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Safety and efficacy of a feed additive consisting of an aqueous extract of *Citrus limon* (L.) Osbeck (lemon extract) for use in all animal species (Nor-Feed SAS)

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Abstract

Following a request from the European Commission, the 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 an aqueous extract of *Citrus limon* (L.) Osbeck (lemon extract) when used as a sensory additive in feed for all animal species. The FEEDAP Panel concluded that the additive under assessment is safe for all animal species up to the maximum proposed use levels of 1,000 mg/kg complete feed and 250 mg/kg water for drinking. No concerns for consumers were identified following the use of lemon extract up to the highest safe level in feed. The additive should be considered a skin and eye irritant, and a potential corrosive. The use of the extract in animal feed under the proposed conditions was not expected to pose a risk for the environment. Lemon 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 limon* L. Osbeck, lemon extract, eriocitrin, hesperidin, limonoids, safety

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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 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 Nor-Feed SAS[2] for re-evaluation of the product lemon extract from *Citrus limon* (L.) Burm. f.[3] when used as a feed additive for all animal species (category: sensory additives; functional group: flavourings).

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 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 12 September 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 the product lemon extract from *C. limon*, when used under the proposed conditions of use (see Section 3.2.4).

1.2. Additional information

Lemon extract from *Citrus limon* (L.) Burm f. 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 feed additive in the EU.

There is no specific EU authorisation for any *C. limon* ssp. preparation when used to provide flavour in food. However, according to Regulation (EC) No 1334/2008[4], flavouring preparations produced from food, may be used without an evaluation and approval as long as 'they do not, on the basis of the scientific evidence available, pose a safety risk to the health of the consumer, and their use does not mislead the consumer'.

The FEEDAP Panel issued an opinion on the safety and efficacy of expressed lemon oil and its fractions from *C. limon* (L.) Osbeck, when used as sensory additives in all animal species (EFSA FEEDAP Panel, 2021).

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[5] in support of the authorisation request for the use of an aqueous extract from *C. limon* (lemon extract) as a feed additive.

The 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 peer-reviewed scientific papers and experts' knowledge, to deliver the present output.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the total polyphenols in lemon extract in animal feed. The Executive Summary of the EURL report can be found in Annex A.[6]

[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] Nor-Feed SAS, 3 rue Amedeo Avogadro, 49070 Beaucouzé, France.
[3] Accepted name: *Citrus limon* (L.) Osbeck, synonym: *Citrus limon* (L.) Burm. F.
[4] 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.
[5] FEED dossier reference: FAD-2010-0130.
[6] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/revised_finrep_fad-2010-0130_lemon_extract.pdf

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of lemon extract from *C. limon* is in line with the principles laid down in Regulation (EC) No 429/2008[7] 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 reported to contain naturally occurring substances of possible concern for human health when used in food and food supplements (EFSA, 2012), Guidance for the preparation of dossiers for sensory additives (EFSA FEEDAP Panel, 2012a), 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 for establishing the safety of additives for the consumer (EFSA FEEDAP Panel, 2017c), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012b), Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018), Guidance document on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals (EFSA Scientific Committee, 2019a), Genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019b) and Guidance on the use of the Threshold of Toxicological Concern approach in food safety assessment (EFSA Scientific Committee, 2019c).

3. Assessment

The additive under assessment, lemon extract, is an aqueous extract derived from the fruit of *Citrus limon* (L.) Osbeck (isonym *C. limon* (L.) Burm. f.; originating from the south of Spain) and 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

Lemons belong to the Rutaceae (citrus family) and are cultivated worldwide in tropical, semi-tropical and warm temperate countries, including the Mediterranean region. They are thought to have originated as a natural hybrid of citron (*Citrus medica*) and the sour orange (*Citrus aurantium*). Commercial production has subsequently resulted in the production of numerous cultivars used for culinary purposes or for the extraction of essential oil. The currently preferred botanical name for lemon is *Citrus limon* (L.) Osbeck, but the isonym *Citrus limon* (L.) Burm. f. is still widely used.

The additive under assessment is an aqueous extract of the material remaining after the extraction of juice from the fruit. [redacted] to give a liquid additive with the required dry matter content (< 55% moisture with > 42% osidic compounds).[8]

3.2. Characterisation

3.2.1. Characterisation of the additive

The extract under assessment is a dark orange to yellow liquid. It has a viscosity of 10–25 mPas (20°C), a pH < 4.0 and a specific gravity of 1.30 g/cm^3. It is miscible with water. Lemon extract from *C. limon* is identified with the single Chemical Abstracts Service (CAS) number 84929-31-7, the EINECS number 284-515-8, the Flavor Extract Manufacturers Association (FEMA) 2623 and the Council of Europe (CoE) number 139a.

The additive is standardised in the content of total polyphenols (selected as a phytochemical marker), eriocitrin (selected as a phytochemical marker) and hesperidin, in order to meet the following

[7] 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.

[redacted]

specification: total polyphenols ≥ 1% (determined by spectrophotometry at 760 nm, expressed as pyrogallol equivalents), eriocitrin ≥ 4,000 mg/kg and hesperidin ≥ 2,000 mg/kg (both determined by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection).[9] Analysis of five batches of lemon extract showed an average content of 1.12% (range: 1.07–1.2%) of total polyphenols.[10] The same batches complied with proposed specifications for eriocitrin ([redacted]) and hesperidin ([redacted]) when determined by HPLC-UV.[11] The additive contains 1% propionic acid as preservative.

The applicant provided the full characterisation of the five batches of the additive (originating from the South of Spain,[12] containing 1% propionic acid).[13] In these batches, the dry matter content was on average 52.05% (range: 51.2–52.8%) and the water content 47.75% (range: 47.19–48.35%). Polar compounds accounted for 51.92% (range: 51.24–52.41%) and apolar compounds (palmitic acid, stearic acid and limonoids) for 0.33% (range: 0.22–0.41%) of the additive. The extract does not contain any essential oil, as demonstrated by gas chromatography–mass spectrometric (GC–MS) analysis.[14]

The polar fraction was characterised by high-performance liquid chromatography using diode array (DAD), evaporative light scattering detector (ELSD) and mass spectrometry (MS) detector. The polar fraction mainly consists of carbohydrates, accounting on average for 44.3% of the extract (43.4–45.1%, quantified by HPLC-ELSD in five batches). [redacted] peaks were identified [redacted] as galacturonans and rhamnogalacturonans,[15] derived from the pectin fraction of the fruit. The content of citric acid determined by titration in five batches of the additive was on average 5.98% ([redacted]).[11] Citric acid determined by ion chromatography with electrochemical detection (IC-EC) was 5.34% in one additional batch.[16]

The composition of the additive (including 1% propionic acid) is summarised in Table 1. The identified constituents account for about 100% of the composition of the additive.

Table 1: Characterisation of the additive consisting of an aqueous extract derived from the fruit of *Citrus limon* (L.) Osbeck based on the analysis of five batches. The results are expressed as % (w/w)

Constituent	Method	Mean	Range
		% (w/w)	% (w/w)
Water		47.8	47.2–48.4
Dry matter	Freeze-drying	52.1	51.2–52.8
Polar compounds		51.9	51.2–52.4
Carbohydrates	HPLC-ELSD	44.3	43.4–45.1
Citric acid	Titrable acidity	5.98	4.84–6.75
Propionic acid		1.0	1.0
Total polyphenols	Spectrophotometry (760 nm)	1.12	1.07–1.2
Phenolic compounds	HPLC-ELSD	0.58	0.51–0.67
Other secondary metabolites	HPLC-ELSD	0.07	0.03–0.11
Non-polar compounds(1)	GC–MS	0.33	0.22–0.41

HPLC: high-performance liquid chromatography; ELSD: evaporative light scattering detector; GC–MS: gas chromatography–mass spectrometry.
(1): Non-polar compounds include palmitic acid, stearic acid and limonoids.

Further analyses were provided to characterise secondary plant metabolites, i.e. flavonoids present in the polar fraction and limonoids present in the non-polar fraction. Seven compounds accounting

[9] Technical dossier/Supplementary information January 2021. The risk assessment is not based on these specifications but on the analytical data reported in Table 2.
[10] Technical dossier/Supplementary information January 2021/Annexes_47 to 51.
[redacted]
[12] At the time of application, the applicant stated that they sourced the raw material from the south of Spain (varieties used: Fino and Verna).
[13] Technical dossier/Supplementary information/January 2021/Annexes_12 to 16.
[14] Technical dossier/Supplementary information January 2020/Supplementary information request on Lemon extract, pp. 8–9.
[redacted]

together for 0.59% of the extract were identified in the polar fraction by liquid chromatography electrospray multi stage mass spectrometry (LC-ESI-MS^n) as phenolic compounds and quantified using HPLC-ELSD in five batches.[17] In the same fraction, unidentified phenolic compounds were detected accounting together for 0.068% of the extract. Among them, eight compounds were individually quantified with average concentrations between 0.0017% and 0.0029%). Limonin (71.2 mg/kg, range: 36.8–91.5 mg/kg) and nomilin (50.9 mg/kg, range: 14.8–112.7 mg/kg) were determined in the non-polar fraction by HPLC in five batches of lemon extract, as representative of the class of limonoids.[18] The results of the analysis of secondary metabolites in five batches of lemon extract are summarised in Table 2.

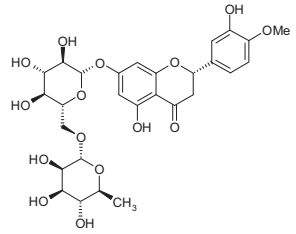
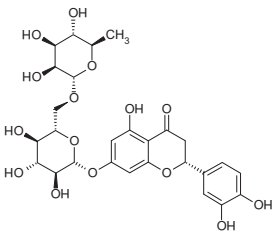
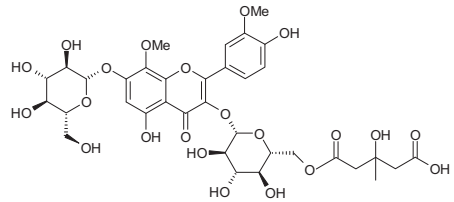
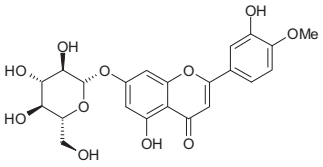
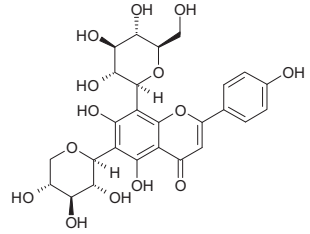
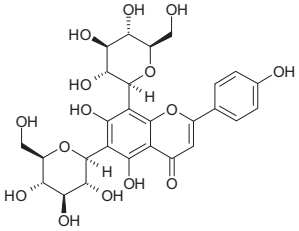
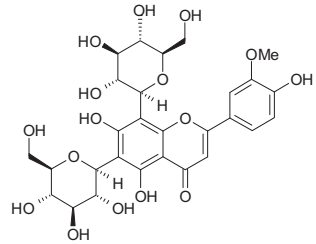
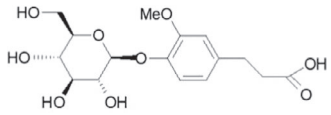
Table 2: Characterisation of the secondary metabolites of an aqueous extract derived from the fruit of *Citrus limon* (L.) Osbeck based on the analysis of five batches

Constituent	CAS no	Mean	Range
		mg/kg	mg/kg
• Secondary metabolites (polar fraction)[(a)]			
o Vicenin-2	23666-13-9	11.6	2.5–16.0
o Stellarin-2	63975-58-6	11.6	2.8–16.0
o Eriocitrin[(b)]	13463-28-0	4,020	3,600–4,600
o Dihydroferulic acid glucoside	47787-63-5	13.1	3.4–18.0
o Diosmetin-7-*O*-glucoside	20126-59-4	9.9	1.2–16.0
o Hesperidin[(b)]	520-26-3	842	400–1,200
o Limocitrin 3-[6″-(3-hydroxy-3-methylglutaryl)glucoside]-7-glucoside	–	980	700–1,300
Sum		5,888	5,149–6,764
• Other secondary unidentified metabolites (sum)	–	680	300–1,100
• Secondary metabolites (apolar fraction)			
o Limonin	1180-71-8	71.2	36.8–91.5
o Nomilin	1063-77-0	50.9	14.8–112.7

CAS: Chemical Abstracts Service.
(a): Analysed and quantified by high-performance liquid chromatography (HPLC) with evaporative light scattering detector (ELSD).
(b): There is a difference between the content of eriocitrin and hesperidin determined by HPLC-UV and HPLC-ELSD. According to the applicant, the difference could be explained considering the different analytical methods, including sample preparation (solvent of extraction), and their purpose: the HPLC-UV method is routinely used to demonstrate compliance with specifications and is based on calibration with an external standard, whereas the HPLC-ELSD method was applied to fully characterise the additive. The results are expressed as the area per cent of the corresponding chromatographic peak (% area), assuming the sum of chromatographic areas of all detected peaks as 100%.

The chemical structures of the compounds identified in the polar fraction are shown in Figure 1, together with vicenin-1, a compound structurally related to vicenin-2.

[17] Technical dossier/Supplementary information/January 2021/Annex_2.
[18] Technical dossier/Supplementary information/January 2021/Annex_11 and supplementary information June 2021.

Hesperidin (CAS 520-26-3); Hesperetin-7-O-rutinoside 	Eriocitrin (CAS 13463-28-0); Eriodictyol-7-O-rutinoside 
Limocitrin 3-[6″-(3-hydroxy-3-methylglutaryl)glucoside]-7-glucoside 	Diosmetin-7-O-glucoside (CAS 20126-59-4) 
Vicenin-1[(a)] 	Vicenin-2 (CAS 23666-13-9) 
Stellarin-2 (CAS 63975-58-6) 	Dihydroferulic acid-4-O-glucoside (CAS 47787-63-5) 

(a): not present in the additive, and structurally related to vicenin-2.

Figure 1: Structures of the secondary metabolites identified in the polar fraction: flavonoid glycosides and dihydroferulic acid-4-*O*-glucoside

Substances of concern

The EFSA Compendium reports the occurrence of phellopterin, 5- and 8-geranoxypsoralen in the peel of *C. limon* and of several furocoumarins (psoralen, 5-methoxypsoralen (bergapten), 8-methoxypsoralen (xanthotoxin), 5,8-dimethoxypsoralen (isopimpellin), imperatorin, oxypeucedanin in the essential oil from the peel of *C. limon* (EFSA, 2012). The applicant performed a literature search to identify coumarins and furocoumarins present in *C. limon* and its extracts.[19] Based on studies investigating the identification and quantitation of coumarins and furocoumarins in *Citrus* species, bergamottin was identified as the major compound in *C. limon* (Dugrand-Judek et al., 2015), lemon juice (Melough et al., 2017) and lemon polar extract (ethanol/water 60/40, %v/v) (Ledesma-Escobar et al., 2015). In addition to bergamottin, the presence of bergaptol has been reported in lemon juice (Melough et al., 2017). The applicant also performed a literature search on the occurrence of limonoids (limonin and nomilin) in *Citrus* species.[20] Limonoids are widely distributed in many *Citrus*

[19] Technical dossier/Supplementary information/January 2021.
[20] Technical dossier/Supplementary information June 2021.

fruits. Limonin and nomilin occur in different parts of citrus fruits, mainly in the seeds. According to Montoya et al. (2019), the total limonoid content (limonin and nomilin) in the seeds of eight *Citrus* fruits ranged from 1,856 to 7,497 mg/kg (average: 3,452 mg/kg). Russo et al. (2016) reported a content of limonin and nomilin of 4,672 mg/kg in *Citrus bergamia* Risso (bergamot) seeds.

Bergamottin was not detected in eight batches of the additive based on HPLC analysis with UV detection and the use of authentic standard (LOD 0.99 μg/mL, LOQ 3.31 μg/mL).[21] Bergaptol (0.29 mg/kg) was determined in one batch of lemon extract by a non-validated method.[22] Subsequent studies using authentic standard did not confirm the presence of bergaptol in five batches of the additive under assessment (LOD 0.03 μg/mL, LOQ 0.1 μg/mL).[23] Synephrine was not detected in three batches of lemon extract by HPLC-UV.[24] The presence of limonin and nomilin was detected in all batches of the extract (see Table 2).[20]

3.2.2. Impurities

Heavy metals and arsenic were not detectable in two batches of the additive.[25]

Mycotoxin analysis on three batches of the additive showed aflatoxin B1 ranging between < 0.1 and 0.2 μg/kg, aflatoxins B2, G1 and G2, each < 0.1 μg/kg.[26] In the same batches, polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (DL-PCBs) and non-DL-PCBs were found below the LOD. The sum of dioxins calculated according to Regulation EU 2017/644 ranged between 0.0767 and 0.0798 ng/kg, the sum of dioxin and DL-PCBs between 0.118 and 0.122 ng/kg and the sum on non-DL-like PCBs 0.0728 and 0.0757 μg/kg.[26] Multiresidue analysis of a recent batch showed that all pesticides were below the LOQ, except fludioxonil (1.2 ± 0.6 mg/kg), imazalil (3.6 ± 1.8 mg/kg), propiconazole (0.59 ± 0.30 mg/kg), pyrimethanil (1.2 ± 0.6 mg/kg), pyriproxyfen (0.061 ± 0.031 mg/kg), thiabendazole (0.057 ± 0.029 mg/kg),[27] which were below the EU maximum residue levels (MRLs) for lemon defined by relevant regulations.[28]

The analysis of recent batches showed that yeasts and moulds were < 10 CFU/g, *Staphylococcus* coagulase positive and *Salmonella* spp. were absent in 25 g, and *Escherichia coli* was < 1 CFU/g.[29]

3.2.3. Stability

The typical shelf-life of the additive is stated to be at least 12 months. However, no evidence supporting this statement was provided.

3.2.4. Conditions of use

Lemon 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 is 1,000 mg/kg in complete feed and 250 mg/kg in water for drinking.

3.3. Safety

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

The additive under assessment mainly contains carbohydrates (up to 45%), which were identified as galacturonans and rhamnogalacturonans and are not considered of concern. The additive also contains secondary plant metabolites, mainly phenolic compounds (up to 0.68%) and minor concentrations of limonoids (about 0.02% or 200 mg/kg).

The applicant provided a literature search on the absorption, distribution, metabolism and excretion (ADME) and on the toxicology of the main secondary metabolites, the flavonoids eriocitrin, hesperidin and vicenin-2,[30] which is summarised in the following sections.

[21] Technical dossier/Supplementary information/January 2021 and supplementary information June 2021.
[22] Technical dossier/Supplementary information/January 2021/Annex_11.
[23] Technical dossier/Supplementary information June 2021/Supplementary information request on Lemon extract_210621, pp. 4–7.
[24] Technical dossier/Supplementary information January 2021.
[25] Technical dossier/Supplementary information/January 2021/Annexes 18 and 19. Limit of quantification (LOQ): lead 0.02 mg/kg, cadmium and mercury 0.005 and arsenic 0.05.
[26] Technical dossier/Supplementary information/January 2021/Annexes 18, 19 and 20.
[27] Technical dossier/Supplementary information/January 2021/Annex 21.
[28] Technical dossier/Supplementary information/January 2021/Annex 22.
[29] Technical dossier/Supplementary information/January 2021/Annexes 23–28.
[30] Technical dossier/Supplementary information January 2020 and Supplementary information January 2021.

Limited information was provided on the bioavailability of limonoids, which were analytically determined in the extract under assessment.

3.3.1. Absorption, distribution, metabolism and excretion

ADME of flavonoids in laboratory animals and humans

Eriocitrin

In vitro, eriocitrin was degraded by several intestinal bacteria up to 25% after 24 h incubation, giving rise to eriodictyol as confirmed by GC–MS and nuclear magnetic resonance (NMR) (Miyake et al., 1997). Eriodyctiol was metabolised to dihydrocaffeic acid and phloroglucinol by some bacteria. Eriocitrin was completely degraded 15 h after incubation in human faeces, with formation of eriodictyol and 3,4-dihydroxyhydrocinnamic acid. Eriodyctiol showed to be able to permeate the intestinal barrier as demonstrated *in vitro* using the Caco-2 cell model (Kobayashi and Konishi, 2008).

In vivo, 4 h after oral administration of eriocitrin to rats at 75 μmol/kg body weight (bw) eriodictyol, homoeriodictyol and hesperetin were found in plasma and urine after enzymatic hydrolysis, indicating its presence as conjugate derivatives. No eriocitrin was detected; small amounts of dihydrocaffeic acid, resulting from eriodictyol degradation by intestinal bacteria were also identified (Miyake et al., 2000). Metabolites in plasma were in the conjugated form and in urine in both free and conjugated form. A similar profile in plasma was found in humans after ingestion of lemon peel preparations containing as principal flavonoids eriocitrin or its aglycon eriodictyol (Miyake et al., 2006). After enzymatic hydrolysis of plasma, eriodictyol, homoeriodictyol and hesperetin were identified, indicating that they circulate in blood as sulfo- and glucuronide conjugates.

Hesperidin

The absorption and metabolic profile of hesperidin was studied in rats after a single oral administration of 10 mg/kg bw (Nectoux et al., 2019). Portal and abdominal blood was collected from 0 up to 24 h after administration for analysis by LC–MS and MALDI-MS. The intact hesperidin was identified in portal blood and as the glucuronide conjugate in the circulating blood. Sulfo- and glucuronide conjugates of homoeriodictyol and eriodictyol were detected in both portal and circulating blood samples. This study showed that in rats' hesperidin can be absorbed as such and conjugated, and both hesperidin and/or hesperetin are susceptible to methylation and demethylation during the intestinal membrane transport process. Sulfate and glucuronide metabolites were detected in both blood systems. An *in vitro* study (Kobayashi and Konishi, 2008) gives support to the findings of this *in vivo* data. By using the Caco-2 cell line model, the authors evaluated the mechanism of absorption of hesperidin and hesperitin through the intestinal barrier. Both compounds were able to permeate the Caco-2 cell monolayer, although hesperitin in a higher extension as compared with hesperidin. Hesperetin is absorbed both by transcellular transport, which occurs mainly via proton-coupled active transport, and by passive diffusion. The transport of hesperidin is via the paracellular pathway.

The kinetics of hesperidin has also been studied in humans. Human volunteers were enrolled in a study to elucidate the fate of hesperidin and hesperitin in the gastrointestinal tract, namely their biotransformation in the intestine and absorption (Actis-Goretta et al., 2015). Equimolar amounts of hesperetin-7-*O*-rutinoside and hesperetin-7-*O*-glucoside were directly perfused into the proximal jejunum. The hesperitin glucoside was rapidly hydrolysed by brush border enzymes without any contribution from pancreatic, stomach or other secreted enzymes, or from bacterial enzymes. Only 3% of the dose was recovered intact in the perfusate, indicating extensive hydrolysis and high absorption. Hesperetin-7-*O*-glucoside was extensively metabolised in the small intestine. About 50% of the metabolites, consisting of hesperitin aglycone and sulfate and glucuronide conjugates attaining altogether a maximum level of 600 nmol/L 30 min after initiation of the perfusion. About 80% of hesperetin-7-*O*-rutinoside was recovered intact in the perfusate showing very limited hydrolysis or absorption; no hesperetin metabolites were detected in plasma (LOD: 10 nmol/L) and only traces were excreted in urine. Some volunteers also received an oral dose of 25 mg of hesperetin-7-*O*-rutinoside or 19 mg of hesperetin-7-*O*-glucoside. Blood was collected at several times up to 24 h as well as the urine of 24 h. Hesperetin-7-*O*-rutinoside administration originated plasma levels of hesperitin metabolites around 100 nM, while after perfusion none were detected. The ingestion of hesperetin-7-*O*-glucoside originated hesperetin metabolites in plasma at similar levels of those obtained after perfusion. Pharmacokinetic analysis of plasma hesperetin metabolites after oral consumption of hesperetin-7-*O*-rutinoside showed delayed absorption (Tmax = 4.8 ± 2.2 h) compared with hesperetin-

7-*O*-glucoside (Tmax = 0.5 ± 0.1 h). The urinary excretion of 3-(3-hydroxy-4-methoxyphenyl) hydracrylic acid was significantly higher after oral administration of the rutinoside compared with the levels after perfusion of the two glucosides or after oral administration of hesperitin-glucoside. These data highlight the role of the microbiota of the colon in the hydrolysis of the rutinoside moiety, the degradation of the aglycone, the absorption of resultant compounds and their excretion in urine.

Brett et al. (2008) evaluated the absorption, metabolism and excretion of flavanones, including hesperidin, in volunteers after ingestion of 300 g of orange juice (containing 72 mg hesperitin as hesperidin) or 150 g of fresh orange fruit (containing 80 mg of hesperitin as hesperidin). After consumption of fruit or juice, conjugates of hesperitin were identified in plasma, namely the 7- and 3'-*O*-monoglucuronides of hesperitin, two hesperetin diglucuronides and a hesperetin sulfo-glucuronide. Free hesperitin and the rutinoside were not detected. Cmax of plasma total hesperitin was about 30 μg/L at Tmax of 6–7 h for both items. Hesperitin sulfate and glucuronide conjugates were also identified in urine. Similar results were obtained in volunteers given two types of orange juices. Hesperetin-7-*O*-glucuronide, hesperetin-3'-*O*-glucuronide and a sulfate conjugate were detected in plasma. In urine, besides the same metabolites found in plasma, a hesperitin glucuronide sulfate was identified (Silveira et al., 2014). Also Aschoff et al. (2016) gave fresh orange fruit or pasteurised orange juice to volunteers and found in urine conjugates of hesperitin and of its catabolite compound 3-(3'-hydroxy-4'-methoxyphenyl)propionic acid.

Vicenin-2

The ADME of apigenin, the aglycone of vicenin-2, was evaluated in rats (males and females, mature and immature animals) after a single intragastric administration of 10 mg of unlabelled apigenin coupled with 10 μCi [^{3}H]apigenin/kg bw (Gradolatto et al., 2005). Some female animals were bile duct cannulated and bile collected each hour up to 48 h after dosing. Urine was also collected and at necropsy kidneys, liver and intestine were removed. Absorption of apigenin showed to be slow, being radioactivity detected in blood only 9 h after dosing, attaining a maximum blood level at 24 h and an elimination half-life of about 92 h. Analysis of bile collected at different time points revealed enterohepatic circulation of the compound. Twenty-four hours after dosing, about 18% of the radioactivity was excreted in urine, and 40% within 5 days. The cumulative radioactivity in urine and faeces of 10 days both of male and female mature rats was 51% and 12%, respectively. In the 10th day of the study, blood, liver, kidney and intestine had about 1%, 1%, 0.3% and 9% radioactivity, respectively, and the radioactivity present in the organism represented about 25% of the dose administered. The LC–MS analysis of urine showed some quantitative differences in the sulfate and glucuronide conjugates between immature and mature rats and between males and females. The data indicate that absorption of apigenin is slow, undergoes enterohepatic circulation, widely distributes in the organism, is mainly eliminated unaltered in urine, being a low portion as sulfate and glucuronide derivatives, and in a minor extension in faeces, and that there is potential to accumulate in the organism. The FEEDAP Panel notes that this ADME study has been made with apigenin, that is the aglycone of vicenin-2, a C-glycosyl flavonoid, supposed to be more stable to hydrolysis than the *O*-glycosyl compounds, and this can impact the bioavailability of vicenin-2. Thus, it is expected that the absorption of apigenin resulting from ingestion of vicenin-2 would be even lower as compared with its direct ingestion. In the Caco-2 cell line model, Gouvea et al. (2014) showed that vicenin-2 did not permeate the cells, suggesting a poor absorption *in vivo*.

Buqui et al. (2015) investigated the absorption of vicenin-2 in male Wistar rats using the *in situ* single-pass intestinal perfusion technique after oral administration of 180 mg/kg bw. The calculated absorption in 30 min was about 40%. The high dose tested and the model used raises some limitations in the interpretation of data.

Two groups of human volunteers were enrolled in a randomised crossover trial to study the absorption and excretion of apigenin after controlled intake of parsley (Nielsen et al., 1999). The mean content of apigenin in the diets was 4.14 and 4.86 mg/MJ corresponding to 20 g parsley/10 MJ per day. Average levels of apigenin in 24 h urine of volunteers on parsley diet was significantly higher (21–5,727 μg) as compared to the control diet (0–1,571 μg). Only 0.58% of the apigenin ingested was excreted in 24 h, being the calculated excretion half-life of about 12 h. It has to be noted that apigenin in parsley is essentially present as apiin, the 7-*O*-apioside of apigenin. The direct comparison between bioavailability of apigenin from apiin and from vicenin-2, a C-glycosyl flavonoid, raises some uncertainty.

For the other compounds, no data were submitted, including for limocitrin 3-[6″-(3-hydroxy-3-methylglutaryl)glucoside]-7-glucoside, one of the major secondary metabolites present in the extract. Its structure suggests a similar metabolic profile as described for the other compounds.

Concluding, the ADME data available for these flavonoids indicate an appreciable hydrolysis of glycosidic bonds at gastrointestinal level, partial biotransformation by intestinal enzymes and by microbiota in the colon with subsequent absorption of the aglycones and of their biotransformation products. For hesperidin, experimental data show partial absorption as such. Some metabolites formed in the gut are absorbed and further metabolised in the liver, circulate mainly in conjugated form and are excreted in urine.

Studies on the metabolism of flavonoids in the target species were not available. However, equivalent routes of metabolism exist in all species routinely exposed to flavonoids and related compounds found in diets (i.e. mammals, poultry and fish, EFSA FEEDAP Panel, 2011). Therefore, it can be assumed that food-producing animals, including fish and birds, have the ability to metabolise and excrete the flavonoids present in lemon extract, which are not expected to accumulate in tissues and products of animal origin.

ADME of limonoids

No experimental data were submitted for ADME of limonoids. A study in human volunteers showed that when limonin glucoside (0.25–2 g) was orally given, the mean concentrations of limonin in plasma ranged from 1.75 to 5.27 nmol/L, indicating that limonin glucoside is bioavailable. The mean time to the maximum concentration was 6 h (Manners et al., 2003, as referenced in Roy and Saraf, 2006 and in Shi et al., 2020).

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. Then, *in vitro* genotoxicity studies performed with the additive under assessment are described.

Flavonoids and structurally related compounds

The secondary metabolites (the flavonoids eriocitrin, hesperidin, limocitrin 3-[6″-(3-hydroxy-3-methylglutaryl)glucoside]-7-glucoside, diosmetin-7-*O*-glucoside, vicenin-2 and stellarin-2, and dihydroferulic acid glucoside structurally related to 3,4-dihydroxyhydrocinnamic acid, metabolite from eriodictyol) were screened with the OECD QSAR Toolbox and no alert was identified for *in vitro* mutagenicity (Ames test), for genotoxic and nongenotoxic carcinogenicity and for other endpoints.[31]

To further support the absence of genotoxicity of the additive, the applicant submitted a literature search on the individual components of lemon extract,[32] which identified relevant studies with hesperidin, one of the major constituents of the additive, glucosyl hesperidin and apigenin, a compound (flavone) structurally related to diosmetin-7-*O*-glucoside, limocitrin 3-[6″-(3-hydroxy-3-methylglutaryl)glucoside]-7-glucoside, vicenin-2 and stellarin-2.

Male NMRI mice were treated with 200 or 400 mg/kg bw of hesperidin by oral gavage. A numerical increase of micronucleated polychromatic erythrocytes occurred in the high dose, which was not statistically significant. Hesperidin significantly reduced the frequency of micronucleated polychromatic erythrocytes induced by cyclophosphamide (50 mg/kg bw) when administered intraperitoneally (Ahmadi et al., 2008).

The potential genotoxic effect of hesperidin was evaluated in human lymphocytes of volunteers. The cells treated with 150 μM hesperidin showed a frequency of micronuclei comparable to the negative controls. Hesperidin (50, 100 or 150 μM) was also tested in combination with 750 μM dianizon, a mutagenic agent, and a significant reduction of dianizon-induced micronuclei was observed (Shokrzadeh et al., 2014).

[31] Technical dossier/Supplementary information/January 2021/Annexes 29 to 42.
[32] Technical dossier/Supplementary information January 2020.

Glucosyl hesperidin did not induce structural or numerical aberrations in a chromosomal aberration test in Chinese hamster lung fibroblast cells up to 5,000 μg/mL, either with or without S9 mix, and did not increase the frequency of micronuclei *in vivo*, in mice when tested at 2,000 mg/kg (Matsumoto et al., 2019).

Apigenin did not induce gene mutation when tested in *Salmonella* Typhimurium strains TA98 and TA100 up to 1,000 μg/plate, with and without metabolic activation. *In vivo*, oral administration of 1, 10 and 100 mg apigenin/kg to CD1 mice was found not to increase the frequency of micronuclei in blood reticulocytes compared to the negative control group, but reduced the frequency of micronuclei induced by cyclophosphamide (Bokulić et al., 2011).

Limonoids

Nomilin and limonin were also screened by QSAR[33] analysis and alerts were identified for several endpoints[34] due to the presence of an epoxide. However, considering the steric hindrance on the oxirane ring in both compounds it is unlikely that the tri-substituted epoxide could react with DNA.

Genotoxicity studies with the additive under assessment

The applicant submitted *in vitro* genotoxicity tests performed with the additive under assessment, lemon extract (*C. limon* (L.) Osbeck).

In order to investigate the potential of lemon extract to induce gene mutations in bacteria, the Ames test was performed according to OECD Test Guideline 471 (1997) and following Good Laboratory Practice (GLP) in [redacted][35] Lemon extract was tested in two independent experiments applying the plate incorporation method and the pre-incubation assay in the presence and absence of metabolic activation (S9-mix). Based on the results of a preliminary cytotoxicity assay, six concentrations ranging from [redacted] μg/plate were selected to evaluate the induction of gene mutations. [redacted] No increase in the mean number of revertant colonies was observed at any tested concentration in any tester strains with or without S9-mix. The FEEDAP Panel notes that the high toxicity of the test item limits the validity of the test.

An *in vitro* micronucleus test was performed according to OECD TG 487 (2016) and following GLP to evaluate the potential of lemon extract to induce chromosome damage [redacted] in the absence and presence of metabolic activation.[36] No significant cytotoxicity was observed at the three concentrations ([redacted]) tested for the analysis of micronuclei after short treatments (3+ 21 h recovery) in the presence and absence of metabolic activation as well as continuous treatment (24 + 0 h recovery) in the absence of metabolic activation. The frequencies of micronuclei detected after treatment with lemon extract were comparable to the values observed in the concurrent negative controls. Therefore, the FEEDAP Panel concluded that lemon extract did not induce structural and numerical chromosomal aberrations in mammalian cells under the experimental conditions applied in the study.

3.3.2.2. Repeated-dose toxicity studies

No subchronic studies or repeated-dose toxicity studies with the additive under evaluation were submitted. The applicant submitted a literature search on the individual components of lemon extract, which identified repeated-dose studies with multiple doses tested with hesperidin and glucosyl hesperidin and a 28-day study with vicenin-1, a compound structurally related to vicenin-2.

A 90-day study with hesperidin (isolated from a methanolic extract of dried peel of the citrus fruit, purity 73%) was performed according to OECD guideline No. 408 (Li et al., 2019). Four groups of rats (15/sex per dose) were administered a daily dose (freshly prepared in water) of hesperidin of 0, 250, 500 and 1,000 mg/kg bw by oral gavage. At the highest dose tested (1,000 mg/kg bw per day), hesperidin showed significant alterations in body and organ weights, haematology, clinical chemistry and tissue histopathology. No effects on body weight, food consumption, clinical signs,

[33] http://www.oecd.org/chemicalsafety/risk-assessment/theoecdqsartoolbox.htm

[34] Alerts for *in vitro* mutagenicity (Ames test), *in vivo mutagenicity* (micronucleus) and carcinogenicity by Istituto Superiore di Sanità (ISS) (Benigni et al., 2010); no alerts for DNA alerts for Ames, chromosomal aberrations and micronucleus and protein bindings for chromosomal aberrations by OASIS (https://oasis-lmc.org/)

[redacted]

ophthalmological and neurological observations, urine analysis, haematology, clinical chemistry, organ weights and gross pathology were detected when hesperidin was administered at 250 and 500 mg/kg bw per day. Therefore, the FEEDAP Panel derived a no observed adverse effect level (NOAEL) of 500 mg hesperidin/kg bw per day from this study.

No adverse effects were observed by Matsumoto et al. (2019) in a 90-day study with glucosyl hesperidin (containing at least 70% hesperidin) up to the highest dose tested, approximately 3,000 mg/kg bw per day. The same authors did not observe adverse effects in a teratogenicity study up to the highest dose tested (1,000 mg/kg bw per day). The FEEDAP Panel considers the toxicological dataset described by Matsumoto et al. (2019) as supportive evidence to the NOAEL identified from the study by Li et al. (2019). The FEEDAP Panel also notes that the same NOAEL of 500 mg/kg bw per day was derived from an 84-day feeding study in rat which showed no adverse effects (Basarkar & Nath, 1981 as referenced in EFSA CEF Panel, 2010).

A repeated-dose toxicity study in Swiss albino mice was available with vicenin-1 (purity 93%, isolated from a hydroalcoholic extract of fenugreek). Four groups of animals (10/sex/dose) were administered a daily oral dose of vicenin-1 of 0, 37.5, 75 and 150 mg/kg bw for 28 days (Kandhare et al., 2016). The study considered a number of endpoints (survival, behaviour, body weight, feed intake; haematology, clinical chemistry and urine analysis; gross pathology and histopathology) and was well reported. At the highest dose tested (150 mg/kg bw per day), a significant increase in the body weight of animals was observed in both sexes and a significant decrease in haematocrit, mean corpuscular volume and platelet in males only. However, due to the limitation in the study design and the limited background knowledge on the use of mice in subchronic studies, this study does not allow to derive a NOAEL.

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 the target species of the flavonoid fraction of the extract. For limonoids, the available data set does 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[37]). Therefore, the assessment of the safety for target species is based on the comparison between limonoids intake via the consumption of citrus by-products as feed material and that via the use of lemon extract as a feed additive.

Flavonoids

The FEEDAP Panel identified a NOAEL of 500 mg/kg bw per day for hesperidin, which was selected as a group NOAEL and applied using read-across to all flavonoids, including the flavanones eriocitrin, which differs from hesperidin for the presence of a methoxy group instead of a hydroxy group in the B-ring, diosmetin-7-*O*-glucoside and limocitrin 3-[6''-(3-hydroxy-3-methylglutaryl)glucoside]-7-glucoside, and the flavones vicenin-2 and stellarin-2 (see Figure 1 for structural similarities and Section 3.3.1 for metabolic similarities). The same NOAEL was applied using read across to dihydroferulic acid-4-*O*-glucoside considering the structural and metabolic similarity of the aglycone with dihydrocaffeic acid, a metabolite from eriodictyol degradation by intestinal bacteria (Miyake et al., 2000). Applying an uncertainty factor (UF) of 100 to the NOAEL, the safe daily dose of PMF for the target species was derived following the EFSA Guidance on the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), and thus the maximum safe feed concentration of flavonoids and relate compounds was calculated (Table 3). Since glucuronidation of the hydroxylated or

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

oxygenated metabolites of the individual constituents of expressed orange oil is an important metabolic pathway facilitating the excretion of these compounds, the calculation of safe concentrations in cat feed needs an additional UF of 5. This factor is due to the unusually low capacity for glucuronidation in cats (Court and Greenblatt, 1997; Lautz et al., 2021).

Table 3: Maximum safe concentration in feed of flavonoids and related compounds for different target animal categories

Animal category	Body weight (kg)	Feed intake (g DM/day)	Daily feed intake (g DM/kg bw)	Maximum safe concentration (mg/kg feed)(1)
Chicken for fattening	2	158	79	56
Laying hen	2	106	53	83
Turkey for fattening	3	176	59	75
Piglet	20	880	44	100
Pig for fattening	60	2,200	37	120
Sow lactating	175	5,280	30	146
Veal calf (milk replacer)	100	1,890	19	233
Cattle for fattening	400	8,000	20	220
Dairy cow	650	20,000	31	143
Sheep/goat	60	1,200	20	220
Horse	400	8,000	20	220
Rabbit	2	100	50	88
Salmon	0.12	2.1	18	251
Dog	15	250	17	264
Cat(2)	3	60	20	55
Ornamental fish	0.012	0.54	5	978

DM: dry matter; bw: body weight.
(1): Complete feed containing 88% DM, milk replacer 94.5% DM.
(2): The uncertainty factor for cats is increased by an additional factor of 5 because of the reduced capacity of glucuronidation.

At the maximum proposed use level in feed of 1,000 mg/kg, the concentration of flavonoids and related compounds would result in 5.9 mg/kg complete feed (range: 5.1-6.7 mg/kg complete feed). This concentration is below the maximum safe concentration of flavonoids calculated for the different animal categories (Table 4) and is considered safe with an additional margin of safety of at least 8 (for cats) and up to 145 (for ornamental fish). This would account for the uncertainty in the read across and for the presence of unidentified compounds in the phenolic fraction (up to 1.1 mg/kg complete feed). The FEEDAP Panel concludes that the presence of flavonoids in the additive does not raise concern for the target species.

Limonoids

Limonin (up to 91 mg/kg) and nomilin (up to 112 mg/kg) were detected in all batches of the additive under assessment. The use of lemon extract at the proposed use levels in feed would result in a limonoid intake up to 18 μg/kg bw for poultry, 10 μg/kg bw for pigs, 7 μg/kg bw for ruminants, 4.5 μg/kg bw for horses, 11 μg/kg bw for rabbit and 4 μg/kg bw for fish (Table 4).[38]

[38] Intake values calculated considering the concentration of limonoids in the additive (0.02%), the default values for feed intake (Table 2), the proposed use levels in feed for the different species (1,000 mg/kg) and that complete feed contains 88% DM except milk replacer for veal calves (94.5%).

Table 4: Target animal intake of limonoids (as μg/kg bw per day) at the maximum proposed use level of the additive in feed (1,000 mg/kg complete feed) and considering that limonoids represents up to 0.02% of the additive

Animal category	Daily feed intake	Body weight	Use level in feed	Limonoids additive	Intake limonoids
	kg DM/day	kg	mg/kg	%	μg/kg bw per day
Chicken for fattening	0.158	2	1,000	0.02	18.0
Laying hen	0.106	2	1,000	0.02	12.0
Turkey for fattening	0.176	3	1,000	0.02	13.3
Piglet	0.88	20	1,000	0.02	10.0
Pig for fattening	2.2	60	1,000	0.02	8.3
Sow lactating	5.28	175	1,000	0.02	6.9
Veal calf (milk replacer)	1.89	100	1,000	0.02	4.3
Cattle for fattening	8	400	1,000	0.02	4.5
Dairy cow	20	650	1,000	0.02	7.0
Sheep/goat	1.2	60	1,000	0.02	4.5
Horse	8	400	1,000	0.02	4.5
Rabbit	0.1	2	1,000	0.02	11.4
Salmon	0.0021	0.12	1,000	0.02	4.0
Dog	0.25	15	1,000	0.02	3.8
Cat	0.06	3	1,000	0.02	4.5
Ornamental fish	0.00054	0.012	1,000	0.02	1.0

bw: body weight; DM: dry matter.

Limonoids occur in citrus by-products, which are used in diets at different concentrations depending on the target species (from 5% up to 30% in ruminants). 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 has been estimated to be 7.9 g dry matter (DM)/kg bw for poultry, 8.8 g DM/kg bw for pigs, 6.2 g DM/kg bw for ruminants, 4 g DM/kg bw for horses, 10 g DM/kg bw for rabbits and 3.6 g DM/kg bw for fish.

Based on the literature data provided by the applicant on the occurrence of limonoids in *Citrus* seeds [e.g. 0.10–0.75% for the sum of limonin and nomilin in eight *Citrus* species, including lemon (0.53%) according to Montoya et al. (2019); 0.68% for the sum of limonoids (0.47% when considering only limonin and nomilin) % in bergamot according to Russo et al. (2016)], and considering that seeds represent 5% of citrus by-products (Bampidis and Robinson, 2006; Zema et al., 2018), the content of limonoids in citrus by-products was estimated to be in the range 0.005–0.04%.[39] Based on citrus by-product intake (see above), the daily intake of limonoids via feed was calculated to be up to 3.2 mg/kg bw for poultry, 3.5 mg/kg bw for pigs, 2.5 mg/kg bw for ruminants, 1.6 mg/kg bw for horses, 4 mg/kg bw for rabbits and 1.4 mg/kg bw for fish.

These daily intake values are about 300-fold higher than those resulting from the high use level of lemon extract in feed as proposed by the applicant (1,000 mg lemon extract/kg feed).

For dogs, cats and ornamental fish, for which comparative intake figures from the use of citrus by-products as feed material are not available, the safety assessment is based on the application of threshold of toxicological concern (TTC). For these species, the intake values (ranging from 1 to 4.5 μg/kg bw per day) are in the same order of the exposure values established for Cramer class III compounds (1.5 μg/kg bw per day), below which there is a low probability of adverse effects. Therefore, exposure to limonoids resulting from the use of the additive under the proposed conditions of use in these species is considered not of concern.

[39] 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 limonoids in citrus by-products is calculated to be in the range 0.005–0.04% (0.10 × 0.05 and 0.75 × 0.05).

The applicant proposed a maximum use level in water for drinking of 250 mg/kg, which would ensure a lower exposure and is considered safe for all animal species (EFSA FEEDAP Panel, 2010).

3.3.3.1. Conclusions on safety for the target species

The FEEDAP Panel concludes that lemon extract under assessment is safe up to the maximum proposed use levels of 1,000 mg/kg complete feed and 250 mg/kg water for drinking.

3.3.4. Safety for the consumer

Lemon extract is added to a wide range of food categories for flavouring purposes. Although individual consumption figures for the EU are not available, the Fenaroli's handbook of flavour ingredients (Burdock, 2009) cites values of 0.032 mg/kg bw per day for lemon extract.

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 lemon extract are expected to be extensively metabolised and excreted in the target species (see Section 3.3.1). Therefore, a relevant increase in the uptake of these compounds by humans consuming products of animal origin is not expected.

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

3.3.5. Safety for user

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

The low pH of the additive (< 4) would indicate a likelihood of skin and eye irritation, and potentially corrosion.[40]

The additive should be considered a skin and eye irritant, and potential corrosive.

3.3.6. Safety for the environment

C. limon is a native species to Europe where it is widely grown both for commercial and decorative purposes. The use of the extract in animal feed under the proposed conditions is not expected to pose a risk for the environment.

3.4. Efficacy

C. limon and its extracts are listed in Fenaroli's Handbook of Flavour Ingredients (Burdock, 2009) and by the Flavour and Extract Manufactures Association (FEMA) with the reference number 2623 (lemon extract).

Since *C. limon* 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 under assessment is safe for all animal species up to the maximum proposed use levels of 1,000 mg/kg of complete feedingstuffs and 250 mg/kg water for drinking.

No concerns for consumers were identified following the use of lemon extract up to the highest safe level in feed.

The additive should be considered a skin and eye irritant, and potential corrosive.

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

Lemon extract is recognised to flavour food. Since its function in feed would be essentially the same as that in food, no further demonstration of efficacy is considered necessary.

[40] According to the classification provided by companies to ECHA in CLP notifications Lemon, ext. causes skin irritation and may cause allergic skin reactions. https://echa.europa.eu/it/substance-information/-/substanceinfo/100.076.805

5. Documentation as provided to EFSA/Chronology

Date	Event
23/09/2010	Dossier received by EFSA. Lemon extract for all animal species and categories. Submitted by Nor-Feed SAS
11/09/2018	Reception mandate from the European Commission
12/09/2018	Application validated by EFSA – Start of the scientific assessment
27/09/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation, safety for the target species, the consumers and the users*
14/12/2018	Comments received from Member States
03/01/2020	Reception of supplementary information from the applicant (partial submission)
16/12/2020	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
04/01/2021	Reception of supplementary information from the applicant - Scientific assessment re-started
21/04/2021	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation, safety*
18/06/2021	Reception of supplementary information from the applicant - Scientific assessment re-started
29/09/2021	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

ADME	absorption, distribution, metabolism and excretion
bw	body weight
CAS	Chemical Abstracts Service
CBA	component based approach
CoE	Council of Europe
CFU	colony forming unit
DAD	diode array detector
DL-PCBs	dioxin-like polychlorobiphenyls
DM	dry matter
EINECS	Existing Commercial Chemical Substances
ELSD	evaporative light scattering detector
ESI	electrospray
EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
GC–MS	gas chromatography–mass spectrometry
GLP	Good Laboratory Practice
HPLC	high-performance liquid chromatography
IUCAP	International Union of Pure and Applied Chemistry
LC-ESI-MS^n	liquid chromatography electrospray multistage mass spectrometry
LC–MS	liquid chromatography–mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
MALDI	matrix assisted laser desorption ionisation
MOE	margin of exposure
MOET	combined margin of exposure (total)
MRL	maximum residue level
MS	mass spectrometry
NMR	nuclear magnetic resonance
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
PCDD/F	polychlorinated dibenzo-*p*-dioxins and dibenzofurans
QSAR	quantitative structure–activity relationship
RH	Relative humidity
SC	EFSA Scientific Committee
TOF	time of flight
UPLC	Ultraperformance liquid chromatography
UV	ultraviolet
WHO	World Health Organization

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Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for total polyphenols in lemon extract

In the current application authorisation is sought under Article 10(2) for *Lemon extract* under the category/functional group 2(b) 'Sensory additives'/'flavouring compounds' according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of the *feed additive* for all animal species.

The *feed additive* is a dark orange-to-yellow liquid extract obtained from the peel and the seeds of the *Citrus limon* (L.) Burm. (CoE 139). *Lemon extract* is to be used through *premixtures* or directly into *feedingstuffs* and *water* at proposed maximum levels of 1000 mg/kg *feedingstuffs* and 0.25 ml/l *water.* According to the Applicant, the phytochemical markers proposed for the characterisation of the *feed additive* (*Lemon extract*) are *total polyphenols* and *eriocitrin. Lemon extract* is a natural product and thus the *total polyphenols* content varies from harvest to harvest, ranging generally from 1 to 4% (w/w).

For the quantification of the *total polyphenols* in the *feed additive* (*Lemon extract*) the Applicant proposed a colourimetric method described in the European Pharmacopoeia monograph 2.8.14. Upon request of the EURL, the Applicant provided experimental data for the analysis of the *total polyphenols* content in two different batches of *Lemon extract.*

Based on the acceptable performance characteristics presented the EURL recommends for official control the colourimetric method described in the European Pharmacopoeia 2.8.14 monograph for the quantification of the *total polyphenols* content in the *feed additive*.

For an additional identification/characterisation of the *feed additive*, the EURL considers a method based on high performance liquid chromatography with spectrophotometric detection (HPLC-UV) suitable for the determination of *eriocitrin* in the *feed additive*.

The Applicant did not provide experimental data or analytical methods for the determination of *Lemon extract* in *premixtures* and *feedingstuffs,* as the unambiguous determination of the *feed additive* added to the matrices is not achievable experimentally. Therefore, the EURL cannot evaluate nor recommend any method for official control for the determination of *Lemon extract* in *premixture*s and *feedingstuffs*.

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.