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Safety and efficacy of a feed additive consisting of a flavonoid-rich dried extract of *Citrus* × *aurantium* L. fruit (bitter orange extract) for use in all animal species (FEFANA asbl)

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Abstract

Following a request from the European Commission, the EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on the safety and efficacy of a dried flavonoid-rich extract of *Citrus* × *aurantium* L. fruit (bitter orange extract), when used as a sensory additive for all animal species. The use of the additive in feed was not expected to increase the exposure to furocoumarins of those target species that are already fed citrus by-products to a relevant extent (< 5%). For dog, cat and ornamental fish, not normally exposed to citrus by-products, no conclusion could be drawn. The FEEDAP Panel concluded that the additive under assessment is safe up to the maximum proposed use level of 400 mg/kg for veal calf (milk replacer), sheep, goat, horse and salmon. For the other species, the calculated maximum safe concentration in complete feed is 102 mg/kg for chicken for fattening, 151 mg/kg for laying hen, 136 mg/kg for turkey for fattening, 182 mg/kg for piglet, 217 mg/kg for pig for fattening, 268 mg/kg for sow, 259 mg/kg for dairy cow and 161 mg/kg for rabbit. The FEEDAP Panel considered that the use in water for drinking is safe provided that the total daily intake of the additive does not exceed the daily amount that is considered safe when consumed via feed, except dog, cat and ornamental fish. No concerns for consumer safety were identified following the use of the additive up to highest safe level in feed for the target animals. The extract under assessment should be considered as irritant to skin, eyes and the respiratory tract, and as a skin sensitiser. Since the additive contains 5-methoxypsoralen, it may cause phototoxicity. The use of the extract in animal feed under the proposed conditions was not expected to pose a risk for the environment. Bitter orange extract was recognised to flavour food. Since its function in feed would be essentially the same as that in food, no further demonstration of efficacy was considered necessary.

Keywords: sensory additives, flavouring compounds, *Citrus* × *aurantium* L., bitter orange extract, flavonoids, 5-methoxypsoralen, (-)-synephrine, safety

Requestor: European Commission

Question number: EFSA-Q-2021-00144

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Declarations of interest: The declarations of interest of all scientific experts active in EFSA's work are available at https://ess.efsa.europa.eu/doi/doiweb/doisearch.

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1. Introduction

1.1. Background and Terms of Reference

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7. In addition, Article 10(2) of that Regulation specifies that for existing products within the meaning of Article 10(1), an application shall be submitted in accordance with Article 7, within a maximum of seven years after the entry into force of this Regulation.

The European Commission received a request from Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG)[2] for authorisation/re-evaluation of 20 preparations (namely buchu leaves oil, amyris oil, olibanum extract (wb), olibanum tincture, lime oil, neroli bigarade oil, petigrain bigarade oil, petitgrain bigarade absolute, bitter orange extract of the whole fruit, lemon oil expressed, lemon oil distilled, orange oil, orange terpenes, mandarin oil, mandarin terpenes, grapefruit oil expressed, grapefruit extract (sb), grapefruit extract, quebracho extract (wb), cashew oil), belonging to botanically defined group (BDG) 8 - *Sapindales*, when used as feed additives for all animal species (category: sensory additives; functional group: flavourings). During the assessment, the applicant withdrew the application for ten preparations.[3,4] During the course of the assessment, this application was split and the present opinion covers only one out of the 20 initial preparations under application: a dried flavonoid-rich extract of *Citrus* × *aurantium* L.[5] fruit (bitter orange extract) for all animal species.

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive) and under Article 10(2) (re-evaluation of an authorised feed additive). EFSA received directly from the applicant the technical dossier in support of this application. The particulars and documents in support of the application were considered valid by EFSA as of 19 March 2018.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of a dried flavonoid-rich extract of *Citrus* × *aurantium* L. fruit (bitter orange extract), when used under the proposed conditions of use (see Section 3.2.4).

The remaining nine preparations belonging to botanically defined group (BDG) 8 – *Sapindales* under application are assessed in separate opinions.

1.2. Additional information

Bitter orange extract of the whole fruit (*Citrus aurantium* ssp.) is currently authorised as a feed additive according to the entry in the European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003 (2b natural products – botanically defined). It has not been assessed as a feed additive in the EU.

There is no specific EU authorisation for any *Citrus* × *aurantium* L. preparation when used to provide flavour in food. However, according to Regulation (EC) No 1334/2008[6] flavouring preparations produced from food, may be used without an evaluation and approval as long as 'they do not, on the

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

[2] On 13/03/2013, EFSA was informed by the applicant that the applicant company changed to FEFANA asbl, Avenue Louise 130 A, Box 1, 1050 Brussels, Belgium.

[3] On 27 February 2019, EFSA was informed about the withdrawal of the application on amyris oil, olibanum tincture, cashew oil, neroli bigarade oil, petigrain bigarade absolute, mandarin terpenes, grapefruit oil expressed, grapefruit extract (sb), grapefruit extract.

[4] On 2 April 2020, EFSA was informed by the applicant about the withdrawal of the application on olibanum extract (wb).

[5] Accepted name: *Citrus* × *aurantium* L., synonyms: *Citrus* × *aurantium* ssp. *amara* (Link) Engl. (PlantList): *Citrus aurantium* L., *Citrus aurantium* L. ssp. *aurantium* L.*; Citrus aurantium* L. ssp. *amara* Engl. (EFSA Compendium, EFSA, 2012).

[6] 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 Regulation (EC) No 1601/91 of the Council, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. OJ L 354, 31.12.2008, p. 34.

basis of the scientific evidence available, pose a safety risk to the health of the consumer, and their use does not mislead the consumer'.

Bitter-orange epicarp and mesocarp (*Aurantii amari* epicarpium et mesocarpium) is described in a monograph of the European Pharmacopoeia 10.1 (PhEur, 2020). It is defined as the dried epicarp and mesocarp of the ripe fruit of *Citrus aurantium* L. ssp. *aurantium* (*C. aurantium* L. ssp. *amara* Engl.) partly freed from the white spongy tissue of the mesocarp and endocarp.

The safety of dried hydro-alcoholic extracts of dried immature fruits and dried peel of the immature and mature fruit of *Citrus* × *aurantium* L. for food supplement use is addressed in the following EFSA documents: 'Advice on the EFSA guidance document for the safety assessment of botanicals and botanical preparations intended for use as food supplements, based on real case studies' (EFSA ESCO, 2009) and 'Scientific Opinion on a Qualified Presumption of Safety (QPS) approach for the safety assessment of botanicals and botanical preparations' (EFSA Scientific Committee, 2014).

Some of the individual components of bitter orange extract and some structurally related flavonoids have been already assessed as chemically defined flavourings for use in feed and food by the FEEDAP Panel and the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF). The list of flavouring compounds currently authorised for food[7] and feed[8] uses together with the EU Flavour Information System (FLAVIS) number, the chemical group as defined in Commission Regulation (EC) No 1565/2000[9] and the corresponding EFSA opinion is given in Table 1.

Table 1: Flavouring compounds already assessed by EFSA as chemically defined flavourings, grouped according to the chemical group (CG) as defined in Commission Regulation (EC) No 1565/2000, with indication of the EU Flavour Information System (FLAVIS) number and the corresponding EFSA opinion

CG	Chemical group	Product – EU register name (common name)	FLAVIS no	EFSA opinion,* year
25	FGE.32	Naringin	16.058	2011a
		Neohesperidin dihydrochalcone	16.061	2011b
		Hesperidin(1)	16.097	2010, CEF
		4',5,7-Trihydroxyflavanone(1) (naringenin)	16.132	2017, CEF

(*): FEEDAP opinion unless otherwise indicated.
(1): Evaluated for use in food.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[10] in support of the authorisation request for the use of a (flavonoid-rich) extract of *Citrus* × *aurantium* fruit (bitter orange extract) as a feed additive.

The FEEDAP Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies, peer-reviewed scientific papers, other scientific reports and experts' knowledge, to deliver the present output.

Many of the components of the extract under assessment have been already evaluated by the FEEDAP Panel as chemically defined flavourings. The applicant submitted a written agreement to refer to the data submitted for the assessment of chemically defined flavourings (dossiers, publications and unpublished reports) for the risk assessment of preparations belonging to BDG 8.[11]

[7] Commission Implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. OJ L 267, 2.10.2012, p. 1.

[8] European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003. Available online: https://ec.europa.eu/food/sites/food/files/safety/docs/animal-feed-eu-reg-comm_register_feed_additives_1831-03.pdf

[9] Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council. OJ L 180, 19.7.2000, p. 8.

[10] FEED dossier reference: FAD-2010-0322.

[11] Technical dossier/Supplementary information/Letter dated 29/04/2021.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the phytochemical markers in the additives. The Executive Summary of the EURL report can be found in Annex A.[12]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of a (flavonoid-rich) extract of *Citrus* × *aurantium* fruit (bitter orange extract) is in line with the principles laid down in Regulation (EC) No 429/2008[13] and the relevant guidance documents: Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements (EFSA Scientific Committee, 2009), Compendium of botanicals that have been reported to contain toxic, addictive, psychotropic or other substances of concern (EFSA, 2012), Guidance for the preparation of dossiers for sensory additives (EFSA FEEDAP Panel, 2012a), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012b), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017a), Guidance on the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c), Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019), Guidance document on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals (EFSA Scientific Committee, 2019a) and Statement on the genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019b).

3. Assessment

The additive under assessment, bitter orange extract, is a dried flavonoid-rich extract obtained from immature fruits of *Citrus* × *aurantium* L. It is intended for use as a sensory additive (functional group: flavouring compounds) in feed and water for drinking for all animal species.

3.1. Origin and extraction

The taxonomy and systematics of the *Citrus* genus are complex, and the exact number of natural species is unclear. Almost all of the common commercially important citrus fruits found today are hybrids derived from three ancestral species now represented by the cultivars described as the mandarin orange, pomelo, and citron. *Citrus* × *aurantium* is considered to have arisen from a cross between the pomelo (*Citrus maxima*) and the mandarin (*Citrus reticulata*). The group *Citrus* × *aurantium* now includes numerous varieties and cultivars as a result of natural and deliberate back-crossing to other parents. These include the orange, bitter orange, grapefruit and clementine. Many varietal names and sub-species have been used to distinguish between members of this hybrid complex but none have current taxonomic standing. The fruits of the plant used in the production of various culinary and medicinal preparations are designated by the name 'bitter orange' as the plant itself.

The additive under assessment is a dried water-methanol extract of dried immature bitter oranges (whole fruit). The plant of origin is identified in the dossier as *Citrus aurantium* L. ssp. *amara*, a subspecies associated with the bitter (sour) orange tree but now recognised only as a synonym of *Citrus* × *aurantium* L.. Immature bitter orange fruits are first disrupted by grinding and then extracted with a methanol-water mixture (80:20 v/v). Insoluble plant residue is removed by centrifugation and the extracted liquor concentrated by vacuum distillation. Methanol is added to the concentrated liquor, which is stirred for 3 days at a temperature < 30°C allowing precipitation to occur. The precipitated material is removed by filtration and the remaining liquor distilled to remove the methanol. Finally, the extract is concentrated by vacuum distillation and then spray-dried to produce the feed additive.

[12] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2010-0322-bdg08.pdf

[13] Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

3.2. Characterisation

3.2.1. Characterisation of a flavonoid-rich dried extract of *Citrus* × *aurantium* L. fruit

The extract is an odourless light brownish hygroscopic powder, with a characteristic flavour and bitter taste. The additive is specified to contain 10–20% neohesperidin and 20–30% naringin (selected as the phytochemical marker for control purposes). Compliance with the specifications was demonstrated in five batches of the additive (originating from Spain, North Africa and Central and South America), with naringin representing 23–26% of the extract and neohesperidin 15–19%.

The applicant provided the full characterisation of the five batches of the additive.[14] Table 2 summarises the results of a proximate analysis. In gross analyses of this type, the difference between the sum of the analysed fractions and the total is conventionally ascribed to carbohydrate but, in practice, will also contain other plant metabolites. A further analysis of the same five batches (Table 3) demonstrated that flavonoids account for 50% of the whole extract, leaving ~ 30% of the extract unidentified. While it is probable that this unidentified fraction consists largely of carbohydrate, particularly galacturonans (e.g. Bracher et al., 2016), other plant-derived compounds may also be present (see Table 3).

Table 2: Proximate analysis of a dried extract of *Citrus* × *aurantium* fruit based on the analysis of five batches (mean and range). The results are expressed as % (w/w)

Constituent	Mean	Range
	% (w/w)	% (w/w)
Proteins	9.34	7.37–10.8
Ash	4.32	3.4–6.15
Fibre	0.44	< 0.1–1.27
Lipids	0.072	< 0.05–0.11
'Carbohydrates'(1)	81.3	77.7–84.6
Moisture	4.12	2.7-4.8
Total	99.63	97.7–100.2

(1): 'Carbohydrates' include secondary plant metabolites, such as flavonoids.

The individual components of the flavonoid fraction were determined by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection (284 nm).[15] Nine identified compounds were quantified accounting together for 49.7% (46.7–52.2%) of the extract. Didymin (CAS number 14259-47-3) was also detected but not quantified. The alkaloid (-)-synephrine was also present in seven batches (ranging from 0.39% to 0.99% of the extract).[16] 5-Methoxypsoralen (5-MOP, also known as bergapten) was quantified in two batches of the additive (0.021-0.034% of the extract).

Table 3: Characterisation of the fraction of secondary plant metabolites of a dried extract of *Citrus* × *aurantium* fruit based on the analysis of five batches (mean and range). The results are expressed as % (w/w) of the extract. (-)-Synephrine was analysed in seven batches, 5-methoxypsoralen in two batches

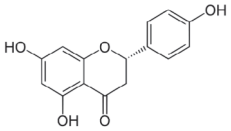
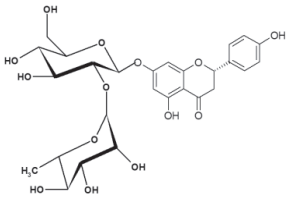
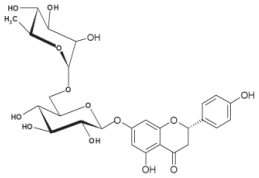
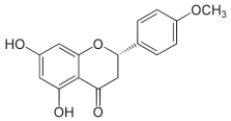
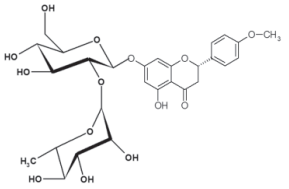
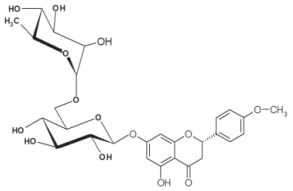
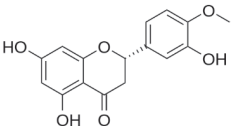
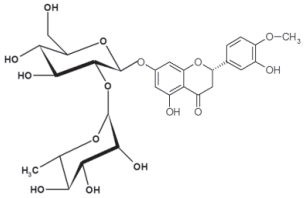
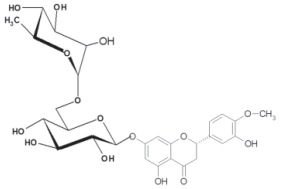
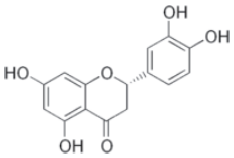
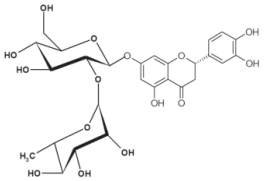
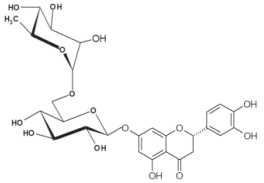
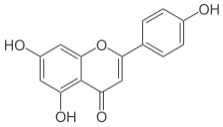
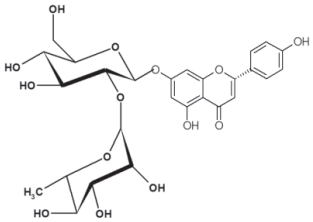
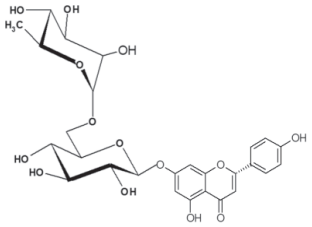
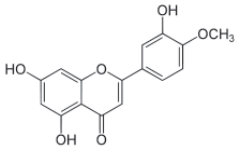
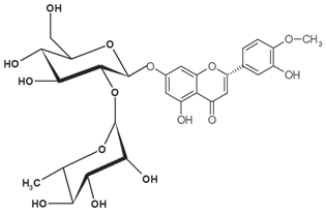
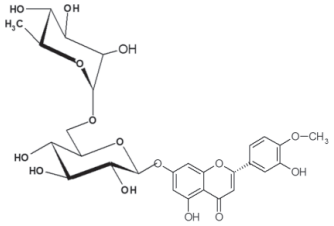
Constituent	CAS no	FLAVIS no	Mean(a)	Range
			% (w/w)	% (w/w)
Flavonoids			52.24	49–55
Eriocitrin	13463-28-0	–	1.22	1.11–1.35
Naringen	14259-46-2	–	1.08	0.94–1.28
Naringin	10236-47-2	16.058	24.3	22.8–25.5

[14] Technical dossier/Supplementary information March 2020/Annex_III_SIn_reply_bitter_orange_extract COA_characterization.
[15] Technical dossier/Supplementary information March 2020/Annex_I_SIn_reply_bitter_orange_extract_HPLC_method.
[16] Technical dossier/Supplementary information March 2020/Annex_V_SIn_reply_bitter_orange_extract_SOC and Annex_VII_SIn_reply_bitter_orange_extract_SOC_further.

Constituent	CAS no	FLAVIS no	Mean[(a)]	Range
			% (w/w)	% (w/w)
Hesperidin	520-26-3	–	1.73	1.24–2.34
Neohesperidin	13241-33-3	–	16.5	14.6–18.5
Rhoifolin	17306-46-6	–	1.00	0.59–1.20
Neodiosmin	38665-01-9	–	0.83	0.34–1.21
4′,5,7-Trihydroxyflavanone (Naringenin)	67604-48-2	16.132	1.38	1.08–1.60
Poncirin	14941-08-3	–	1.56	1.22–1.80
Total identified			49.7	46.7–52.2
(-)-Synephrine	94-07-5	–	0.77	0.39–0.99
5-Methoxypsoralen (bergapten)	484-20-8	–	0.028	0.021–0.034

CAS no.: Chemical Abstracts Service number; FLAVIS number: EU Flavour Information System numbers.
(a): Mean calculated on five batches, except (-)-synephrine (7 batches) and 5-methoxypsoralen (2 batches).

The chemical structures of the compounds identified in the extract are shown in Figure 1 (in bold), together with the corresponding aglycones and other structurally related compounds, to highlight the structural similarity, which together with the metabolic similarity is the basis for the read across (see Section 3.3.3). Eight out of the 10 flavonoids identified in the extract are flavanones, two are flavones (rhoifolin and neodiosmin). All flavonoids except naringenin are glycosides, five are 7-O-neohesperosides and four are 7-O-rutinosides.

Aglycones(1)	**7-*O*-Neohesperidosides**	**7-*O*-Rutinosides**
Naringenin (CAS 480-41-1) 	**Naringin** (CAS 10236-47-2) Naringenin 7-*O*-neohesperidoside 	Isonaringin (narirutin, **naringen**) (CAS 14259-46-2) Naringenin 7-*O*-rutinoside 
Isosakuranetin (CAS 48043-3) 	**Poncirin** (CAS 14941-08-3) Isosakuranetin-7-neoesperidoside 	**Didymin** (CAS 14259-47-3) Isosakuranetin-7-*O*-rutinoside 
Hesperitin (CAS 520-33-2) 	**Neohesperidin** (CAS 13241-33-3) Hesperetin 7-*O*-neohesperidoside 	**Hesperidin** (CAS 520-26-3) Hesperetin 7-*O*-rutinoside 
Eriodictyol (CAS 555-58-9) 	Neoeriocitrin (CAS 13241-32-2) Eriocitidol-7-*O*-neohesperidoside 	**Eriocitrin** (CAS 13463-28-0) Eriodictyol-7-*O*-rutinoside 
Apigenin (CAS 520-36-5) 	**Rhoifolin** (CAS 17306-46-6) Apigenin 7-*O*-neohesperoside 	Isorhoifolin (CAS 209-015-9) Apigenin-7-*O*-rutinoside 
Diosmetin (CAS 520-34-3) 	**Neodiosmin** (CAS 38665-01-9) Diosmetin-7-*O*-neohesperidoside 	Diosmin (CAS 520-27-4) Diosmetin-7-*O*-rutinoside 

(1): Naringenin, isosakuranetin, hesperitin, eriodictyol and their related 7-O-neohesperosides 7-O-rutinosides are flavanones; apigenin and diosmetin and their related O-neohesperosides 7-O-rutinosides are flavones

Figure 1: Molecular structures, Chemical Abstract System (CAS) numbers and synonyms of the ten flavonoids under assessment (in bold) and of structurally related glycosides and aglycones

Substances of concern

The EFSA Compendium reports the occurrence of 5-MOP (also known as bergapten) in the essential oil from the aerial parts of *C. aurantium* L. and that of synephrine[17] in the unripe whole fruit and in the pericarp as chemicals of concern (EFSA, 2012). The applicant performed a literature search regarding substances of concern and chemical composition of the plant species *Citrus* × *aurantium* L. and its preparations.[18] The search identified 83 references, the vast majority of them dealing with general information. Nine references reported the occurrence of substances of concern and two were considered of relevance for the present assessment and are briefly described. In four hydroalcoholic extracts from *Citrus* × *aurantium* (parts of the plant not specified) containing 6%, 10%, 30% and 50% (-)-synephrine, 5-MOP and bergamottin determined by liquid chromatography–mass spectrometry (LC–MS) were each below 5 mg/kg and 6,7-dihydroxybergamottin below 10 mg/kg (Stohs et al., 2014). Coumarins and furocoumarins (scopoletin, scoparone, oxypeudanin and isoimperatorin) were detected by UPLC–MS/MS at concentrations between 20 and 90 mg/kg in an extract of comparable botanical origin and manufacturing (Hwang and Ma, 2018). In the same preparation, the content of terpenoids limonin and nomilin, the major limonoids present in *Citrus* seeds, was determined. Only free limonin was detected (3 mg/g), whereas nomilin was not detected (neither as free nor as glycoside).

The presence (-)-synephrine was detected in all batches of the extract, whereas 5-MOP was detected in two batches (see Table 3).[19] In addition, the content of other furocoumarins, namely psoralen, xanthoxin, isopimpinellin, imperatorin, oxypeucedanin, phellopterin, 8-geranoxypsoralen and 5-geranoxypsoralen (also known as bergamottin), was analysed in other five batches.[20] All furocoumarins including 5-MOP were each below the limit of detection (LOD, 20 mg/kg).

3.2.2. Impurities

Data on chemical and microbial impurities were provided in at least three batches of the additive under assessment.[21] The concentrations of heavy metals were below the corresponding limit of quantification (LOQ) in all the batches, with the exception of arsenic in one batch (0.068 mg/kg). In the same batches, aflatoxins B1, B2, G1 and G2 were below the LOQ and pesticides were not detected in a multiresidue analysis. Polychlorinated dibenzo-*p*-dioxin (PCDD), polychlorinated dibenzofuran (PCDF) and dioxin-like polychlorinated biphenyls (PCBs) were below the corresponding LOQ, and the calculated upper bond for the sum of WHO (2005) PCCD/F+PCB TEQ ranged between 3.4 and 20 pg/kg wet weight in three batches. Methanol analysed in one batch was 13.6 mg/kg.[22] None of the data on chemical impurities raised concerns.

Analysis of microbial contamination (seven batches) indicated that *Salmonella* spp. was not detected in 25 g, Enterobacteriaceae was not detected in 1 g, total viable count was $< 10^3$ colony forming unit (CFU)/g, yeasts and moulds $< 10^2$ CFU.[23]

3.2.3. Shelf-life

The typical shelf-life of the additive is stated to be at least 12 months, when stored in tightly closed containers under standard conditions (in a cool, dry place protected from light).[24]

[17] '(-)-synephrine' is used in the document, when the compound is naturally occurring and 'synephrine', when the origin is unclear and the compound may be the synthetic racemate. From the available studies, Stohs and Preuss (2012) concluded that the biological activity of (-)-synephrine is roughly twice that of the racemate.
[18] Technical dossier/Supplementary information March 2020/Literature_search_bitter_orange_extract.
[19] Technical dossier/Supplementary information March 2020/Annex_V_SIn_reply_bitter_orange_extract_SOC.
[20] Technical dossier/Supplementary information March 2020/Annex_VII_SIn_reply_bitter_orange_extract_SOC_further.
[21] Technical dossier/Supplementary information March 2020/Annex X_ SIn_reply_bitter_orange_extract_impurities_analysis. Limits of quantification (LOQs) for heavy metals and arsenic: cadmium 0.01 mg/kg, lead 0.04 mg/kg, mercury 0.005 mg/kg and arsenic 0.03 mg/kg (cadmium, lead and arsenic 0.250 mg/kg, and mercury 0.05 mg/kg for one batch analysed in different laboratory); LOQ for mycotoxins: 2 μg/kg for aflatoxins B1, B2, G1 and G2. LOQ for individual pesticides: 0.01 mg/kg.
[22] Technical dossier/Supplementary information March 2020/Annex_XI_SIn_reply_bitter_orange_extract_methanol and Annex_XII_SIn_reply_bitter_orange_extract_methanol_batch.
[23] Technical dossier/Supplementary information March 2020/Annex_II_SIn_reply_bitter_orange_extract COA_batch and Annex VI_SIn_reply_bitter_orange_extract_SOC_batch.
[24] Technical dossier/Section II.

3.2.4. Conditions of use

Bitter orange extract is intended to be added to feed and water for drinking for all animal species without a withdrawal time. The maximum proposed use level in complete feed is 254 mg/kg for chicken for fattening, 379 mg/kg for laying hen, 340.5 mg/kg for turkey for fattening and 400 mg/kg for all the other species. No specific use level has been proposed by the applicant for the use in water for drinking.

3.3. Safety

The assessment of the safety of the additive is based on the maximum use levels proposed by the applicant.

The additive under assessment mainly contains flavonoids (up to 55%). The unidentified fraction of the additive is likely to consist of carbohydrates, particularly galacturonans, and is not considered of concern. The additive also contains minor concentrations of (-)-synephrine (up to 1%) and trace amounts of 5-MOP (up to 0.03%).

Some of the flavonoids in the extracts belongs to FGE.32 and were already evaluated by the EFSA Panels (see Section 1.2, Table 1). In particular, two of the major identified components of the additive (naringin and neohesperidin), were assessed and considered safe for use as flavourings, and are currently authorised for food[7] and feed[8] uses. The FEEDAP Panel notes that naringin and naringenin are structurally related (naringenin is the aglycone of naringin) as well as hesperidin and neohesperidin, which are the 7-*O*-neohesperidoside and the 7β-rutinoside of hesperetin, respectively.

The applicant provided a literature search on the absorption, distribution, metabolism and excretion (ADME)[25] and on the toxicology of flavonoids, which is summarised in the following sections.

Information on (-)-synephrine and 5-MOP, which were analytically determined in the extract under assessment and for which a quantitative risk assessment is performed, is briefly summarised below. A more extensive safety evaluation of furocoumarins is available in the EFSA opinion on expressed lemon oil and its fractions and on lime oil (EFSA FEEDAP Panel, 2021). For synephrine, reference is made to the assessment by Bundesinstitut für Risikobewertung (BfR, 2012).

Other components like the limonoids, limonin and nomilin, whose presence in extracts similar to the additive under assessment has been reported in the literature (see Section 3.2.1), are ubiquitous in natural feed and foods and not further addressed.

3.3.1. Absorption, distribution, metabolism and excretion

Flavonoids

ADME in laboratory animals and humans

The flavonoids occurring in the additive belong to the classes of flavanones and flavones, most of them are present in their glycoside form as 7-*O*-neohesperidosides and 7-*O*-rutinosides. The flavonoids present in the extract are listed in Table 3, and the structures of flavanones and flavones are shown in Figure 1. The major compounds are the flavanones naringin and neohesperidin. The other flavanones present at low levels (1–2% of the extract) are hesperidin, naringen (narirutin), eriocitrin and poncirin, the flavones are rhoifolin and neodiosmin. The aglycone present in the extract is naringenin.

The ADME of some of these flavonoids including the glycosides **naringin** and **hesperidin** and their respective aglycones (naringenin and hesperitin) was already reviewed by EFSA (EFSA CEF Panel, 2010, 2017). For both compounds, the glycoside is hydrolysed by the intestinal microbiota of experimental animals (rats and dogs) and humans, originating the respective aglycones. The aglycones are partially absorbed and conjugated with glucuronic acid and/or sulfate and may also undergo hydroxylation, dehydroxylation and/or dealkylation before urinary or biliary excretion. The bacterial reductive cleavage of the flavanone C-ring can also occur in intestine, originating phenolic compounds, which are further metabolised and excreted with faeces or absorbed and then excreted via the bile and urine, either as such or as their glucuronide, sulfate, glycine or other conjugates. The conjugates eliminated through bile can be hydrolysed in the intestine and reabsorbed, entering enterohepatic circulation, and/or eliminated in faeces.

Although it is commonly accepted that the glycosides are hydrolysed in the gut before the corresponding aglycones are systemically absorbed, there are studies demonstrating that the intact

[25] Technical dossier/Supplementary information November 2020.

glycoside form can also be absorbed as such. This is the case in the study carried out with dogs that were orally administered 70 mg of flavanones present in grapefruit extract (42.1 mg naringin, 24 mg narirutin and 4.3 mg naringenin). Naringin, and its metabolites naringenin and naringenin glucuronide, were analysed in plasma by liquid chromatography–tandem mass spectrometry (LC–MS/MS, the LOD and the LOQ were 0.74 and 2.48 nmol/L for naringin, respectively, and 2.06 and 6.91 nmol/L for naringenin) (Mata-Bilbao et al., 2007). Plasma C_{max} of naringin was attained at around 80 min, whereas for naringenin and naringenin glucuronide their C_{max} were at around 20 and 30 min, respectively. Mean C_{max} plasma concentrations of naringin, naringenin and naringenin glucuronide were 0.24, 0.02 and 0.09 μmol/L, respectively, and the corresponding mean $AUCs_{0\text{-}24}$ were 23.16 min μmol/L, 1.78 min μmol/L and 22.5 min μmol/L. The median and range values for mean residence time were 3.3 (1.5–9.3), 2.8 (0.8–11.2) and 8.0 (2.3–13.1) h for naringin, naringenin and naringenin glucuronide, respectively.

Recently, the kinetics of naringin and naringenin were studied in rats, dogs and humans after single (i.v. and oral) or oral repeated doses of naringin (Bai et al., 2020). Naringin and naringenin were quantified by LC–MS/MS in blood and urine, with and without enzymatic hydrolysis. After single oral administration, the C_{max} blood levels of naringin in rat (doses: from 10.5 to 168 mg/kg body weight (bw)) and dog (doses: from 3.1 to 49.6 mg/kg bw) were in the range of 30–100 ng/mL, at about 1–3 h after administration, and the half-life was between 0.5 and 2.0 h. In humans (doses: from 40 mg to 480 mg per day), C_{max} ranged from 2.2 to 11 ng/mL at about 2 h after administration. For naringenin (same doses as for naringin), C_{max} in rat and dog ranged from 0.03 to 1.8 ng/mL at 2.5–6 h, depending on the dose. In humans, naringenin levels were from 12 to 100 ng/mL, at 7 to 13 h after administration. The bioavailability of naringin was about 44% in rat and 34% in dog. In the same study, twelve compounds were identified by *in vitro* incubation of naringin with liver and kidney microsomes of rats and humans: naringin, naringenin, rhoifolin, neoeriocitrin, hesperidin, apigenin, eriodictyol, hesperitin, conjugates of naringenin, and 5,7-dihydroxychromone. The study showed that *in vivo* naringin is bioavailable and that both naringin and naringenin are extensively metabolised *in vitro*. After a single oral dose of 10 mg/kg bw to rats, intact hesperidin in portal vein blood was found with a t_{max} of 2 h. Hesperidin glucuronide, and homoeriodictyol and eriodictyol conjugates were found both in portal and central blood (Nectoux et al., 2019). The data showed that hesperidin is absorbed in its intact glycosidic form as well as that the methylation/demethylation pathways occur for the hesperitin molecule.

The tissue distribution of naringenin was evaluated in rats after gastric instillation of 10 or 50 mg/kg bw ^{3}H-naringenin (Mohsen et al., 2004). About 87% of the radioactivity disappeared from the gastrointestinal tract 18 h after administration, being 42% excreted in urine, 7% in faeces, and 9% present in plasma. Liver was the organ with the highest level, 1.3% for both doses, and for the remaining tissues, levels were from 0.07% to 0.95%. For almost all of the tissues, there were no differences in radioactivity levels between the two administered doses. In parallel, the distribution of metabolites of naringenin was evaluated in rats at 2 and 18 h after oral administration of 50 mg/kg bw of the non-labelled compound. At 2 h, the predominant plasma and tissues metabolites were naringenin glucuronides. In tissues, the aglycone naringenin level was higher than in plasma. The highest levels of metabolites were in the small intestine, followed by kidney, liver, and lung. At 18 h, both in plasma and tissues, naringenin was the main compound. Naringenin, naringenin glucuronides and *p*-hydroxyphenyl propionic acid were excreted in urine and faeces.

Two studies evaluated the distribution of naringin in rats after a single oral administration of 42 mg/kg bw (Zou et al., 2012; Zeng et al., 2019). In the first study, both naringin and naringenin (measured as total naringenin) were highly distributed in tissues and organs, the highest values in liver (AUC and C_{max} of 634 ng.h/g and 231 ng/g for naringin at 0.08 h and 2,212 ng.h/g and 507 ng/g for naringenin at 2 h). For naringin the second highest level (111 ng/g) was found at 0.08 h in trachea, followed by lung and kidney (35 ng/g, at 1 and 0.5 h, respectively); for naringenin, the second highest level was 110 ng/g in kidney at 2 h, followed by lung and trachea (about 50 ng/g, at 2 at 4 h, respectively). In brain, the concentrations of the compounds were lower than the LOQ (10 ng/g, LC–MS/MS). After 12 h, both compounds were not detected in the analysed tissues (Zou et al., 2012).

Zeng et al. (2019) carried out a study in aged rats with naringin to evaluate its pharmacokinetics and metabolic profile after oral administration of a single dose of 42 mg/kg bw. Globally, they verified that absorption and excretion were delayed as compared with adult rats, being C_{max} and AUC about the double in aged animals. Naringin and naringenin metabolites were broadly distributed in tissues 24 h after administration with the highest concentrations of naringin in lung, followed by trachea, liver, kidney, muscle, fat, spleen, heart, and brain, while that of total naringenin was higher in liver followed by kidney, lung, trachea, heart, fat, spleen, muscle, and brain. Some differences were noted in the

distribution of the compounds as compared with adult rats. Thirty-nine flavonoid metabolites (*O*-glucuronide, *O*-diglucuronide, *O*-sulfate, *O*-disulfate, *O*-glucuronide-sulfate, *O*-glucoside-*O*[1]glucuronide, *O*-glucoside-*O*-sulfate derivatives of naringenin, apigenin, eriodictyol and hesperetin) were identified or partially identified in urine and/or faeces and 46 microbial-derived phenolic metabolites, such as phenylpropenoic acid, phenylpropionic acid, phenylacetic acid, benzoic acid, benzenetriol and benzoylglycine derivatives, including free phenolics and sulfate and glucuronide metabolites. Analysis of urine and faeces collected during 48 h post-dose showed that the excretion of naringin and its metabolites is rapid and complete.

After repeated oral administration of a high dose of naringin to rats (210 mg/kg bw, twice daily for 8 days), the distribution of naringin metabolites was evaluated in liver, kidney, heart, spleen and brain (Lin et al., 2014). Six hours after the last dose, free naringin and naringenin were not detected in the analysed organs (HPLC-UV; LOQ and LOD for naringenin were 0.40 and 0.02 μg/mL). Liver contained the highest concentration of naringenin sulfates (8.2 nmol/g), followed by spleen, heart, brain and kidney. Naringenin glucuronides were present in serum (3.2 nmol/mL), liver (2.6 nmol/g) and kidney (0.9 nmol/g), but not detected in spleen, brain and heart. Despite the limitations in terms of sensitivity and specificity of the analytical method, this study showed the distribution of some naringin metabolites at very low levels after repeated high dose administration of naringin.

Yang et al. (2012) also investigated the toxicokinetics of naringin and naringenin in dogs after repeated oral administration of naringin at the doses of 20, 100, or 500 mg/kg bw per day for 1, 30, 90, and 180 days. A rapid resolution liquid chromatography–electrospray ionisation-tandem mass spectrometry (RRLC–ESI-MS/MS) was used. A plasma dose-dependent increase for both naringin and naringenin was verified, without accumulation of the compounds (e.g. a C_{max} of naringin of 470 and 459 μg/L at 1 and 180 days, respectively, after oral administration of 500 mg/day). T_{max} of naringin was about 1 h for all doses. Naringenin was present in plasma at similar levels as naringin, although the T_{max} for naringenin was higher (5–10 h) than that for naringin (about 1 h). $T_{1/2}$ of naringin ranged from 1 h for the dose of 20 mg/kg bw to about 7 h for 500 mg/kg bw. For naringenin $T_{1/2}$ was higher and similar among doses and time (2.3–7.4 h). $AUC_{0\text{-}24h}$ (μg h/L) increased with the dose and generally attained a plateau at day 30 of administration of the compounds.

The same metabolic pathways described for naringin can be anticipated for **neohesperidin**, the second most abundant flavonoid in the additive after naringin. Both are neohesperosides, the only structural difference consisting of a second hydroxyl group in the 5′ position of the B ring of neohesperidin, and of a methoxy group in the 4′ position. This feature (the methoxy group in 4′-position) is shared by other flavanones present in the additive (hesperidin, poncirin and didymin) and will not impact the final fate of the resultant aglycones, as demethylation is a common metabolic pathway, making the hydroxyl group prone to further conjugation (EFSA CEF Panel, 2010). The same metabolic pathways observed for hesperidin, which is metabolised in humans to hesperitin 3′-glucuronide, hesperitin 7-glucuronide and hesperitin 3′-sulfate (Brett et al., 2009), are therefore expected for neohesperidin.

Other minor flavanone glycosides (e.g. eriocitrin, poncirin and didymin) are expected to share the same ADME profile as naringin and hesperidin. For eriocitrin, this is supported by several publications. Myiake et al. (2000) identified in both plasma and urine of rats orally given **eriocitrin** at 75 μmol/kg bw, eriodictyol, homoeriodictyol and hesperetin and small amounts of 3,4-dihydroxyhydrocinnamic acid, resulting from eriodictyol degradation by intestinal bacteria. Metabolites were present in plasma in conjugated form and in urine in both free and conjugated form. A similar profile in plasma was found in humans after ingestion of eriocitrin or its aglycon (Myiake et al., 2006), with the detection of eriodictyol, homoeriodictyol and hesperetin. The metabolism of eriocitrin was recently studied *in vitro* and *in vivo* (Li et al., 2019). Eriocitrin was administered by gavage to rats at 50 mg/kg bw and blood, urine, faeces and bile collected at several time points. The *in vitro* studies were made by incubating eriocitrin with rat liver microsomes and with rat intestinal microbiota. Analysis of all samples performed by LC–MS/MS identified 32 metabolites *in vivo* and 27 metabolites *in vitro* (12 in microsomes and 20 in intestinal microbiota). Several metabolic pathways were proposed for *in vivo* and *in vitro* metabolism, consisting of hydrolysis of the glycosidic bound, oxidations, reductions, formation of ketones and loss of water with epoxide formation, as well as conjugations by methylation, sulfation, acetylation and glycine and glutamine conjugation.

No ADME data were available for **poncirin** and **didymin**. As they share the same aglycon, isosakuranetin, the only difference expected is the extension and time for hydrolysis of the glycosidic bond in intestine as poncirin is a neohesperidoside and dydimin a rutinoside. In fact, there is evidence that the α-L-rhamnosidase of intestinal bacteria is more effective in hydrolysing (1→6) bonds than (1→2)

bonds of rhamnoglycosides (Bang et al., 2015). Hydrolysis of C-ring, with formation of phenolic compounds, their absorption together with the respective aglycone, and subsequent demethoxylation, hydroxylations, and conjugations and excretion through bile and urine are expected. The incubation of poncirin in human intestinal microbiota pointed to its effective hydrolysis as shown by its rapid disappearance in the incubation medium (Bang et al., 2015). Isosakuranetin, the aglycon of the two flavanones, was proved to be formed *in vivo* from naringenin.

Some flavone glycosides are present in the extract under assessment, namely **rhoifolin** and **neodiosmin**, which by hydrolysis lead to the aglycones apigenin and diosmetin. Chen et al. (2019) studied the metabolic profile of **diosmin** and its aglycon **diosmetin** in rat after intragastric administration (dose not specified). Blood, urine and faeces were analysed by ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS). Forty-six diosmetin metabolites and 64 diosmin metabolites were proposed to be present in the biological samples. The metabolic pathways methylation, demethylation, hydroxylation, glycosylation, glucuronidation, diglucuronidation and sulfation were proposed for diosmetin, while demethoxylation, decarbonylation, dihydroxylation and dehydroxylation were proposed for diosmin. Diosmin and diosmetin-7-*O*-glucoside identified in urine and faeces as well as their subsequent metabolites accounted for a substantial part of all the diosmin metabolic products. Diosmetin and diosmin were mainly excreted in urine probably after phase II metabolism. Diosmetin was not detected in any sample (LOD not given) suggesting a rapid biotransformation by gut microbiota or hepatic metabolism, originating a plethora of phenolic compounds. On the contrary, diosmin was present in urine, demonstrating its bioavailability. A similar behaviour is expected for neodiosmin, the 7-*O*-neohesperidoside of diosmetin. For **rhoifolin**, with the same backbone structure of the flavone diosmin lacking the methoxy group in ring B, the same metabolic pathways as for diosmin are expected, that is, hydrolysis by gut microbiota, absorption, conjugations, and excretion in bile and urine.

Importantly, there is evidence, both *in vitro* and *in vivo,* that some flavones are converted into flavanones by reduction of the C2 = C3 bond in the C-ring. *In vitro*, this was shown for luteolin after incubation with *Eubacterium ramulus*, an anaerobic microorganism very abundant in human faeces (Braune et al., 2001, as referenced in Erlund, 2004). The subsequent metabolites resulting from fission of the C-ring, very well known for flavanone compounds, were identified. The same degradation pathways were proposed for apigenin. *Clostridium orbiscindens* is another anaerobic microorganism that degrades the flavones apigenin and luteolin to the flavanones naringenin (further degraded to the dihydrochalcone phloretin) and eriodictyol, respectively (Schoefer et al., 2003, as referenced in Erlund, 2004). *In vivo*, the biotransformation of apigenin-7-glucoside to apigenin and naringenin was postulated in rats inoculated with human microbiota (Hanske et al., 2009). The same pathway (hydrolysis followed by dehydrogenation) is proposed for luteolin. As the aglycone of rhoifolin and isorhoifolin is apigenin, it is expected that it is reduced to naringenin in the gut for which the metabolism was above described. The opposite conversion of flavanones into flavones was postulated from observations made in humans, rats and dogs after oral administration (p.o.) and intravenous (i.v.) injection with naringenin, which was dehydrated to apigenin (Orrego-Lagarón et al., 2016, Bai et al., 2020). Zeng et al. (2019) also showed in rats that apigenin is one of the main metabolites of naringenin excreted in urine and faeces. The metabolic interconversion of flavone and flavanone aglycons is shown in Figure 2.

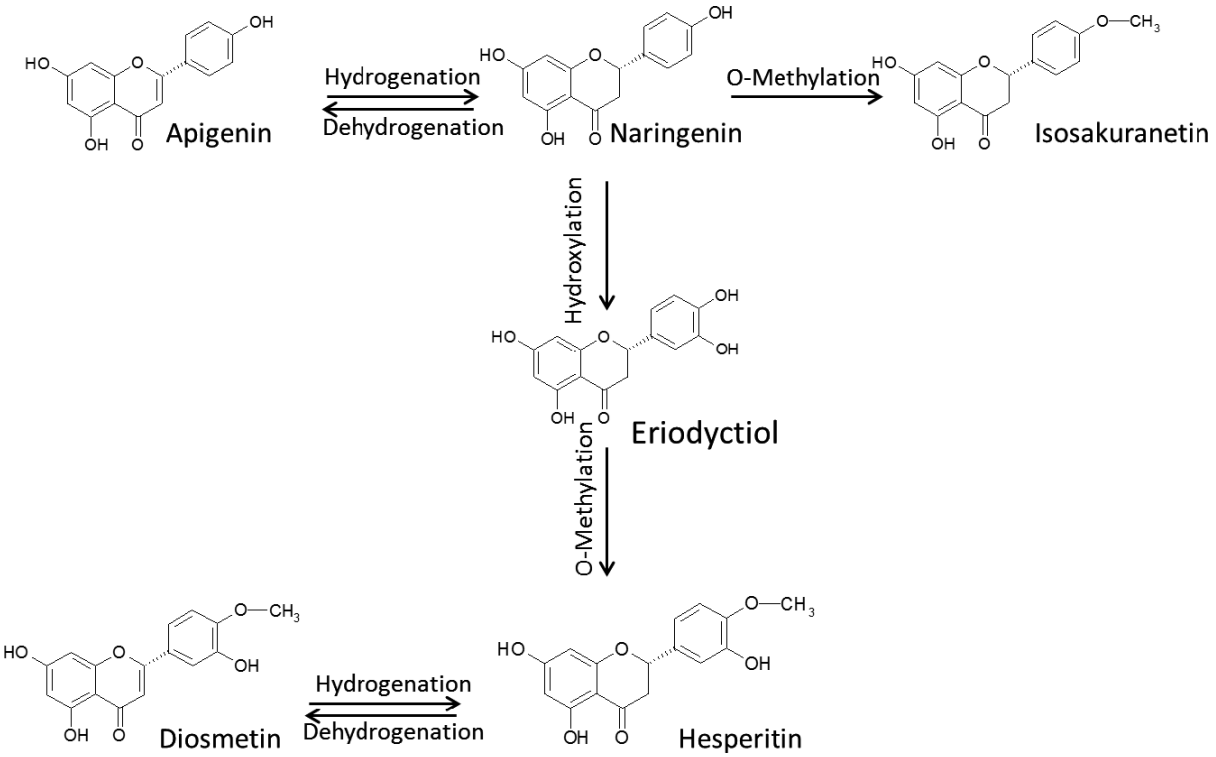


Figure 2: Metabolic interconversion of flavone and flavanone aglycons derived after hydrolysis of glycosides present in *Citrus* × *aurantium* fruit extract

There are plenty of published data in humans on absorption, metabolism and excretion of some of the flavonoids present in the additive. A similar profile has been identified as that commonly found in experimental animals. Pereira-Caro et al. (2016) gave 500 mL of orange juice (containing, among other compounds, hesperidin, narirutin, didymin, and eriocitrin) to human volunteers and analysed blood and urine in the following 24 h. A total of 19 flavanone metabolites, including di-*O*-glucuronide, *O*-glucuronide, *O*-glucuronyl-sulfate and sulfate derivatives of hesperetin, naringenin and eriodictyol, and 65 microbial-derived phenolic compounds, such as phenylpropanoid, phenylpropionic, phenylacetic, benzoic, and hydroxycarboxylic acids and benzenetriol and benzoylglycine derivatives, including free phenolics and phase II sulfate, glucuronide, and methyl metabolites, were identified or partially identified in plasma and/or urine samples. The same group carried out a similar study in humans and evaluated the bioavailability of the components of the fruit juice and their recovery in urine (Pereira-Caro et al., 2014). Taking into account the conjugated derivatives of flavanones excreted in urine resulting from the C-ring fission of the flavanones by microbiota, including the hippuric acid, the recovery was about 100%. The reliability of the data is supported by analysing the urine of the same subjects given a placebo after a washout period of 2 weeks.

ADME in the target species

Data on ADME of the flavonoids present in the extract under assessment in the target species is very scarce. A commercial naringenin extract was administered orally to 2-year-old lambs with the proposal of testing its anticoccidial activity (Pérez-Fonseca et al., 2016). Animals were given a daily dose of 5 mg/kg bw of a commercial naringenin extract at 98% purity for 90 days. Blood was collected at several times up to 96 h after administration. Maximum plasma concentration of naringenin was 1.940 ± 0.408 μg/mL at about 2 h after administration, decreasing in the following 8 h.

Some flavonoids, including naringenin and hesperitin, were incubated in pig caecum microbiota under anaerobic conditions (Labib et al., 2004). Naringenin was transformed into 3-(4-hydroxyphenyl)-propionic acid and 3-phenylpropionic acid. Hesperitin was degraded into eriodictyol and subsequently to 3-(3-hydroxyphenyl)-propionic acid and phloroglucinol. The data confirm the ability of gut microbiota of this target species to metabolise flavanones. In the EFSA FEEDAP opinion on naringin (EFSA FEEDAP Panel, 2011a,b), reference is made to the bovine rumen microorganisms' ability to degrade flavonoid glycosides including naringin, and hesperidin.

Neohesperidin dihydrochalcone, a structurally related compound of hesperidin, was evaluated in 2014 by the FEEDAP Panel. Because of lack of data on the ADME of the compound, a comprehensive review of ADME of several related flavonoids in fish was made. The FEEDAP Panel concluded that the gastrointestinal tract of fish has the capacity to hydrolyse the glycosidic bond of flavonoids and both, the phase I and II enzymes responsible for metabolism of flavonoids, including metabolic activities of UDP-glucuronosyltransferases, sulfotransferases, catechol-*O*-methyltransferases and glutathione-S-transferases, are present in fish (EFSA FEEDAP Panel, 2014).

Altogether, the flavonoids present in the extract under assessment are well absorbed, in the glycosidic form and after intestinal metabolism, are extensively metabolised after absorption, mainly by conjugation, but also phase I reactions can occur. Metabolism shows interconversion between flavanones and flavones. The formation of toxic metabolites has not been reported in the literature made available to the FEEDAP Panel. A similar profile of metabolism was identified for experimental animals and humans. Both the flavonoids and their metabolites are widely distributed in the organism of animals and excretion occurs in urine, and through bile in faeces. There is no evidence of accumulation of the compounds and their metabolites in the organism.

For target species data is scarce, but *in vitro* studies with intestinal and rumen microbiota indicate that flavonoids are degraded, and the enzymes involved in subsequent metabolism after absorption are present in all target species (EFSA FEEDAP Panel, 2016).

(-)-Synephrine

No studies on the ADME of (-)-synephrine were made available to the FEEDAP Panel. However, from an NTP review it is known that after oral administration of synephrine to 10 patients, its biological half-life was about 2 h and excretion was mainly via urine with 2.5% of the dose recovered as unchanged synephrine and the main metabolite being *p*-hydroxymandelic acid (NTP, 2004).

Furocoumarins (5-methoxypsoralen)

As regards 5-MOP, reference is made to the general knowledge on the ADME of furocoumarins as reviewed by the FEEDAP Panel (EFSA FEEDAP Panel, 2021). In mammals, furocoumarins are almost completely absorbed from the gastrointestinal tract and metabolised predominantly in the liver via cytochrome P450, the main excretion route being the urine.

3.3.2. Toxicology

3.3.2.1. Genotoxicity

For mixtures containing a substantial fraction of substances that have not been chemically identified, the EFSA Scientific Committee recommends that first the chemically defined substances be assessed individually for their potential genotoxicity using all available information, including read across and quantitative structure–activity relationship (QSAR) considerations about their genotoxic potential (EFSA Scientific Committee, 2019b). Therefore, the potential genotoxicity of identified constituents is first considered.

(-)-Synephrine

The absence of genotoxicity of (-)-synephrine was stated in a review of Stohs (2017).

Furocoumarins (5-methoxypsoralen)

The genotoxicity and carcinogenicity data of psoralens (linear furocoumarins) have been summarised in IARC monographs 40 (IARC, 1986) and 100 (IARC, 2012) and in the report of the Senate Commission of the Deutsche Forschungsgemeinschaft (SKLM, 2006) and reviewed by the FEEDAP Panel (EFSA FEEDAP Panel, 2021).

Furocoumarins are genotoxic *in vitro* and *in vivo*. In the absence of UVA, the potency is low but increases greatly under UVA irradiation. Linear furocoumarins (5-MOP) are carcinogenic in rats in the absence of UVA (only males) and in male and female rats and mice after concomitant UVA irradiation. There is evidence in humans that oral intake of linear furocoumarins together with UVA can cause skin tumours (IARC, 2012). The available carcinogenicity data do not allow to define a benchmark dose (BMD) lower confidence limit for a benchmark response of 10% ($BMDL_{10}$) that can be used as reference point in a quantitative risk assessment of furocoumarins.

3.3.2.2. Repeated dose toxicity studies

Flavonoids

For flavonoids, the applicant identified reference points for hesperidin and naringin from studies previously evaluated by EFSA. Particularly, the EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (EFSA CEF Panel) identified a no observed adverse effect level (NOAEL) of 500 mg hesperidin/kg bw per day from an 84-day feeding study in rat which showed no adverse effects (Basarkar and Nath, 1981 as referenced in EFSA CEF Panel, 2010) and a NOAEL of 1,250 mg naringin/kg bw per day identified by the FEEDAP Panel in its assessment of naringin as feed additive (based on feed intake depression observed at the highest dose tested of 2,000 mg/kg bw per day) (EFSA FEEDAP Panel, 2011a).

(-)-Synephrine

The applicant identified a 90-day rat study performed with a bitter orange extract of immature fruit standardised to contain 50% (-)-synephrine. Although the content of (-)-synephrine in the test material used in the 90-day study is different compared to the additive under application (< 1%), the FEEDAP Panel considers this study relevant for the present assessment with respect to the evaluation of (-)-synephrine. The study is summarised below.

A bitter orange (*Citrus aurantium L.*) extract (origin not specified; 'extract' of dried immature fruits) standardised to contain 50% (-)-synephrine was examined in a 90-day study in rats (Deshmukh et al., 2017) following OECD TG 408 (OECD, 1998). Doses of 100, 300 and 1,000 mg extract/kg bw per day were administered, by gavage to male and female rats (10 rates of each sex per group). No adverse effects were observed with respect to any of the observed parameters, including clinical signs, functional observations, ophthalmology, body weight, food consumption, haematology, urinalysis, organ weights, gross and microscopic pathology, at any of the doses, in either sex. At the highest dose tested (1,000 mg extract/kg bw per day), several non-adverse effects were reported, including fully reversible signs of repetitive head burrowing in the bedding material and piloerection for short periods of time in both sexes immediately after administration. Furthermore, a slight and reversible elevation of blood urea nitrogen and urea levels in male rats, and slight to mild increase in the relative but not absolute heart weights of male and female rats were observed at the highest dose. The authors of the study identified a no observed effect level (NOEL) for this bitter orange extract standardised to 50% (-)-synephrine of 300 mg/kg, while the NOAEL was 1,000 mg/kg bw per day (Deshmukh et al., 2017). The FEEDAP Panel notes that the significant increase in the relative heart weights observed at the highest dose tested in males and females, although not correlated with histopathology and considered non-adverse by the authors of the study, could be related to the pharmacological effects of (–)–synephrine. Therefore, the FEEDAP Panel selected the intermediate dose of 300 mg/kg bw per day as the NOAEL for the extract, which corresponds to a NOAEL of 150 mg (-)-synephrine/kg bw per day.

Furocoumarins (5-methoxypsoralen)

As summarised in the toxicological assessment by the FEEDAP Panel (EFSA FEEDAP Panel, 2021), limited data are available on the sub-chronic toxicity of furocoumarins. Studies with 5-MOP in dogs and rats were considered insufficient to derive a NOAEL. From the available data set, the SKLM (2006) concluded that a NOAEL for the repeated intake of furocoumarins could not be specified.

3.3.3. Safety for the target species

Tolerance studies and/or toxicological studies made with the extract under application were not submitted.

In the absence of these data, the approach to the safety assessment of a mixture whose individual components are known is based on the safety assessment of each individual component (component-based approach, EFSA Scientific Committee, 2019a). This approach requires that the mixture is sufficiently characterised. The individual components can be grouped into assessment groups, based on structural and metabolic similarity. The combined toxicity can be predicted using the dose addition assumption within an assessment group, taking into account the relative toxic potency of each component.

As the additive under assessment is sufficiently characterised, the FEEDAP Panel applied a component-based approach to assess the safety for target species of the flavonoid fraction of the extract. For furocoumarins and particularly for 5-MOP, their toxicological properties and the available

dataset do not allow to identify a reference point for the risk assessment or to derive a safe level. On the other hand, feeding citrus by-products to food-producing animals is a common practice with no report of adverse effects (Bampidis and Robinson, 2006; Feedipedia[26]). Therefore, the assessment of the safety for target species is based on the comparison between the combined furocoumarin and 5-MOP intake via the consumption of citrus by-products as feed material and that via the use of bitter orange extract as a feed additive.

Flavonoids and (-)-synephrine

Based on considerations related to structural and metabolic similarities (see Figure 1), the flavonoids were allocated to the same assessment group, as shown in Table 4.

For each component in the assessment group, exposure in target animals was estimated considering the use levels in feed, the percentage of the component in the extract and the default values for feed intake according to the guidance on the safety of feed additives for target species (EFSA FEEDAP Panel, 2017b). Default values on body weight are used to express exposure in terms of mg/kg bw per day. The intake levels of the individual components calculated for chicken for fattening, the species with the highest ratio of feed intake/body weight per day, are shown in Table 4.

For hazard characterisation, each component of an assessment group was first assigned to the structural class according to Cramer classification. For some components in the assessment group toxicological data were available to derive NOAEL values. Structural and metabolic similarity among the components in the assessment groups were evaluated to explore the application of read-across allowing extrapolation from a known NOAEL of a component of an assessment group to the other components of the group with no available NOAEL or, if sufficient evidence were available for members of an assessment group, to derive an assessment group NOAEL.

Toxicological data for subchronic studies were available, from which NOAEL values could be derived for naringin (EFSA FEEDAP Panel, 2011a), hesperidin (EFSA CEF Panel, 2010) and (-)-synephrine (see Section 3.3.2).

Considering the structural and metabolic similarities within the assessment group of flavonoids, the lowest NOAEL of 500 mg/kg bw per day for hesperidin was selected as a group NOAEL and applied using read-across to all flavanones (naringin, naringenin, naringen, eriocitrin, neohesperidin and poncirin) and flavones (rhoifolin and neodiosmin). The extrapolation from flavanones to flavones is justified by the observation of a metabolic interconversion of the two types of flavonoids (see Section 3.3.1).

As the result of the hazard characterisation, a reference point was identified for each component in the assessment group based on the toxicity data available (NOAEL from *in vivo* toxicity study or read across). Reference points selected for each compound/group are shown in Table 4.

For risk characterisation, the margin of exposure (MOE) was calculated for each component as the ratio between the reference point and the exposure. For each assessment group, the combined (total) margin of exposure (MOET) was calculated as the reciprocal of the sum of the reciprocals of the MOE of the individual substances (EFSA Scientific Committee, 2019a). A MOET $>$ 100 allowed for interspecies- and intra-individual variability (as in the default 10 $\times$ 10 uncertainty factor).

The approach to the safety assessment of bitter orange extract expressed for the target species is summarised in Table 4. The calculations were done for chicken for fattening, the species with the highest ratio of feed intake/body weight and represent the worst-case scenario at the use level of 254 mg/kg complete feed.

[26] https://www.feedipedia.org/node/680

Table 4: Compositional data, intake values, reference points and margin of exposure (MOE) for the individual components of a dried extract of *Citrus* × *aurantium* fruit classified according to assessment groups

Extract composition			Exposure		Hazard characterisation		Risk characterisation	
Assessment group	**CAS No**	**Max conc. extract**	**Max Feed conc.**	**Intake[a]**	**Cramer class**	**NOAEL[b]**	**MOE**	**MOET**
Constituent		**%**	**mg/kg**	**mg/kg bw per day**	–	**mg/kg bw per day**	–	–
Flavonoids								
Naringin	10236-47-2	25.5	64.8	5.815	II	500	86	
Neohesperidin	13241-33-3	18.5	47.0	4.218	III	500	119	
Hesperidin	520-26-3	2.34	5.94	0.534	II	**500**	937	
Poncirin	14941-08-3	1.80	4.57	0.410	III	500	1,218	
Naringenin	67604-48-2	1.61	4.09	0.367	III	500	1,362	
Eriocitrin	13463-28-0	1.35	3.43	0.308	III	500	1,624	
Naringen	14259-46-2	1.28	3.25	0.292	III	500	1,713	
Rhoifolin	17306-46-6	1.20	3.05	0.274	III	500	1,827	
Neodiosmin	38665-01-9	1.08	2.74	0.246	III	500	2,030	
								40
(-)-Synephrine		0.99	2.52	0.226		**150**	664	

bw: body weight.

(a): Intake calculations for the individual components are based on the use level of 254 mg/kg in feed for chicken for fattening, the species with the highest ratio of feed intake/body weight. The MOE for each component is calculated as the ratio of the reference point (NOAEL) to the intake. The combined margin of exposure (MOET) is calculated for each assessment group as the reciprocal of the sum of the reciprocals of the MOE of the individual substances.

(b): Values **in bold** refer to those components for which the NOAEL value was available, other values (plain text) are NOAELs extrapolated by using read-across.

As shown in Table 4, the MOET for the assessment group 'flavonoids' was 40 resulting from the use of the additive in chicken for fattening at the proposed use levels (254 mg/kg). For the other target species, the MOET was calculated considering the respective daily feed intake and conditions of use. The results are summarised in Table 5.

Table 5: Combined margin of exposure (MOET) for the assessment group 'flavonoids' calculated for the different target animal categories at the proposed use level in feed and maximum safe use levels in feed calculated to ensure a MOET ≥ 100 (500 for cats)

Animal category	Body weight (kg)	Feed intake (g DM/day)	Proposed use level (mg/kg feed)	Lowest MOET	Maximum safe use level (mg/kg feed)[1]
Chicken for fattening	2	158	254	40	102
Laying hen	2	106	379	40	151
Turkey for fattening	3	176	340.5	40	136
Piglet	20	880	400	46	182
Pig for fattening	60	2,200	400	54	217
Sow lactating	175	5,280	400	67	268
Veal calf (milk replacer)	100	1,890	400	113	–
Cattle for fattening	400	8,000	400	100	–
Dairy cow	650	20,000	400	65	259
Sheep/goat	60	1,200	400	100	–

Animal category	Body weight (kg)	Feed intake (g DM/day)	Proposed use level (mg/kg feed)	Lowest MOET	Maximum safe use level (mg/kg feed)(1)
Horse	400	8,000	400	100	–
Rabbit	2	100	400	40	161
Salmon	0.12	2.1	400	111	–
Dog	15	250	400	118	–
Cat	3	60	400	100	80
Ornamental fish	0.012	0.54	400	401	–

DM: dry matter.

Considering the magnitude of the MOET, the additive is safe at the proposed use level of 400 mg/kg complete feed for veal calf (milk replacer), cattle for fattening, sheep, goat, horse, salmon, dog and ornamental fish. For the other species, the maximum safe use levels in feed were calculated in order to ensure a MOET $\geq$ 100 and $>$ 500 for cats, considering their unusually low capacity for glucuronidation (Court and Greenblatt, 1997; Lautz et al., 2021). The calculated maximum safe levels in feed are shown in Table 5.

No specific proposals have been made by the applicant for the use level in water for drinking. The Panel considers that the use in water for drinking is safe provided that the total daily intake of the additive does not exceed the daily amount that is considered safe when consumed via feed (EFSA FEEDAP Panel, 2010).

Furocoumarins (5-MOP).

Low concentrations of 5-MOP (0.021–0.034%) were detected in the additive under assessment and other furocoumarins were below the limit of detection (< 20 mg/kg, 0.002%). The use of bitter orange extract at the proposed use level in feed would result in an intake of 5-MOP up to 7.8 μg/kg bw for poultry, 6.8 μg/kg bw for pigs, 4.8 μg/kg bw for ruminants, 3.1 μg/kg bw for horse, 7.7 μg/kg bw for rabbit and 2.7 μg/kg bw for fish.[27]

Furocoumarins occur in citrus by-products, which are used in diets at concentrations up to 30% depending on the target species.[28] Taking into account an inclusion level of 10% for poultry and 20% for the other species and considering the default values for feed intake according to the guidance on the safety of feed additives for target species (EFSA FEEDAP Panel, 2017b), the daily intake of citrus by-products[29] has been estimated to be 7.9 g DM/kg bw for poultry, 8.8 g DM/kg bw for pigs, 6.2 g DM/kg bw for ruminants, and 4 g DM/kg bw for horse, 10 g DM/kg bw for rabbit and 3.6 g DM/kg bw for fish.

Based on the literature data on the occurrence of furocoumarins in citrus peel (e.g. 0.003% in lemon, 0.02% in grapefruit, 0.057% in mandarin and 0.009% in orange according to Russo et al., 2014; Mercolini et al., 2013; Ramirez-Pelayo et al., 2019) in citrus pulp (e.g. 0.0003% in grapefruit and 0012% in mandarin as reported by Chebrolu et al., 2016; Scordino et al., 2011), and in citrus seeds (0.001% in lemon, Miyake et al., 1999), the occurrence of furocoumarins and methoxycoumarins in citrus by-products was estimated to be 0.0018%.[30] Based on citrus by-product intake (see above), the intake of furocoumarins and methoxycoumarins via feed was calculated to be 140 μg/kg bw per day for poultry, 160 μg/kg bw per day for pigs, 100 μg/kg bw per day for ruminants and 70 μg/kg bw per day for horse, 180 μg/kg bw per day for rabbit and 65 μg/kg bw per day for fish.

These concentrations are about 20-fold higher than those resulting from the high use level of bitter orange extract in feed as proposed by the applicant. For dog, cat and ornamental fish not normally exposed to citrus by-products, no conclusion can be drawn.

[27] Intake values calculated considering the maximum concentration of 5-MOP in the additive (0.034%), the default values for feed intake (Table 5), the proposed use levels in feed for the different species (Table 5) and that complete feed contains 88% DM except milk replacer for veal calves (94.5%).

[28] Technical dossier/Supplementary information July 2020/SIn FAD-2010-0322-request of clarification.

[29] Composition of fresh citrus by-products: 62.5% citrus peel, 32.5% pulp and 5% seeds. Similar proportions are assumed in dried citrus by-products.

[30] Composition of fresh citrus by-products: 62.5% citrus peel, 32.5% pulp and 5% seeds. Assuming similar proportions in dried citrus by-products, the occurrence of furocoumarins in citrus by-products is calculated to be 0.0018%. (0.003% × 0.625 + 0 × 32.5 + 0.0001 × 0.05). The value for lemon peel was selected for the calculations, as most data collected from the literature are for this *Citrus* species.

Conclusions on safety for the target species

The FEEDAP Panel concludes that the extract of *Citrus* × *aurantium* fruit under assessment is safe up to the maximum proposed use levels of 400 mg/kg for veal calf (milk replacer), cattle for fattening, sheep, goat, horse and salmon. For the other species, the calculated maximum safe concentration in complete feed is 102 mg/kg for chicken for fattening, 151 mg/kg for laying hen, 136 mg/kg for turkey for fattening, 182 mg/kg for piglet, 217 mg/kg for pig for fattening, 268 mg/kg for sow, 259 mg/kg for dairy cow and 161 mg/kg for rabbit. These target species are fed citrus by-products as part of normal daily feed. For these species, the use of the bitter orange extract in feed is not expected to increase the exposure to furocoumarins to a relevant extent (< 5%). For dog, cat and ornamental fish not normally exposed to citrus by-products, no conclusion can be drawn.

The Panel considers that the use in water for drinking is safe provided that the total daily intake of the additive does not exceed the daily amount that is considered safe when consumed via feed, except for dog, cat and ornamental fish.

3.3.3.1. Safety for the consumer

The whole mature or immature fruits of *Citrus* × *aurantium* and their preparations are used traditionally for producing foods, e.g. preserves (especially bitter orange marmalade), syrups, liqueurs and candied orange peel (EFSA ESCO, 2009; EFSA Scientific Committee, 2014). Due to its specific tart flavour, the dried peel of the fruit is used as a seasoning and to flavour food (Burdock, 2009; EFSA ESCO, 2009). Although individual consumption figures for the EU are not available, the Fenaroli's handbook of flavour ingredients (Burdock, 2009) cites values of individual consumption of 2.4 mg/kg bw per day for the peel.

Data on the natural occurrence of the glycosides naringin and hesperidin and the aglycon naringenin in citrus fruits or vegetables are presented in an EFSA CEF Panel opinion (EFSA CEF Panel, 2010). For example, for naringenin and hesperidin levels of 73–865 mg/L and 36–528 mg/L have been reported for grapefruit juice and orange juice, respectively. Concentrations of naringenin in tomatoes amount to 8–42 mg/kg (EFSA CEF Panel, 2010). For (-)-synephrine, concentration ranges of 55–160 mg/kg and 37–85 mg/kg have been reported for mandarin juice and orange juice, respectively, in a BfR-opinion (BfR, 2012).

Concerning furocoumarins intake from food, in the UK, the furocoumarin intake has been estimated by the Committee on Toxicity (COT) to be up to 1.2 mg per person per day corresponding to approximately 0.02 mg/kg bw per day for 60 kg bw (COT, 1996 as cited by SKLM, 2010). In Germany, the estimated average total daily intake of furocoumarins from non-flavoured and flavoured foods is approximately 0.56 mg/person per day (approximately 0.01 mg/kg bw per day for 60 kg bw), flavoured foods contributing to about 10% to the total intake (SKLM, 2006, 2010). Reported exposure levels for furocoumarin intake from food for the general population are below the phototoxicity threshold dose (0.25 mg/kg bw for 60 kg bw) and have not been associated with an additional risk of skin cancer.

No data on residues in products of animal origin were made available for any of the constituents of the extract. However, the FEEDAP Panel recognises that the individual constituents of bitter orange extract are expected to be extensively metabolised and excreted in the target species and are not expected to accumulate in animal tissues and products (see Section 3.3.1). Therefore, a relevant increase of the uptake of these compounds by humans consuming products of animal origin is not expected.

Considering the direct food use of bitter orange fruits and their preparations and the natural occurrence of their major components in food, it is unlikely that consumption of products from animals given bitter orange extract at the proposed maximum use level would significantly increase human background exposure.

Consequently, no safety concern would be expected for the consumer from the use of bitter orange extract up to the highest safe use level in feed for the target animals.

3.3.3.2. Safety for user

No specific data were provided by the applicant regarding the safety of the additive for users.

The applicant produced a safety data sheets[31] for bitter orange extract where hazards for users have been identified.

The extract under assessment should be considered as irritant to skin and eyes and the respiratory tract and as a skin sensitiser. Since the additive contains 5-MOP, the additive may cause phototoxicity after skin contact (NTP, 2000).

3.3.3.3. Safety for the environment

Citrus × *aurantium* is a native species to Europe where it is widely grown both for commercial and decorative purposes. Use of the extract under the proposed conditions of use in animal production is not expected to pose a risk for the environment.

3.4. Efficacy

Citrus aurantium L. subspecies *amara* L. (orange bitter) and its extracts are listed in Fenaroli's Handbook of Flavour Ingredients (Burdock, 2009).

Since *Citrus* × *aurantium* and its extracts are recognised to flavour food and their function in feed would be essentially the same as that in food, no further demonstration of efficacy is considered necessary.

4. Conclusions

The additive is safe up to the maximum proposed use levels of 400 mg/kg for veal calf (milk replacer), sheep, goat, horse and salmon. For the other species, the calculated maximum safe concentration in complete feed is 102 mg/kg for chicken for fattening, 151 mg/kg for laying hen, 136 mg/kg for turkey for fattening, 182 mg/kg for piglet, 217 mg/kg for pig for fattening, 268 mg/kg for sow, 259 mg/kg for dairy cow and 161 mg/kg for rabbit. These target species are fed citrus by-products as part of normal daily feed. For these species, the use of the bitter orange extract in feed is not expected to increase the exposure to furocoumarins to a relevant extent (< 5%). For dog, cat and ornamental fish not normally exposed to citrus by-products, no conclusion can be drawn.

The FEEDAP Panel considers that the use in water for drinking is safe provided that the total daily intake of the additive does not exceed the daily amount that is considered safe when consumed via feed, except for dogs, cats and ornamental fish.

No concerns for consumer safety were identified following the use of the additive up to highest safe level in feed for the target animals.

The extract under assessment should be considered as irritant to skin and eyes and the respiratory tract and as a skin sensitiser. Since the additive contains 5-MOP, the additive may cause phototoxicity after skin contact.

The use of the extract in animal feed under the proposed conditions is not expected to pose a risk for the environment.

Since bitter orange extract from *Citrus* × *aurantium* is recognised to flavour food, and its function in feed would be essentially the same as that in food, no further demonstration of efficacy is considered necessary.

5. Documentation as provided to EFSA/Chronology

Date	Event
05/11/2010	Dossier received by EFSA. Botanically defined flavourings from Botanical Group 08 – Sapindales for all animal species and categories. Submitted by Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG) and registered with the Question number EFSA-Q-2010-01517
14/12/2010	Reception mandate from the European Commission
26/02/2011	EFSA informed the applicant (EFSA ref. 7150727) that, in view of the workload, the evaluation of applications on feed flavourings would be re-organised by giving priority to the assessment of the chemically defined feed flavourings, as agreed with the European Commission

[31] Technical dossier/Supplementary Information March 2020/Annex_XIV_SIn reply_bitter_orange_extract_MSDS. The product is not classified as dangerous according to Regulation (EC) 1272/2008 (CLP). In solid form (dust) possible eye and respiratory irritant.

Date	Event
24/06/2015	Technical hearing during risk assessment with the applicant according to the "EFSA's Catalogue of support initiatives during the life-cycle of applications for regulated products": data requirement for the risk assessment of botanicals
17/06/2016	Technical hearing during risk assessment with the applicant according to the "EFSA's Catalogue of support initiatives during the life-cycle of applications for regulated products". Discussion on the ongoing work regarding the pilot dossiers BDG08 and BDG 09
27/04/2017	Trilateral meeting organised by the European Commission with EFSA and the applicant FEFANA on the assessment of botanical flavourings: characterisation, substances of toxicological concern present in the botanical extracts, feedback on the pilot dossiers
19/03/2018	Application validated by EFSA – Start of the scientific assessment
20/06/2018	Comments received from Member States
27/02/2019	Partial withdrawal by applicant (EC was informed) for the following additives: Amyris oil, Cashew oil, Neroli bigarade oil, Petitgrain bigarade absolute, Mandarin terpenes, Grapefruit oil expressed, Grapefruit extract (sb), Grapefruit extract.
03/05/2019	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterization, safety for the target species, safety for the consumer, safety for the user, safety for the environment*
26/03/2020	Reception of supplementary information from the applicant (partial submission)
03/11/2020	Reception of supplementary information from the applicant (partial submission)
12/03/2021	The application was split and a new EFSA-Q-2021-00144 was assigned to the preparation included in the present assessment
17/03/2021	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives - Scientific assessment re-started for the preparation included in the present assessment
02/04/2021	Partial withdrawal by applicant (EC was informed) for the following additive: olibanum extract (wb)
23/06/2021	Opinion adopted by the FEEDAP Panel on bitter orange extract (EFSA-Q-2021-00144). End of the Scientific assessment for the preparation included in the present assessment.

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Abbreviations

ADME	absorption, distribution, metabolism and excretion
AUC	area under the curve
BDG	botanically defined group
BfR	Bundesinstitut für Riskobewertung
BMD	benchmark dose
$BMDL_{10}$	BMD lower confidence limit for a benchmark response of 10%
bw	body weight
CAS	Chemical Abstracts Service
CBA	component-based approach
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
COT	Committee on Toxicology
CG	chemical group
CFU	colony forming unit
Cmax	maximum (peak) concentration
DM	dry matter
EEIG	European economic interest grouping
ESCO	EFSA Scientific Cooperation
EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
FEMA	Flavor Extract Manufacturers Association
FFAC	Feed Flavourings authorisation Consortium of (FEFANA) the EU Association of Specialty Feed Ingredients and their Mixtures
FLAVIS	the EU Flavour Information System
FL-No	FLAVIS number
HPLC	high-performance liquid chromatography
LC-MS	liquid chromatography–mass spectrometry
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
MOE	margin of exposure
MOET	combined margin of exposure (total)
5-MOP	5-methoxypsoralen
NOAEL	no observed adverse effect level
NOEL	no observed adverse effect level
NTP	National Toxicology Program
PCBs	polychlorobiphenyls
PCDD/F	polychlorinated dibenzo-p-dioxins and dibenzofurans
PhEur	European Pharmacopoeia
QPS	Qualified Presumption of Safety
QSAR	quantitative structure-activity relationship
RRLC-ESI-MS/MS	rapid resolution liquid chromatography–electrospray ionisation-tandem mass spectrometry
SC	EFSA Scientific Committee
SKLM	Senate Commission of the Deutsche Forschungsgemeinschaft
TEQ	toxic equivalent
UHPLC–HRMS	ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry
UPLC-MS/MS	ultra performance liquid chromatography tandem mass spectrometry
UV(A)	ultraviolet (A)
WHO	World Health Organization

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for buchu leaves oil, olibanum extract (wb), lime oil, petigrain bigarade oil, bitter orange extract of the whole fruit, lemon oil expressed, lemon oil distilled (residual fraction), lemon oil distilled (volatile fraction), orange oil cold pressed, orange terpenless (concentrated 4 times), orange terpenless (concentrated 10 times), orange terpenless (folded), orange terpenes, mandarin oil and quebracho extract (wb) from botanically defined flavourings Group (BDG 08) – Sapindales

In the current grouped application an authorisation is sought under Articles 4(1) and 10(2) for *buchu leaves oil, olibanum extract (wb), lime oil, petigrain bigarade oil, bitter orange extract of the whole fruit, lemon oil expressed, lemon oil distilled (residual fraction), lemon oil distilled (volatile fraction), orange oil cold pressed, orange terpenless (concentrated 4 times), orange terpenless (concentrated 10 times), orange terpenless (folded), orange terpenes, mandarin oil and quebracho extract (wb)* from *botanically defined flavourings group 08 (BDG 08)1*, under the category/functional group 2(b) 'sensory additives'/flavouring compounds', according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for all animal species. For each preparation the Applicant indicated the corresponding phytochemical marker(s) and the corresponding range of content. The *feed additives* are intended to be incorporated into *feedingstuffs* or drinking *water* directly or through flavouring *premixtures* with no proposed minimum or maximum levels. However, the Applicant suggested the typical maximum inclusion level of the *feed additives* of 25 mg/kg *feedingstuffs*.

For the quantification of the phytochemical markers *d-limonene* and *d,l-isomenthone* in *buchu leaves oil* and *d-limonene* in *orange terpenless (concentrated 10 times)* oil, the Applicant submitted a method using gas chromatography coupled with flame ionisation detection (GC-FID) based on the generic standard ISO 11024. The quantification is performed by using the normalisation approach for the estimation of the area percentage of individual components. The Applicant tested the method, following an experimental design proposed by the EURL, and obtained satisfactory performance characteristics.

For the quantification of the phytochemical markers *11-keto-β-boswellic acid* and *3-O-acetyl-11-keto-β-boswellic acid* in *olibanum extract (wb)*, the Applicant submitted a method using high performance liquid chromatography (HPLC) with spectrophotometric (UV) detection at 250 nm described in the European Pharmacopeia monograph for Indian Frankincense (Olibanum indicum). The quantification of *11-keto-β-boswellic acid* and *3-O-acetyl-11-keto-β-boswellic acid* is performed by means of specific expressions and is indicated as percentage content (absolute value). The Applicant, using the HPLC-UV method, analysed 5 batches of the *feed additive* obtaining results within the proposed specifications.

For the quantification of the phytochemical marker *d-limonene* in *lime oil* the Applicant submitted a GC-FID method based on the corresponding standard ISO 3519:2005 for the characterisation of the "oil of lime distilled, Mexican type (Citrus aurantifolia [Christm.] Swingle)". The quantification is performed using the normalisation approach for the estimation of the area percentage of individual components. The Applicant presented a chromatogram and the specific analytical procedure for the analysis of *d-limonene* in *lime oil*.

For the quantification of the phytochemical markers *linalyl acetate* and *linalool* in *petigrain bigarade oil* the Applicant submitted a GC-FID method based on the corresponding standard ISO 8901:2003 for "Oil of bitter orange petitgrain, cultivated (Citrus aurantium L.)". The quantification is performed using the normalisation approach for the estimation of the area percentage of individual components. The Applicant presented a chromatogram and the specific analytical procedure for the analysis of *linalyl acetate* and *linalool* in *petigrain bigarade oil*.

For the quantification of the phytochemical marker *naringin* in *bitter orange extract of the whole fruit* the Applicant submitted a single-laboratory validated and further verified method based on HPLC-UV (284 nm). The method has been developed for the determination of total flavonoids (including *naringin* alone) in a mixture of citrus flavonoids. The quantification of *naringin* is performed using the normalisation approach for the estimation of the area percentage of individual components. The Applicant provided validation and verification studies demonstrating the applicability of the method for

the analysis of pure *naringin*. Furthermore, *naringin* has been satisfactory quantified in the *feed additive* by the proposed method in 5 different lots of *bitter orange extract of the whole fruit*.

For the quantification of the phytochemical marker *d-limonene* in *lemon oil expressed, lemon oil distilled (residual fraction)* and *lemon oil distilled (volatile fraction)* the Applicant submitted a GC-FID method based on the corresponding standard ISO 855:2003 for "Oil of lemon (Citrus limon (L.) Burm. f.), obtained by expression". The quantification is performed using the normalisation approach for the estimation of the area percentage of individual components. The Applicant presented a chromatogram and the specific analytical procedure for the analysis of *d-limonene* in *lemon oil expressed, lemon oil distilled (residual fraction)* and *lemon oil distilled (volatile fraction)*.

For the quantification of the phytochemical marker *d-limonene* in *orange oil cold pressed*, *orange terpenless (concentrated 4 times)* oil, *orange terpenless (folded)* oil and *orange terpenes* oil the Applicant submitted a GC-FID method based on the corresponding standard ISO 3140:2019 for "Essential oil of sweet orange expressed (Citrus sinensis (L.))". The quantification is performed using the normalisation approach for the estimation of the area percentage of individual components. The Applicant presented a chromatogram and the specific analytical procedure for the analysis of *d-limonene* in *orange oil cold pressed*, *orange terpenless (concentrated 4 times)* oil, *orange terpenless (folded)* oil and *orange terpenes* oil.

For the quantification of the phytochemical marker *d-limonene* in *mandarin oil* the Applicant submitted a GC-FID method based on the corresponding standard ISO 3528:2012 for "Essential oil of mandarin, Italian type (Citrus reticulate Blanco)". The quantification is performed using the normalisation approach for the estimation of the area percentage of individual components. For *mandarin oil*, the Applicant presented a chromatogram and the specific analytical procedure for the analysis of the *d-limonene* in *mandarin oil*.

For the quantification of the phytochemical marker *tannins* in *quebracho extract (wb)* the Applicant submitted the method ISO 14088:2020 "Leather - Chemical tests - Quantitative analysis of tanning agents by filter method". The method proposed is suitable for the determination of tanning agents in all vegetable tanning products and it is based on indirect gravimetric analysis of tanning agents with fixing of the absorbent compounds in low chromed hide powder. The quantification of *tannins* in *quebracho extract (wb)* is performed by means of specific expressions and is indicated as percentage content (absolute value). Furthermore, the Applicant provided satisfactory results for the analysis of *tannins* in 3 batches of *quebracho extract (wb)*.

The accurate quantification of the *feed additives* in *premixtures* and *feedingstuffs* is not achievable experimentally and the Applicant did not provide experimental data to determine the *feed additives* in *water*. Therefore, the EURL cannot evaluate nor recommend any method for official control to quantify the *feed additives* in *premixtures*, *feedingstuffs* and *water*.

Based on the information above, the EURL recommends for official control: (i) the GC-FID method based on the generic standard ISO 11024 for the quantification of *d-limonene* and *d,l-isomenthone* in *buchu leaves oil* and *d-limonene* in *orange terpenless (concentrated 10 times)* oil; (ii) the HPLC-UV method described in the European Pharmacopeia monograph "Indian Frankincense (Olibanum indicum)" for the quantification of *11-keto-β-boswellic acid* and *3-O-acetyl-11-keto-β-boswellic acid* in *olibanum extract (wb)*; (iii) the GC-FID method based on the standard ISO 3519:2005 for the quantification of *d-limonene* in *lime oil*; (iv) the GC-FID method based on the standard ISO 8901:2003 for the quantification of *linalyl acetate* and *linalool* in *petigrain bigarade oil*; (v) the HPLC-UV single-laboratory validated and further verified method for the quantification of *naringin* in *bitter orange extract of the whole fruit*; (vi) the GC-FID method based on the standard ISO 855:2003 for the quantification of *d-limonene* in *lemon oil expressed, lemon oil distilled (residual fraction)* and *lemon oil distilled (volatile fraction)*; (vii) the GC-FID method based on the standard ISO 3140:2019 for the quantification of *d-limonene* in *orange oil cold pressed*, *orange terpenless (concentrated 4 times)* oil, *orange terpenless (folded)* oil and *orange terpenes* oil; (viii) the GC-FID method based on the standard ISO 3528:2012 for the quantification of *d-limonene* in *mandarin oil*; and (ix) the indirect gravimetric analysis of tanning agents with fixing of the absorbent compounds in low chromed hide powder described in ISO 14088:2020 for the quantification of *tannins* in *quebracho extract (wb)*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761) is not considered necessary.