children, expressed per kg/bw per day, should be used as the basis for the safety evaluation of [FL-no: 16.129], i.e. the value of 36.5 μg/kg bw per day for a 15-kg child should be compared to the appropriate no-observed-adverse-effect-level (NOAEL) for [FL-no: 16.129].

Combined Dietary Exposure

This is an estimate of total dietary exposure derived from both the addition of the flavouring substance to foods and beverages and other dietary sources. Since there is no exposure to the substance from other dietary sources, the combined dietary exposure is equal to the chronic dietary exposure derived from the intentional addition of [FL-no: 16.129] as flavouring substance in food.

Consequently, the total combined APET for adults will be 870 μg/adult per day corresponding to 14.5 μg/kg bw per day for a 60-kg person and the total combined APET for children will be 548 μg/child per day corresponding to 36.5 μg/kg bw per day for a 15-kg child.

Acute Dietary Exposure

The calculation is based on the maximum use levels and large portion size, i.e. three times standard portion size (see Table C.1). The APET calculation for children applies the portion sizes listed in Table C.1 adjusted by a factor 0.63 to take into account the smaller portion sizes consumed in this case.

Adults

The highest acute intake is assumed to result from the consumption of three portions of 14.1 (Other non-alcoholic ('soft') beverages (expressed as liquid)) and is 3 × 300 g × 6 mg/kg = 5.4 mg/person per day.

The total APET will be 5.4 mg/adult per day corresponding to 90 μg/kg bw per day for a 60-kg person.

[5] The APET has been calculated based on the occurrence levels in the food subcategories reported in the above table, with the exclusion of categories 13.2 (complementary foods for infants and young children).

Children[6]

The highest acute intake is assumed to result from the consumption of three portions of 14.1 (Other non-alcoholic ('soft') beverages (expressed as liquid)) and is 0.63 × 3 × 300 g × 6 mg/kg = 3.4 mg/person per day.

The total APET will be 3.4 mg/child per day corresponding to 227 μg/kg bw per day for a 15-kg child.

Cumulative Dietary Exposure

Since no structurally related substances have been identified to justify a supporting read-across with respect to toxicity data, the Panel concluded that cumulative dietary exposure did not need to be calculated.

[6] Based on the same considerations as for adults but using the special factors used for chronic exposure to infants.

Appendix D – Absorption, Distribution, Metabolism and Elimination

D.1. *In vitro* metabolic profiling using human and rat hepatic microsomes

A qualitative metabolic profiling study of the candidate substance was performed using rat and human hepatic microsomes (Senomyx, 2013a) in which only phase I metabolism can be monitored. LC/MS analysis showed that [FL-no: 16.129] was only slightly metabolised by the rat or human microsomes with > 97% of [FL-no: 16.129] remaining intact after a 60 min incubation period. Two metabolites were identified as monohydroxylation products of [FL-no: 16.129], but the position of hydroxylation was not determined.

D.2. Single dose pharmacokinetic and *in vivo* metabolism study of [FL-no: 16.129] in rats

The pharmacokinetic parameters and oral bioavailability of [FL-no: 16.129] in plasma were determined following either a single intravenous or oral administration in male and female Sprague–Dawley rats (Senomyx, 2013b). Plasma samples were also analysed (using LC MS/MS) for the presence of the two monohydroxylated metabolites observed in incubations of [FL-no: 16.129] with rat liver microsomes (Senomyx, 2013c). For intravenous (i.v.) administration, rats (four animals per sex per group) were dosed at 1 mg/kg. For oral administration, rats (four animals per sex per group) were administered the candidate substance [FL-no: 16.129] at either 10, 30 or 100 mg/kg as a suspension in 1% methylcellulose by oral gavage. Samples were collected from groups given oral exposure untill 24-h post-dose, while samples from i.v. administration were collected untill 8-h post-dose. The area under the curve (AUClast[7]) and the terminal half-life were calculated; the maximum plasma concentration (Cmax) and the corresponding peak time (Tmax) were determined.

The candidate substance [FL-no: 16.129] was poorly orally bioavailable. The absolute bioavailability (%F) ranged from 0.44 ± 0.31 to 1.7 ± 1.2% in female rats and 0.20 ± 0.06 to 0.67 ± 0.20% in male rats. It was rapidly eliminated after intravenous administration (average T1/2 in females 0.29 h; in males 0.23 h). After oral administration, the elimination half-life was variable, ranging from 1.2 to 7.6 h for males and from 1.2 to 6.3 h in females, but on average was less than 3.85 h. The systemic exposure to [FL-no: 16.129] was relatively low. Based on AUClast and Cmax, the exposure to [FL-no: 16.129] in plasma after oral administration tended to be higher in female rats than in males (1.3–2.1 fold). Both AUClast and Cmax increased with increasing dose, but tended to be less than dose proportional in both male and female Sprague–Dawley rats. The Panel noted that the AUC (and thus %F) may have been underestimated, because blood sampling was only continued for 24 h, when considerable levels of the substance could still be determined in plasma, especially at the higher dose levels. None of the observed microsomal metabolites of [FL-no: 16.129] were detected in the plasma samples of rats dosed orally with 100 mg/kg of [FL-no: 16.129] at any time point.

In order to determine if the two forms of the compound were equally absorbed, a comparative oral bioavailability study was conducted using both [FL-no: 16.129] and its sodium salt (Senomyx, 2016a). Four male and four female Sprague–Dawley rats per group were administered a single oral dose of 30 mg/kg of either [FL-no: 16.129] or its sodium salt (equivalent to 28.4 mg [FL-no: 16.129]) in 1% MC by oral gavage. Blood samples were taken from a jugular catheter at approximately 0-, 15-, 30-min, 1-, 2-, 4-, 8-, and 24-h post-dose and analysed by LC-MS/MS. The lower limit of quantification for [FL-no: 16.129] was 0.1 ng/ml. The [FL-no: 16.129] sodium salt is water soluble and completely dissolved in 1% MC at 3 mg/mL, while the parent compound [FL-no: 16.129] is a suspension in 1% MC. The dose-normalised AUC's for the sodium salt were 5.4-fold higher for the males and 6.3-fold higher for the females when compared to those of the parent compound [FL-no: 16.129]. Using the AUC's from the previous intravenous study (Senomyx, 2013b), the oral bioavailability (%F) of the sodium salt ranged from 5.29% to 10.0% in male and female rats, while that of [FL-no: 16.129] ranged from 0.98–1.59%. The data suggest that the oral bioavailability of [FL-no: 16.129] may be limited by its rate of dissolution.

D.3. Pharmacokinetics in a repeated dose 90-day oral toxicity study

A toxicokinetic study was performed where the candidate substance [FL-no: 16.129] was administered in the diet (Meal Lab Diet® Certified Rodent Diet #5002) to rats (3 animals per sex per

[7] AUC was determined until the last sampling point (8 h for intravenous studies, 24 h for oral studies)

group) at dose levels of 0 (control), 10, 30 and 100 mg/kg per day for 90 days. Blood for toxicokinetic analyses was collected on days 7 and 90 predose and at approximately, 1, 3, 6, 12 and 24 h post the start of the dark cycle from [FL-no: 16.129] dosage. Blood samples were collected at alternating time points (0, 3 and 12 h, and 1, 6 and 24 h) from two subgroups of three animals/sex per group. The toxicity data from this study (MPI Research, 2013) are described in more detail in Appendix F.

Systemic exposure to the candidate substance was highly variable as assessed by plasma concentrations of [FL-no: 16.129] following dietary administration of [FL-no: 16.129] to male and female rats. Consistent with results from pharmacokinetic studies in rats (described above), systemic exposure to [FL-no: 16.129] was low. Individual and mean plasma concentration data dose-normalised AUC ratios were not indicative of any consistent gender difference (female to male exposure ratio ranged from 0.81 to 1.37) and therefore, were combined for pharmacokinetic analysis. Systemic exposure generally increased with increasing dose in a less than dose proportional manner; it appeared to be greater on day 90 than on day 7 at 30 and 100 mg/kg per day. Dose-normalised accumulation ratios were 1.1, 2.2 and 2.9 for the 10, 30 and 100 mg/kg per day dose groups, respectively.

A significant food effect on the absorption of [FL-no: 16.129] was observed in this toxicokinetic study compared to the single dose studies described above (Senomyx, 2013b; 2016a). The oral bioavailability was calculated using the data from the previous intravenous pharmacokinetic study (Senomyx, 2013b). The dose-normalised AUC's and %F of [FL-no: 16.129] administered as a food ad-mix were comparable, if not higher, to those seen with the sodium salt administered as solution in 1% MC by oral gavage. Apparently, the presence of food in the gastrointestinal tract (GI) of the rats significantly increases the rate of dissolution or slows the transit time of [FL-no: 16.129] in the GI, when coadministered with food and results in levels available for absorption comparable to those achieved with aqueous solutions of the sodium salt.

The applicant concluded that based on these toxicokinetic studies, the systemic exposures (AUC's) achieved in the 28- and 90-day food ad-mix toxicology studies (MPI Research, 2012, 2013) on [FL-no: 16.129] in rats are representative of the maximum exposure that can be obtained in this species, even relative to when the compound is administered as a homogenous solution of the corresponding sodium salt.

Appendix E – Genotoxicity

The genotoxic potential of [FL-no: 16.129] has been investigated through the following *in vitro* genotoxicity tests: bacterial reverse mutation assays, chromosome aberration test and micronucleus test.

In addition, the applicant performed genotoxicity tests on a synthetic intermediate (S1116) and on photodegradation products of [FL-no: 16.129]. The studies are described in this Appendix.

E.1. Genotoxicity studies on the candidate substance

E.1.1. Bacterial Reverse mutation assays

A screening Ames test was performed on the candidate substance [FL-no: 16.129] (purity > 98.5%) (WIL Research, 2012) to evaluate its potential to induce base pairs substitution or frameshift mutations in *Salmonella* Typhimurium strains, TA98 and TA100 in a plate incorporation assay in the presence or absence of metabolic activation with rat liver S9 from rats induced with Aroclor 1254. Precipitates were observed at 5,000 μg/plate in both strains with and without metabolic activation. Neither bacterial toxicity nor mutagenic activity was demonstrated in either strain with or without metabolic activation. Positive and vehicle controls yielded the expected results. It was concluded that [FL-no: 16.129] was not mutagenic under these test conditions.

[FL-no: 16.129] (purity > 99%) was evaluated for its potential to induce point mutations in *S.* Typhimurium strains, TA98, TA100, TA1535, TA1537 and *Escherichia coli* strain WP2 uvrA in the presence and absence of metabolic activation with rat liver S9 from rats induced with Aroclor 1254 (Nucro-Technics, 2012a). The experimental design followed the OECD test guideline 471 (OECD, 1997a) and was performed according to good laboratory practice (GLP). Five concentrations of [FL-no: 16.129], from 310 to 5,000 μg/plate, were tested with the plate incorporation and the preincubation method.

In the plate incorporation assay, a slight precipitate was visible at 5,000 μg/plate. In the preincubation assay, precipitate was visible at both 2,500 and 5,000 μg/plate. Under both conditions, toxicity was not observed at any concentration evident by a normal background lawn and colony counts similar to the concurrent negative controls. Therefore, at this concentration, the test article was evaluated at the limit of solubility in the test system and at the maximum recommended concentration for a non-toxic substance. For the plate incorporation test, without metabolic activation, [FL-no: 16.129] produced one statistically significant increase ($p < 0.01$) in colony counts over the concurrent negative control at 2,500 μg/plate in TA1535. Despite this increase, a dose-response was not observed. No other changes in colony counts were noted in the other strains with and without metabolic activation. In the preincubation test, with or without metabolic activation, [FL-no: 16.129] did not produce any statistically significant increases ($p > 0.01$) in colony counts over the concurrent negative controls and a dose-response was not observed. Therefore, the preincubation test confirmed the negative results of the plate incorporation test for genotoxicity. The negative controls for each tester strain were all within the historical negative control and/or spontaneous reversion ranges. All concurrent positive controls induced a significant increase in colony counts when compared to the corresponding negative controls and were at levels similar to the historical positive control data.

Thus, it was concluded that [FL-no: 16.129] was not mutagenic to *S.* Typhimurium strains TA98, TA100, TA1535, TA1537 and *E. coli* strain WP2 *uvrA* at concentrations up to 5,000 μg/plate, in the absence and presence of metabolic activation. The Panel confirms this conclusion.

E.1.2. Chromosome aberration test

[FL-no: 16.129] (purity > 99%) was tested in the *in vitro* mammalian chromosome aberration test using isolated frozen preserved human peripheral blood lymphocytes in the absence and presence of liver preparations (S9-mix) from rats treated with phenobarbital and 5,6-benzoflavone (Nucro-Technics, 2012b).

The experimental design followed the OECD test guideline 473 (OECD, 1997b) and GLP guideline. A preliminary toxicity test was performed to establish the range of concentrations for testing in the cytogenetic test. In the chromosome aberration assay, human lymphocytes were treated for 3 h or 20 h in the absence of S9-mix and for 3 h in the presence of S9-mix. For all conditions, cultures were harvested approximately 20 h after the initiation of treatment. Solvent and positive control cultures were also included.

Test article precipitate was observed at 2,500 μg/mL. For the 3-h exposure experiment without S9-mix, the concentrations 310, 630, 1,300 and 2,500 μg/mL induced a Relative Mitotic Index (RMI) of 87%, 81%, 82% and 44%, respectively. The same [FL-no: 16.129] concentrations tested for 3 h with S9-mix induced a RMI of 104%, 107%, 93% and 69%, respectively. For the 20-h treatment without S9-mix, the tested concentrations 130, 250, 500 and 1,000 μg/mL induced RMI of 100%, 94%, 59% and 35%, respectively. The number of each type of aberration was analysed in 200 cells per concentration. All conditions were tested at the limit of test article toxicity evaluated by RMI levels. Precipitate was observed in each treatment condition at the highest concentration tested. Low levels of chromosome aberrations were observed at all concentrations, including the solvent control. All concurrent positive controls induced a statistically significant increase in the number of cells with chromosome aberrations.

Under these test conditions, no structural or numerical chromosome aberrations were observed in the [FL-no: 16.129]-treated cultures beyond those seen in the concurrent solvent controls. The Panel concluded that, in this *in vitro* test system, [FL-no: 16.129] did not induce chromosome aberrations in human peripheral blood lymphocytes in both the absence and presence of metabolic activation.

E.1.3. *In vitro* micronucleus test

The clastogenic and/or aneugenic potential of [FL-no: 16.129] (purity > 99%) was evaluated through an *in vitro* micronucleus assay in Chinese hamster ovary cells (CHO-WB_L) in both the absence and presence of liver preparations (S9-mix) from rats treated with phenobarbital and 5,6-benzoflavone (Nucro-Technics 2012c). The experimental design followed the OECD test guideline 487 (OECD, 2010) and GLP guideline. Cultures of CHO-WB_L cells were treated with [FL-no: 16.129] under three different conditions: 3-h exposure in the absence or presence of S9-mix or 20.5-h exposure in the absence of S9-mix. The same range of [FL-no: 16.129] concentrations was tested in the three different test conditions: 156, 313, 625, 1250 and 2,500 μg/mL. Cytochalasin B at 3 μg/mL was present in the medium after the short-term exposure to [FL-no: 16.129] and during the extended exposure in its entirety. Cells were harvested approximately 20–21 h from the beginning of the treatment (i.e. approximately 1.5-fold the normal cell cycle length). Test article precipitate was observed only at 2,500 μg/mL the highest concentration tested. For all treatment conditions, [FL-no: 16.129] was tested at the limit of its solubility and the same concentrations were chosen for micronuclei (MN) scoring: 625, 1250 and 2,500 μg/mL. The MN frequency was analysed in 2,000 binucleated cells per concentration.

For the 3-h exposure without S9-mix, the concentrations 625, 1,250 and 2,500 μg/mL yielded up to 35% of cells proliferation inhibition at the highest concentration. For the 20.5-h treatment without S9-mix, up to 43% of cells proliferation inhibition was observed at the highest concentration. For the 3-h treatment with S9-mix, up to 18% of cells proliferation inhibition was observed. Low frequencies of binucleated cells with micronuclei were observed at all concentrations, including the solvent control. All positive controls induced a statistically significant increase in the number of binucleated cells with micronuclei over the concurrent solvent controls.

Under these test conditions, no micronuclei were observed in the [FL-no: 16.129]-treated cultures beyond those seen in the concurrent solvent controls. The Panel concluded that, in this test system, [FL-no: 16.129] did not increase the incidence of micronuclei in CHO-WB_L cells in both the absence and presence of S9-mix.

The genotoxicity studies described above are summarised in Table E.1.

E.1.4. Genotoxicity *in vivo*

No data available.

E.2. Other studies

E.2.1. Synthetic intermediate

The synthetic intermediate S1116 ((*S*)-2-amino-6-((1-(3- methylbutanoyl) piperidin-3-yl) methoxy) benzonitrile) has been observed as a minor impurity in some batches of [FL-no: 16.129]. It has not been detected in commercial batches of [FL-no: 16.129]. A screening Ames test was performed on S1116 to evaluate its genotoxic potential to induce base pairs substitution or frameshift mutations in

S. Typhimurium strains, TA98 and TA100 in a plate incorporation assay in the presence or absence of S9-mix (BioReliance, 2013a).

No precipitate was observed with S1116 under any of the test conditions at concentrations up to 5,000 μg/plate. Neither bacterial toxicity nor mutagenic activity was demonstrated in either strain with or without metabolic activation. Positive and vehicle controls yielded the expected results indicating that criteria for a valid assay were met. S1116 was not mutagenic under these test conditions.

However, the Panel considered that the Ames assay, limited to *S.* Typhimurium strains, TA98 and TA100, is inadequate to conclude that S1116 is devoid of mutagenic potential in bacteria.

E.3. Genotoxicity studies on photodegradation products

In vitro genotoxicity tests were conducted on the 1,2- and 1,4-dihydroxy (S7859 and S4462, respectively) and 1,2- and 1,4-quinone (S7863 and S4465, respectively) photo-breakdown products of [FL-no: 16.129] as well as photo-breakdown product S8900 (see structures in Section 3.4.3, Figure 2). In addition, an *in vivo* combined micronucleus and comet assay in rats were conducted on the 1,2- and 1,4-dihydroxy (S7859 and S4462, respectively) photo-breakdown products of the candidate substance. Individual studies and tests are summarised below and in Tables E.2 and E.3. No genotoxicity study was performed on the photoproduct 3b and on S9465.

E.3.1. Genotoxicity Studies on the photo-breakdown product 1a (S7859)

E.3.1.1. Bacterial Reverse Mutation Screening Test (TA98 and TA100)

S7859 was tested up to 5,000 μg/plate in a bacterial reverse mutation test in two *S.* Typhimurium strains, TA98 and TA100, with and without metabolic activation. The plate incorporation method was applied. S7859 showed no evidence of mutagenic activity under the test conditions employed (Huntingdon, 2009c). However, this study was not in accordance with OECD test guideline 471 and not performed under GLP.

The Panel considered that that the Ames assay, limited to *S.* Typhimurium strains, TA98 and TA100, is inadequate to conclude that S7859 is devoid of mutagenic potential in bacteria.

E.3.1.2. Bacterial Reverse Mutation assay in *S.* Typhimurium and *E. coli*

S7859 was evaluated for mutagenic activity in an *in vitro* bacterial reverse mutation assay in *S.* Typhimurium (strains TA1537, TA98, TA100 and TA1535) and *E. coli* WP2 *uvrA* (WIL Research, 2013d). In the initial assay, S7859 was tested at 25, 50, 100, 250, 500, 1,000, 2,500 and 5,000 μg/plate. Precipitates were not observed in any strain either with or without metabolic activation. Cytotoxicity was observed at concentrations above 1,000 μg/plate in TA1537, TA100 and TA1535 without metabolic activation; at concentrations above 2,500 μg/plate in TA98 with and without metabolic activation and at 5,000 μg/plate in WP2 *uvrA* with metabolic activation. In the confirmatory assay, S7859 was tested at: 50, 100, 250, 500, 1,000 and 2,500 μg/plate in TA100 and TA1535 without metabolic activation, at 100, 250, 500, 1,000, 2,500 and 5,000 μg/plate in strains TA100 and TA1535 with metabolic activation and in strains TA1537, TA98 and WP2 *uvrA* with and without metabolic activation. Cytotoxicity was observed at concentrations above 1,000 μg/plate in TA100 and TA1535 without metabolic activation. Cytotoxicity was observed at concentrations above 2,500 μg/plate in TA1537 without metabolic activation and TA98 with and without metabolic activation and at 5,000 μg/plate in WP2 *uvrA* with metabolic activation. The study was performed according to OECD test guideline 471 and GLP.

Positive and vehicle controls yielded the expected results indicating that criteria for a valid assay were met. Mean increases in the number of revertant colonies indicative of a positive response were not observed in the *S.* Typhimurium strains TA1537, TA98, TA100 and TA1535 and in the *E. coli* strain WP2 *uvrA*, with and without metabolic activation, under the conditions of this assay. Therefore, the Panel concluded that S7859 does not induce mutagenicity under these test conditions.

E.3.1.3. *In vitro* chromosome aberration test in cultured human peripheral blood lymphocytes

S7859 was evaluated for the potential to induce chromosome aberrations in human peripheral blood lymphocytes (HPBL) during short (3 h) and long-term (22 h) incubations with or without an exogenous metabolic activation system of an Aroclor 1254-induced rat liver S9 microsomal fraction (S9-mix). The study was performed according to OECD test guideline 473 and GLP.

For the short treatment in the presence or absence of metabolic activation, the concentrations selected for the analysis of chromosome aberrations were 500, 1,000 and 1750 μg/mL with a cytotoxicity of 7%, 29% and 50% (without S9-mix) and 2%, 15% and 55% (with S9-mix), respectively. For the treatment at 22 h without metabolic activation, the concentrations selected were 250, 400 and 500 μg/mL with a cytotoxicity of 5%, 33% and 60%, respectively (WIL Research, 2013 h).

Statistically significant increases ($p \leq 0.01$) in the percentage of cells with structural chromosome aberrations were observed in the 3-h treatment without metabolic activation at 1,000 and 1750 μg/mL, in the 22-h treatment without metabolic activation at 250, 400 and 500 μg/mL and in the 3-h treatment with metabolic activation at 1,750 μg/mL. In addition, statistically significant increases ($p \leq 0.01$) in the percent of cells with more than one aberration were noted in the 3-h treatments with and without metabolic activation at 1,750 μg/mL and in the 22-h treatment without metabolic activation at 400 and 500 μg/mL. There were no statistically significant increases in numerical aberrations (endoreduplication or polyploidy). The aberrations in vehicle and positive control cultures were comparable to acceptable historical control ranges (WIL Research, 2013 h).

The Panel concluded that S7859 induces structural chromosome aberrations in HPBL with and without metabolic activation under the conditions of this test system.

E.3.1.4. *In Vivo* Micronucleus/Comet Study of S7859 in Rats

S7859 was tested in a combined *in vivo* micronucleus test and comet assay in Sprague–Dawley rats. The micronucleus assay was based on OECD test guideline 474 (OECD, 1997c), and the comet assay design is based on JaCVAM protocol version 14.2 and compliant with GLP. The micronucleus test was performed in bone marrow cells. The comet assay was performed on liver and duodenum. S7859 was tested in a dose range-finding assay at 125, 250, 500, 1,000 and 2,000 mg/kg bw per day. Clinical signs of piloerection and lethargy were seen after 3 days of treatment at all dose levels. Based upon these results, the highest dose of S7859 was selected as 2,000 mg/kg bw per day (the limit dose for this assay). Since no differences in the clinical signs of toxicity were observed between the sexes, only male rats were used for the definitive assay. All groups evaluated had five male rats assigned to each group. Doses evaluated in the definitive assay were 0 (vehicle), 500, 1,000 and 2,000 mg/kg bw per day. All doses were administered by oral gavage once per day over three consecutive days. The positive control (ethyl methanesulfonate, EMS) was administered at a dose of 200 mg/kg bw per day. All animals were euthanised approximately 3–4 h after the final dose. Femoral bone marrow was collected from all animals for the micronucleus assay. A section of the liver and sections of the duodenum were removed from all animals for the comet assay (BioReliance, 2013e).

S7859 (tested up to 2,000 mg/kg bw per day) did not cause any significant increase in DNA damage in liver cells or duodenal cells and did not induce any significant increase in the incidence of micronucleated polychromatic erythrocytes (MNPCEs) in bone marrow relative to the concurrent vehicle control group. Therefore, the authors of the study report concluded that S7859 was negative in the *in vivo* Comet assays and in the *in vivo* bone marrow micronucleus assay. However, no proof of systemic exposure was provided: no clinical symptoms, no evidence of toxicity from serum chemistry and histopathology analysis, no increase of cloud cells in the comet assay and no decrease of the ratio PCE/NCE was observed in the bone marrow. The Panel concluded that without evidence of liver and bone marrow exposure to S7859, the results of this study are considered as inconclusive for these organs.

Although negative results of comet assay in duodenum indicate no induction of primary DNA damage *in vivo*, the potential aneugenicity of S7859 has not been adequately investigated.

E.3.1.5. Conclusions on genotoxicity studies for S7859

S7859 (photo-breakdown product 1a) is not mutagenic in bacteria. It induces clastogenic effects *in vitro* in human peripheral blood lymphocytes with and without metabolic activation. An *in vivo* combined micronucleus test in bone marrow and comet assay in liver and duodenum with S7859 in rats was negative for both tests. The Panel considered that these *in vivo* studies are not adequate to rule out the concern for genotoxicity resulting from *in vitro* studies because there is no proof of target tissues exposure.

E.3.2. Genotoxicity Studies on the photo-breakdown product 1b (S4462)

E.3.2.1. Bacterial Reverse Mutation Screening Test (TA98 and TA100)

S4462 was tested up to 5,000 μg/plate in a bacterial reverse mutation test in two *S*. Typhimurium strains, TA98 and TA100, with and without metabolic activation. The plate incorporation method was applied. S4462 showed no evidence of mutagenic activity under the test conditions employed (Huntingdon, 2009a). However, this study was not in accordance to OECD test guideline 471 and not performed under GLP.

The Panel considered that the Ames assay, limited to *S.* Typhimurium strains TA98 and TA100, is inadequate to conclude that S4462 is devoid of mutagenic potential in bacteria.

E.3.2.2. Bacterial Reverse Mutation assay in *S.* Typhimurium and *E. coli*

S4462 was evaluated for mutagenic activity in an *in vitro* bacterial reverse mutation assay in *S.* Typhimurium (*strains* TA1537, TA98, TA100 and TA1535) *and E. coli* WP2 *uvrA* (WIL Research, 2013c). In the initial assay, S4462 was tested at 25, 50, 100, 250, 500, 1,000, 2,500 and 5,000 μg/plate in all strains with and without metabolic activation in two independent experiments. Precipitates were not observed in any strain either with or without metabolic activation. Cytotoxicity was observed at concentrations above 1,000 μg/plate in TA1537, TA98, TA100 and TA1535 without metabolic activation and at 5,000 μg/plate in E. Coli WP2 *uvrA* without metabolic activation. In the confirmatory experiment, S4462 was tested at 50, 100, 250, 500, 1,000 and 2,500 μg/plate in TA1537, TA98 and TA1535 without metabolic activation; at 50, 100, 250, 500, 1,000, 2,500 and 5,000 μg/plate in TA100 without metabolic activation and at 100, 250, 500, 1,000, 2,500 and 5,000 μg/plate in strains TA1537, TA98, TA100 and TA1535 with metabolic activation and E. Coli WP2 uvrA with and without metabolic activation. The study was performed according to OECD test guideline 471 (OECD, 1997a) and GLP. Precipitates were not observed in any strain either with or without metabolic activation. Cytotoxicity was observed at concentrations above 500 μg/plate in TA1537 without metabolic activation, at concentrations above 1,000 μg/plate in TA100 and TA1535 without metabolic activation; at concentrations at and above 2,500 μg/plate in TA98 without metabolic activation and at 5,000 μg/plate in E. Coli WP2 uvrA without metabolic activation.

Positive and vehicle controls yielded the expected results indicating that criteria for a valid assay were met. In both assays, increases in the mean number of revertant colonies indicative of a positive response were observed with S4462 in the *S.* Typhimurium strains TA1537 and TA98 with metabolic activation. An increase in colonies was also observed in the initial assay in TA100 with metabolic activation, but this result was not replicated in the confirmatory assay.

Therefore, S4462 is considered to be positive for inducing mutagenicity in strains TA1537 and TA98 with metabolic activation; equivocal for inducing mutagenicity in strain TA100 with metabolic activation and negative for inducing mutagenicity in strains TA1535 and E. Coli WP2 uvrA with and without metabolic activation and TA1537, TA98 and TA100 without metabolic activation.

The Panel concluded that S4462 induces gene mutation in bacteria in strains TA1537 and TA98 and is equivocal in strain TA100 under these test conditions.

E.3.2.3. Repeated Reverse Mutation Assay on S4462 in *S.* Typhimurium Strains TA98, TA100 and TA1537

S4462 was tested for mutagenic activity in a new *in vitro* reverse mutation assay in *S.* Typhimurium strains TA98, TA100 and TA1537 in the presence of either Aroclor-induced rat liver S9 or phenobarbital/5,6-benzoflavone-induced rat liver S9, using the plate incorporation method (BioReliance, 2013b). This test was not performed according to GLP. S4462 was tested at the following concentrations: 25, 50, 100, 250, 500, 1,000, 2,500 and 5,000 μg/plate. Concentration-related increases in revertant counts (1.7- and 1.8-fold maximum increases) were observed with tester strain TA100 in the presence of both S9 activation systems. These increases did not meet all criteria required for evaluation as positive (i.e. a maximum response of at least 2-fold the mean vehicle control value), but suggest the presence of a low mutagenic activity. No significant increase was observed in the strains TA1537 and TA98 in the presence of both S9 activation systems.

The Panel noted that the mutagenic effects in strains TA1537 and TA98 were not reproducible and that equivocal effects in strain TA100 were observed in both experiments. Thus, overall, the outcome of the studies on the induction of gene mutations in bacteria is equivocal for S4462.

E.3.2.4. *In Vitro* L5178Y/TK$^{+/-}$ Mouse Lymphoma Assay on S4462

The substance S4462 was tested in the L5178Y/TK$^{+/-}$ mouse lymphoma assay in the absence and presence of Aroclor-induced rat liver S9 using the methodology of agar cloning. The study was performed according to OECD test guideline 476 (OECD, 1997d) and GLP. The mammalian mutation assay was conducted by exposing L5178Y/TK$^{+/-}$ cells to S4462 using duplicate cultures for 4 h in the presence and absence of S9 or for 24 h in the absence of S9. Based on the results of a preliminary toxicity assay, the concentrations used ranged from 5 to 250 μg/mL for the cultures without metabolic activation and from 1.5 to 250 μg/mL for the cultures with metabolic activation (BioReliance, 2013c).

Cloned cultures exhibited induced mutant frequencies ≥ 90 mutants per 10^6 clonable cells at concentrations higher than 75 μg/mL in the absence of S9 activation. There was a concentration-related increase in mutant frequency in the absence of S9 activation. With S9, no cloned cultures exhibited induced mutant frequencies ≥ 90 mutants per 10^6 clonable cells. There was no concentration-related increase in mutant frequency. The data on colony size distributions showed an increase in the frequency of small and large colonies when the test article-treated cultures were compared to the solvent control cultures. This observation demonstrates that the test article induced both gene mutations and chromosomal breaks. The authors of this study concluded that S4462 was positive after 4-h and 24-h exposure in the absence of S9 activation and negative after 4-h exposure in the presence of S9 activation in the L5178Y/TK+/− mouse lymphoma assay.

The Panel concluded that S4462 is genotoxic in mammalian cells under these test conditions *in vitro*.

E.3.2.5. *In vitro* chromosome aberration test in cultured human peripheral blood lymphocytes

S4462 was evaluated for the potential to induce chromosome aberrations in HPBL during short (3 h) and long (22 h) incubations with or without an exogenous metabolic activation system of an Aroclor 1254-induced rat liver S9 microsomal fraction (S9-mix). The study was performed according to OECD test guideline 473 (OECD, 1997b) and GLP.

The concentrations selected for evaluation of chromosome aberrations were based on cytotoxicity and are as follows (with percentage cytotoxicity): for 3-h treatment without S9-mix, 150 μg/mL (4%), 200 μg/mL (39%) and 225 μg/mL (51%); for 3-h treatment with S9-mix, 150 μg/mL (no cytotoxicity), 200 μg/mL (21%) and 250 μg/mL (51%) and for 22-h treatment without S9-mix, 100 μg/mL (no cytotoxicity), 150 μg/mL (29%) and 175 μg/mL (52%).

Statistically significant increases in the percentage of cells with structural chromosome aberrations were observed in the 3-h treatment without S9-mix at 200 μg/mL ($p \leq 0.05$) and 225 μg/mL ($p \leq 0.01$), in the 22-h treatment without S9-mix at 150 and 175 μg/mL ($p \leq 0.01$) and in the 3-h treatment with S9-mix at 200 and 250 μg/mL ($p \leq 0.01$). In addition, statistically significant increases in the percentage of cells with more than one aberration were noted in the 22-h treatment without S9-mix at 175 μg/mL ($p \leq 0.01$) and in the 3-h treatment with S9-mix at 250 μg/mL ($p \leq 0.01$). Statistically significant increases in polyploidy were observed in the 22-h treatment without metabolic activation at 175 μg/mL ($p \leq 0.01$) and in the 3-h treatment with metabolic activation at 250 μg/mL ($p \leq 0.01$). The aberrations in vehicle and positive control cultures were comparable to historical control ranges (WIL Research, 2013 g).

The authors of this study concluded that S4462 was considered positive for inducing structural chromosome aberrations in HPBL with and without metabolic activation under the conditions of this test system. In addition, S4462 produced statistically significant increases in the percentage of polyploid cells in the 3-h incubation with metabolic activation and the 22-h incubation without metabolic activation.

The Panel concluded that S4462 induces chromosomal aberrations in HPBL under the conditions of this study.

E.3.2.6. *In Vivo* Micronucleus/Comet Study of S4462 in Rats

S4462 was tested in a combined *in vivo* micronucleus test and Comet assay in Sprague–Dawley rats. The micronucleus assay was based on OECD test guideline 474 (OECD, 1997c) and the Comet assay design was based on JaCVAM protocol version 14.2 and compliant with GLP. The micronucleus test was performed in bone marrow cells. The comet assay was performed on liver and duodenum. Based upon the results of a dose range-finding assay, the highest dose of S4462 was selected as 2,000 mg/kg bw per day (the limit dose for this assay). Since no differences in the clinical signs of

toxicity were observed between the sexes, only male rats were used for the definitive assay. All groups evaluated had five male rats assigned to each group. Doses evaluated in the definitive assay were 0 (vehicle), 500, 1,000 and 2,000 mg/kg bw per day. All doses were administered by oral gavage once per day over three consecutive days. The positive control EMS was administered at a dose of 200 mg/kg bw per day. All animals were euthanised approximately 3–4 h after the final dose. Femoral bone marrow was collected from all animals for the micronucleus assay. A section of the liver and sections of the duodenum were removed from all animals for the comet assay (BioReliance, 2013d).

S4462 (tested up to 2,000 mg/kg bw per day) did not cause any significant increase in DNA damage in liver cells or duodenal cells and did not induce any significant increase in the incidence of MNPCEs in bone marrow relative to the concurrent vehicle control group. Therefore, the authors of the study report concluded that S4462 was negative in the *in vivo* comet assay and in the *in vivo* bone marrow micronucleus assay. However, no proof of systemic exposure was provided: no clinical symptoms (except piloerection at the top dose), no changes in serum chemistry and histopathology, no increase of cloud cells in the comet assay and no decrease of the ratio PCE/NCE were observed in the bone marrow. The Panel concluded that without evidence of liver and bone marrow exposure to S4462, the results of this study are considered as inconclusive for these organs. Although negative results of comet assay in duodenum indicate no induction of primary DNA damage *in vivo*, the potential aneugenicity of S4462 has not been adequately investigated.

E.3.2.7. Conclusions on genotoxicity studies for S4462

S4462 is equivocally mutagenic in bacteria, with metabolic activation. It induced gene mutations in the mouse lymphoma assay (L5178Y/TK$^{+/-}$) in the absence of metabolic activation. S4462 induces chromosomal aberrations suggesting a clastogenic activity and, a possible aneugenic effect *in vitro* in human peripheral blood lymphocytes in the presence and absence of metabolic activation. In rats, S4462 did not induce primary DNA damage in liver and duodenum following oral administration as demonstrated in the comet assay. In the bone marrow, no change in the frequency of micronuclei was observed. However, in the absence of demonstration of the exposure of liver and bone marrow, the Panel considered that it is not possible to assess the genotoxic potential of S4462 *in vivo*.

E.3.3. Genotoxicity Studies on the photo-breakdown product 2a (S7863)

E.3.3.1. Bacterial Reverse Mutation Screening Test (TA98 and TA100)

S7863 was tested up to 5,000 μg/plate in a bacterial reverse mutation test in two *S.* Typhimurium strains, TA98 and TA100, with and without metabolic activation. The plate incorporation method was applied. S7863 showed no evidence of mutagenic activity under the test conditions employed (Huntingdon, 2009d). However, this study was not in accordance to OECD TG 471 (OECD, 1997a) and not performed under GLP.

The Panel considered that the Ames assay, limited to *S.* Typhimurium strains TA98 and TA100, is inadequate to conclude that S7863 is devoid of mutagenic potential in bacteria.

E.3.3.2. Bacterial Reverse Mutation assay in *S.* Typhimurium and *E. coli*

S7863 was evaluated for mutagenic activity *in vitro* in a bacterial reverse mutation assay in *S.* Typhimurium (TA1537, TA98, TA100, and TA1535) and *E. coli* WP2 uvrA (WIL Research, 2013f). S7863 was tested at 50, 100, 250, 500, 1,000, 2,500 and 5,000 μg/plate in all strains with and without metabolic activation in two separate experiments. Cytotoxicity was observed at concentrations above 1,000 μg/plate in TA98 and WP2 uvrA with and without metabolic activation; and at concentrations above 2,500 μg/plate in TA1537, TA100, and TA1535 with and without metabolic activation. The study was performed according to OECD test guideline 471 (OECD, 1997a) and GLP.

In both assays, criteria for a negative response were met for all tester strains with and without metabolic activation. Mean number of revertant colonies was comparable to historical control ranges at all concentrations for all tester strains with and without metabolic activation.

Positive and vehicle controls yielded the expected results indicating that criteria for a valid assay were met. S7863 is negative for mutagenic activity in *S.* Typhimurium strains TA1537, TA98, TA100 and TA1535 and in the *E. coli* strain WP2 uvrA, with and without metabolic activation, under the conditions of this assay.

The Panel concluded that S7863 does not induce mutagenicity under these test conditions.

E.3.3.3. *In vitro* chromosome aberration test in cultured human peripheral blood lymphocytes

S7863 was evaluated *in vitro* for the potential to induce chromosome aberrations in HPBL during short (3 h) and long-term (22 h) incubations with or without an exogenous metabolic activation system of an Aroclor 1254-induced rat liver S9 microsomal fraction. The study was performed according to OECD test guideline 473 (OECD, 1997b) and GLP.

The concentrations selected for the chromosome aberration assay were based on cytotoxicity and are as follows (with percentage of cytotoxicity): for 3-h treatment without metabolic activation, 550 μg/mL (no cytotoxicity), 700 μg/mL (20%) and 1100 μg/mL (59%); for 3-h treatment with activation, 500 μg/mL (2%), 600 μg/mL (29%) and 850 μg/mL (50%) and for 22-h treatment without metabolic activation, 10 μg/mL (no cytotoxicity), 200 μg/mL (30%) and 300 μg/mL (52%).

Statistically significant increases in the percentage of cells with structural chromosome aberrations were observed in the 3-h treatment without metabolic activation at 550 μg/mL ($p \leq 0.05$) and at 700 and 1100 μg/mL ($p \leq 0.01$), in the 22-h treatment without metabolic activation at 200 μg/mL ($p \leq 0.05$) and at 300 μg/mL ($p \leq 0.01$), and in the 3-h treatment with metabolic activation at 500, 600 and 850 μg/mL ($p \leq 0.01$). In addition, statistically significant increases in the percentage of cells with more than one aberration were noted in the 22-h treatment without metabolic activation at 300 μg/mL ($p \leq 0.01$). There were no statistically significant increases in numerical aberrations (endoreduplication or polyploidy). The aberrations in vehicle and positive control cultures were comparable to historical control ranges except that the percentage of cells with aberrations in the positive control for the 3-h treatment with metabolic activation was higher than the historical control range (WIL Research, 2013j).

The authors of the study concluded that S7863 was positive for inducing structural chromosome aberrations in HPBL with and without metabolic activation under the conditions of this test system.

The Panel concluded that S7863 induces structural chromosome aberrations in HPBL with and without metabolic activation under the conditions of this test system.

E.3.3.4. Conclusions on genotoxicity studies for S7863

S7863 is not mutagenic in bacteria. In the *in vitro* chromosomal aberration test in human peripheral blood lymphocytes, S7863 presents a clastogenic effect both with and without metabolic activation. The same study does not suggest a potential aneugenicity for S7863. The Panel concluded that S7863 is genotoxic *in vitro*. Based on the available data, the concern for genotoxicity could not be ruled out.

E.3.4. Genotoxicity Studies on the photo-breakdown product 2b (S4465)

E.3.4.1. Bacterial Reverse Mutation Screening Test (TA98 and TA100)

S4465 was tested up to 5,000 μg/plate in a bacterial reverse mutation test in two *S.* Typhimurium strains, TA98 and TA100, with and without metabolic activation. The plate incorporation method was applied. S4465 showed no evidence of mutagenic activity under the test conditions employed (Huntingdon, 2009b). However, this study was not in accordance with OECD test guideline 471 (OECD, 1997a) and not performed under GLP.

The Panel considered that the Ames assay, limited to *S.* Typhimurium strains TA98 and TA100, is inadequate to conclude that S4465 is devoid of mutagenic potential in bacteria.

E.3.4.2. Bacterial Reverse Mutation assay in *S.* Typhimurium and *E. coli*

S4465 was tested for mutagenic activity *in vitro* in a bacterial reverse mutation assay in *S.* Typhimurium (TA1537, TA98, TA100, and TA1535) and *E. coli* WP2 uvrA (WIL Research, 2013e). In the initial assay, S4465 was tested at 25, 50, 100, 250, 500, 1,000, 2,500 and 5,000 μg/plate. Precipitates were not observed in any strain either with or without metabolic activation. Cytotoxicity was observed at concentrations above 500 μg/plate in TA98 with and without metabolic activation; at concentrations above 2,500 μg/plate in TA100 and TA1535 with metabolic activation and at 5,000 μg/plate in TA100, TA1535 and *E. coli* WP2 uvrA without metabolic activation and TA1537 with and without metabolic activation. In the confirmatory assay, S4465 was tested at 100, 250, 500, 1,000, 2,500 and 5,000 μg/plate with and without metabolic activation. Cytotoxicity was observed at concentrations above 1,000 μg/plate in TA1537 without metabolic activation and in TA98 and *E. coli* WP2 uvrA with and without metabolic activation and at 5,000 μg/plate in TA100 and TA1535 with and without metabolic activation and in TA1537 with metabolic activation. The study was performed according to OECD test guideline 471 (OECD, 1997a) and GLP.

Positive and vehicle controls yielded the expected results indicating that criteria for a valid assay were met. The Panel concluded that S4465 is negative for mutagenic activity in *S.* Typhimurium strains TA1537, TA98, TA100 and TA1535 and in the *E. coli* strain WP2 uvrA, with and without metabolic activation, under the conditions of this assay.

E.3.4.3. *In vitro* chromosome aberration test in cultured human peripheral blood lymphocytes

S4465 was evaluated for the potential to induce chromosome aberrations in HPBL during short (3 h) and long-term (22 h) incubations with or without an exogenous metabolic activation system of an Aroclor 1254-induced rat liver S9 microsomal fraction (WIL Research, 2013i). The study was performed according to OECD test guideline 473 (OECD, 1997b) and GLP.

The concentrations selected for evaluation of chromosome aberrations were based on cytotoxicity and are as follows (with percentage of cytotoxicity): for 3-h treatment without metabolic activation, 100 μg/mL (12%), 200 μg/mL (23%) and 450 μg/mL (52%); for 3-h treatment with activation, 200 μg/mL (no cytotoxicity), 400 μg/mL (27%) and 700 μg/mL (56%) and for 22-h treatment without metabolic activation, 45 μg/mL (no cytotoxicity), 100 μg/mL (25%) and 185 μg/mL (60%).

Statistically significant increases ($p \leq 0.01$) in the percentage of cells with structural chromosome aberrations were observed in the 3-h treatment without metabolic activation at 450 μg/mL, in the 22-h treatment without metabolic activation at 100 and 185 μg/mL and in the 3-h treatment with metabolic activation at 200, 400 and 700 μg/mL. In addition, statistically significant increases in the percentage of cells with more than one aberration were noted in the 3-h treatment without metabolic activation at 450 μg/mL ($p \leq 0.05$), in the 22-h treatment without metabolic activation at 185 μg/mL ($p \leq 0.01$) and in the 3-h treatment with metabolic activation at 700 μg/mL ($p \leq 0.01$). A statistically significant increase ($p \leq 0.05$) in polyploidy was observed at 185 μg/mL in the 22-h treatment without metabolic activation.

The Panel concluded that S4465 induces chromosome aberrations in HPBL with and without metabolic activation under the conditions of this test system.

E.3.4.4. Conclusions on genotoxicity studies for S4465

The Panel considered that S4465 is not mutagenic in bacteria, but it presents a clastogenic effect in human peripheral blood lymphocytes with and without metabolic activation and a possible aneugenic effect *in vitro* without metabolic activation. The Panel concluded that S4465 is genotoxic *in vitro*. Based on the available data, the concern for genotoxicity could not be ruled out.

E.3.5. Genotoxicity Studies on the photo-breakdown product 3a (S8900)

S8900 was tested in a very limited test in *S. Typhimurium* strains TA98 and TA100, using a micromethod in multiwells plates. Results showed that S8900, tested up to 250 μg/ well (considered by the authors as equivalent to 5,000 μg/plate), with or without metabolic activation, was not mutagenic in both strains (Midwest BioResearch, 2009).

E.3.5.1. Conclusions on genotoxicity studies for S8900

S8900 (photo-breakdown product 3a) was not mutagenic in a very limited test in bacteria. The Panel considered that these data are too limited to assess the genotoxic potential of S8900. The Panel considered that a complete battery of assays performed according to EFSA scientific committee opinion on genotoxicity testing strategies (EFSA Scientific Committee, 2011) should be provided.

E.3.6. Conclusions on genotoxicity of the photo-breakdown products

Photo-breakdown products S7859, S4462, S7863 and S4465 are positive in at least one genotoxicity assay *in vitro*. S8900 has been tested in a limited microAmes study. For S9665 and photo-breakdown product 3b, no data are available. S7859 and S4462 have been tested in rats, in a combined bone marrow micronucleus assay and comet assay in liver and duodenum that gave negative results. However, in this study, there is no evidence of systemic exposure to the test substance. Therefore, it is only possible to conclude that S7859 and S4462 do not induce primary DNA damage locally in the duodenum.

Based on the available data, the concern for genotoxicity cannot be ruled out for the photo-breakdown products.

Table E.1: Summary of *in vitro* Genotoxicity Studies

Test material	Test System	Test Object	Concentration	Result	Reference	Comments
[FL-no: 16.129]	Bacterial reverse mutation screening test	*S.* Typhimurium TA98, TA100	Up to 5,000 μg/plate[(a)]	Negative	WIL Research, 2012	Plate incorporation assay. Precipitates were observed at 5,000 μg/plate. No cytotoxicity
	Bacterial reverse mutation assay	*S.* Typhimurium TA98, TA100, TA1535, TA1537 *E. coli* WP2uvrA	0, 310, 630, 1,300, 2,500 and 5,000 μg/plate[(a),(b)]	Negative	Nucro-Technics, 2012a	No cytotoxicity was observed up to 5,000 μg/plate. Slight precipitate at 5,000 μg/plate in the plate incorporation assay and at both 2,500 and 5,000 μg/plate in the preincubation assay.
	Chromosome aberration test	Human peripheral blood lymphocytes	310, 630, 1,300 and 2,500 μg/mL[(c)] 130, 250, 500 and 1,000 μg/mL[(d)]	Negative	Nucro-Technics, 2012b	Precipitate was observed at 2,500 μg/mL. Cytotoxicity was observed at 1,000 μg/mL in the treatment at 20 h
	Micronucleus test	Chinese hamster ovary cells	0, 625, 1,250 and 2,500 μg/mL[(e),(f)]	Negative	Nucro-Technics, 2012c	Test article precipitate was observed at the highest concentration tested
S1116	Bacterial reverse mutation screening test	*S.* Typhimurium TA98, TA100	Up to 5,000 μg/plate[(a)]	Negative	BioReliance, 2013a	Plate incorporation assay. Precipitates were not observed. No cytotoxicity

(a): with and without metabolic activation.
(b): test performed both with the plate incorporation and preincubation methods.
(c): 3-h exposure with and without metabolic activation.
(d): 20-h exposure without metabolic activation.
(e): 3 + 21-h exposure with and without metabolic activation.
(f): 21-h exposure without metabolic activation.

Table E.2: Summary of *in vitro* Genotoxicity Studies on photodegradation products

Test material	Test System	Test Object	Concentration	Result	Reference	Comments
S7859	Bacterial reverse mutation screening test	*S.* Typhimurium TA98, TA100	Up to 5,000 μg/plate	Negative[a]	Huntingdon, 2009c	Bacterial toxicity (reduction of the background lawn) was observed at 1,500 and 5,000 μg/plate in the absence of S9-mix and at 5,000 μg /plate in the presence of S9-mix.
	Bacterial reverse mutation assay	*S.* Typhimurium TA1537, TA98, TA100, and TA1535 *E. coli WP2uvrA*	100, 250, 500, 1,000, 2,500 and 5,000 μg/plate[a] 50, 100, 250, 500, 1,000 and 2,500 μg/plate[b] 100, 250, 500, 1,000, 2,500 and 5,000 μg/plate[c] 100, 250, 500, 1,000, 2,500 and 5,000 μg/plate[a]	Negative	WIL Research, 2013d	Cytotoxicity was observed at concentrations ≥ 1,000 μg/plate in TA100 and TA1535 without metabolic activation; at ≥ 2,500 μg/plate in TA1537 without metabolic activation and TA98 with and without metabolic activation; and at 5,000 μg/plate in WP2 uvrA with metabolic activation.
	Chromosome aberration test	Human peripheral blood lymphocytes	500, 1,000 and 1750 μg/mL[a],[d] 250, 400 and 500 μg/mL[b],[e]	Positive	WIL Research, 2013h	The test article induced structural chromosomal aberrations both in the presence and absence of metabolic activation. There were no statistically significant increases in numerical aberrations (endoreduplication or polyploidy).
S7863	Bacterial reverse mutation screening test	*S.* Typhimurium TA98, TA100	Up to 5,000 μg/plate	Negative[a]	Huntingdon, 2009d	No evidence of cytotoxicity at concentrations up to 5,000 μg/plate
	Bacterial reverse mutation assay	*S.* Typhimurium TA1537, TA98, TA100, TA1535 *E. coli WP2uvrA*	50, 100, 250, 500, 1,000, 2,500, and 5,000 μg/plate[a]	Negative[a]	WIL Research, 2013f	Cytotoxicity was observed at concentrations ≥ 1,000 μg/plate in TA98 and WP2uvrA with and without metabolic activation; and at concentrations ≥ 2,500 μg/plate in TA1537, TA100, and TA1535 with and without metabolic activation.
	Chromosome aberration test	Human peripheral blood lymphocytes	550, 700, 1100 μg/mL[b],[d] 500, 600, 850 μg/mL[c],[d] 10, 200, 300 μg/mL[b],[e]	Positive	WIL Research, 2013j	The test article induced structural chromosomal aberrations both in the presence and absence of metabolic activation. There were no statistically significant increases in numerical aberrations (endoreduplication or polyploidy).
S8900	microAmes screening test	*S.* Typhimurium TA98, TA100	Up to 250 μg/well	Negative[a]	Midwest BioResearch, 2009	No evidence of cytotoxicity at concentrations up to 250 μg /well

Test material	Test System	Test Object	Concentration	Result	Reference	Comments
S4462	Bacterial reverse mutation screening test	*S.* Typhimurium TA98, TA100	Up to 5,000 μg/plate	Negative[a]	Huntingdon 2009a	Bacterial toxicity (reduction of the background lawn) was observed at 1,500 and 5,000 μg/plate in the absence of S9-mix, and at 5,000 μg/plate in the presence of S9-mix.
	Bacterial reverse mutation assay	*S.* Typhimurium TA1537, TA98, TA1535 TA100 TA1537, TA98, TA100, and TA1535 *E. coli WP2uvrA*	50, 100, 250, 500, 1,000, and 2,500 μg/plate[b] 50, 100, 250, 500, 1,000, 2,500, and 5,000 μg/plate[b] 100, 250, 500, 1,000, 2,500, and 5,000 μg/plate[c] 100, 250, 500, 1,000, 2,500, and 5,000 μg/plate[a]	Negative Negative Positive Equivocal Negative	WIL Research, 2013c	Cytotoxicity was observed at concentrations ≥ 500 μg/plate in TA1537 without metabolic activation; at concentrations ≥ 1,000 μg/plate in TA100 and TA1535 without metabolic activation; at concentrations ≥ 2,500 μg/plate in TA98 without metabolic activation; at 5,000 μg/plate in WP2uvrA without metabolic activation. Positive results in strains TA1537 and TA98 with metabolic activation. Equivocal results in TA100 with metabolic activation. The study was performed according to GLP.
	Bacterial reverse mutation assay	*S.* Typhimurium TA1537, TA98 TA100	25, 50, 100, 250, 500, 1,000, 2,500 and 5,000 μg/plate	Negative Equivocal[a]	Bioreliance, 2013b	Each strain was tested with two different metabolic activation systems: aroclor-induced rat liver S9 or phenobarbital-benzoflavone-induced rat liver S9. Concentration-related increases in revertant counts (1.7- and 1.8-fold maximum increases) were observed with tester strain TA100 in the presence of both S9 activation systems. The study was not performed according to GLP.
	L5178Y/TK$^{+/-}$ Mouse Lymphoma Assay	L5178Y cells	5–250 μg/mL[b],[f] or [g] 1.5–250 μg/mL[c],[f]	Positive Negative	Bioreliance 2013c	The test article induced gene mutations (increase in the frequency of small and large colonies).
	Chromosome aberration test	Human peripheral blood lymphocytes	150, 200, 225 μg/mL[b],[d] 150, 200, 250 μg/mL[c],[d] 100, 150, 175 μg/mL[b],[e]	Positive	WIL Research, 2013g	The test article induced structural chromosomal aberrations both in the presence and absence of metabolic activation. A statistically significant increase in the percentage of polyploid cells was observed in the 3-h treatment with metabolic activation and in the 22-h treatment without metabolic activation.

Test material	Test System	Test Object	Concentration	Result	Reference	Comments
S4465	Bacterial reverse mutation screening test	*S.* Typhimurium TA98, TA100	Up to 5,000 μg/plate	Negative[(a)]	Huntingdon, 2009b	No evidence of cytotoxicity at concentrations up to 5,000 μg/plate.
	Bacterial reverse mutation assay	*S.* Typhimurium TA1537, TA98, TA100, TA1535 *E. coli WP2uvrA*	100, 250, 500, 1,000, 2,500 and 5,000 μg/plate[(a)]	Negative[(a)]	WIL Research, 2013e	Cytotoxicity was observed at concentrations ≥ 1,000 μg/plate in TA1537 without metabolic activation, in TA98 and WP2uvrA with and without metabolic activation and at 5,000 μg/plate in TA100 and TA1535 with and without metabolic activation and TA1537 with metabolic activation.
	Chromosome aberration test	Human peripheral blood lymphocytes	100, 200, 450 μg/mL[(b),(d)] 200, 400, 700 μg/mL[(c),(d)] 45, 100, 185 μg/mL[(b),(e)]	Positive	WIL Research, 2013i	The test article induced structural chromosomal aberrations both in the presence and absence of metabolic activation. Statistically significant increase in polyploidy cells in the 22-h treatment without metabolic activation.

(a): with and without metabolic activation.
(b): without metabolic activation.
(c): with metabolic activation.
(d): 3-h treatment.
(e): 22-h treatment.
(f): 4-h treatment.
(g): 24-h treatment.

Table E.3: Summary of *in vivo* Genotoxicity Studies on photodegradation products

Test material	Test System	Test Object	Doses	Route of exposure	Result	Reference	Comments
S7859	Micronucleus assay	Male rats bone marrow	0, 500, 1,000, 2,000 mg/kg bw per day	gavage	Negative	BioReliance, 2013e	No evidence of bone marrow cytotoxicity
	Comet assay	Male rats liver and duodenum	0, 500, 1,000, 2,000 mg/kg bw per day	gavage	Negative		No evidence of liver toxicity
S4462	Micronucleus assay	Male rats bone marrow	0, 500, 1,000, 2,000 mg/kg bw per day	gavage	Negative	BioReliance, 2013d	No evidence of bone marrow cytotoxicity
	Comet assay	Male rats liver and duodenum	0, 500, 1,000, 2,000 mg/kg bw per day	gavage	Negative		No evidence of liver toxicity

Appendix F – Toxicity

F.1. 28-day dose-range finding toxicity study in rats

A 28-day dose-range finding toxicity study with dietary administration of the candidate substance [FL-no: 16.129] was performed in CD® [Crl:CD®(SD)] rats (MPI Research, 2012).

Three treatment groups of rats (eight animals/ sex per group) were administered a diet with the candidate substance [FL-no: 16.129] to provide doses of 10, 30 and 100 mg/kg bw per day. One additional group of eight animals/sex served as the control and received the vehicle diet. The vehicle or diet with the candidate substance was available *ad libitum* for 28 consecutive days. Fresh diet formulations were prepared weekly and stored at room temperature. The study was conducted in compliance with the United States Food and Drug Administration Toxicological Principles for the Safety of Food Ingredients (US FDA, 2000). Observations for morbidity, mortality, injury and the availability of food and water were conducted twice daily for all animals. Observations for clinical signs were conducted pretest and weekly. Body weights were measured and recorded on days -1, 1, 4, 5, 11, 14, 21 and 28. Food consumption was measured and recorded on days 4, 7, 11, 14, 21 and 28, and compound consumption was calculated. Ophthalmoscopic examinations were conducted pretest and prior to the terminal necropsy. Blood and urine samples for evaluation of clinical chemistry were collected from all animals prior to the terminal necropsy. At study termination, necropsy examinations were performed. Only the liver from animals dosed at 0 and 100 mg/kg bw per day was microscopically examined.

Average daily compound consumption for animals given 10 mg/kg bw per day was 8.9 and 9.4 mg/kg bw per day; for 30 mg/kg bw per day, 26.0 and 28.2 mg/kg bw per day; for 100 mg/kg bw per day, 87.1 and 93.2 mg/kg bw per day, for males and females, respectively. Formulation analysis showed that the formulation technique used produced homogeneous preparations (relative standard deviation (RSD) < 20%). Mean food consumption in males across all treated groups was lower beginning at week 1 (5–8% lower) when compared to controls. The lower means coincide with lower body weight means across all treatment groups when compared to controls. There were no test article-related effects among hematology or urine parameters, or clinical chemistry analytes in either sex at termination. There were no macroscopic or microscopic findings or changes in organ weights. As a result, the no-observed-adverse-effect-level (NOAEL) following 28 days of dietary administration of [FL-no: 16.129] was 100 mg/kg bw per day, the highest dose level tested.

F.2. 90-day feeding administration systemic toxicity study in rats

A 90-day feeding study was carried out in CD® [Crl:CD®(SD)] rats with the candidate substance [FL-no: 16.129] (MPI Research, 2013).

The study was conducted in accordance with the United States Food and Drug Administration (FDA) Good Laboratory Practice (GLP)[8] Regulations and in compliance with the United States FDA Toxicological Principles for the Safety of Food Ingredients (US FDA, 2000). The candidate substance [FL-no: 16.129] was administered in the diet to rats (20 animals per sex per group) at dose levels of 0 (control), 10, 30 and 100 mg/kg bw per day. Additionally, one control group of three animals/sex and three treated groups of six animals/sex per group were used for toxicokinetic investigation and received the vehicle or [FL-no: 16.129] in the diet in the same manner as the main study groups at respective dose levels of 0 (control), 10, 30 and 100 mg/kg bw per day (see Section 3.10). The feed with the candidate substance was prepared separately for each sex once weekly at nominal concentrations based on the estimated mean body weight and food consumption per sex per group. The vehicle or test article diet was available *ad libitum* for 13 weeks. Average daily compound consumption for animals in the 10 mg/kg bw per day group was 10.2 and 10.4 mg/kg bw per day; for the 30 mg/kg bw per day group 30.7 and 30.8 mg/kg bw per day; for the 100 mg/kg bw per day group 102.5 and 103.9 mg/kg bw per day, for males and females, respectively. Formulation analysis demonstrated that the formulation preparation method produced homogeneous preparations (RSD < 20%).

Observations for morbidity, mortality, injury and the availability of food and water were conducted twice daily for all animals. Clinical observations were conducted once daily for main study animals. A detailed neurobehavioral examination was performed pretest and weekly during the study. Functional Observation Battery (FOB) and Motor Activity (MA) evaluations were conducted pretest and again at

[8] United States Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations, 21 CFR Part 58.

the same time of day during the 13th week for all animals. Body weights were measured and recorded on Days -1 and then weekly throughout the study. Food consumption was measured and recorded weekly. Ophthalmoscopic examinations were conducted pretest on all animals and prior to scheduled necropsy for main study animals. Haematology and clinical chemistry were evaluated on Days 14 and 45 and again prior to termination. Urine and blood samples for coagulation evaluations were collected prior to termination only. At study termination, necropsy examinations were performed and organ weights were recorded for all animals, and appropriate organ weight ratios were calculated (relative to body and brain weights). Microscopic examination of fixed haematoxylin and eosin-stained paraffin sections were performed on sections of all investigated tissues from the control and high-dose (100 mg/kg bw per day) groups.

There were no test article-related deaths. Mortality was limited to a single control male on Day 36 and a low dose male on Day 65. All other animals survived to their scheduled termination. There were no effects among haematology parameters, coagulation times or clinical chemistry analytes in either gender at any dose level. There were no alterations observed among urinalysis parameters. No changes in organ weight, macroscopic or microscopic were observed at any dose level. A neoplasm (malignant schwannoma) was observed in the brain of one male at 100 mg/kg bw per day which correlated with swollen/thickened meninges noted macroscopically. Another neoplasm (malignant adenocarcinoma) was noted in the mammary gland of one female at 30 mg/kg bw per day. Both neoplasms were considered incidental/spontaneous, as there were no related preneoplastic lesions or similar neoplasms present in any other animals, and both of these neoplasms have been observed in control Sprague–Dawley rats from previous studies conducted at MPI Research. There were no test article-related effects on FOB or MA parameters in males or females administered any dietary concentration of the test article. Sporadic variations or statistically significant changes were noted prior to test article administration or at week 13. However, due to the limited occurrence, lack of dose dependency and/or lack of other corresponding findings, these observations were considered incidental and not exposure- related.

The NOAEL following 13 weeks of dietary administration was 100 mg/kg bw per day, the highest dose level tested, in male and female rats.

F.3. Dose range-finding study for a developmental toxicity test in rats

The objective of the study (WIL Research, 2013a) was to determine dosage levels of [FL-no: 16.129] to be used in a definitive developmental toxicity study conducted in accordance with the OECD test guideline 414 (OECD, 2001) and the United States FDA Redbook 2000.[9] The test article [FL-no: 16.129] in the vehicle (1% methyl cellulose, 400 cps) was administered orally by gavage to four groups of eight bred female Crl:CD®(SD) rats once daily from gestation Days 6 through 20, with dosage levels of 125, 250, 500 and 1,000 mg/kg bw per day. A concurrent control group composed of eight bred females received the vehicle on the same regimen. All animals were observed for mortality, moribundity, clinical observations, body weights and food consumption. On gestation Day 21, a laparohysterectomy was performed on each female. The uteri, placentae and ovaries were examined, and the numbers of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The fetuses were weighed, sexed and examined for external malformations and developmental variations.

All females survived to the scheduled necropsy on gestation Day 21. No remarkable clinical findings were observed, and mean body weights, body weight gains, net body weights, net body weight gains, gravid uterine weights and food consumption in the 125, 250, 500 and 1,000 mg/kg bw per day groups were unaffected by test article administration. No macroscopic findings were noted at the scheduled necropsy on gestation Day 21. Intrauterine growth and survival were unaffected by test article administration at all dosage levels. In addition, there were no external malformations or developmental variations observed at 125, 250, 500 and 1,000 mg/kg bw per day. Based on the lack of toxicity noted at all dose levels, doses of 250, 500 and 1,000 mg/kg bw per day were selected for a definitive embryo/fetal development study on [FL-no: 16.129] administered orally by gavage to bred Crl:CD®(SD) rats.

[9] United States FDA Redbook 2000: IV.C.9.b Guidelines for Developmental Toxicity Studies, January 2001.

F.4. Developmental Toxicity (Prenatal) Study in Rats

A developmental toxicity study was performed with maternal exposure from implantation to one day prior to expected parturition to study maternal and developmental toxicity (WIL Research, 2013b). The study was conducted in accordance with the OECD test guideline 414 (OECD, 2001) and the United States FDA Redbook (US FDA, 2000) Guidelines for Reproduction and Development Studies.[10]

The candidate substance [FL-no: 16.129] was administered orally by gavage in 1% methyl cellulose to Crl:CD®(SD) rats (25 bred females per group) once daily from gestation days 6 through 20, at dosage levels 250, 500 and 1,000 mg/kg bw per day. A concurrent control group composed of 25 bred females received the vehicle on a comparable regimen. All animals were observed for mortality, moribundity, clinical observations, body weights and food consumption. On gestation Day 21, a laparohysterectomy was performed on each female. The uteri, placentae and ovaries were examined, and the numbers of fetuses, early and late resorptions, total implantations and corpora lutea were recorded. Gravid uterine weights were recorded, and net body weights and net body weight changes were calculated. The fetuses were weighed, sexed and examined for external, visceral and skeletal malformations and developmental variations.

All females survived to the scheduled necropsy on gestation Day 21. No test article-related clinical or macroscopic findings were noted at any dosage level. Mean maternal body weights, body weight gains, gravid uterine weights and food consumption were unaffected by administration of the candidate substance at all dose levels. With the exception of one female each in the 250 and 1,000 mg/kg bw per day groups, all females were determined to be gravid. No treatment-related findings were noted on intrauterine growth, survival and fetal morphology at any dose level. Parameters evaluated included post-implantation loss, live litter size, mean fetal body weights and fetal sex ratios. Mean numbers of corpora lutea and implantation sites and the mean litter proportions of preimplantation loss were similar across all groups. Differences from the control group were slight and not statistically significant. The numbers of fetuses (litters) available for morphological evaluation were 360(25), 343(24), 353(25) and 345(24) in the control, 250, 500 and 1,000 mg/kg bw per day groups, respectively. There were no test article-related external malformations noted for fetuses at any dosage level. Only a single malformation of conjoined twinning was noted at 1,000 mg/kg bw per day and was considered spontaneous in origin. There were no test article-related soft tissue or skeletal malformations noted for fetuses at any dose level. Skeletal developmental variations that were noted in all groups, consisted of 14th rudimentary rib(s), vertebral centra not fully ossified, 7th cervical rib(s) and reduced ossification of the 13th rib(s). These findings were noted similarly in the concurrent control group and/or the mean litter proportions were not statistically significantly different from the concurrent control group and were within the historical control data ranges of the laboratory. Based on the lack of adverse maternal toxicity or effects on intrauterine growth, survival and fetal morphology at any dosage level, 1,000 mg/kg bw per day (the highest dosage level evaluated) was considered to be the NOAEL for maternal toxicity and embryo/fetal development when [FL-no: 16.129] was administered orally by gavage to bred Crl:CD®(SD) rats.

The toxicity studies are summarised in Table F.1.

Table F.1: Summary of Toxicity Studies

Test material	Species; Sex No/group	Route of administration	Dose level mg/kg bw per day	Duration days	Result mg/kg bw per day	Reference	Comments
[FL-no: 16.129]	Crl:CD®(SD) rats; M + F 8/4	Diet	0, 10, 30 and 100	28	87.1 M 93.2 F	MPI Research, 2012	Performed in accordance with United States Food and Drug Administration Toxicological Principles for the Safety of Food Ingredients (US FDA, 2000)
	Crl:CD®(SD) rats; M + F 20/4	Diet	0, 10, 30 and 100	90	102.5 M 103.9 F	MPI Research, 2013	Performed in accordance with United States Food and Drug Administration Toxicological Principles for the Safety of Food Ingredients (US FDA, 2000) and FDA Good Laboratory Practice (GLP) Regulations, 21 CFR Part 58
	Crl:CD®(SD) rats; F 25/4	Gavage	0, 250, 500 and 1,000	Gestation days 6 through 20	Maternal 1,000 Fetal 1,000	WIL Research, 2013b	Performed in accordance with OECD TG 414 (OECD, 2001) and United States Food and Drug Administration Toxicological Principles for the Safety of Food Ingredients (US FDA, 2000)

M: Male; F: Female.