SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 27 January 2022

doi: 10.2903/j.efsa.2022.7160

Safety and efficacy of a feed additive consisting of an essential oil from the leaves of *Agathosma betulina* (P.J. Bergius) Pillans (buchu leaf oil) 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 an essential oil from the leaves of *Agathosma betulina* (P.J. Bergius) Pillans (buchu leaf oil), when used as a sensory additive (flavouring) in feed and water for drinking for all animal species. The FEEDAP Panel concluded that the essential oil under assessment is safe up to the maximum proposed use levels in complete feed of 0.1 mg/kg for chickens for fattening, 0.15 mg/kg for laying hens, turkeys for fattening and rabbits, 0.20 mg/kg for piglets, 0.25 mg/kg for pigs for fattening, 0.30 mg/kg for sows and dairy cows, 0.45 mg/kg for cattle for fattening, sheep, goats and horses, 0.5 for veal calves (milk replacer), fish, ornamental fish and dogs. For cats, the calculated maximum safe level in feed is 0.2 mg/kg complete feed. 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. No concerns for consumer safety were identified following the use of the additive up to the highest safe levels in feed. The essential oil under assessment should be considered as irritant to skin and eyes, and as a skin and respiratory sensitiser. The use of the additive in animal feed under the proposed conditions was not expected to pose a risk for the environment. Buchu leaf oil 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, *Agathosma betulina* (P.J. Bergius) Pillans, buchu leaf oil, d,l-isomenthone, diosphenol, methyleugenol, pulegone and menthofuran, thujones

Requestor: European Commission

Question number: EFSA-Q-2010-01517, new EFSA-Q-2021-00597

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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.

Acknowledgements: The Panel wishes to thank the following for the support provided to this scientific output: Jaume Galobart, Jordi Ortuño and Maria Vittoria Vettori.

Suggested citation: EFSA FEEDAP Panel (EFSA Panel on Additives, Products or Substances used in Animal Feed), Bampidis V, Azimonti G, Bastos ML, Christensen H, Fašmon Durjava M, Kouba M, López-Alonso M, López Puente S, Marcon F, Mayo B, Pechová A, Petkova M, Ramos F, Sanz Y, Villa RE, Woutersen R, Brantom P, Chesson A, Westendorf J, Manini P, Pizzo F and Dusemund B, 2022. Scientific Opinion on the safety and efficacy of a feed additive consisting of an essential oil from the leaves of *Agathosma betulina* (P.J. Bergius) Pillans (buchu leaf oil) for use in all animal species (FEFANA asbl). EFSA Journal 2022;20(3):7160, 30 pp. https://doi.org/10.2903/j.efsa.2022.7160

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, a European agency funded by the European Union.

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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 7 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 (buchu leaves oil, amyris oil, olibanum extract (water based, wb), olibanum tincture, lime oil, neroli bigarade oil, petitgrain 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 nine preparations.[3,4] In addition, during the course of the assessment, the application was split and the present opinion covers only one out of the 20 initial preparations under application: an essential oil from the leaves of *Agathosma betulina* (P.J. Bergius) Pillans[5] (buchu leaf oil) 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 an essential oil from *A. betulina* (buchu leaf oil), when used under the proposed conditions of use (see Section 3.2.3).

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

1.2. Additional information

'Buchu leaves oil' from *A. betulina* (P.J. Bergius) Pillans is currently authorised as 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 *A. betulina* preparation when used to provide flavour in food.

Many of the individual components of buchu leaf oil 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

[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, cashew oil, neroli bigarade oil, petitgrain bigarade absolute, mandarin terpenes, grapefruit oil expressed, grapefruit extract (sb), grapefruit extract.
[4] On 2 April 2021, EFSA was informed by the applicant about the withdrawal of the application on olibanum tincture.
[5] Accepted name: *Agathosma betulina* (P.J. Bergius) Pillans; synonym: *Barosma betulina* Bartl.
[6] 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.

authorised for food and feed uses together with the EU Flavour Information System (FLAVIS) number, the chemical group as defined in Commission Regulation (EC) No 1565/2000[8], 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
04	Non-conjugated and accumulated unsaturated straight chain and branched chain aliphatic primary alcohols/ aldehydes/acids, acetals and esters	Citronellol	02.011	2016a
05	Saturated and unsaturated aliphatic secondary alcohols, ketones and esters with esters containing secondary alcohols	Isopulegol	02.067	2020
06	Aliphatic, alicyclic and aromatic saturated and unsaturated tertiary alcohols and esters with esters containing tertiary alcohols ethers	Linalool	02.013	2012a
		α-Terpineol	02.014	
		4-Terpinenol	02.072	
07	Primary alicyclic saturated and unsaturated alcohols, aldehydes, acids, acetals and esters with esters containing alicyclic alcohols	Myrtenyl acetate[(a)]	09.302	2017, CEF
		p-Menth-1-en-9-yl acetate[(a)]	09.615	JECFA
08	Secondary alicyclic saturated and unsaturated alcohols, ketones, ketals and esters with ketals containing alicyclic alcohols or ketones and esters containing secondary alicyclic alcohols	Menthol	02.015	2016b
		d,l-Isomenthone	07.078	
		Bornyl acetate	09.017	
		Carvyl acetate[(b)]	09.215	
		Isopulegone	07.067	2020
		Sabinene hydrate[(a),(c)]	02.085	JECFA
		p-Menthan-3-one[(a)]	07.059	CoE
		Dihydrocarvone[(a),(d)]	07.128	JECFA
		p-Menth-1-en-3-one[(a)]	07.175	JECFA; 2011a, CEF
		Pin-2-en-4-one[(a)]	07.196	2011a, CEF 2012, CEF
13	Furanones and tetrahydrofurfuryl derivatives	Linalool oxide[(e)]	13.140	2012b
16	Aliphatic and alicyclic ethers	1,8-Cineole	03.001	2012c, 2021a
18	Allylhydroxybenzenes	Eugenol	04.003	2011a
20	Aliphatic and aromatic mono- and di-thiols and mono-, di-, tri- and polysulfides with or without additional oxygenated functional groups	8-Mercapto-*p*-menthan-3-one[(f)]	12.085	2019a
23	Benzyl alcohols, aldehydes, acids, esters and acetals	Benzyl benzoate	09.727	2012d
27	Anthranilate derivatives	Methyl N-methyl anthranilate		2011b

[7] 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

[8] 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.

CG	Chemical Group	Product – EU register name (common name)	FLAVIS No	EFSA opinion* Year
30	Miscellaneous substances	2-Hydroxypiperitone (diosphenol, buchu camphor) 1-Methyl-4-isopropyl-1-cyclohexen-2-ol-3-one	07.168	2011b, CEF
31	Aliphatic and aromatic hydrocarbons and acetals containing saturated aldehydes	1-Isopropyl-4-methylbenzene (p-cymene)	01.002	2015
		1-Isopropenyl-4-methylbenzene	01.010	
		Terpinolene	01.005	
		α-Terpinene	01.019	
		γ-Terpinene	01.020	
		d-Limonene	01.045	
		Pin-2(10)-ene (β-pinene)	01.003	2016c
		Pin-2(3)-ene (α-pinene)	01.004	
		Myrcene	01.008	
		Camphene	01.009	
		4(10)-Thujene (sabinene)(a)	01.059	2015a, CEF
		cis-3,7-Dimethyl-1,3,6-octatriene (cis-β-ocimene)(a)	01.064	

*: FEEDAP opinion unless otherwise indicated.
(a): Evaluated for use in food. According to Regulation (EC) 1565/2000, flavourings evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) before 2000 are not required to be re-evaluated by EFSA.
(b): EFSA evaluated carvyl acetate [09.215] as a mixture of isomers (related to (1R,5R)-carvyl acetate or cis-l-carvyl acetate).
(c): EFSA evaluated sabinene hydrate [02.085] as a mixture of cis- and trans-sabinene hydrate.
(d): JECFA evaluated dihydrocarvone [07.128] as a mixture of cis- and trans-dihydrocarvone.
(e): EFSA evaluated linalool oxide [13.140] as a mixture of cis- and trans-linalool oxide (5-ring).
(f): EFSA evaluated 8-mercapto-*p*-menthan-3-one [12.038] as a mixture of cis- and trans-isomers.

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[9] in support of the authorisation request for the use of buchu leaf oil from *A. betulina* 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 essential oil under assessment have been already evaluated by the FEEDAP Panel as chemically defined flavourings. The applicant submitted a written agreement to use 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.[10]

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.[11]

[9] FEED dossier reference: FAD-2010-0322.
[10] Technical dossier/Supplementary information/Letter dated 29/04/2021.
[11] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2010-0322-bdg08.pdf

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of buchu leaf oil from *A. betulina* is in line with the principles laid down in Regulation (EC) No 429/2008[12] and the relevant guidance documents: Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements (EFSA SC, 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, 2012e), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012f), 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 efficacy of feed additives (EFSA FEEDAP Panel, 2018), Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019b), Guidance on the use of the benchmark dose approach in risk assessment (EFSA SC, 2017), Guidance document on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals (EFSA SC, 2019a), Statement on the genotoxicity assessment of chemical mixtures (EFSA SC, 2019b), Guidance on the use of the Threshold of Toxicological Concern approach in food safety assessment (EFSA SC, 2019c) and General approach to assess the safety for the target species of botanical preparations which contain compounds that are genotoxic and/or carcinogenic (EFSA FEEDAP Panel, 2021b).[13]

3. Assessment

The additive under assessment, buchu leaf oil, is obtained from the leaves of *Agathosma betulina* (P.J. Bergius) Pillans. It is intended for use as sensory additive (functional group: flavouring compounds) in feed and in water for drinking for all animal species.

3.1. Origin and extraction

Agathosma betulina (syn. *Barosma betulina* Bartl.) is an evergreen shrub which belongs to the Rutaceae family. It is native to South Africa but is now cultivated in other parts of Africa and in South America. *A. betulina* is one of two *Agathosma* species to which the common name 'buchu' may be applied. The other (*Agathosma crenulata* (L.) Pillans) is also an evergreen shrub native to South Africa with a similar habitat and is also used in the production of an essential oil. The common name 'short buchu' is sometimes used for *A. betulina* and 'oval leaf buchu' for *A. crenulata*. Extracts of *A. betulina* have a history of medicinal use by the indigenous peoples of South Africa, predominately in the treatment of kidney and urinary tract infections. The powdered dried leaf was also used as an insecticide.

The dried leaves of *A. betulina* are extracted by steam distillation without a pre-extraction process. The plants are cut by hand, dried and then transferred to the distillery where they undergo the distillation process. The essential oil is separated from the aqueous layer by decantation.

3.2. Characterisation

3.2.1. Characterisation of buchu leaf oil

The essential oil under assessment is a yellow to orange mobile liquid. In six batches of the additive (all originating from South Africa), the density (20°C) ranged between 910 and 923 kg/m^3 (specification: 910–980 kg/m^3) and the refractive index (20°C) between 1.472 and 1.481 (specification: 1.460–1.485).[14] Buchu leaf oil is identified with the Flavor Extract Manufacturers Association (FEMA) number 2169.

[12] 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.

[13] https://www.efsa.europa.eu/sites/default/files/2021-05/general-approach-assessment-botanical-preparations-containing-genotoxic-carcinogenic-compounds.pdf

[14] Technical dossier/Supplementary information June 2019/Annex_II_ SIn_Reply_buchu_leaves_oil_expressed_CoA.

The product specifications are based on the concentrations of selected components of the essential oil, analysed by gas chromatography with flame ionisation detection (GC-FID) and expressed as % of gas chromatographic peak area (% GC area). These components are d,l-isomenthone (19–27%, selected as a phytochemical marker), d-limonene (19–26%, selected as a phytochemical marker), 2-hydroxypiperitone (also known as diosphenol, 8–17%), p-menthan-3-one (5–12%) and pulegone (1.5–8%). Analysis of six batches of the additive by gas chromatography-mass spectrometry (GC-MS) showed compliance with these specifications except for d-limonene,[16] which was below the proposed specification in four batches and 2-hydroxypiperitone, which was above the proposed specification in one batch. These five compounds account for about 70% on average (range 65.9–74.0%) of % GC area (Table 2).

Table 2: Constituents of the essential oil from the leaves of *Agathosma betulina* (P.J. Bergius) Pillans as defined by specification (based on the analysis of six batches). The content of each constituent is expressed as the area per cent of the corresponding chromatographic peak (% GC area), assuming the sum of chromatographic areas of all detected peaks as 100%

Constituent EU register name	CAS No	FLAVIS No	% GC area		
			Specification	Mean[(a)]	Range
(d,l)-Isomenthone	491-07-6	07.078	19–27	24.4	20.3–26.2
d-Limonene	5989-27-5	01.045	19–26	18.6	16.9–20.7
2-Hydroxypiperitone (diosphenol)[(b)]	490-03-9	07.168	8–17	13.6	10.4–20.8
p-Menthan-3-one	10458-14-7	07.059	5–12	9.26	7.38–10.2
Pulegone[(c)]	89-82-7	–	1.5–8	5.03	2.13–7.81
Total				70.7	65.9–74.0

EU: European Union; CAS no.: Chemical Abstracts Service number; FLAVIS number: EU Flavour Information System numbers.
(a): Mean calculated on six batches.
(b): 2-Hydroxy-p-menth-1-en-3-one (2-hydroxypiperitone). Due to a keto-enol tautomerism, this substance can exist as two isomers: the keto-isomer, p-menthan-2,3-dione, is an α-diketone.
(c): Substance which shall not be added as such to food (Annex III), maximum level in food is set by Regulation (EC) No 1334/2008, including mint/peppermint containing confectionery (250 mg/kg), chewing gum (350 mg/kg) and mint/peppermint containing non-alcoholic (20 mg/kg) and alcoholic (100 mg/kg) beverages.

The applicant provided a full characterisation of the six batches obtained by GC-MS.[17] In total, up to 102 constituents were detected, 52 of which were identified and accounted on average for 97.8% (97.6–98.0%) of the % GC area. Besides the five compounds indicated in the product specifications, 18 other compounds were detected at individual levels > 0.1% and are listed in Table 3. These 23 compounds > 0.1% together account on average for 96.9% (96.4–97.3%) of the % GC area. The remaining 29 compounds (ranging between 0.003% and 0.1%) and accounting for 1.03% are listed in the footnote.[18] Based on the available data on the characterisation, buchu leaf oil is considered a fully defined mixture.

[15] Technical dossier/Supplementary information June 2019.
[16] Differences in the values determined by GC with different detectors are due to the fact that GC-MS method underestimates d-limonene.
[17] Technical dossier/Supplementary information June 2019/Annex_III_SIn _Reply_lemon_oil_expressed_chromatograms.
[18] Additional constituents: constituents (n = 10) between < 0.1 and ≥ 0.05%: α-terpinene, isopulegol, cis-3,7-dimethyl-1,3,6-octatriene (β-ocimene), trans-1-methyl-4-(1-methylvinyl)cyclohex-2-en-1-ol, bicyclogermacrene, p-menth-1-en-3-one, terpinolene, (1R,5R)-carvyl acetate, citronellol, bornyl acetate; constituents (n = 12) between < 0.05 and > 0.01%: α-thujene, trans-sabinene hydrate, eugenol, cis-para-2,8-menthadien-1-ol, 1-isopropyl-4-methylbenzene, trans-dihydrocarvone, menthol, beta-thujone, pin-2-en-4-one, camphene, p-menth-1-en-9-yl acetate, (E)-4,8-dimethyl-1,3,7-nonatriene; constituents (n = 6) ≤ 0.01%: cis-menthone-8-thioacetate, 1-isopropenyl-4-methylbenzene, methyl N-methylanthranilate, α-thujone, thuja-2,4(10)-diene and benzyl benzoate.

Table 3: Other constituents of the essential oil from the leaves of *Agathosma betulina* (P.J. Bergius) Pillans accounting for > 0.1% of the composition (based on the analysis of six batches) not included in the specification. The content of each constituent is expressed as the area per cent of the corresponding chromatographic peak (% GC area), assuming the sum of chromatographic areas of all detected peaks as 100%

Constituent EU register name	CAS No	FLAVIS No	% GC area	
			Mean[(a)]	Range
Pseudo-diosphenol	54783-36-7	–	11.57	8.95–15.20
Isopulegone	29606-79-9	07.067	3.58	3.09–4.42
cis-8-Mercapto-p-menthan-3-one[(b)]	33284-96-7	–	1.90	1.28–3.00
Myrcene	123-35-3	01.008	1.78	1.48–2.02
1,8-Cineole	470-82-6	03.001	1.68	1.49–2.11
α-Pinene (pin-2(3)-ene)	80-56-8	01.004	1.14	0.81–1.33
Sabinene (4(10)-thujene)	3387-41-5	01.059	1.13	0.86–1.38
trans-3,7-Dimethyl-1,3,6-octatriene	3779-61-1	–	0.64	0.58–0.69
Linalool	78-70-6	02.013	0.48	0.34–0.59
trans-2-(1-Mercapto-1-methylethyl)-5-methylcyclohexan-1-one	35117-85-2	–	0.35	0.22–0.53
4-Terpinenol	562-74-3	02.072	0.34	0.25–0.52
β-Pinene (pin-2(10)-ene)	127-91-3	01.003	0.33	0.24–0.43
Menthofuran[(c)]	494-90-6	13.035	0.28	0.22–0.37
trans-Menthone-8-thioacetate	166022-17-9	–	0.28	0.27–0.29
Myrtenyl acetate	1079-01-2	09.302	0.18	0.12–0.24
cis-Dihydrocarvone[(d)]	3792-53-8	–	0.16	0.14–0.18
α-Terpineol	98-55-5	02.014	0.14	0.10–0.16
Methyleugenol[(e)]	93-15-2	04.012	0.12	0.09–0.17
γ-Terpinene	99-85-4	01.020	0.12	0.08–0.18
Total			26.2	22.5–30.7

EU: European Union; CAS no.: Chemical Abstracts Service number; FLAVIS number: EU Flavour Information System numbers.
(a): Mean calculated on six batches.
(b): 8-Mercapto-*p*-menthan-3-one [12.038]: a mixture of cis- and trans-isomers was evaluated.
(c): Substance which shall not be added as such to food (Annex III), maximum level in food is set by Regulation (EC) No 1334/2008, including mint/peppermint containing confectionery (500 mg/kg), chewing gum (1,000 mg/kg) and mint/peppermint containing alcoholic beverages (200 mg/kg).
(d): Dihydrocarvone [07.128]: a mixture of cis- and trans-isomers was evaluated.
(e): Substance which shall not be added as such to food (Annex III), maximum level in food is set by Regulation (EC) No 1334/2008, including dairy products (20 mg/kg), meat products (15 mg/kg), fish products (10 mg/kg), soups and sauces (60 mg/kg), ready-to eat savouries (20 mg/kg) and non-alcoholic beverages (1 mg/kg).

The applicant performed a literature search for the chemical composition of *A. betulina* and its preparations and the identity of any recognised substances of concern.[19]

The occurrence of pulegone in leaf essential oil is reported in the EFSA Compendium (EFSA, 2012).[20] Analysis of the six batches[15] showed detectable concentrations of pulegone (2.13–7.81%) in all batches. Buchu leaf oil was shown to contain α-thujone (0.003–0.010%), β-thujone (0.015–0.038%), menthofuran (0.22–0.37%) and methyleugenol (0.09–0.17%). Pulegone, α- and β-thujone, menthofuran and methyleugenol are included in the list of substances which shall not be added as such to food according to Annex III of Regulation (EC) No 1334/2008, and for which maximum levels in food are set by Regulation (EC) No 1334/2008.[21]

[19] Technical dossier/Supplementary information June 2019/Literature Buchu_leaves_oil.
[20] Online version: https://www.efsa.europa.eu/en/data-report/compendium-botanicals
[21] Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. OJ L 354, 31.12.2008, p. 34.

3.2.1.1. Impurities

The applicant makes reference to the 'periodic testing' of some representative flavourings premixtures for heavy metals (mercury, cadmium and lead), arsenic, fluoride, dioxins and polychlorinated biphenyls (PCBs), organo-chloride pesticides, organo-phosphorous pesticides, aflatoxin B1, B2, G1, G2 and ochratoxin A. However, no data have been provided on the presence of these impurities. Since buchu leaf oil is produced by steam distillation, the likelihood of any measurable carry-over of heavy metals is low except for mercury.

3.2.2. 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).[22] However, no data supporting this statement were provided.

3.2.3. Conditions of use

Buchu leaf oil is intended to be added to feed and water for drinking for all animal species without a withdrawal period. The maximum proposed use levels in complete feed for the different target species are reported in Table 4. No use level has been proposed by the applicant for the use in water for drinking.

Table 4: Conditions of use for the essential oil from the leaves of *Agathosma betulina* (P.J. Bergius) Pillans: maximum proposed use levels in complete feed for the different target species

Animal category	Maximum use level (mg/kg complete feed)
Chicken for fattening	0.10
Laying hen	0.15
Turkey for fattening	0.15
Piglet	0.20
Pig for fattening	0.25
Sow	0.30
Veal calf (milk replacer)	0.50
Cattle for fattening	0.45
Dairy cow	0.30
Sheep/goat	0.45
Horse	0.45
Rabbit	0.15
Fish	0.50
Dog	0.50
Cat	0.50
Ornamental fish	0.50

3.3. Safety

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

Many of the components of buchu leaf oil, accounting for about 78% of the % GC peak areas, have been previously assessed and considered safe for use as flavourings, and are currently authorised for food[6] and feed[7] uses. The list of the compounds already evaluated by the EFSA Panels is given in Table 1 (see Section 1.2).

Pseudo-diosphenol, a constituent accounting for 11.6% of the % GC area, is structurally related to 2-hydroxypiperitone [07.168], for which EFSA overruled the genotoxicity concern due to the presence of ketones and α,β-unsaturated carbonyls based on negative Quantitative Structure–Activity Relationship (QSAR) predictions and data submitted for the representative substances in Flavouring Group Evaluation 213 (EFSA CEF Panel, 2014). Similarly to diosphenol, for pseudo-diosphenol, the

[22] Technical dossier/Section II.

alerts due to the presence of ketones and α,β-unsaturated carbonyls were discounted by the FEEDAP Panel based on read-across analysis.[23]

Several volatile components accounting for < 0.2% of the % GC area (*cis*- and *trans*-dihydrocarvone, (1R,5R)-carvyl acetate, trans-1-methyl-4-(1-methylvinyl)cyclohex-2-en-1-ol, cis-para-2,8-menthadien-1-ol, *trans*-sabinene hydrate, trans-3,7-dimethyl-1,3,6-octatriene, (E)-4,8-dimethyl-1,3,7-nonatriene, α-thujene, thuja-2,4(10)diene and bicyclogermacrene) have not been previously assessed for use as flavourings. The FEEDAP Panel notes that they are aliphatic mono- or sesquiterpenes structurally related to flavourings already assessed in CG 8 and 31 and a similar metabolic and toxicological profile is expected. Because of their lipophilic nature, they are expected to be rapidly absorbed from the gastro-intestinal tract, oxidised to polar oxygenated metabolites, conjugated and excreted (EFSA FEEDAP Panel, 2015, 2016b,c).

These compounds together with four compounds belonging to CG 20 (cis-8-mercapto-p-menthan-3-one, trans-2-(1-mercapto-1-methylethyl)-5-methylcyclohexan-1-one, trans-menthone-8-thioacetate and cis-menthone-8-thioacetate) were screened with the Organization for Economic Co-operation and Development (OECD) QSAR Toolbox and no alerts were identified for *in vitro* mutagenicity, for genotoxic and non-genotoxic carcinogenicity and for other endpoints, or if alerts were identified they were discounted based on read-across analysis.[24]

The following sections focus on those compounds not previously assessed or not structurally related to flavourings previously assessed, particularly on substances of concern: methyleugenol, pulegone and its metabolite menthofuran, based on the evidence provided by the applicant in the form of several literature searches. For α- and β-thujone, reference is made to the FEEDAP opinion on expressed lemon oil and its fractions and on lime oil (EFSA FEEDAP Panel, 2021a).

3.3.1. Absorption, distribution, metabolism and excretion

Methyleugenol

Methyleugenol is a highly lipophilic compound and as such readily and completely absorbed from the gastrointestinal tract. Phase I metabolism is catalysed by CYP450 enzymes mainly in the liver. Demethylation of the 4-methoxygroup with formation of 4-allylphenol is followed by conjugation with glucuronic acid or sulfate and renal excretion. Oxidation of the allyl-side chain leads to methyleugenol-2′,3′-epoxide, which is hydrolysed to the corresponding diol with subsequent glucuronidation. Both metabolic pathways represent detoxification of methyleugenol. The formation of genotoxic metabolites is initiated by oxidation of the side chain with formation of 1′-hydroxy-methyleugenol. Sulfate conjugation of the hydroxyl group leads to 1′-sulfooxymethyleugenol, which is highly unstable and breaks down to form a highly reactive carbonium ion, which can react covalently with DNA (as reviewed in EMA, 2005, IARC, 2018a). The occurrence of DNA adducts of methyleugenol in liver samples of humans obtained at liver surgery as a result of exposure to this compound via normal food has been demonstrated (Herrmann et al., 2013).

Pulegone and menthofuran

The gastrointestinal absorption of pulegone is high as demonstrated in rats after oral gavage of the labelled compound (Chen et al., 2001). One day after dosing, 44–71% of pulegone-derived radioactivity was present in urine. Chen et al. (2003a) evaluated the comparative disposition of labelled pulegone orally administered to mice and rats after a single oral dose of 0.8, 8, 80 mg/kg bw or an i.v. dose of 0.8 mg/kg bw. In mice, the bioavailability of pulegone was 80% and in the rat about 60%. Pulegone was broadly distributed in the tissues, the liver being the organ containing the highest concentration in both species and sexes, and the liver together with the kidney in the case of male rats. The administration of increasing doses resulted in higher levels of radioactivity in tissues and

[23] Technical dossier/Supplementary information June 2019/Annex IV_SIn_reply_buchu_leaves_oil_QSAR. 'For pseudodiospheol structural alerts were due to the presence of ketones and α,β-unsaturated carbonyls. Predictions of Ames mutagenicity was made by "read-across" analyses of data available for similar substances to the target compounds (i.e. analogues obtained by categorisation). Categories were defined using general mechanistic and endpoint profilers as well as empirical profilers. Ames test (with and without S9) read across predictions were found negative in all cases'.

[24] Technical dossier/Supplementary information June 2019/Annex IV_SIn_reply_buchu_leaves_oil_QSAR. 'For trans-1-methyl-4-(1-methylvinyl)cyclohex-2-en-1-ol and cis-para-2,8-menthadien-1-ol to the presence of vinyl/allyl alcohol groups, for trans- and cis-menthone-8-thioacetate to the presence of thiols radicals and esters. Predictions of Ames mutagenicity was made by "read-across" analyses of data available for similar substances to the target compounds (i.e. analogues obtained by categorisation). Categories were defined using general mechanistic and endpoint profilers as well as empirical profilers. Ames test (with and without S9) read across predictions were found negative in all cases'.

organs, although rarely with statistical significance. The daily oral administration of 80 mg/kg bw for 4 consecutive days resulted in significantly increased levels of radioactivity in tissues, showing the possibility of accumulation of pulegone and its metabolites.

Pulegone is rapidly and extensively excreted in mice, 85–100% of the dose in 24 h. Rats excrete 59–81% of the administered radioactivity in the same period, both in urine and faeces (Chen et al., 2003a). In rats, about 10% of radioactivity remained in gut 24 h after administration, justifying the lower amount excreted in urine as compared with mice.

The metabolism of pulegone has been considered by several scientific bodies, i.e. the EFSA Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Foods (AFC; EFSA, 2005), by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA; WHO, 2001), and more recently by the European Medicines Agency (EMA, 2016). Metabolism of menthofuran was examined in parallel because it is a metabolite of pulegone and plays a role in the toxicity of pulegone.

As summarised by the EMA in the public statement on the use of herbal medicinal products containing pulegone and menthofuran (EMA, 2016), the metabolism of pulegone and menthofuran has been elucidated in detail in *in vivo* and *in vitro* studies. The metabolism of pulegone is rather complex and involves several pathways, the major being: (i) the pathway leading to the formation of menthofuran involving the 9-hydroxylation with a subsequent reduction of carbon–carbon double bond and furan ring formation; (ii) hydroxylation at C-5 or methyl (9- or 10-) to hydroxylated metabolites, followed by conjugation with glucuronic acid; (iii) formation of piperitenone (p-mentha-1,4(8)-dien-3-one) after 5-hydroxylation followed by dehydration; piperitenone is further metabolised by ring and side-chain hydroxylations (4, 5, 7, 10-positions); (iv) reduction of pulegone to menthone and isomenthone, followed by hydroxylation in ring or side chain and subsequent conjugation with glucuronic acid; (v) direct glutathione (GSH) conjugation with pulegone and with metabolites resulting from oxidation of menthofuran and subsequent metabolism to mercapturic acids.

Many of the metabolites of pulegone are derived from menthofuran and piperitenone. A total of approximately 14 phase I metabolites and approximately 10 phase II metabolites have been identified in *in vivo* rodent studies on pulegone metabolism (Thomassen et al., 1991; Chen et al., 2001; Zhou et al., 2005). When menthofuran was administered to rats (6 or 60 mg/kg bw), three sulfonic acid metabolites and several glucuronide conjugates of hydroxylated mint lactones were identified. Four of the metabolites were identical to pulegone metabolites (Chen et al., 2003b). There is some evidence that in the metabolism of pulegone, conjugation reactions predominate over the menthofuran pathway at lower doses of pulegone (Chen et al., 2001), i.e. the formation of menthofuran would not be significant at lower doses. The only available human study (Engel, 2003) seems to point to a similar scenario. Studies with the expressed human CYP enzymes and human liver microsomes indicate that pulegone is metabolised by human liver CYP2E1, CYP1A2 and CYP2C19 to menthofuran (Khojasteh-Bakht et al., 1999). Menthofuran is metabolised by the same human liver CYPs involved in the metabolism of pulegone and additionally by CYP2A6. Menthofuran inhibits human CYP2A6 irreversibly, possibly by covalent adduction (Khojasteh-Bakht et al., 1998).

GSH plays a relevant role in the metabolism/detoxication of pulegone and its metabolite menthofuran. In *in vivo* studies, it was demonstrated the direct conjugation of GSH with pulegone, being 9–13% of metabolites derived from direct Michael addition of GSH to the α,β-unsaturated ketone of pulegone (Chen et al., 2001). The addition of GSH to pulegone and to menthofuran after bioactivation *in vitro* with human S9 fraction emphasised the role of this tripeptide in the detoxication of both compounds (Lassila et al., 2016). In this study, the formation of mono- and di-conjugates of pulegone-GSH was demonstrated, being the conjugation at the S and N atoms of GSH. The furan epoxide and γ-ketoenal formed by oxidation of menthofuran are conjugated with GSH, neutralising these reactive metabolites (Lassila et al., 2016).

Gordon et al. (1982) showed *in vivo* that the hepatotoxicity of pulegone depends to a large extent on the GSH contents of liver, and its depletion caused a higher hepatotoxicity as observed by histopathology.

The effect of GSH depletion on pulegone hepatotoxicity was evaluated by administration of pulegone to buthionine sulfoximine (BSO)-pretreated and control rats. Five hours following pretreatment with BSO the plasma levels of GSH showed a 59% decrease from control. Administration of an intraperitoneal dose of 150 mg/kg bw pulegone resulted in $>$ 50% hepatocellular necrosis and plasma alanine aminotransferase (ALT) levels of 2,743 $\pm$ 1,346 U/L in the BSO group, compared with $>$ 6% hepatocellular necrosis and 163 $\pm$ 59 U/L, respectively, in controls. Thus, depletion of GSH increased the hepatotoxicity of pulegone by about 10-fold (Thomassen et al., 1990).

Khojasteh et al. (2012), demonstrated the formation of protein adducts in liver of rats given intraperitoneally menthofuran at 200 mg/kg bw. These authors concluded that GSH would not be very efficient in preventing the conjugation of proteins with menthofuran and/or its metabolites. Menthofuran was not detected in the Chen et al. (2001) study in rats given single or multiple 80 mg/kg bw oral doses of pulegone. Menthofuran was also not detected in a study in humans given a single dose of up to 70 mg pulegone. These data suggest that at lower, non-hepatoxic doses, pulegone is metabolised forming glucuronic acid and GSH conjugates (Cohen et al., 2020).

As generally assumed, metabolism of pulegone is very complex, although some pathways were consistently clarified. Important metabolic pathways of pulegone led to its detoxication: (i) direct conjugation of GSH at the α,β-unsaturated ketone of pulegone, as well as with menthofuran resulting from metabolism of pulegone; (ii) hydroxylation at several points of the molecule followed by glucuronidation; and (iii) reduction of pulegone originating menthone and isomenthone. The formation of furan epoxide and γ-ketoenal by oxidation of the furan ring of menthofuran is considered a bioactivation pathway, but some metabolic studies support their conjugation with GSH, neutralising their reactivity. Moreover, at realistic levels, it is not expected that menthofuran is extensively formed.

α-Thujone and β-thujone

The metabolism of thujone has been described by the European Medicines Agency (EMA) in the public statement on the use of herbal medicinal products containing thujone (EMA, 2012) and summarised by the FEEDAP Panel in the EFSA opinion on expressed lemon oil (EFSA FEEDAP Panel, 2021a).

Briefly, hydroxylation of thujones occurs at various positions, mainly at 7 and 4 positions, and is followed to a different extent by glucuronidation, and reductions as minor reactions are main metabolic pathways, with some differences in the *in vitro* and *in vivo* metabolic profiles for the two isomers. In mice, 2-hydroxy-α-thujone (mostly as a glucuronide) was the main urinary metabolite of α-thujone, whereas 7-hydroxy-β-thujone was the most abundant urinary metabolite after β-thujone administration. In the rat, 4-hydroxythujones were the main urinary metabolites after administration of thujones.

3.3.2. Toxicology

3.3.2.1. Genotoxicity and carcinogenicity

For fully defined mixtures, the EFSA Scientific Committee (EFSA SC) recommends applying a component-based approach, i.e. assessing all components individually for their genotoxic potential (EFSA SC, 2019b).

Methyleugenol

Buchu leaf oil contains methyleugenol (0.12% on average, range: 0.09–0.17%), a compound with experimentally proven genotoxicity and carcinogenicity in rodents (as reviewed in EMA, 2005; IARC, 2018a).

Methyleugenol was not mutagenic in the bacterial mutagenicity assay with Salmonella Typhimurium and *Escherichia coli* WP-*uvrA* in the presence and absence of S9-mix. However, positive results were obtained in a modified strain of S. Typhimurium (TA100-hSULT1C2) expressing sulfotransferase (Honda et al., 2016), indicating that the formation of sulfate esters plays a key role in the genotoxicity of alkenylbenzenes. In Chinese hamster ovary (CHO) cells, sister chromatid exchange (SCE) was induced by methyleugenol exposure in the presence and absence of microsomal activation and chromosomal aberrations only in the presence of microsomal activation (NTP, 2000). The induction of malignant transformation by methyleugenol was demonstrated in Syrian hamster ovary cells (Kerkaert et al., 1996). DNA repair was induced by methyleugenol in primary hepatocytes from rats and mice (Howes et al., 1990; Chan and Caldwell, 1992; Burkey et al., 2000). The DNA damaging effects could be inhibited by addition of sulfotransferase inhibitors. DNA adducts were detected after i.p. injection of methyleugenol in the livers of female CD-1 mice and treatment of human HepG2 cells *in vitro* with methyleugenol (Zhou et al., 2007).

The carcinogenicity of methyleugenol was investigated in a 2-year National Toxicology Program (NTP) carcinogenicity study in rats and mice (NTP, 2000) using doses of 0, 37, 75 or 150 mg/kg bw per day in both species and a higher dose of 300 mg/kg bw per day in rats. Rats of both sexes receiving methyleugenol had dose-related increased incidences of hepatocellular carcinomas and neuroendocrine tumours of the glandular stomach.[25] Higher incidences of kidney neoplasms, malignant mesothelioma, mammary gland fibroadenoma and subcutaneous fibroma and fibrosarcoma were observed in male rats only.[26] Increased incidence of hepatocellular carcinomas was seen in both sexes of mice although the incidence was not related to dose. Neuroendocrine tumours of the glandular stomach were also observed in male mice but only at the highest dose. The NTP concluded that there was clear evidence for the carcinogenicity of methyleugenol in rats and mice.

Suparmi et al. (2019) performed an evaluation of the available evidence using BMD approach and found that dose-response modelling, applying model averaging as recommended by the EFSA Scientific Committee (EFSA SC, 2017) on the long-term chronic toxicity study (NTP, 2000) using hepatocellular carcinomas in male rats as a response, yielded a BMD lower confidence limit for a benchmark response of 10% ($BMDL_{10}$) of 22.2 mg/kg bw per day.

Pulegone and menthofuran

The genotoxicity studies of pulegone and its metabolite menthofuran have been reviewed by IARC (2018b) and EMA (2016). *In vitro* studies were generally negative, with few (weak) positive findings in the Ames test. IARC regarded pulegone as non-genotoxic *in vitro*. EMA considered the genotoxic potential of pulegone and menthofuran *in vitro* unlikely. Pulegone, menthofuran and peppermint oil were tested *in vivo* in rat (NTP, 2011a), in a combined micronucleus test and Comet assay (with liver, kidney and urinary bladder urothelium as target organs). The results were consistently negative, except for menthofuran in the Comet assay, which was slightly positive in liver cells most probably due to high-dose cytotoxicity. Overall, EMA concluded that 'pulegone is devoid of genotoxic potential also in those studies in which the production of short-lived reactive intermediates and their scavenging by cellular protection mechanisms has been taken into consideration. A slight increase in tail intensity by high-dose menthofuran in the Comet assay is most likely due to cytotoxicity. Despite some (weak) positive findings in some studies the overall conclusion is that pulegone and menthofuran do not possess genotoxic potential'.

The carcinogenicity of pulegone was investigated in a 2-year NTP carcinogenicity study in mice (dose levels 0, 37.5, 75 or 150 mg/kg bw per day) and rats (dose levels[27] 0, 18.75 (males only) 37.5, 75 or 150 (females only) mg/kg bw per day) (NTP, 2011a). Both NTP (2011a) and IARC (2018b) concluded that there is clear evidence of carcinogenicity of pulegone and its metabolite menthofuran in male and female mice (liver)[28] and in female rats (bladder).[29] IARC classified pulegone and menthofuran as possibly carcinogenic to humans (2B). Based on a weight of evidence approach, EMA concluded that cell cytotoxicity and regenerative proliferation are driven by reactive metabolites and GSH depletion as a probable mechanism of action, with the overall conclusion that toxicity and carcinogenicity of pulegone have a threshold (EMA, 2016).

[25] Male rats: hepatocellular adenoma (5/50, 12/50, 23/50, 38/50, 32/50), hepatocellular carcinoma (2/50, 3/50, 14/50, 25/50, 36/50), hepatocellular adenoma or carcinoma combined (7/50, 14/50, 28/50, 43/50, 45/50), hepatocholangioma or hepatocolangiocarcinoma (0/50, 0/50, 1/50, 2/50, 13/50); glandular stomach (0/50, 0/50, 0/50, 7/50, 4/50). Female rats: hepatocellular adenoma (1/50, 8/50, 11/49, 33/49, 43/50), hepatocellular carcinoma (0/50, 0/50, 4/49, 8/49, 22/50), hepatocellular adenoma or carcinoma combined (1/50, 8/50, 14/49, 34/49, 43/50), hepatocholangioma or hepatocolangiocarcinoma (0/50, 0/50, 0/50, 3/50, 13/17); glandular stomach (0/50, 1/50, 25/50, 34/50, 41/50).

[26] Male rats: kidney neoplasms (4/50, 6/50, 17/50,13/50, 20/50), malignant mesothelioma (1/50, 3/50, 5/50, 12/50, 5/50), mammary gland fibroadenoma (5/50, 5/50, 15/50, 13/50, 6/50), subcutaneous fibroma or fibrosarcoma (1/50, 12/50, 8/50, 8/50, 4/50).

[27] Dosing of males at the highest dose (75 mg/kg bw) and females at the highest dose (150 mg/kg bw) was stopped after 60 weeks because of high morbidity and mortality.

[28] Male mice: hepatoblastoma (1/50, 3/50, 7/50*, 2/50, *p = 0.04), hepatocellular adenoma (22/50, 31/50, 35/50*, 28/50, p = 0.008), hepatocellular carcinoma (13/50, 11/50, 18/50, 15/50), hepatocellular adenoma or carcinoma combined (29/50, 36/50, 42/50*, 35/50, *p = 0.004), hepatocellular adenoma or carcinoma or hepatoblastoma combined (29/50, 37/50, 42/50*, 36/50, *p = 0.004). Female mice: hepatoblastoma (0/50, 1/50, 2/50, 2/50), hepatocellular adenoma (13/49, 15/50, 13/50, 27/50*, *p < 0.002), hepatocellular carcinoma (5/50, 1/50, 4/50, 8/50), hepatocellular adenoma or carcinoma combined 17/49, 15/50, 15/50, 32/50*, *p = 0.002), hepatocellular adenoma or carcinoma or hepatoblastoma combined (17/49, 15/50, 15/50, 33/50*, *p < 0.001). Although statistically significant, increases in incidences of hepatocellular adenomas, carcinomas and hepatomas in mice are not considered very relevant for humans, considering the highly variability in the background incidences hepatocellular tumours (adenomas as well as carcinomas) in mice.

[29] Female rats: urinary bladder papilloma (0/50, 0/49, 1/50, 3/47*, *p = 0.044), urinary bladder carcinoma (0/50, 0/49, 0/50, 2/47), urinary bladder papilloma or carcinoma combined (0/50, 0/49, 1/50, 5/47*, *p = 0.005).

α-Thujone and β-thujone

The genotoxicity and carcinogenicity data of thujone (α- and β-thujone) have been summarised in the EMA public statement on the use of herbal medicinal products containing thujone (EMA, 2012) and reviewed by the FEEDAP Panel in the EFSA opinion on expressed lemon oil (EFSA FEEDAP Panel, 2021a). Briefly, α-thujone and an isomeric mixture thujone were not mutagenic in an Ames test in the presence or absence of the activating enzyme system. *In vivo*, racemic thujone (6.25, 12.5, 25, 50 or 75 mg α,β-thujone/kg bw) induced a small but significant increase in micronucleated erythrocytes in the peripheral blood at the end of the 3-month study in female B6C3F1 mice but not males.

Some neoplastic lesions were reported in the 2-year study with α,β-thujone in rat (dose levels 12.5, 25 and 50 mg/kg) and mice (dose levels 3, 6, 12, 25 mg/kg) (NTP, 2011b). However, the increase of tumours in tissues referred in the NTP report (some evidence of carcinogenicity in the preputial gland observed in male rats only at the dose of 25 mg/kg, not dose-related)[31] is unlikely to arise by a genotoxic mechanism and therefore was not considered of concern.

3.3.2.2. Subchronic toxicity studies

Methyleugenol

Methyleugenol was tested in a short-term toxicity assay by repeated dosing over a period of 14 weeks in rats and mice dosed with 10, 30, 100, 300 and 1,000 mg/kg bw by gavage for 5 days per week for 14 weeks (NTP, 2000). Weight reduction and haematological changes were seen. Changes of organ weight and function, including effects on liver and the glandular stomach, were observed at doses of 100 mg/kg bw and higher. A no observed adverse effect level (NOAEL) of 30 mg/kg bw could be derived from the rat study. Similar observations were obtained in a study in mice with the same dosing and time schedule. Increased liver weights and lesions of the glandular stomach occurred at a dose of 30 mg/kg bw and above. Thus, the NOAEL for non-neoplastic lesions identified in the mouse study was 10 mg/kg bw per bw.

Pulegone

Pulegone was tested in the framework of the NTP of the US Department of Health and Human Services (TR 563, NTP, 2011a).

Groups of 10 male and 10 female F344/N rats were administered 0, 9.375, 18.75, 37.5, 75 or 150 mg pulegone/kg bw in corn oil by gavage, 5 days per week for 14 weeks. No treatment-related mortality was observed. Several adverse effects were observed at the two highest doses (75 and 150 mg/kg bw): weight reduction, increased absolute and relative liver and kidney weights, hyaline glomerulopathy, bile duct hyperplasia, hepatocyte hypertrophy and many others. Some of these effects were seen also in lower doses. When the same dosages were administered to B6C3F1 mice, the only treatment-related observations were the increase of liver weight and glutathione levels at the highest dose of 150 mg/kg bw in males and at the two highest doses of 75 and 150 mg/kg bw in females (NTP, 2011a). From the rat study, an NOAEL value of 9.375 mg/kg bw was derived based on a dose-dependent reduction of red blood cells starting at 18.75 mg/kg bw.

Two earlier 28-day studies in rat (Thorup et al., 1983; Mølck et al., 1998, as referenced in EMA, 2016) indicate an NOAEL of 20 mg pulegone/kg bw per day.

Thujones

The subchronic toxicity studies of thujone (α- and β-thujone) have been summarised in the EMA public statement on the use of herbal medicinal products containing thujone (EMA, 2012) and reviewed by the FEEDAP Panel in the EFSA opinion on expressed lemon oil (EFSA FEEDAP Panel, 2021a). Briefly, a no observed effect level (NOEL) was derived from a chronic study with an isomeric mixture of thujone[30] administered by gavage to B6C3F1 mice at doses of 0, 3, 6, 12 and 25 mg/kg bw per day and to Fischer 344 rats at doses of 0, 12.5, 25 and 50 mg/kg bw per day for 2 years. In the rat, the NOEL was 12.5 mg/kg bw for mortality and tonic seizures (no NOEL for clonic seizures). In the mouse, the NOEL was 12 mg/kg bw for seizures and mortality.

For α-thujone, the FEEDAP Panel retains a $BMDL_{10}$ of 8 mg/kg bw per day, recalculated from the $BMDL_{10}$ of 11 mg/kg bw per day derived from the long-term chronic toxicity study in mice and rats

[30] α,β-Thujone mixture containing 70% α-thujone, 11% β-thujone, 16% fenchone, 2% camphor and 0.5% of unidentified impurities.

[31] Male rats only: Preputial gland: incidences of carcinoma (1/49, 0/49, 5/50); adenoma or carcinoma (3/49, 1/49, 9/50).

using clonic seizures as a response (EFSA FEEDAP Panel, 2021a) as described in EMA (2012). In this dose range, β-thujone is described to have a much lower neurotoxic activity compared to α-thujone (NTP, 2011b). As a worst-case assumption, the $BMDL_{10}$ of 8 mg/kg bw per day is also applied to β-thujone.

3.3.3. Safety for the target species

Tolerance and/or toxicological studies made with the essential oil 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). 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 (> 97.6%), the FEEDAP Panel applied a component-based approach to assess the safety for target species of the essential oil. Substances for which a concern for genotoxicity has been identified (methyleugenol) are assessed separately.

Components other than methyleugenol

Based on considerations related to structural and metabolic similarities, the components were allocated to 13 assessment groups, which correspond to the chemical groups (CGs) 4, 5, 6, 7, 8, 14, 16, 18, 20, 23, 27, 30 and 31 (and related subgroups), as defined in Annex I of Regulation (EC) No 1565/2000. For chemical group 31 ('aliphatic and aromatic hydrocarbons'), the application of subassessment groups as defined in Flavouring Group Evaluation 25 (FGE.25) and FGE.78 was applied (EFSA CEF Panel, 2015a,b). The allocation of the components to the (sub-)assessment groups is shown in Table 5 and in the corresponding footnote.

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 oil 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. The intake levels of the individual components calculated for chickens for fattening, the species with the highest ratio of feed intake/body weight, are shown in Table 5.

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 was assessed 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 a (sub-) assessment group, to derive a (sub-)assessment group NOAEL.

Toxicological data of subchronic studies, from which NOAELs could be derived, were available for citronellol [02.011] in CG 4 (EFSA FEEDAP Panel, 2016a), isopulegol [02.067] in CG 5 (EFSA FEEDAP Panel, 2020), linalool [02.013] and terpineol[32] [02.230] in CG 6 (EFSA FEEDAP Panel, 2012a), menthol [02.015] and isopulegone [07.067] in CG 8 (EFSA FEEDAP Panel, 2016b, 2020), linalool oxide [13.140] in CG 13 (EFSA FEEDAP Panel, 2012b), 1,8-cineole [03.001] in CG 16 (EFSA FEEDAP Panel, 2012c, 2021a), eugenol [04.003] in CG 18 (EFSA FEEDAP Panel, 2011a), methyl N-methyl anthranilate [09.781] in CG 27 (EFSA FEEDAP Panel, 2011b), myrcene [01.008], d-limonene [01.045], 1-isopropyl-4-benzene [01.002] and β-caryophyllene [01.007] in CG 31 (EFSA FEEDAP Panel, 2015, 2016c).

For α-thujone and β-thujone, the FEEDAP Panel retains a $BMDL_{10}$ of 8 mg/kg bw per day (see Section 3.3.2.2). The NOAEL value of 9.375 mg/kg bw per day for pulegone is applied to menthofuran in the same assessment group.

Considering the structural and metabolic similarities, for the subgroup of terpinyl derivatives in CG 6, i.e. α-terpineol [02.014] and 4-terpinenol [02.072], the reference point was selected based on the NOAEL of 250 mg/kg bw per day available for terpineol [02.230] and d-limonene [01.045].

[32] Terpineol is a mixture of four isomers: α-terpineol [02.014], a mixture of *(R)*-(+)-α-terpineol and *(S)*-(–)-α-terpineol, β-terpineol, γ-terpineol and 4-terpinenol [02.072] (or δ-terpineol). The specification for terpineol [02.230] covers α-, β-, γ and δ-terpineol. Composition of mixture: 55–75% α-terpineol, 16–23% γ-terpineol, 1–10% cis-β-terpineol, 1–13% trans-β-terpineol and 0–1% δ-terpineol (EFSA CEF Panel, 2015c) FGE.18Rev 3.

Similarly, the NOAELs for the representative compounds of CG 31, myrcene [01.008], d-limonene [01.045], 1-isopropyl-4-benzene [01.002] and β-caryophyllene [01.007] were applied as group NOAELs to the subassessment group II, III, IVe and V, respectively (EFSA CEF Panel, 2015a,b).

For the remaining 21 compounds, p-menth-1-en-9-yl acetate [09.615], myrtenyl acetate [09.302], d,l-isomenthone [07.078], p-menthan-3-one [07.059], cis-dihydrocarvone, trans-dihydrocarvone, (1R,5R)-carvyl acetate, bornyl acetate [09.017], pin-2-en-4-one [07.196], p-menth-1-en-3-one [07.175], trans-sabinene hydrate [02.085], trans-1-methyl-4-(1-methylvinyl)cyclohex-2-en-1-ol, cis-para-2,8-menthadien-1-ol, cis-8-mercapto-p-menthan-3-one, trans-2-(1-mercapto-1-methylethyl)-5-methylcyclohexan-1-one, trans-menthone-8-thioacetate, cis-menthone-8-thioacetate, 2-hydroxypiperi tone [07.168], thuja-2,4(10)diene, bicyclogermacrene and pseudo-diosphenol, toxicity studies and NOAEL values performed with the compounds under assessment were not available and read-across was not possible. Therefore, the threshold of toxicological concern (TTC) approach was applied (EFSA FEEDAP Panel, 2017b). These compounds belong to Cramer classes I, II and III. According to the assessment by JECFA, 2-hydroxypiperitone [07.168] belongs to Cramer class II (WHO, 2011). The same allocation to Cramer class II is applied to pseudo-diosphenol and the two compounds are assessed together in CG 30.

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) or from the 5th percentile of the distribution of NOAELs of the corresponding Cramer Class (i.e. 3, 0.91 and 0.15 mg/kg bw per day for compounds belonging to Cramer Class I, II and III, respectively). Reference points selected for each compound are shown in Table 5.

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 SC, 2019a). An MOET > 100 allowed for interspecies- and intra-individual variability (as in the default 10x10 uncertainty factor). The compounds resulting individually in an MOE > 50,000 were not further considered in the assessment group as their contribution to the MOE(T) is negligible.[33]

The approach to the safety assessment of buchu leaf oil for chickens for fattening is summarised in Table 5.

Table 5: Compositional data, intake values (calculated for chickens for fattening at 0.1 mg/kg complete feed), reference points and margin of exposure (MOE) for the individual components of buchu leaf oil classified according to assessment groups

Essential oil composition			**Exposure**		**Hazard characterisation**		**Risk characterisation**	
Assessment group	**FLAVIS-No**	**Max conc. in the oil**	**Max Feed conc.**	**Intake[a]**	**Cramer Class[b]**	**NOAEL[c]**	**MOE**	**MOET**
Constituent	–	**%**	**µg/kg**	**µg/kg bw per day**	–	**mg/kg bw per day**	–	–
CG 30								
2-Hydroxypiperitone (Diosphenol)	07.168	20.80	20.80	1.87	II[d]	*0.91*	487	
Pseudo-diosphenol	n.a.	15.20	15.20	1.365	II	*0.91*	667	

[33] Compounds included in the assessment groups but not reported in the table: citronellol (CG 4); isopulegol (CG 5); linalool, 4-terpineol, α-terpineol trans-1-methyl-4-(1-methylvinyl)cyclohex-2-en-1-ol and cis-para-2,8-menthadien-1-ol (CG 6); p-menth-1-en-9-yl acetate and myrtenyl acetate (CG 7); isopulegone, cis dihydrocarvone, trans-dihydrocarvone, (1R,5R)-carvyl acetate, bornyl acetate, pin-2-en-4-one, menthol, p-menth-1-en-3-one and trans sabinene hydrate (CG 8); 1,8-cineole (CG 16); eugenol (CG 18); cis-menthone-8-thioacetate (CG 20); benzyl benzoate (CG 23); methyl N-methyl anthranilate (CG 27); myrcene, trans-3,7-dimethyl-1,3,6-octatriene, cis-3,7-dimethyl-1,3,6-octatriene and (E)-4,8-dimethyl-1,3,7-nonatriene (CG 31, II); d-limonene, γ-terpinene, α-terpinene and terpinolene (CG 31, III); p-cymene and 1-isopropenyl4-methylbenzene (CG 31, IV); sabinene, α-pinene, β-pinene, α-thujene, camphene, thuja-2,4(10)-diene (CG 31, V); bicyclogermacrene (CG 31, VI); α-thujone and β-thujone.

Essential oil composition			Exposure		Hazard characterisation		Risk characterisation	
Assessment group	**FLAVIS-No**	**Max conc. in the oil**	**Max Feed conc.**	**Intake[a]**	**Cramer Class[b]**	**NOAEL[c]**	**MOE**	**MOET**
Constituent	–	**%**	**µg/kg**	**µg/kg bw per day**	–	**mg/kg bw per day**	–	–
MOET								282
CG 8								
d,l-Isomenthone	07.078	26.20	26.20	2.070	II	*0.91*	387	
p-Menthan-3-one	07.059	10.20	10.20	0.806	II	*0.91*	994	
MOET CG 8								274
Pulegone and menthofuran								
Pulegone	n.a.	7.81	7.81	0.617	(II)	**9.375**	13,371	
Menthofuran	13.035	0.37	0.37	0.033	(II)	9.375	285,329	
MOET								12,773
CG 20								
cis-8-Mercapto-p-menthan-3-one	n.a.	3.00	3.00	0.269	III	*0.15*	557	
trans-2-(1-Mercapto-1-methylethyl)-5-methylcyclohexan-1-one	n.a.	0.53	0.53	0.047	III	*0.15*	3,159	
Menthone-8-thioacetate, trans-	n.a.	0.29	0.29	0.026	III	*0.15*	5,762	
MOET CG 20								437

(a): Intake calculations for the individual components are based on the use level of 0.1 mg/kg in feed for chickens 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): When an NOAEL value is available or read-across is applied, the allocation to the Cramer class is put into parentheses.
(c): Values **in bold** refer to those components for which the NOAEL value was available, values *in italics* are the 5th percentile of the distribution of NOAELs of the corresponding Cramer Class, other values (plain text) are NOAELs extrapolated by using read-across.
(d): Allocated to Cramer class II, according to JECFA.

As shown in Table 5, for all the assessment groups and individual constituents, the MOE(T) was ≥ 274. Therefore, no safety concern was identified for the oil under assessment (without considering the presence of methyleugenol) when used as a feed additive for chickens for fattening at the proposed use levels (0.1 mg/kg). From the lowest MOET of 274 (for CG 8) in chickens for fattening, the MOET was calculated for the other target species considering the respective daily feed intake and conditions of use. The results are summarised in Table 6.

Table 6: Combined margin of exposure (MOET) for the assessment group CG 8 calculated for the target animal categories at the proposed use level

Animal category	Body weight (kg)	Feed intake (g DM/day)	Proposed use level (mg/kg feed)[a]	Lowest MOET
Chicken for fattening	2	158	0.10	274
Laying hen	2	106	0.15	272
Turkey for fattening	3	176	0.15	245
Piglet	20	880	0.20	246
Pig for fattening	60	2,200	0.25	234
Sow	175	5,280	0.30	241

Animal category	Body weight (kg)	Feed intake (g DM/day)	Proposed use level (mg/kg feed)[a]	Lowest MOET
Veal calf (milk replacer)	100	1,890	0.50	228
Cattle for fattening	400	8,000	0.45	241
Dairy cow	650	20,000	0.30	233
Sheep/goat	60	1,200	0.45	241
Horse	400	8,000	0.45	241
Rabbit	2	100	0.15	289
Salmon	0.12	2.1	0.50	241
Dog	15	250	0.50	255
Cat	3	60	0.50	216[b]
Ornamental fish	0.012	0.54	0.50	866

(a): Complete feed containing 88% DM, milk replacer 94.5% DM.
(b): The MOET for cats is increased to 500 because of the reduced capacity of glucuronidation.

Considering the magnitude of the MOET, the additive is safe at the proposed use level of 0.5 mg/kg complete feed for all animal species except cats. For cats, the maximum safe use levels in feed were calculated in order to ensure an MOET > 500, considering their unusually low capacity for glucuronidation (Court and Greenblatt, 1997; Lautz et al., 2021). The calculated maximum safe level in feed for cats is 0.2 mg/kg complete feed.

No specific proposals have been made by the applicant for the use level in water for drinking. The Panel considers that the use of the additive 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).

Methyleugenol

Low concentrations of methyleugenol were detected in all batches of the additive under assessment (average: 0.12%, range: 0.09–0.17%). The use of buchu leaf oil at the proposed use levels in feed for the different target species (ranging from 0.1 to 0.5 mg/kg complete feed, see Section 3.2.3), would result in concentrations ranging from 0.17 to 0.85 μg methyleugenol/kg complete feed.

The maximum daily intake of methyleugenol in μg/kg bw was calculated at the maximum proposed use level of the additive in feed for the different target animal categories and considering the maximum analysed value in the additive (0.17%). The calculated intake values range between 0.004 μg/kg bw per day (in ornamental fish) and 0.018 μg/kg bw per day (in pigs for fattening and dairy cows, see Appendix A).

When the estimated exposures for the different animal categories are compared to the $BMDL_{10}$ of 22.2 mg/kg bw per day, derived by Suparmi et al. (2019) from a rodent carcinogenicity study (NTP, 2000), an MOE ranging between 1,200,000 and 5,100,000 is calculated. The magnitude of this MOE is indicative of a low concern for the target species (see Appendix A).

3.3.3.1. Conclusions on safety for the target species

The FEEDAP Panel concludes that buchu leaf oil under assessment is safe up to the maximum proposed use levels in complete feed of 0.1 mg/kg for chickens for fattening, 0.15 mg/kg for laying hens, turkeys for fattening and rabbits, 0.20 mg/kg for piglets, 0.25 mg/kg for pig for fattening, 0.30 mg/kg for sows and dairy cows, 0.45 mg/kg for cattle for fattening, sheep, goats and horses, 0.5 for veal calves (milk replacer), fish, ornamental fish and dogs. For cats, the maximum safe level in fed is 0.2 mg/kg complete feed.

The Panel considers that the use of the additive 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.

3.3.4. Safety for the consumer

Buchu leaf oil is added to a wide range of food categories for flavouring purposes. Although individual consumption figures are not available, the Fenaroli's handbook of flavour ingredients (Burdock, 2009) cites values of 0.0003 mg/kg bw per day (FEMA 2169). Fenaroli also reports use levels in food and beverages in the range of 1 mg/kg up to 15 mg/kg.

Many of the constituents of the essential oil under assessment are currently authorised as food flavourings without limitations and have been already assessed for consumer safety when used as feed additives in animal production (see Table 1, Section 1.2).

No data on residues in products of animal origin were made available for any of the constituents of the essential oil. However, the Panel recognises that the constituents of buchu leaf oil are expected to be extensively metabolised and excreted in the target species. Also for methyleugenol, the available data indicate that it is absorbed, metabolised and rapidly excreted and is not expected to accumulate in animal tissues and products, consequently residues in food products are unlikely (see Section 3.3.1). Therefore, a relevant increase of the uptake of the individual constituents by humans consuming products of animal origin is not expected.

Considering the reported human exposure due to direct use of buchu leaf oil in food (Burdock, 2009), it is unlikely that consumption of products from animals given buchu leaf oil at the proposed maximum use level would increase human background exposure.

Consequently, no safety concern would be expected for the consumer from the use of buchu leaf oil 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 applicant produced a safety data sheet[34] for buchu leaf oil where hazards for users have been identified. The essential oil under assessment should be considered as irritant to skin and eyes, and as a dermal and respiratory sensitiser.

When handling the essential oil, exposure of unprotected users to methyleugenol cannot be excluded. Therefore, to reduce the risk, the exposure of the users should be minimised.

3.3.6. Safety for the environment

A. betulina is not native to Europe. Therefore, the safety for the environment is assessed based on the individual components of the essential oil.

Several main constituents included in Tables 2 and 3 have been already evaluated by EFSA as sensory additives in animal feed and were considered to be safe for the environment at use individual levels higher than those resulting from the use of the essential oil in feed at the proposed use levels. At the proposed conditions of use (0.1–0.5 mg/kg complete feed), the main constituent, d,l-isomenthone [07.078] would result in a concentration of 0.026–0.13 mg/kg complete feed, which is below the concentration which was considered safe for the environment when evaluated as sensory additive in animal feed (EFSA FEEDAP Panel, 2016b). For the other components, the concentrations in feed would be lower.

For other major components, e.g. 2-hydroxypiperitone [07.168] and pulegone, there is evidence that they are naturally occurring at concentrations higher than those resulting from the use of the essential oil in feed at the proposed use levels (2-hydroxypiperitone: 36 mg/kg in black currant (buds) EFSA CEF Panel, 2011b; EMA, 2016).

Several major and minor constituents present in buchu leaf oil have not been evaluated by EFSA with respect to its safety for the environment. All the other components of the essential oil will be below < 0.5 mg/kg complete feed, the threshold below which the trigger value for the predicted environmental concentration (PEC_{soil}) of 10 μg/kg is not exceeded.

Therefore, the FEEDAP Panel concludes that the use of buchu leaf oil as a flavour in animal feed is not expected to pose a risk for the environment.

3.4. Efficacy

Buchu leaf oil is listed in Fenaroli's Handbook of Flavour Ingredients (Burdock, 2009) and by FEMA with the reference number 2169.

Since the oil from the leaves of *A. betulina* 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.

[34] Technical dossier/Supplementary Information June 2019/Annex_IX_SIn reply_Buchu leaves_oil_MSDS. Aspiration hazard (H304, category 1), Hazards for skin corrosion/irritation (H315, category 2), skin sensitisation (H317, category 1).

4. Conclusions

Since buchu leaf oil from *Agathosma betulina* (P.J. Bergius) Pillans may be produced from plants of different origins and by various processes resulting in preparations with different composition and toxicological profiles, the following conclusions apply only to buchu leaf oil which contains ≤ 0.17% methyleugenol and is produced by steam distillation from *A. betulina*.

The FEEDAP Panel concludes that buchu leaf oil under assessment is safe up to the maximum proposed use levels in complete feed of 0.1 mg/kg for chickens for fattening, 0.15 mg/kg for laying hens, turkeys for fattening and rabbits, 0.20 mg/kg for piglets, 0.25 mg/kg for pigs for fattening, 0.30 mg/kg for sows and dairy cows, 0.45 mg/kg for cattle for fattening, sheep, goats and horses, 0.5 for veal calves (milk replacer), fish, ornamental fish and dogs. For cats, the calculated maximum safe level in feed is 0.2 mg/kg complete feed. 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.

No concerns for consumers were identified following the use of the additive at the use level in feed considered safe for the target animals.

The essential oil under assessment should be considered as irritant to skin and eyes, and as a dermal and respiratory sensitiser. When handling the essential oil, exposure of unprotected users to methyleugenol cannot be excluded. Therefore, to reduce the risk, the exposure of the users should be minimised.

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

Buchu leaf oil 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.

5. Recommendation

The specification should ensure that the methyleugenol concentration should be as low as possible and should not exceed 0.17% of the essential oil.

6. Documentation as provided to EFSA/Chronology

Date	Event
05/11/2010	Dossier received by EFSA. Chemically 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)
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
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
03/05/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. Issues: characterization, safety for the target species, safety for the consumer, safety for the user, safety for the environment
20/06/2018	Comments received from Member States
13/07/2018	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: Method of analysis*

Date	Event
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
03/06/2019	Reception of supplementary information from the applicant (partial submission: buchu leaves oil)
01/07/2020	Reception of supplementary information from the applicant (partial submission: clarification request)
17/03/2021	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
02/04/2021	Partial withdrawal by applicant (EC was informed) for the following additive: olibanum tincture
09/11/2021	The application was split and a new EFSA-Q-2021-00597 was assigned to the preparation included in the present assessment. Scientific assessment re-started for the preparation included in the present assessment
27/01/2022	Opinion adopted by the FEEDAP Panel on buchu leaf oil. End of the Scientific assessment for the preparation included in the present assessment. The assessment of one preparation is still ongoing

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Abbreviations

AFC	EFSA Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Foods
BDG	botanically defined group
BMD	benchmark dose
$BMDL_{10}$	benchmark dose lower confidence limit for a benchmark response of 10%
bw	body weight
CAS	Chemical Abstracts Service
CD	Commission Decision
CDG	chemically defined group
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CG	chemical group
DM	dry matter
EEIG	European economic interest grouping
EINECS	European Inventory of Existing Chemical Substances
EURL	European Union Reference Laboratory
FAO	Food and Agriculture Organization of the United Nations
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
FGE	Flavouring Group Evaluation
FLAVIS	the EU Flavour Information System
FL-No	FLAVIS number
GC	gas chromatography
GC-FID	gas chromatography with flame ionisation detector

GC–MS	gas chromatography–mass spectrometry
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
LOD	limit of detection
MOE	margin of exposure
MOET	combined margin of exposure (total)
NOAEL	no observed adverse effect level
PEC_{soil}	Predicted environmental concentration for soil
TTC	threshold of toxicological concern
UF	uncertainty factor
WHO	World Health Organization

Appendix A – Methyleugenol: Maximum daily intake and margin of exposure for the different target species

The maximum daily intake of methyleugenol for the different target species and categories was calculated based on

- the default values for body weight and feed intake (EFSA FEEDAP Panel, 2017b)
- the maximum proposed use level of the additive in feed for the different target animal categories (ranging from 0.10 to 0.50 mg/kg complete feed) and
- assuming that methyeugenol is present at a concentration corresponding to the maximum analysed value in the additive (0.17%).

The margin of exposure (MOE) for each animal category is calculated as the ratio of the reference point (the $BMDL_{10}$ of 22.2 mg methyeugenol/kg bw per day, see Section 3.3.1) to the intake.

According to the general approach to assess the safety for the target species of botanical preparations which contain compounds that are genotoxic and/or carcinogenic (EFSA FEEDAP Panel, 2021b),[13] 'for substances for which carcinogenicity studies in rodents are available, from which a $BMDL_{10}$ can be derived, the MOE approach (EFSA, 2005; EFSA Scientific Committee, 2012) can be applied. Similarly to human risk assessment, a margin of exposure (MOE) with a magnitude of $\geq$ 10,000, when comparing estimated exposure to genotoxic and/or carcinogenic substances with a $BMDL_{10}$ from a rodent carcinogenicity study, would be indicative of a low concern for the target species (EFSA SC, 2019a)'.

The maximum daily intake of methyleugenol for the different target animal categories and the corresponding MOE are reported in Table A.1.

Table A.1: Target animal intake of methyleugenol (as μg/kg bw per day) and margin of exposure (MOE) at the maximum proposed use level of the additive in feed for target animal category

Animal category	Daily feed intake	Body weight	Use level	Methyleugenol intake[(a)]	MOE[(b)]
	kg DM/day	kg	mg/kg	μg/kg bw per day	–
Chicken for fattening	0.158	2	0.10	0.015	1,454,654
Laying hen	0.106	2	0.15	0.015	1,445,505
Turkey for fattening	0.176	3	0.15	0.017	1,305,882
Piglet	0.88	20	0.20	0.017	1,305,882
Pig for fattening	2.2	60	0.25	0.018	1,253,647
Sow lactating	5.28	175	0.30	0.017	1,269,608
Veal calf (milk replacer)	1.89	100	0.50	0.017	1,305,882
Cattle for fattening	8	400	0.45	0.017	1,276,863
Dairy cow	20	650	0.30	0.018	1,244,941
Sheep/goat	1.2	60	0.45	0.017	1,276,863
Horse	8	400	0.45	0.017	1,276,863
Rabbit	0.1	2	0.15	0.014	1,532,235
Salmon	0.0021	0.12	0.50	0.017	1,313,345
Dog	0.25	15	0.50	0.016	1,379,012
Cat	0.06	3	0.20	0.008	2,872,941
Ornamental fish	0.00054	0.012	0.50	0.004	5,107,451

(a): The values of methyleugenol in feed are calculated considering the maximum analysed value in the additive.
(b): The MOE for methyleugenol is calculated as the ratio of the reference point ($BMDL_{10}$) to the intake.

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 four 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 four 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 five 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 five 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 four 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 four 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 three 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 four 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.