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Scientific Opinion of Flavouring Group Evaluation 411 (FGE.411): 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide from chemical group 30 (miscellaneous substances)

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Abstract

EFSA was requested to deliver a scientific opinion on the implications for human health of the flavouring substance 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide [FL-no: 16.133], in the Flavouring Group Evaluation 411 (FGE.411), according to Regulation (EC) No 1331/2008 of the European Parliament and of the Council. The substance has not been reported to occur in natural source materials of botanical or animal origin. It is intended to be used as a flavouring substance in specific categories of food but not intended to be used in beverages, except for milk and dairy based beverages that are opaque. The chronic dietary exposure to the substance estimated using the added portions exposure technique (APET), is calculated to be 225 μg/person per day for a 60-kg adult and 142 μg/person per day for a 15-kg 3-year-old child. A 90-day oral gavage study in rats showed no adverse effects at doses up to 100 mg/kg body weight (bw) per day, providing an adequate margin of safety. Developmental toxicity was not observed in a study with rats at the dose levels up to 1,000 mg/kg bw per day. The Panel concluded that there is no safety concern for [FL-no: 16.133], when used as a flavouring substance at the estimated level of dietary exposure calculated using the APET approach and based on the recommended uses and use levels as specified in Appendix B. This conclusion does not apply for use in beverages where the substance can be subject to phototransformation.

Keywords: flavouring, 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide, FGE.411, [FL-no: 16.133]

Requestor: European Commission

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1. Introduction

The present scientific opinion on Flavouring Group Evaluation 411 (FGE.411) covers the safety assessment of the flavouring substance 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide [FL-no: 16.133], with cooling sensation properties.

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

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 the 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.

An application for authorisation as a new flavouring substance of the substance: 2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide has been submitted to the Commission.

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 the safety assessment of this substance as a new flavouring substance.

1.1.1. Terms of Reference

The European Commission requests the European Food Safety Authority to carry out a safety assessment on: 2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide as a new flavouring substance in accordance with Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

2. Data and methodologies

The present evaluation is based on data on 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide [FL-no: 16.133] provided by the applicant in a dossier submitted in support of its application for authorisation as a new flavouring substance (Senomyx, 2015). Additional information was sought from the applicant during the risk assessment process (Senomyx, 2017, 2018). Use and use levels for a wide range of food categories were provided by the applicant (see Appendix B). Based on these use levels, the Panel calculated the intakes in adults and children that were subsequently used in the assessment.

The safety assessment of 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide [FL-no: 16.133] is carried out by EFSA 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 flavouring substances is given in Appendix A.

3. Assessment

3.1. Identity of the substance

The candidate substance has been allocated the FLAVIS number [FL-no: 16.133]. The IUPAC name of the flavouring substance is 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide and its CAS name is 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(2-thienylmethyl)acetamide.

3.2. Organoleptic characteristics

The candidate substance provides a cooling sensation.

[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 L354, 31.12.2008, p. 1–6.

3.3. Existing authorisations and evaluations

2-(4-Methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide [FL-no: 16.133] has the status 'Generally Regarded As Safe' (GRAS) by the Flavour and Extract Manufactures Associations (FEMAs) expert Panel (FEMA no 4809). It has been recently evaluated by the Joint FAO/WHO Expert Committee on Food Additives to be of no safety concern based on the current estimated dietary exposure (JECFA, 2018).

3.4. Technical data

The specifications of the flavouring substance are reported in Table 1. They refer to the commercial quality of [FL-no: 16.133] for which no impurities were detected when the material was analysed by high-performance liquid chromatography (HPLC) (Senomyx, 2015).

3.4.1. Information on the configuration of the flavouring substance

The substance may occur in two tautomeric forms depending on the position of the hydrogen atom on the pyrazole ring. The CAS number covers both tautomers. The candidate substance [FL-no: 16.133] does not possess chiral centres and does not have geometrical or optical isomers (Senomyx, 2015).

3.4.2. Manufacturing process

2-(4-Methylphenoxy)-*N*-1*H*-pyrazol-3-yl-*N*-(thiophen-2-ylmethyl)acetamide [FL-no: 16.133] was chemically synthesised from 1*H*-pyrazol-3-amine, thiophene-2-carbaldehyde and methyl or ethyl 2-(4-methylphenoxy)acetate. The commercial material is produced according to the reaction sequence below with purity ≥ 99% (Figure 1) (Senomyx, 2015).

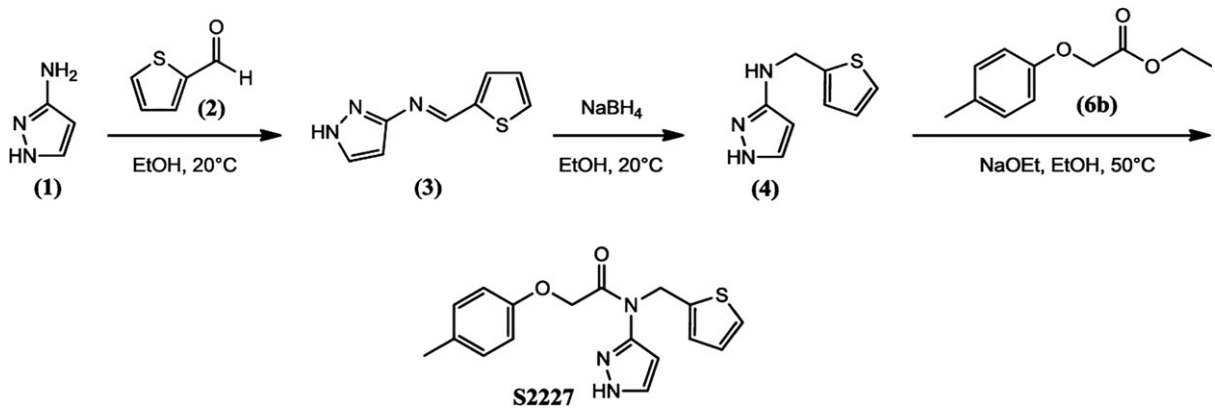
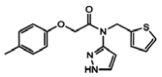


Figure 1: 'Large scale' manufacturing process of [FL-no: 16.133]. S2227 is the company code of this substance. For the 'lab scale' synthesis, the methyl ester of 6b was used

Table 1: Specifications for 2-(4-methylphenoxy)-*N*-1*H*-pyrazol-3-yl-*N*-(thiophen-2-ylmethyl)acetamide [FL-no: 16.133]

FL-no	Chemical name	Structural formula	JECFA no FEMA no CoE no CAS no EINECS no E no	Odour 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]	Specification comments
16.133	2-(4-Methylphenoxy)-*N*-1*H*-pyrazol-3-yl-*N*-(thiophen-2-ylmethyl) acetamide		2237 4809 – 1374760-95-8 – –	Solid $C_{17}H_{17}N_3O_2S$ 327.4	No impurity to exceed 0.1% peak area (UPLC-UV, 254 nm)	0.12 mM at pH = 7 > 308 mM 40.47 mM in triacetine	–[f] 115–116.5 IR, NMR, MS 99%	n.a. n.a.	Purity (UPLC-UV, 254 nm) ≥ 99% peak area

JECFA: The Joint FAO/WHO Expert Committee on Food Additives; FEMA: Flavor and Extract Manufacturers Association; CoE: Council of Europe; CAS: Chemical Abstract Service; EINECS: European Inventory of Existing Commercial Chemical Substances; UPLC-UV: ultra performance liquid chromatography coupled with ultraviolet detection; IR: infrared; NMR: nuclear magnetic resonance; MS: mass spectrometry.
(a): Solubility in water, if not otherwise stated.
(b): Solubility in 95% ethanol, if not otherwise stated.
(c): At 1,013.25 hPa (1 atm), if not otherwise stated.
(d): At 20°C, if not otherwise stated.
(e): At 25°C, if not otherwise stated.
(f): The substance decomposes at 226°C ± 2°C.

3.4.3. Stability and decomposition products

Dry powder stability

The dry powder of [FL-no: 16.133] was found to be stable when heated for 24 h at 110°C (Senomyx, 2015).

Hydrolytic stability

The percentage of [FL-no: 16.133] remaining after 24 h at 100°C in aqueous citric (pH 2.8 and 4.0) and phosphoric acid (pH 7.1) buffers was 90.1%, 97.2% and 83.5%, respectively. In all cases, the amide function of the substance is hydrolysed to the corresponding acid and amine (Senomyx, 2015) (Figure 2).

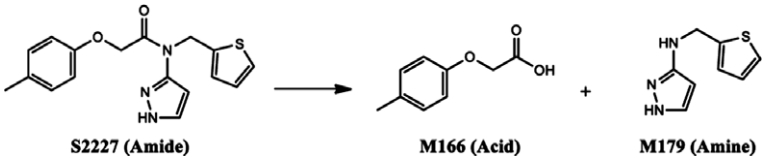


Figure 2: Hydrolysis of [FL-no: 16.133] (S2227) in aqueous media

Stability in the presence of light

The photostability of the substance was determined using a Q-Sun Xenon Test Chamber that reproduces the spectrum of natural sunlight. In citric acid buffers of [FL-no: 16.133] (pH 2.8 and 4.0) containing 30 mg/kg of ethylenediaminetetraacetic acid (EDTA), the major phototransformation product identified was the amide M357 (see Figure 3).

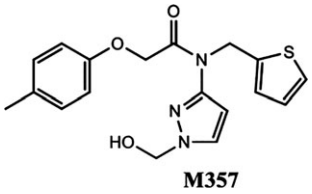


Figure 3: Phototransformation product of [FL-no: 16.133]

Interaction with food components

This was not investigated but data are available on thermostability, hydrolytic stability and phototransformation (see above). The potential formation of nitrosamines from M179 in the gastrointestinal (GI) tract and in foods is considered in Section 3.8.1.

3.4.4. Particle size[3]

The mean particle size of the compound was found approximately 6 μm (Senomyx, 2015). The substance is not to be considered as a nanomaterial, as defined by Commission Recommendation 2011/696/EU.

3.4.5. Conclusion on specifications and chemical characteristics

The Panel considered the data on the manufacturing, specifications and stability of [FL-no: 16.133] as dry powder, as sufficient. When incorporated in aqueous solutions, the amide function of the substance is hydrolysed to the corresponding acid and amine. Following tests on photostability conducted on citric acid buffers of [FL-no: 16.133] (pH 2.8 and 4.0) containing 30 mg/kg of EDTA, the major photobreakdown product identified was the amide M357.

[3] Commission Recommendation 2011/696/EU of 18 October 2011 on the definition of nano-materials. Official Journal of the European Union L257/38-40 on 20.10.2010 (http://ec.europa.eu/environment/chemicals/nanotech/pdf/commission_recommendation.pdf).

3.5. Structural/metabolic similarity to substances present in existing FGEs

No clear structural/metabolic similarity to other flavouring substances evaluated in an existing FGE was identified for [FL-no: 16.133]. Therefore, the Panel decided to assess the flavouring substance on its own (see Appendix A).

3.6. Exposure assessment

All data necessary for the calculation of exposure estimates (i.e. normal and maximum use levels as recommended by the producer for refined sub categories of foods and beverages) are reported in Appendix B.

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

The flavouring substance is not reported to occur naturally in food according to extensive searches of the literature (Senomyx, 2015).

3.6.2. Non-food sources of exposure

The substance was registered according to Article 10 of the REACH Regulation (https://echa.europa.eu/fr/registration-dossier/-/registered-dossier/22065/3/1/2). No non-food uses of the substance are reported on the ECHA website apart from a general statement informing that the substance is contained in consumer products. No further details have been provided.

3.6.3. Chronic dietary exposure

The exposure assessment to be used in the Procedure for the safety evaluation of the flavouring substance is the chronic added portions exposure technique (APET) estimate (EFSA CEF Panel, 2010). The chronic APET has been calculated for adults and children (see Table 2), and these values, expressed per kg body weight (bw), will be used in the Procedure (see Appendix B).

Table 2: APET – chronic dietary exposure

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]	3.8	225	na	na	3.8	225
Children[e]	9.5	142	na	na	9.5	142

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 Added is 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 flavouring substance as such.

3.6.4. Acute dietary exposure

The calculation was based on the maximum use levels and large portion size – i.e. three times standard portion size (see Appendix B, Table 3).

Table 3: APET – acute dietary exposure

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]	30	1,800	na	na	30	1,800
Children[e]	75.6	1,134	na	na	75.6	1,134

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 Added 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

Not applicable.

3.7. Genotoxicity

Both the flavouring substance [FL-no: 16.133] and its secondary amine hydrolysis product (M179) were tested for genotoxicity both *in vitro* and *in vivo* (Appendix C). All tests were performed according to GLP and OECD test guidelines. Although the potential phototransformation product, the amide M357, might raise concerns with respect to genotoxicity due to the presence of a structural alert for genotoxicity, the Panel considered that a request of additional studies is not required since [FL-no: 16.133] is not intended to be used in beverages (where the phototransformation product could be formed) except for milk and dairy based beverages that are opaque.

3.7.1. *In vitro* studies

The flavouring substance [FL-no: 16.133] does not contain structural alerts for genotoxicity (Benigni et al., 2008). The potential of the substance to show genotoxicity for any of the three endpoints (i.e. gene mutations, structural chromosomal aberrations and numerical chromosomal aberrations) has been explored experimentally.

3.7.1.1. Bacterial reverse mutation test on [FL-no: 16.133]

The flavouring substance (purity > 97%) was tested for its potential to induce gene mutations in *Salmonella* Typhimurium strains TA1535, TA1537, TA98 and TA100 and *Escherichia coli* strain WP2uvrA tested using the plate incorporation and preincubation methods both with and without Aroclor induced rat liver S9 (Nucro-techniques, 2013a). The concentrations tested ranged from 63 to 1,000 µg/plate. Appropriate positive controls were included for all five strains, and the assay was performed in accordance with OECD TG 471 (OECD, 1997a).

No statistically significant or biological relevant increases in revertant colonies were observed in any of these tester strains at any test conditions.

3.7.1.2. Bacterial reverse mutation test on S1140 (hydrochloride salt of the secondary amine M179)

N-(Thiophen-2-ylmethyl)-1*H*-pyrazol-3-amine, hydrochloride salt (purity > 97%) (see Figure 2), was tested in the bacterial reverse mutation test using *S.* Typhimurium strains TA98, TA100, TA1535 and TA1537 and *E. coli* strain WP2 *uvr*A (BioReliance, 2013a). The assay was performed with the plate incorporation method. Concentrations up to 5,000 µg/plate were tested in the absence and presence of metabolic activation by S9-mix. In both experiments, *N*-(thiophen-2-ylmethyl)-1*H*-pyrazol-3-amine, hydrochloride salt did not show mutagenicity in bacteria.

Overall, the hydrochloride salt of M179 did not show mutagenicity in the bacterial reverse mutation assay.

3.7.1.3. *In vitro* chromosome aberration tests

Both the flavouring substance [FL-no: 16.133] (Nucro-techniques, 2013b) and the hydrochloride salt of the amine M179 (see Figure 2) (Bioreliance, 2013b) were investigated for their potential to induce structural chromosome aberrations in mammalian cells, both in the presence and absence of metabolic activation. The experimental design followed the OECD TG 473 (OECD, 1997b, 1998).

Chromosome aberration test on the flavouring substance [FL-no: 16.133]

The flavouring substance [FL-no: 16.133] (purity > 97%) was tested in cultured human peripheral blood lymphocytes (HPBL) in a chromosome aberration test (Nucro-techniques, 2013b) at concentrations up to 270 μg/mL. It produced no statistically significant increases in the frequency of cells with aberrations in the absence and presence of metabolic activation (see Appendix C).

Chromosome aberration test on S1140 (hydrochloride salt of the amine M179)

N-(Thiophen-2-ylmethyl)-1*H*-pyrazol-3-amine, hydrochloride salt (see Figure 2) was tested in the chromosome aberration assay using HPBL, with and without metabolic activation (S9-mix). Two different batches were tested in the first and second experiments (purity > 97.0% and > 98.0%, respectively). The HPBL were tested for 4 + 16 h in the absence or in the presence of S9-mix) and for 20 + 0 h (in the absence of S9-mix) with duplicate cultures (BioReliance 2013b). Based on a range-finding study, the concentrations in the main assay ranged from 280 to 2,160 μg/mL (in the absence of S9-mix), from 100 to 1,840 μg/mL (in the presence of S9-mix) in the 4 + 16 h treatment and from 25 to 280 μg/mL (in the absence of S9-mix) for the 20 + 0 h treatment.

In the first test, statistically significant increases were observed at the highest concentration tested (2,160 μg/mL) without metabolic activation. In the second test, statistically significant and concentration-related increases in structural aberrations were observed at concentrations of 2,000 and 2,160 μg/mL. No significant or concentration-related increases in polyploidy were observed in any of the treatment conditions. Due to scorer variability, the slides were re-analysed (see Appendix C). The results of the re-analysis confirmed the positive outcome observed in the original scoring.

The Panel concluded that the hydrochloride salt of the secondary amine M179 induced chromosomal aberrations in HPBL at the short-term treatment in the absence of metabolic activation.

3.7.2. *In vivo* data

3.7.2.1. Mouse micronucleus study on the flavouring substance [FL-no: 16.133]

The result of an *in vivo* micronucleus study in mice did not show any evidence of genotoxicity (no statistically significant increases in the incidence of micronucleated polychromatic erythrocytes (MNPCEs) at dose levels of 1,000, 1,500 and 2,000 mg/kg bw, administered by gavage. There was no direct indication that the test substance did reach the bone marrow since no toxicity was observed in the bone marrow (Wil Research, 2013b). However, toxicokinetic (TK) studies (see Section 3.8) have shown exposure to the flavouring substance and its two main metabolites: the acid and the secondary amine (see Appendix C and Section 3.8). The Panel considered the results of this study as negative.

The secondary amine (M179) is considered to be of main relevance for genotoxicity, and therefore, it was tested both *in vitro* and *in vivo* for all three genetic endpoints.

3.7.2.2. Combined comet and micronucleus assay on the hemisulfate salt of the amine metabolite (M179)

The genotoxic potential of the hemisulfate salt of *N*-(thiophen-2-ylmethyl)-1*H*-pyrazol-3-amine (M179) was assessed in mice using the bone marrow micronucleus assay combined with the comet assay (BioReliance, 2014).

In a range-finder assay, doses up to 2,000 mg/kg bw per day were tested and a maximum tolerated dose (MTD) of 500 mg/kg bw per day in male mice and 1,000 mg/kg bw per day in female mice was identified. In the main study, by oral gavage, groups of five male mice were administered doses of 0% (vehicle used was 1% methylcellulose in deionised water), 125, 250 and 500 mg/kg bw per day and groups of five female mice were administered doses of 0, 250, 500 and 1,000 mg/kg bw per day for three consecutive days. The positive control, methyl methanesulfonate, was administered at a dose of 40 mg/kg bw per day on days 2 and 3.

Micronucleus assay

There was no statistically significant increase of micronuclei in bone marrow polychromatic erythrocytes (PCE) of treated mice compared to the vehicle control animals.

The Panel concluded that oral administration of the hemisulfate salt of M179 did not induce micronuclei in male and female mice. Although there was no indication that the target organ was exposed, since no toxicity was observed in the bone marrow, TK studies showed that the substance was systemically available and indicate that both the liver and the bone marrow were exposed (see Section 3.8).

Overall, the Panel concluded that the secondary amine did not induce structural or numerical aberrations in the bone marrow of mice.

Comet assay

No statistically significant increase in the mean % tail DNA was observed in liver cells from the test article dosed animals compared to the vehicle control mice. No increase in the percentage of clouds was observed at any dose level compared to control.

The Panel concluded that the secondary amine metabolite did not induce DNA damage in the liver and did not induce micronuclei in mice bone marrow. Although there was no direct indication that the target organ was exposed, TK studies showed that the substance is systemically available and indicate that both the liver and the bone marrow were exposed (see also Section 3.8). There was no increase in tail intensity in liver cells analysed through the comet assay. The Panel concluded that M179 is not genotoxic *in vivo*.

3.7.3. Conclusion on genotoxicity

The flavouring substance [FL-no: 16.133] and its amine metabolite were investigated for genotoxicity both *in vitro* and *in vivo*. All three genetic endpoints (i.e. gene mutations, numerical and structural chromosomal aberrations) were investigated.

The flavouring substance [FL-no: 16.133] did not induce gene mutations in bacteria or chromosomal aberrations in mammalian cells. An *in vivo* micronucleus assay was negative. There was no direct indication of bone marrow exposure since there was no toxicity in the bone marrow. However, TK studies showed that the substance and two of its main metabolites, the acid M166 and the amine M179, were systemically available.

The amine did not induce gene mutations in bacteria; it did induce chromosomal aberrations in mammalian cells after short term exposure without metabolic activation.

In a combined micronucleus and comet assay in mice, the amine metabolite was not genotoxic. Although there was no direct indication that the target organ was exposed, TK studies showed that the substance was systemically available and indicated that both the liver and the bone marrow were exposed.

The Panel noted that, in TK studies, the concentration of the amine in blood of mice was low after administration of the flavouring substance. However, even if the amine concentration was higher there would be no concern with respect to genotoxicity, because the amine was tested separately *in vivo* in mice and the result was negative.

The Panel noted that a phototransformation product (amide M357) might raise concerns with respect to genotoxicity due to the presence of a structural alert and that no data on genotoxicity are available in the technical dossier for this amide, except a screening bacterial reverse mutation assay (see Appendix C). Considering however, that [FL-no: 16.133] is not intended to be used in beverages (where the phototransformation product could be formed) except opaque dairy products, the Panel considered that additional studies on the above-mentioned phototransformation product M357 are not required in this case.

Based on the available data, the Panel concluded that there is no concern with respect to genotoxicity for the flavouring substance [FL-no: 16.133] under the intended conditions of use.

3.8. Absorption, distribution, metabolism and excretion

Absorption, distribution, metabolism and elimination (ADME) studies on [FL-no: 16.133] are available including *in vitro* (profiling concerning hepatic phase I metabolism in five species including human) (Senomyx, 2014a), *ex vivo* (plasma stability in six species) (Senomyx, 2014b) and *in vivo* (single dose kinetic and metabolic study in rats, dogs and mice) (Charles River, 2012; Senomyx, 2013a,b,c, 2014c,d, 2017; Karanewsky et al., 2015).

Together these studies indicate that: (1) the flavouring substance [FL-no: 16.133] (S2227) is absorbed after oral administration; (2) it is rapidly hydrolysed to the corresponding acid M166 and

secondary amine M179, both identified as the two major metabolites of the flavouring substance in all *in vivo* studied species (i.e. dog, rat and mice); (3) it generates other phase I metabolic products resulting from among others hydroxylation. The latter metabolic transformations occur in minor extend and do not involve cleavage of the amide bond of the parent compound.

Among the purposes of the TK studies performed was to investigate which of the different species would be more suitable for toxicological studies. The available data support the rat as an appropriate species for the toxicological evaluation of the candidate substance, since the metabolite picture of the *in vivo* rat study to a high extent matched the *in vitro* microsomal metabolites in humans (Senomyx, 2013a, 2014a,c). Based on the above, the subchronic and developmental toxicity studies for [FL-no: 16.133] were conducted in rats (see Sections 3.9.2 and 3.9.4).

The *in vivo* genotoxicity studies for both the parent compound and its amine metabolite however, were conducted in mice and not in rat (see Section 3.7 and Appendix C). To investigate the relevance of these studies, the Panel investigated the systemic exposure of [FL-no: 16.133] and amine M179 in mice.

After oral administration in rats and mice at a dose level of 30 mg/kg bw, [FL-no: 16.133] (S2227) was rapidly hydrolysed to the corresponding carboxylic acid (M166) and secondary amine (M179) (see Figure 4). This resulted to a low systemic exposure to the parent amide in both species. It was observed that exposure to the carboxylic acid metabolite M166 was significantly greater than either [FL-no: 16.133] or the amine M179 in both species. The levels of M179 in the mice plasma, however, were considerably lower than the ones observed in rats indicating that the amine metabolite M179, which is of main relevance for the evaluation of the genotoxic potential of [FL-no: 16.133], is less systemically available in mice than in rat (see Figure 4).

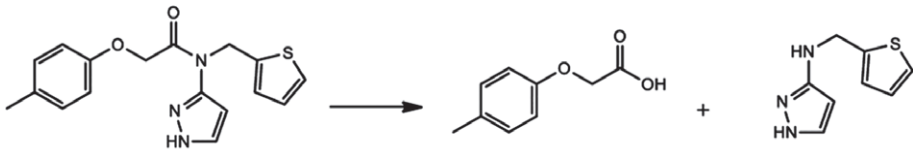
To demonstrate systemic exposure to the substance M179 in the genotoxicity studies, the oral exposure to the hydrochloric salt of the amine metabolite was studied only in the mice at a dose level of 20 mg/kg bw. The results showed that the salt is rapidly absorbed (T_{max} = 0.25 h) and eliminated ($t_{1/2}$ = 0.46 h). Additionally, much higher levels of the amine metabolite were found in plasma than the ones measured with an equimolar dose of the amide [FL-no: 16.133] in the previous study (see Figure 4) (Karanewsky et al., 2015; Senomyx, 2017).

The data demonstrate that the amine M179, which is of main relevance for the evaluation of the genotoxic potential for the flavouring substance [FL-no: 16.133] (S2227) and which did not induce genotoxicity in an *in vivo* study in mice, is systemically available also in mice.

The Panel noted that although based on Figure 4 the amine M179 would be expected to be further metabolised, no conjugation/transformation product of M179 was identified in the plasma of rats or mice.

Considering the potential sites where hydrolysis of [FL-no: 16.133] may occur, i.e. the lumen of the GI tract, the intestinal wall or the liver, potential biotransformation products (i.e. the secondary amine M179) may undergo nitrosamine formation. This possibility was investigated by the Panel (see Section 3.8.1).

The Panel concluded from the data on kinetics and biotransformation that it cannot be anticipated that the flavouring substance [FL-no: 16.133] will be metabolised to innocuous products and therefore has to be evaluated via the B-side of the Procedure.



S2227 (Amide) **M166 (Acid)** **M179 (Amine)**

Compound (dose)	Species (sex)	S2227		M166		M179	
		C_{max} (ng/mL)	AUC_{last} (ng × hr/mL)	C_{max} (ng/mL)	AUC_{last} (ng × hr/mL)	C_{max} (ng/mL)	AUC_{last} (ng × hr/mL)
S2227 (30 mg/kg bw)	Rat (M)	6.22	6.22	4,843	14,845	290	614
	Rat (F)	33.1	29.1	9,732	25,920	1,538	2,511
	Mouse (M)	125	128	11,290	61,730	27.8	27.6
M179 × HCl (20 mg/kg bw)	Mouse (M)	-	-	-	-	9,780	4,370

Figure 4: Pharmacokinetics of [FL-no: 16.133] (S2227) and hydrolysis products after oral exposure in rats and mice

3.8.1. Endogenously produced *N*-nitrosamines

The Panel noted that nitrosamines can be formed endogenously in the GI tract by reaction of the secondary amine metabolite M179 with nitrites (the actual reactant is N_2O_3) present in the stomach and/or by action at the level of the intestinal flora. Considering that this endogenously formed nitrosamine might be carcinogenic to humans, the Panel estimated the maximum expected quantity of nitrosamine in the stomach based on the following conservative scenario (see also EFSA AFC Panel, 2008):

- The flavouring substance is completely hydrolysed to the amine (M179) in the stomach, where it can react with the endogenously present N_2O_3 to form the corresponding nitrosamine. Note that for the formation of N_2O_3 two nitrite ions are necessary (Mirvish, 1975).
- The rate of nitrosation depends on the concentration of the unprotonated amine which is governed by the pH and its pKa, decreasing with the increase of the amine's basicity. Based on data by SciFinder, the predicted pKa_{M179} for amine M179 is pKa_{M179} = 14.83 and is the one used in the calculations.
- Considering the acute exposure estimate (acute APET) of 1,800 μg/person per day for the parent compound [FL-no: 16.133], its total hydrolysis to the secondary amine M179 will result in an amine intake of 984 μg/person per day.
- The nitrosation of the M179 follows reaction kinetics similar to those of dimethylamine (DMA). For the stomach pH of 3.4, the rate constant of DMA is $k_{DMA} = 0.0017 M^{-2}s^{-1}$ (Mirvish, 1975). The reaction rate constant for M179 is estimated from this figure via a correction based on the difference between pKa of DMA and the pKa of M179.
- The gastric juice volume assumed is 0.3 L.
- The assumed nitrite concentration in the stomach juice [nitrite] = 3.7 x 10^{-5}M (see Minutes of the 30th meeting of the AFC Panel, 2008).
- The assumed reaction time of the nitrosation reaction is 2 h (or 7,200 s).

Based on the above assumptions, the maximum amount of nitrosamine formed in the stomach was calculated as follows:

- Estimation of the rate constant of M179. The pKa of M 179 is 14.8 (Scifinder predicted value), while that of DMA is 10.7. This means that at pH 3.4 the concentration of the unprotonated form of DMA is approx. 10^4-fold higher than that for M179. The rate constant of M179 can therefore be estimated at $k_{M179} = 0.0017 \times 10^{-4}\ M^{-2}s^{-1}$.
- Estimation of the concentration of the amine [M179], produced by hydrolysis of 1,800 μg [FL-no: 16.133]/person per day to 984 μg M179/person per day in 0.3 L of gastric volume:
  $[M179] = (3.3 \times 984 \times 10^{-6})/179$ or
  $[M179] = 1.8 \times 10^{-5}$ M.
- Estimation of the rate of nitrosamine formation, R_{R2NNO} (M/s):
  $R_{R2NNO} = k_{M179} \times [M179] \times [nitrite]^2$ or $R_{R2NNO} = 0.0017 \times 10^{-4} \times 1.8 \times 10^{-5} \times (3.7 \times 10^{-5})^2$ or $R_{R2NNO} = 4.2 \times 10^{-21}\ M\ s^{-1}$.
- Estimation of the concentration of the nitrosamine formed in the gastric juice in 2 h (7,200 s):
  $[R_2NNO] = R_{R2NNO} \times 7{,}200$ or $[R_2NNO] = 4.2 \times 10^{-21} \times 7{,}200$ or
  $[R_2NNO] = 3.0 \times 10^{-17}$ M, corresponding to 9.1×10^{-18} mol R_2NNO in 0.3 L of gastric juice.
- Estimation of the maximum amount of nitrosamine (MW_{R2NNO} = 208) formed in the stomach: 1.9×10^{-15} μg per person per day or 0.03×10^{-15} μg per kg bw per day, which is far below the TTC of 0.0025 μg per kg bw per day for substances that are anticipated to be genotoxic carcinogens.

In addition, assuming that the nitrosamine formed has a carcinogenic potential equal to that of dimethylnitrosamine ($BMDL_{10\ DMNA}$ = 27 μg/kg bw per day; EC SCCS, 2012, EFSA ANS Panel, 2017) a margin of exposure (MoE) of 0.9×10^{12} can be estimated for the nitrosamine produced by M179 in the stomach, which is higher than the MoE of 10,000 set by the EFSA Scientific Committee on genotoxic carcinogens (EFSA Scientific Committee, 2012).

Some of the default values used in this estimation vary from the ones used in the ANS Panel opinion (EFSA ANS Panel, 2017). Nitrosation reaction time was set to 7,200 s instead of 3,600 s and stomach volume was set to 0.3 L instead of 0.5 L. The values were chosen in order to be aligned with the ones used in previous flavouring opinions where similar calculations took place (EFSA CEF Panel, 2015) and the use of the values used in the ANS Panel opinion would not change the results to a notable extent.

The Panel noted that additional nitrosamine formation might occur in the heterocyclic ring of M179 and that the candidate substance might also be endogenously converted to its corresponding nitrosamide. However, considering that in the case of the amide the electron withdrawing carbonyl group attached to the nitrogen decreases the reactivity toward common nitrosating agents (EC SCCS, 2012) the likelihood of formation of nitrosamines and nitroamides is very low.

Based on the above and the high MoE estimated for the potential nitrosamine of M179, the Panel concluded that no safety concern would be expected from possible formation of nitroso compounds in the GI tract at the estimated levels of acute exposure to the flavouring substance.

The possibility of formation of nitrosamines in the food matrix was also considered weak since the substance is not intended to be used in meat or vegetables where a high concentration of nitrate (that could be converted into nitrite) is expected.

3.9. Toxicity data

3.9.1. Acute toxicity

No acute toxicity studies were performed (Senomyx, 2015).

3.9.2. Short-term and subchronic toxicity

28-day dose-range finding toxicity study of the candidate substance in rat

A non-GLP 28-day study evaluated the potential toxicity of the candidate substance. The candidate substance was administered in the diet of four groups of eight male and eight female CD®[Crl:CD®(SD)] rats at intended dose levels of 0 (control), 10, 30 and 100 mg/kg bw per day. Based on a no-observed-adverse-effect-level (NOAEL) of 100 mg/kg bw per day, the doses for the 90-day study were selected (Charles River, 2013).

90-day oral toxicity study of the candidate substance in rats

In a 90-day study conducted in compliance with the United States Food and Drug Administration Toxicological Principles for the Safety of Food Ingredients which is comparable to OECD Guideline 408, the candidate substance was administered as a suspension in 1% MC by oral gavage to four groups of 20 male and 20 female CD®[Crl:CD®(SD)] rats at intended dose levels of 0 (control), 10, 30, and 100 mg/kg bw per day. There were no test article-related effects noted for any parameter examined. There were no macroscopic or microscopic findings or toxicologically significant organ weight changes noted at any dose level. As a result, 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 (Charles River, 2014). The Panel agreed with this NOAEL. The study has also been published by Karanewsky et al., 2015.

3.9.3. Chronic toxicity and carcinogenicity

The highest exposure estimates are less than the EFSA decision tree threshold of 900 μg/person per day; therefore, no study longer that 13 weeks has been performed, which is in compliance with the EFSA guidance (EFSA CEF Panel, 2010).

3.9.4. Reproductive and developmental toxicity study in rats

A dose-range study for the developmental toxicity has been performed in rats with dose levels 125, 250, 500 and 1,000 mg/kg bw per day. Based on the results of this study, the dosage levels of 125, 300 and 1,000 mg/kg bw per day were selected for a definitive embryo/fetal development study of the candidate substance administered orally by gavage to inbred Crl:CD(SD) rats (WIL Research, 2013b).

A developmental toxicity study was conducted in accordance with the OECD Test Guideline 414 (OECD, 2001),. The candidate substance was administered by oral gavage in female rats at intended dose levels of 0 (control), 125, 300 and 1,000 mg/kg bw per day.

In absence of any signs of maternal toxicity and embryo or fetal development effects, the dose level of 1,000 mg/kg bw per day is the NOAEL from this study (WIL Research, 2015). The Panel agreed with this NOAEL. The study has also been published by Karanewsky et al., 2015.

3.9.5. Other studies

Skin sensitisation potential

In order to assess the potential of the candidate substance to act as a skin sensitiser in certain product applications (e.g. edible ice), [FL-no: 16.133] was evaluated in two series of *in vitro* assays, namely the direct peptide reactivity assay (DPRA) (CiToxLab, 2014) and the KeratinoSens Assay (IIVS, 2013), followed by an investigation in humans using the human repeat insult patch test (HRIPT) in a study enrolling 120 subjects of which 112 completed the test (Harrison Research Laboratories, 2014a,b). The Panel noted that even though the results of the two *in vitro* tests indicate that the substance may exhibit a skin sensitisation potential, the negative result seen in the human test rules out the concern with respect to skin sensitisation.

***In vitro* receptor and cytochrome P450 interaction profiling**

In vitro tests were done to provide an early indication of whether the candidate compound interacts with P450 enzymes that might cause adverse or unexpected effects or affect drug metabolism. Also, *in vitro* studies were done to investigate possible interaction of the substance with several drug receptors.

In vitro screening for the potential interaction of the candidate substance with drug receptors, included 67 receptor binding assays for G-protein coupled receptors (GPCRs), ion channels, nuclear receptors and transporters. No significant responses ($\geq$ 50% inhibition or stimulation) in the lead profiling receptor screen are reported (Ricerca, 2012).

In an *in vitro* hERG electrophysiology (patch clamp) assay, [FL-no: 16.133] did not significantly inhibit the hERG ion channel current ($<$ 10%) at a concentration of 10 μM (AVIVA, 2012).

The candidate substance tested at a concentration of 10 μM ($\sim$ 3.3 mg/L) on a panel of CYP enzymes utilising pooled human liver microsomes and CYP-specific substrates demonstrated significant inhibition of CYP2C19 and CYP3A4 (midazolam substrate) (Absorption Systems, 2013a). In another study, [FL-no: 16.133] was evaluated for time-dependent inhibition of CYP2C19 and CYP3A4 using human liver microsomes and CYP-specific substrates (Absorption Systems, 2013b). The IC_{50}s of [FL-no: 16.133] on CYP2C19 using *S*-mephenytoin as substrate and CYP3A4 using midazolam as a substrate were found 1.3 and 15 μM, respectively. No evidence of time-dependent CYP inhibition was seen on CYP2C19. For CYP3A4, there was indication of time-dependent inhibition as a significant increase in inhibition was observed when the microsomes were pre-incubated with the substance in the presence of NADPH.

Using the method of Schmitt (2008) to estimate blood-tissue partitioning coefficients, estimation of liver concentrations following acute exposure to the flavouring substance in humans, based on the use levels provided, gives a maximum liver concentration, which is lower than the lowest IC_{50} determined *in vitro* for any of the CYP enzymes or receptors examined.

Phototoxicity

Balb/c 3T3 cells were exposed to [FL-no: 16.133] in concentrations up to 100 μg/mL in the presence and absence of UVA irradiation (5 J/cm^2) (CeeTox, 2013). The test follows the INVITTOX 3T3 NRU Phototoxicity test guideline (EURL ECVAM DB-ALM (INVITTOX) Protocol no 78, updated Dec 2008). The cell viability dropped below 68% viability, both in the presence and absence of UVA.

Based on the results from the study, the Panel does not anticipate that [FL-no: 16.133] has a phototoxic potential.

3.10. Exposure compared with TTC

By comparison of the APET exposure estimate with the threshold of toxicological concern (TTC, or in short threshold of concern) and TTC $\times$ 10 (see Table 4), it follows from the Procedure (see Appendix A) that for the evaluation of the candidate flavouring substance the results of a 90-day oral toxicity study and a developmental toxicity study are necessary. These studies have been submitted by the applicant.

Table 4: Summary table on calculated chronic APET and threshold of concern

Consumer	Structural class	Add APET[a]	Threshold of concern	Threshold of concern x 10
		µg/person per day	µg/person per day	
Adult[b]	III	225	90	900
Child[c]		142		

APET: added portions exposure technique.
(a): The APET figure to be used in the Procedure is based on the chronic dietary 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. Procedure for the safety assessment

The Procedure is schematically represented in Appendix A.

The candidate substance 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide [FL-no: 16.133] is not considered to be of concern with respect to genotoxicity.

3.11.1. Safety assessment for acute exposure

Estimates of maximum acute dietary exposure indicate that this would be about 0.08 mg/kg bw for a 3-year-old child. The doses of 100 mg/kg bw per day (NOAEL) are well tolerated in rats without adverse effects. From these data, a MoE of more than 1,000 for children can be calculated, which indicates no concern for acute effects, taking into account that the NOAEL was derived from a 90-day toxicity study. In a developmental toxicity study with an exposure period of 14 days, no overt maternal toxicity was observed at a dose levels up to 1,000 mg/kg bw per day, which supports this conclusion.

3.11.2. Safety assessment for long-term exposure

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: 16.133], according to the EFSA Guidance (EFSA CEF Panel, 2010) see Appendix A.

Procedure steps

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

The candidate substance 2-(4-methylphenoxy)-*N*-1*H*-pyrazol-3-yl-*N*-(thiophen-2-ylmethyl)acetamide [FL-no: 16.133] is not considered to be of concern with respect to genotoxicity (see Section 3.7).

Step 1

On the basis of the chemical structure, the candidate substance [FL-no: 16.133] is classified in structure class III (Cramer et al., 1978). The threshold of toxicological concern for a structural class III substance is 90 µg/person per day.

Step 2

Are there data available to demonstrate that the metabolites can be considered innocuous?

It cannot be concluded that the candidate substance or the metabolites are innocuous and the candidate substance therefore proceeds via the B-side of the Procedure.

Step B3

Does the dietary exposure exceed the respective Cramer class threshold?

The chronic exposure (based on APET) is 225 µg/person per day for adults and 142 µg/person per day for children (see Section 3.6.1), and therefore exceeds the Cramer Class III Threshold of 90 µg/person per day. Therefore, the safety evaluation proceeds to step B4.

Step B4

Does the dietary exposure exceed the respective Cramer class threshold x 10?

The Cramer class threshold x 10 is 900 μg/person per day and the chronic dietary exposure is 225 μg/person per day for adults and 142 μg/person per day for children (see Section 3.6.1).

Following the Procedure (see Appendix A), a 90-day study and a developmental study is required to finalise the safety evaluation of the candidate substance.

Both a 90-day feeding study in rats and a developmental study in female rats have been performed with the candidate substance and these studies provide NOAELs of 100 and 1,000 mg/kg bw per day, respectively.

3.12. Margins of safety

The NOAEL of the 90-day oral toxicity study (100 mg/kg bw per day), the highest dose tested, was considered in the risk assessment of the flavouring substance (see Section 3.9). For acute exposure, a margin of safety of 1,300 for children could be derived based on the APET of 0.076 mg/kg bw per day for a 3-year-old child, which is considered sufficient. Based on the chronic exposure of 9.5 μg/kg bw per day (APET) and the NOAEL of 100 mg/kg bw per day, a lowest margin of safety of 10,500 could be derived for long-term exposure in children (Table D.1).

Table 5: Summary table of calculated margins of safety

	Study type	NOAEL mg/kg bw per day	Add APET μg/kg bw per day (mg/kg bw per day)	Margin of safety
Adult	A 90-day feeding study in rats on the candidate substance [FL-no: 16.133]	100	3.8 (0.0038)	26,300
Child			9.5 (0.0095)	10,500
Adult	Developmental toxicity study in the rat	1,000	3.8 (0.0038)	263,000
Child			9.5 (0.0095)	105,000

NOAEL: no observed adverse effect level; bw: body weight; APET: added portions exposure technique.

A developmental study is also available providing a NOAEL of 1,000 mg/kg bw per day (highest dose tested) for maternal toxicity and embryo/fetal development. Based on the Procedure, the Panel concluded that there is no safety concern for the use of [FL-no: 16.133] 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 B.

Discussion

Following a request from the European Commission, EFSA was asked to deliver a scientific opinion on the implications for human health of a chemically defined flavouring substance used in or on foodstuffs in the Member States. In particular, EFSA was requested to evaluate 2-(4-methylphenoxy)-*N*-(1*H*-pyrazol-3-yl)-*N*-(thiophen-2-ylmethyl)acetamide [FL-no: 16.133], allocated in the Flavouring Group Evaluation 411 (FGE. 411), using the procedure as referred to in Regulation (EC) No 1334/2008 of the European Parliament and of the Council.

The candidate substance has no structural similarity to flavouring substances in the existing chemical groups. Consequently, the Panel decided to assess this substance on its own.

2-(4-Methylphenoxy)-*N*-1H-pyrazol-3-yl-*N*-(thiophen-2-ylmethyl)acetamide [FL-no: 16.133] is chemically synthesised and has not been reported to occur in natural materials of botanic or animal origin.

Specifications

Specifications including complete purity criteria and identity for the material of commerce have been provided and considered adequate. The candidate substance does not possess chiral centres and does not have geometrical isomers. It may occur in two tautomeric forms, both assigned the same CAS number. The information provided on the manufacturing process, the composition and the stability of the flavouring substance was considered sufficient.

Use and exposure

2-(4-Methylphenoxy)-*N*-1*H*-pyrazol-3-yl-*N*-(thiophen-2-ylmethyl)acetamide [FL-no: 16.133] is intended to be used as a flavouring substance in specific food categories.

The chronic dietary exposure to the candidate substance has been estimated using the APET. It is calculated to be 225 μg/person per day (3.8 μg/kg bw per day) for a 60-kg adult and 142 μg/person (9.5 μg/kg bw) per day for a 15-kg 3-year-old child.

The highest acute intake of the candidate substance is calculated to be 1.8 mg/person per day (or 30 μg/kg bw per day) for a 60-kg adult and 1.13 mg/person per day (or 75.6 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 on [FL-no: 16.133] are available including *in vitro* (profiling concerning hepatic phase I metabolism in five species including human), *ex vivo* (plasma stability in six species) and *in vivo* (single dose kinetic and metabolic study in rats, dogs and mice). Together these studies indicate that: (1) the flavouring substance is absorbed after oral administration and (2) the compound is rapidly eliminated from the blood due to (a) degradation in the blood and (b) hepatic metabolism leading to the two products of hydrolysis of the amide bond as supplemented by some other phase I metabolic products resulting from among others hydroxylation.

It cannot be concluded that the candidate substance or the metabolites are innocuous.

The Panel considered the possibility that the hydrolysis breakdown product M179 might give rise to the formation of nitrosamines however, the estimated formation of this nitrosamine applying a conservative scenario is negligible.

Genotoxicity

Based on the available data, the Panel concluded that there is no concern with respect to genotoxicity for the flavouring substance [FL-no: 16.133] under the intended conditions of use.

However, the Panel noted that a phototransformation product (amide M357) might raise concerns with respect to genotoxicity, due to the presence of a structural alert and that no data on genotoxicity are available in the technical dossier for this amide, except a screening bacterial reverse mutation assay. Considering however, that [FL-no: 16.133] is not intended to be used in beverages (where the phototransformation product could be formed) except opaque dairy product, the Panel considered that additional studies on the above-mentioned phototransformation product M357 are not required in this case.

Subchronic toxicity studies

Two subchronic toxicity studies were performed with the candidate substance. These were a 28-day dose range-finding study and a 90-day oral toxicity study. In the 90-day study, oral administration of [FL-no: 16.133] by gavage for 13 weeks to CD rats 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, the highest dose tested, in both sexes.

Developmental toxicity

In a developmental toxicity study, rats were administered oral doses of 125, 300, and 1,000 mg/kg bw per day. There were no statistically significant differences between the treated and control groups. Therefore, there is no concern for developmental toxicity of [FL-no: 16.133] in rats at dose levels up to 1,000 mg/kg bw per day, the highest dose tested.

Skin sensitisation potential

Experimental data provided, including data obtained in humans, indicated that the candidate substance is not of safety concern with respect to skin sensitisation.

In vitro *receptor and cytochrome P450 interaction profiling*

In vitro tests were done to provide an early indication of whether the candidate compound interacts with P450 enzymes that might cause adverse or unexpected effects or affect drug metabolism. Also, *in vitro* studies were done to investigate possible interaction of the substance with several drug receptors. A conservative estimate of blood concentrations following acute exposure to the flavouring substance in humans, based on the use levels provided, gives a value lower than the lowest IC_{50}

determined *in vitro* for any of the CYP enzymes or receptors investigated. Consequently, the substance would not be expected to interact with CYP enzymes at the estimated levels of dietary exposure.

Safety assessment for acute exposure

Estimates of maximum acute dietary exposure indicate that this would be about 75.6 μg/kg bw for a 3-year-old child, which is higher than the acute exposure estimate for an adult (30 μg/kg bw per day). From these data, a margin of exposure of more than 1,000 for children can be calculated, which indicates no concern for acute effects, taking into account that the NOAEL was derived from a 90-day toxicity study. In addition, no toxicity was observed at dose levels up to 1,000 mg/kg bw per day in a developmental toxicity study.

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: 16.133], according to the EFSA Guidance (EFSA CEF Panel, 2010).

Based on its chemical structure, the substance has been assigned to Cramer 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 Cramer class III threshold, a 90-day study and a developmental toxicity study were required and carried out for this substance. Adequate margins of safety of 26,300 for adults and 10,500 for 3-year-old children have been calculated on the basis of the exposure estimates calculated and the NOAEL from the 90-day toxicity study.

Conclusion

Overall, the Panel concluded that there is no safety concern for [FL-no: 16.133], when used as a flavouring substance at the estimated level of dietary exposure calculated using the APET approach based on the recommended uses and use levels as specified in Appendix B, including milk and dairy based beverages that are opaque.

This conclusion does not apply for use in beverages where the substance can be subject to phototransformation.

Documentation provided to EFSA

1) Absorption Systems, 2013a. Inhibition of Cytochrome P450 Enzymes in Human Liver Microsomes by S2227 (Study No. 13F131).
2) Absorption Systems, 2013b. IC50 and Time-Dependent Inhibition of S2227 for CYP2C19 and CYP3A4 in Human Liver Microsomes.
3) AVIVA, 2012. hERG Inhibition Data Report (S2227).
4) Bioreliance, 2013a. Bacterial Reverse Mutation Assay (Test Article S1140; BioReliance Study Number AD65CV.503.BTL)
5) Bioreliance, 2013b. *In Vitro* Mammalian Chromosome Aberration Assay in Human Peripheral Blood Lymphocytes (HPBL) (Test Article S1140; BioReliance Study Number AD65CV.341.BTL)
6) Bioreliance, 2013c. *In Vivo* Micronucleus and Comet Assay in Mice (Test Article S2312, S2225 hemisulfate salt; BioReliance Study Number AD82AC.431.BTL)
7) Bioreliance, 2017. Bacterial Reverse Mutation Assay in 6-well Plates (Test Article S9513, BioReliance Study Number AF03CT.501008.BTL)
8) CeeTox, 2013. Assessment of Phototoxicity Using the Balb/c 3T3 Cell Line (Test Article S2227; CeeTox Study No. 9044-101010).
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Abbreviations

ADME	absorption, distribution, metabolism and elimination
APET	added portions exposure technique
BMD	benchmark dose
$BMDL_{10}$	lower confidence limit (90%; two-sided) of the BMD, associated with 10% extra risk
bw	body weight
CAS	Chemical Abstract Service
CEF	Panel on Scientific Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CoE	Council of Europe
DMA	dimethylamine
DMSO	dimethyl sulfoxide
DPRA	direct peptide reactivity assay
EDTA	ethylenediaminetetraacetic acid
EINECS	European Inventory of Existing Commercial chemical Substances
EP	European Parliament
FAO	Food and Agriculture Organization of the United Nations
FEMA	Flavor and Extract Manufacturers Association
FGE	Flavouring Group Evaluation
FL-no	FLAVIS number

FLAVIS	Flavour Information System database
GC	gas chromatography
GI	gastrointestinal
GLP	Good Laboratory Practice
GPCR	G-protein coupled receptors
GRAS	Generally Regarded As Safe
GSFA	General Standard for Food Additives
HPBL	human peripheral blood lymphocytes
HPLC	high-performance liquid chromatography
HRIPT	human repeat insult patch test
IC_{50}	half maximal inhibitory concentration
IR	infrared
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LC	liquid chromatography
MC	methyl cellulose
MMS	methyl methanesulfonate
MNPCE	micronucleated polychromatic erythrocytes
MoE	margin of Exposure
MS	mass spectrometry
MSDI	Maximised Survey-derived Daily Intake
MTD	maximum tolerated dose
NCE	normochromatic erythrocyte
NMR	nuclear magnetic resonance
NOAEL	no observed adverse effect level
NOEL	no observed effect level
OECD	Organisation for Economic Co-operation and Development
PCE	polychromatic erythrocytes
RCG	relative cell growth
RMI	Relative Mitotic Index
SPET	single portion exposure technique
TK	toxicokinetic
TTC	threshold of toxicological concern
UPLC-UV	ultra performance liquid chromatography coupled with ultraviolet detection
WHO	World Health Organization

Appendix A – Procedure for the evaluation of a new flavouring substance

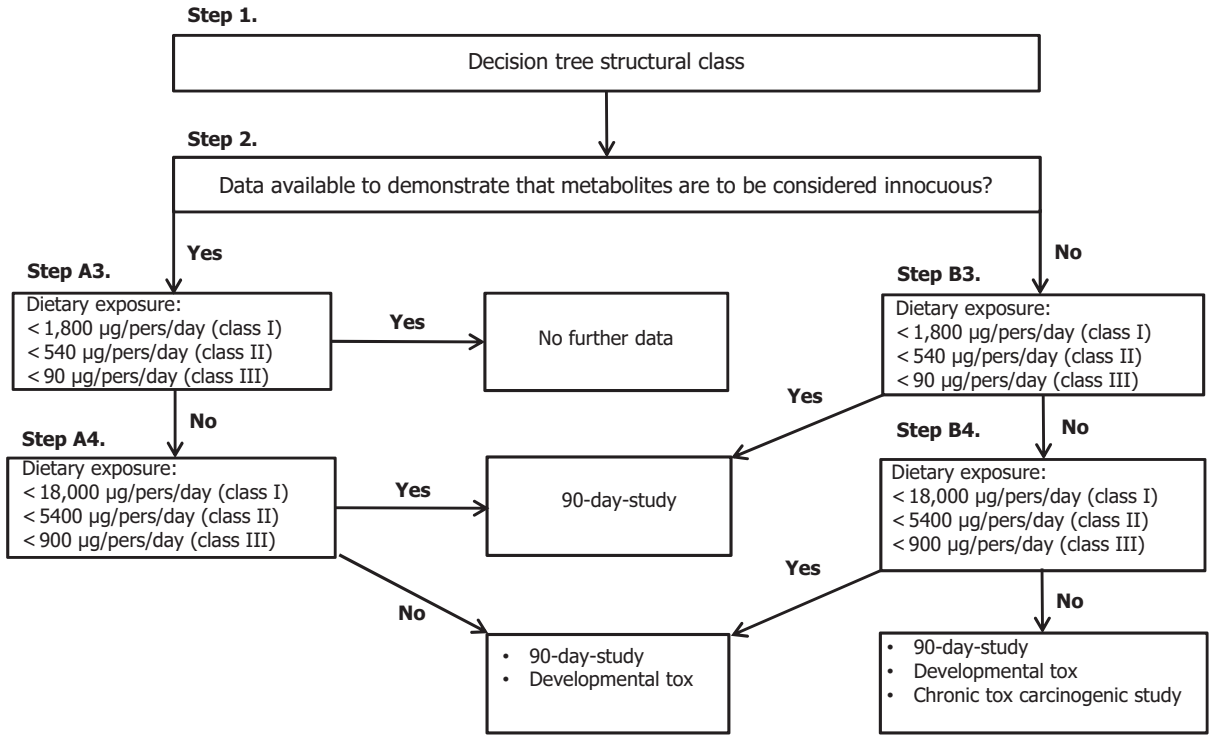

Figure A.1: The stepwise procedure for evaluation of new flavouring substances according to Commission Regulation (EC) No 1334/2008

Appendix B – Use levels and exposure calculations

Table B.1: Normal and maximum use levels for refined categories of foods and beverages

	Food categories[a]	Standard portions[b] (g)	Use 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	1	3			1	3
01.7	Dairy-based desserts (e.g. pudding, fruit or flavoured yoghurt)	125	1	3			1	3
02.4	Fat-based desserts excluding dairy-based dessert products of category 1.7	50	1	3			1	3
03.0	Edible ices, including sherbet and sorbet	50	1	3			1	3
05.1	Cocoa products and chocolate products, including imitations and chocolate substitutes	40	5	15			5	15
05.2	Confectionery, including hard and soft candy, nougats, etc., other than 05.1, 05.3 and 05.4	30	5	15			5	15
05.3	Chewing gum	3	75	150			75	150
05.4	Decorations (e.g. for fine bakery wares), toppings (non-fruit) and sweet sauces	35	5	15			5	15
06.5	Cereal and starch-based desserts (e.g. rice pudding, tapioca pudding)	200	1	3			1	3
10.4	Egg-based desserts (e.g. custard)	125	1	3			1	3

	Food categories[a]	Standard portions[b] (g)	Use 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
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	5	15			5	15
11.4	Other sugars and syrups (e.g. xylose, maple syrup, sugar toppings)	30	5	15			5	15
12.5	Soups and broths	200	1	3			1	3
12.6	Sauces and like products	30	5	15			5	15

(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 - APET

Chronic dietary exposure

Adults ('Added Portions Exposure Technique' (APET)[4])

On the basis of normal use 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 maximum intake will be from one of the three categories 01.1 (Milk and dairy-based drinks), 05.1 (Cocoa products and chocolate products, including imitations and chocolate substitutes) and 12.5 (Soups and broths) to which the candidate substance is added; they have the same normal combined occurrence level of 225 μg/adult per day.

Beverage: The flavouring substance is not intended to be added in beverages.

The total APET will be 225 μg/adult per day corresponding to 3.8 μg/kg bw per day for a 60-kg person.

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

Food subcategories resulting in the highest potential dietary exposure:
Solid food: The maximum intake will be from one of the three categories 01.1 (Milk and dairy-based drinks), 05.1 (Cocoa products and chocolate products, including imitations and chocolate substitutes) and 12.5 (Soups and broths) to which the candidate substance is added. The chronic APET for children is issued from the one of adults adjusted by a factor 0.63 to take into account the smaller portion sizes consumed by the child. In a child, the normal combined occurrence level will be 225 × 0.63 = 142 μg/child per day.
Beverage: The flavouring substance is not intended to be added in beverages.
The total APET will be 142 μg/child per day corresponding to 9.5 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 the candidate substance, i.e. the value of 9.5 μg/kg bw per day for a 15-kg child should be compared to the appropriate NOAEL for the candidate substance.

Acute dietary exposure

The acute APET is the highest value calculated at the maximum combined occurrence level in three portions of either a food or a beverage. As for the chronic APET calculation, the acute APET for children is adjusted by a factor 0.63 to take into account the smaller portion sizes consumed by the child.

Adults

The highest contribution comes from three portions of one of the four categories 01.1 (Milk and dairy-based drinks), 05.1 (Cocoa products and chocolate products, including imitations and chocolate substitutes), 06.5 (Cereal and starch based desserts) and 12.5 (Soups and broths) and is 3 × 0.2 mg × 3 mg/kg = 1.8 mg/adult for categories 0.01 and 12.5 and 3 × 0.04 mg × 15 mg/kg = 1.8 mg/adult for category 05.1. The highest acute exposure level for adults is 1,800 μg/person per day, which is equivalent to 30 μg/kg bw per day.

Children (3-year-old child of 15 kg body weight)[5]

The highest contribution comes from three portions of one of the four categories 01.1 (Milk and dairy-based drinks), 05.1 (Cocoa products and chocolate products, including imitations and chocolate substitutes), 06.5 (Cereal and starch based desserts) and 12.5 (Soups and broths) and is 1.8 × 0.63 = 1.13 mg/child. The highest acute exposure level for children is 1,134 μg/person per day, which is equivalent to 75.6 μg/kg bw per day.

[4] The APET has been calculated based on the use levels in the food subcategories reported in the above table, with the exclusion of categories 13.2 (complementary foods for infants and young children).
[5] Based on the same considerations as for adults but using the special factors used for chronic exposure to infants.

Cumulative dietary exposure

There is no other flavouring substance structurally and metabolically related to [FL-no: 16.133]. Moreover, the candidate substance is chemically synthesised and is not expected to occur naturally in food. Consequently, the cumulative dietary exposure estimate is not applicable in this case.

Appendix C – Genotoxicity

The candidate substance [FL: 16.133] does not contain any structural alert for genotoxicity based on a QSAR-toolbox Benigni et al., 2008.

Genotoxicity studies on the candidate substance [FL-no: 16.133] (S2227)

Bacterial reverse mutation assays on [FL-no: 16.133] (S2227)

In a preliminary Ames test, the candidate substance (purity 99.06%) was evaluated for the potential to induce frame shifts or point 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-mix from rats induced with Aroclor™ 1254 (WIL Research, 2012). Dimethyl sulfoxide (DMSO) was used as a solvent. Precipitates were observed at 5,000 μg/plate in both strains with and without metabolic activation. Cytotoxicity (reduction in the mean number of revertant colonies and/or background lawn) was observed at 5,000 μg/plate in TA100 with and without metabolic activation. There was no increase in the number of revertant colonies as compared with the vehicle control in either strain with or without metabolic activation. Positive and vehicle (DMSO) controls yielded the expected results demonstrating that the plate incorporation assay is sufficiently sensitive to detect mutagens.

In the main test, the flavouring substance [FL-no: 16.133] (purity > 97%) was evaluated for its potential to induce gene 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-mix from rats induced with Aroclor™ 1254 (Nucro-Technics, 2013a). The experimental design followed the OECD TG 471 (OECD, 1997a) and was in compliance with GLP. The concentrations of the candidate substance investigated for both the plate incorporation and preincubation tests, were 0, 63, 130, 250, 500 and 1,000 μg/plate. The highest concentration used was based on toxicity.

In the plate incorporation assay, once plated and at the end of the incubation period, a slight precipitate was visible from 250 to 1,000 μg/plate; toxicity was not observed at any concentration tested. Therefore, at this concentration, the test article was evaluated at the limit of solubility in the test system. In the pre-incubation assay, precipitate was visible only at 1,000 μg/plate. Toxicity was similar to the plate incorporation test when compared to the concurrent negative controls with one exception.

For TA1537 without S9-mix, the colony counts were slightly reduced at the highest concentration of 1,000 μg/plate. At the same concentration, also, the background lawns were slightly reduced both in the presence and in the absence of metabolic activation. Therefore, the candidate substance was evaluated at the limit of toxicity.

For the plate incorporation test, with or without metabolic activation, the candidate substance did not produce any statistically significant increase ($p > 0.01$) in colony counts over the concurrent negative control. For the preincubation test with TA1535, without metabolic activation, there were two slight (1.25- and 1.44-fold), but statistically significant increases ($p < 0.01$) in colony counts at 130 and 1,000 μg/plate over the concurrent negative control. However, these increases were not concentration-related, not reproduced in the plate incorporation assay and within the historical negative control range. Therefore, the authors of this study considered that this small increase was not biologically relevant. The negative controls for each tester strain were all within the historical negative control and/or spontaneous reversion ranges. All concurrent positive controls induced at least a 3.3-fold increase in colony counts when compared to the corresponding negative controls and were at levels similar to the historical positive control data. The Panel agreed with the evaluation of the authors.

Thus, the Panel concluded that the candidate substance was not mutagenic in the bacterial reverse mutation assay under the conditions applied in this test.

In vitro chromosome aberration test on [FL-no: 16.133] (S2227)

The flavouring substance [FL-no: 16.133] (purity > 97%) was tested in the *in vitro* mammalian chromosome aberration test using human peripheral blood lymphocytes (HPBL) in both the absence and presence of liver preparations (S9-mix) from rats treated with phenobarbital and 5,6-benzoflavone (Nucro-Technics 2013b). The chromosome aberration assay was used to evaluate the clastogenic potential of the test article. The experimental design followed the OECD TG 473 (OECD, 1997b) and was in compliance with GLP. A preliminary cytotoxicity 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 for 20 h in the absence of S9-mix and for 3 h in the presence S9-mix.

Sampling was performed 20 h after the start of the treatment. Solvent (DMSO) and positive control (mitomycin C and cyclophosphamide) cultures were also included.

The concentrations of [FL-no: 16.133] tested for the main experiment were from 35 to 270 μg/mL for the 3-h treatment without S9-mix, from 1.3 to 160 μg/mL for the 3-h treatment with S9-mix, from 23 to 180 μg/mL for the 20-h treatment without S9-mix.

The treated cultures were considered to be in acceptable condition with respect to pH and osmolality.

A test article precipitate was observed in the 3-h (without S9-mix) exposure at the highest concentration of 270 μg/mL. According to the study report, for the 3-h exposure experiment without S9-mix, the concentrations 35, 58, 97, 160, and 270 μg/mL yielded relative cell growth (RCG) (test vs. solvent control) of 108, 93, 98, 60 and 18%, and the four lowest concentrations yielded relative mitotic index (RMI) (test vs. solvent control) of 102, 88, 110 and 48%, respectively. For 20-h exposure without S9 the concentrations 23, 39, 65, 110 and 180 μg/mL, yielded RCGs of 100, 97, 60, 56 and 19%, and the four lowest concentrations yielded RMIs of 106, 82, 57 and 0%, respectively. For the 3-h exposure experiment with S9-mix, the concentrations 1.3, 2.5, 5.0, 10, 20, 40, 80 and 160 μg/mL yielded RCGs of 109, 100, 121, 130, 97, 84, 60 and 11%, respectively. The lowest three concentrations yielded RMIs of 61, 55 and 45%, respectively. [FL-no: 16.133] was tested at the limit of its toxicity evaluated by RCG and RMI levels, for all the testing conditions applied. The low levels of chromosome aberrations observed in the cultures treated with the candidate substance were similar to the solvent control. All concurrent positive controls induced a statistically significant increase of chromosomal aberrations.

No statistically significant increase in structural or numerical chromosome aberrations were observed in the cultures treated with the candidate substance [FL-no: 16.133] compared to the negative controls.

The Panel concluded that [FL-no: 16.133] did not induce chromosome aberrations under the test conditions applied in this study.

In vivo micronucleus study on [FL-no: 16.133] (S2227)

[FL-no: 16.133] (purity > 97%) was tested in a micronucleus assay in the bone marrow of CD-1 mice (WIL Research 2013a). The study design is based on OECD TG 474 (OECD, 1997c) and complies with GLP. Animals were treated with the candidate substance suspended in vehicle (1% methylcellulose (MC) in purified water) and administered at a volume of 10 mL/kg bw by oral gavage for both the dose range-finding test and in the main experiment. 1% MC was also used as the vehicle (negative) control. Cyclophosphamide monohydrate (CP) was used as the positive control (at a dose of 60 mg/kg bw per day). During the course of the study, animals were observed for signs of toxicity.

In the dose range-finding study, mice (3 animals/sex per group) were dosed at 500, 1,000, 1,500 and 2,000 mg/kg bw per day for three consecutive days. Animals were euthanised at 18–24 h following the last dose administration. Since no toxicity was observed up to 2,000 mg/kg bw per day, dose levels of 1,000, 1,500 and 2,000 mg/kg bw per day (6 animals/sex/group), were used for the main experiment.

No clinical signs of toxicity were observed in all animals administered with [FL-no: 16.133]. Neither body weights nor food consumption were affected by the treatment.

In the main experiment, bone marrow from five animals/sex per group was analysed for MN. A total of 1,000 erythrocytes (both polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs)) per animal were counted. No decrease in the ratio PCEs/TE (total erythrocytes) was observed in the treated groups compared to the vehicle control group, indicating that [FL-no: 16.133] did not induce toxicity in the bone marrow. Two thousand PCEs per animal were examined microscopically for the presence of micronuclei.

No statistically significant increases in the incidence of micronucleated polychromatic erythrocytes (MNPCEs) were observed in the groups treated with [FL-no: 16.133] compared to the negative control group. A statistically significant increase in the frequency of MNPCEs was observed in the positive control (CP) group. The group mean values for both MNPCEs and PCE:TE ratios for the vehicle and positive control groups were within the respective historical control ranges for the laboratory.

The authors of the study concluded that under the test conditions used the candidate substance was not clastogenic/aneugenic in bone marrow of mice. However, the Panel noted that no toxicity was observed in the bone marrow, and therefore there was no direct indication that the substance did reach the bone marrow. Nevertheless, toxicokinetic (TK) studies have shown exposure to the flavouring substance and its two main metabolites (see Figure 4). The secondary amine is considered to be the most relevant substance for genotoxicity testing, and it was therefore tested both *in vitro* and *in vivo* for all the three genetic endpoints. The results of these studies are described below.

Genotoxicity studies on S1140 (hydrochloride salt of the secondary amine M179)

Bacterial reverse mutation test on the S1140 (hydrochloride salt of the secondary amine M179)

N-(Thiophen-2-ylmethyl)-1*H*-pyrazol-3-amine, hydrochloride salt (purity > 97%) (see Figure 2) was tested in the bacterial reverse mutation test using *S.* Typhimurium strains TA98, TA100, TA1535 and TA1537 and *E. coli* strain WP2 *uvr*A (BioReliance, 2013a). The assay was performed using the plate incorporation method in the absence and presence of metabolic activation by S9-mix (from Aroclor 1254-induced rat livers). Two separate experiments were performed, with duplicate plating in the first toxicity-mutation test and with triplicate plating in the main experiment. DMSO was used as a solvent and as a negative control. In the first experiment, S1140 was tested at concentrations of 1.5, 5, 15, 50, 150, 500, 1,500 and 5,000 μg/plate (in the absence or in the presence of S9-mix). Neither precipitate nor background lawn toxicity was observed. Due to technical issues, the first experiment with *E. coli* strain WP2 *uvr*A was repeated (50, 150, 500, 1,500 and 5,000 μg/plate). Also, in this test, neither precipitation nor background lawn toxicity was observed. Based on results in the first experiment, concentrations of 50, 150, 500, 1,500 and 5,000 μg/plate, in the absence or in the presence of S9-mix, were applied for all strains in experiment two. There was no induction of revertants in any of the tester strains.

Appropriate positive controls were included for all five strains, and the assay was performed in accordance with OECD TG 471 (OECD, 1997a).

In the confirmatory mutagenicity assay, no positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9-mix activation. Neither precipitate nor background lawn toxicity was observed in the two studies performed.

In both experiments, S1140 did not show mutagenicity in bacteria.

Overall, the hydrochloride salt of M179 did not show mutagenicity in the bacterial reverse mutation assay.

Chromosome aberration test on S1140 (hydrochloride salt of the secondary amine M179)

N-(Thiophen-2-ylmethyl)-1*H*-pyrazol-3-amine, hydrochloride salt (see Figure 2), was tested in the chromosome aberration assay using human peripheral blood lymphocytes (HPBL), with and without metabolic activation provided by Aroclor-induced rat liver S9-mix) (BioReliance 2013b). Two different batches were tested in the first and second experiments (purity > 97.0% and > 98.0%, respectively). The assay was performed in accordance with GLP and OECD TG 473 (OECD, 1998).

DMSO was used as the vehicle. Cyclophosphamide and mitomycin C were evaluated as the concurrent positive controls for treatments with and without S9-mix, respectively. A range-finding test was performed using concentrations of 0.216 to 2,160 μg/mL (corresponding to 10 mM). HPBL were tested for 4 + 16 h (in the absence or in the presence of S9-mix) and for 20 + 0 h (in the absence S9-mix) with duplicate cultures. Two hours prior to harvest, colcemid was added to all the treatment conditions. Cells were harvested 20 h after treatment initiation.

The mitotic index was recorded as the percentage of cells in mitosis per 500 cells counted. At least 50% reduction in mitotic index compared to the vehicle control, was observed at 2,160 μg/mL in the 4 + 16 h treatment (in the absence or in the presence of S9-mix) and at a concentration ≥ 216 μg/mL in the 20 + 0 h treatment (in the absence of S9-mix). Based on these findings, the concentrations chosen for the main assay in the 4 + 16 h treatment ranged from 280 to 2,160 μg/mL (in the absence of S9-mix) and from 100 to 1,840 μg/mL (in the presence of S9-mix). The concentrations chosen for the 20 + 0 h treatment (in the absence of S9-mix) were from 25 to 280 μg/mL. One hundred metaphase cells were scored for structural aberrations and polyploidy from each duplicate culture (consistent with the OECD TG 473 (OECD, 1998) that was applicable at the time when the study was performed).

The vehicle control values of chromosomal aberrations were within historical control ranges. The positive controls induced statistically significant increases in the percentage of aberrant metaphases. No statistically significant or concentration-related increases in structural chromosomal aberrations were induced in the 4 + 16 h treatment (in the presence of S9-mix) and 20 + 0 h treatment (in the absence of S9-mix). In the 4 + 16 h treatment (in the absence of S9-mix), a statistically significant increase in structural aberrations was observed only at the highest concentration of 2,160 μg/mL ($p \leq 0.05$).

Based on this result, the 4 + 16 h treatment without S9-mix was repeated with a new batch (purity > 98%) of the test article and a narrower concentrations range (500–2,160 μg/mL). The highest concentration tested produced an ~ 50% reduction in mitotic index which met the concentration limit as recommended by testing guidelines for this assay.

Statistically significant and concentration-related increases in structural aberrations were observed at concentrations of 2,000 and 2,160 μg/mL ($p \leq 0.01$, Fisher's Exact test and $p \leq 0.05$, Cochran-Armitage test, respectively). No statistically significant or concentration-related increases in polyploidy were induced in any of the treatment conditions. Due to scorer variability, the slides were re-analysed and the results of this second analysis confirmed the positive outcome observed in the original scoring.

The Panel concluded that the hydrochloride salt of the secondary amine M179 induced chromosomal aberrations in HPBL at the short-term treatment in the absence of metabolic activation.

Combined comet and micronucleus assay on the hemisulfate salt of the amine metabolite (M179)

The genotoxic potential of the hemisulfate salt of *N*-(thiophen-2-ylmethyl)-1*H*-pyrazol-3-amine (S2312, purity > 98%) was assessed in Hsd:ICR (CD-1) mice using the bone marrow micronucleus assay combined with the comet assay (BioReliance, 2014). The comet assay design was based on the JaCVAM protocol version 14.2. The study was GLP compliant and the micronucleus test was in accordance with OECD TG 474 (OECD, 1997c).

In a dose range-finder assay, doses up to 2,000 mg/kg bw per day were tested (via oral gavage) and a maximum tolerated dose (MTD) of 500 mg/kg bw per day in male mice and 1,000 mg/kg bw per day in female mice was identified. In the main study, groups of five male mice were administered doses of 0 (vehicle used was 1% methylcellulose in deionised water), 125, 250 and 500 mg/kg bw per day and groups of five female mice were administered doses of 0, 250, 500 and 1,000 mg/kg bw per day for three consecutive days. The positive control, methyl methanesulfonate (MMS), was administered at a dose of 40 mg/kg bw per day on days 2 and 3.

Animals were examined for signs of toxicity before the administration of S2312 and 1 and 2 h after S2312 administration. No mortality was observed at any dose level. Clinical signs observed included: piloerection (at all dose levels), lethargy (at 500 and 1,000 mg/kg bw per day), prostration, irregular breathing and crusty eyes (at 1,000 mg/kg bw per day in female mice). All other animals did not show signs of toxicity throughout the observation period.

All animals were euthanised approximately 3–4 h after the final dose for tissue collection. Femoral bone marrow was collected for the micronucleus assay, while liver was analysed for the comet assay.

Micronucleus assay

The frequency of MNPCEs and the proportion of PCEs to total erythrocytes were determined for each animal and treatment group. Two thousand PCEs per mouse were scored for micronucleus analysis.

There was no statistically significant increase of micronuclei in bone marrow polychromatic erythrocytes of mice treated with the hemisulfate salt of M179 compared to the vehicle control.

The positive controls (MMS) induced statistically significant increases in the frequency of MNPCEs. The study authors concluded that M179 did not induce micronuclei in male and female CD-1 mice. Although there was no indication that the target organ was exposed since no toxicity was observed in the bone marrow, TK studies showed that the substance was systemically available and indicate that both the liver and the bone marrow were exposed.

Overall, the Panel concluded that the secondary amine did not induce structural or numerical aberrations in the bone marrow of mice.

Comet assay

For the comet assay in the liver, two slides per animal were analysed. Fifty randomly selected, non-overlapping cells per slide were scored resulting in a total of 100 cells evaluated for DNA damage. Each slide was also examined for indications of cytotoxicity, i.e. presence of 'clouds'.

No statistically significant increase in the mean % tail DNA was observed in liver cells from the test article dosed animals compared to the vehicle control mice. No increase in the percentage of clouds was observed at any dose level compared to control.

The Panel concluded that the secondary amine metabolite did not induce DNA damage in the liver. In addition, the liver might be the most relevant organ to study genotoxicity of M179, since

experimental data (see Section 3.8) indicate that the majority of the amide bond hydrolysis of [FL-no: 16.133] occurs post-absorption and not within the intestinal lumen.

The Panel concluded that the hemisulfate salt of the secondary amine M179 did not induce micronuclei in mice bone marrow. Although there was no direct indication that the target organ was exposed, TK studies showed that the substance was systemically available and indicate that both the liver and the bone marrow were exposed. There was no increase in % tail intensity in liver cells analysed through the comet assay. The Panel concluded that M179 is not genotoxic *in vivo*.

Bacterial reverse mutation assay on M357 (phototransformation product of [FL-no: 16.133])

M357 (the major phototransformation product of [FL-no: 16.133]) was tested for its ability to induce mutations in the Ames test using *S.* Typhimurium strains TA97a, TA98, TA100, TA1535 and *E. coli* strain WP2 *uvr*A (BioReliance 2017). DMSO was used as a solvent and as a negative control, and the assay was performed using a modified plate incorporation method (six-well plating with two wells per concentration of test, vehicle or positive control). The test was performed with and without metabolic activation by S9-mix (from Aroclor 1,254-induced rat livers) at concentrations of 0, 0.3, 1.0, 3.0, 10, 30, 100, 300 and 1,000 μg/well. Appropriate positive controls were included in the test. In strain TA98 both with and without metabolic activation and in *E. coli* strain WP2 uvrA with metabolic activation an increase in revertant frequency was observed (greater than twofold). All increases, however, were within the historical control range and not concentration responsive. In strains TA100 and TA97a, without metabolic activation, increase in revertant frequency was greater than twofold and concentration responsive, but they were within the historical range. The author of the study concluded that the tested substance is not mutagenic.

Since the substance M357 is not expected to be produced under the intended conditions of use of [FL-no: 16.133], the Panel did not evaluate further this phototransformation product.

Table C.1: Summary of *in vitro* genotoxicity studies

Chemical name [FL-no]	Test system	Test object	Concentration	Result	Reference	Comments
2-(4-Methylphenoxy)-*N*-1*H*-pyrazol-3-yl-*N*-(thiophen-2-ylmethyl) acetamide [FL-no: 16.133]	Abbreviated Ames test	*Salmonella* Typhimurium TA98, TA100	Up to 5,000 μg/plate	Negative[(a)]	WIL Research (2012)	Cytotoxicity was observed at 5,000 μg/plate in TA100 with and without metabolic activation
	Bacterial reverse mutation	*Salmonella* Typhimurium TA98, TA100, TA1535, TA1537, *Escherichia coli strain WP2 uvrA*	0, 63, 130, 250, 500 and 1,000 μg/plate	Negative[(a)]	Nucro-Technics (2013a)	A slight precipitate was visible from 250 to 1,000 μg/plate
	Chromosome aberration	Human peripheral blood lymphocytes	35, 58, 97, 160, and 270 μg/mL[(b)] 1.3, 2.5, 5.0, 10, 20, 40, 80, and 160 μg/mL[(c)] 23, 39, 65, 110 and 180 μg/mL[(d)]	Negative	Nucro-Technics (2013b)	Test article precipitate was observed in the 3-hr (without S9-mix) exposure at the highest concentration of 270 μg/mL
N-(Thiophen-2-ylmethyl)-1*H*-pyrazol-3-amine, hydrochloride salt (M179)	Bacterial reverse mutation	*Salmonella* Typhimurium TA98, TA100, TA1535, TA1537, *Escherichia coli strain WP2 uvrA*	1.5, 5, 15, 50, 150, 500, 1,500 and 5,000 μg/plate 50, 150, 500, 1,500 and 5,000 μg/plate	Negative[(a)]	BioReliance (2013a)	Neither precipitate nor background lawn toxicity was observed in the two studies performed
	Chromosome aberration	Human peripheral blood lymphocytes	280, 563, 1,130, 1,560, 1,840 and 2,160 μg/mL[(e)] 100, 280, 563, 1,130, 1,325, 1,560 and 1,840 μg/mL[(f)] 25, 50, 100, 200, 225, 250 and 280 μg/mL[(g)] 500, 1,000, 1,500, 1,600, 1,850, 2,000 and 2,160 μg/mL[(e)]	Positive[(e)]	BioReliance (2013b)	Two different batches were tested. The positive results were confirmed in the repeated test

(a): With and without metabolic activation.
(b): 3-h treatment without S9-mix.
(c): 3-h treatment with S9-mix.
(d): 20-h treatment without S9-mix.
(e): 4 + 16 h in the absence of S9-mix.
(f): 4 + 16 h in the presence of S9-mix.
(g): 20 + 0 h in the absence of S9-mix.

Table C.2: Summary of *in vivo* genotoxicity studies

Chemical name [FL-no]	Test system	Test object	Route	Dose mg/kg bw per day	Result	References	Comments
2-(4-Methylphenoxy)-*N*-1*H*-pyrazol-3-yl-*N*-(thiophen-2-ylmethyl)acetamide [FL-no: 16.133]	Micronucleus assay	CD-1 mice	Gavage	1,000, 1,500 and 2,000	Negative	WIL Research (2013a)	No direct evidence of bone marrow exposure TK studies indicate exposure
N-(Thiophen-2-ylmethyl)-1*H*-pyrazol-3-amine, hemisulfate salt (M179)	Micronucleus assay in bone marrow	CD-1 female mice	Gavage	250, 500 and 1,000	Negative	BioReliance (2014)	No direct evidence of bone marrow exposure TK studies indicate exposure
	Comet assay in liver	CD-1 male mice		125, 250 and 500	Negative		TK studies indicate exposure to the liver

FL-no: FLAVIS number; bw: body weight; TK: toxicokinetic.

Appendix D – Toxicity

Table D.1: Summary of toxicity studies considered by the Panel

FL-no	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
16.133	2-(4-Methylphenoxy)-*N*-1*H*-pyrazol-3-yl-*N*-(thiophen-2-ylmethyl) acetamide	Sprague–Dawley Crl: CD(SD) rats; males and females 8/group	Gavage	10, 30 and 100	28 days	NOEL: ≥ 100	Charles River (2013)	The study was conducted according to the General Guidelines for the Designing and Conducting Toxicity Studies. Redbook 2000 Toxicological Principles for the Safety Assessment of Food Ingredients (November 2003)
		Sprague–Dawley Crl: CD(SD) rats; males and females 20/group	Gavage	10, 30 and 100	90 days	NOEL: ≥ 100	Charles River (2014)	The study was conducted according to the General Guidelines for the Designing and Conducting Toxicity Studies. Redbook 2000 Toxicological Principles for the Safety Assessment of Food Ingredients (November 2003). The study is GLP compliant

FL-no.: FLAVIS number; bw: body weight; NOEL: no observed effect level; GLP: Good Laboratory Practice.

Table D.2: Reproductive and developmental toxicity study considered by the Panel

FL-no	Test material	Study type duration	Species; sex no/group	Route	Dose level mg/kg bw per day	NOAEL (mg/kg bw per day) including information on possible maternal toxicity	Reference	Comments
16.133	2-(4-Methylphenoxy)-*N*-1*H*-pyrazol-3-yl-*N*-(thiophen-2-ylmethyl)acetamide	Dose range-finding Gestation days 6 through 20	Sprague–Dawley Crl: CD(SD) rats; female 8/group	Gavage	125, 250, 500, and 1,000	–	WIL Research (2013b)	Non-GLP study
		Developmental toxicity study Gestation days 6 through 20	Sprague–Dawley Crl: CD(SD) rats; female 25/group	Gavage	125, 300, and 1,000	1,000	WIL Research (2015)	Study compliant with GLP, OECD TG 414 and US FDA Redbook 2000

FL-no: FLAVIS number; bw: body weight; NOAEL: no observed adverse effect level; GLP: Good Laboratory Practice.