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Safety and efficacy of a feed additive consisting of an extract of olibanum from *Boswellia serrata* Roxb. ex Colebr. for use in dogs and horses (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 olibanum extract from *Boswellia serrata* Roxb. ex Colebr., when used as a sensory additive (flavouring) in feed for all dogs and horses. The FEEDAP Panel concluded that the additive under assessment is safe for horses at the maximum proposed use level of 100 mg/kg in complete feed. For dogs, the calculated safe concentration in feed is 330 mg/kg complete feed. The additive is considered safe for consumers when used at the proposed conditions of use in horses. The additive under assessment should be considered as non-irritant to skin and eyes, but in the absence of data, no conclusion can be drawn on its potential to be a dermal and respiratory sensitiser. The use of the additive under the proposed conditions of use in feed for horses was not expected to pose a risk for the environment. *Boswellia* species and their preparations were recognised to flavour food. Since their 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, olibanum extract, *Boswellia serrata* Roxb. ex Colebr., boswellic acids, tirucallic acids, methyleugenol, estragole, safety

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1. Introduction

1.1. Background and Terms of Reference

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

The European Commission received a request from the Feed Flavourings Authorisation Consortium European Economic Interest Grouping (FFAC EEIG)[2] for authorisation/re-evaluation of 20 preparations (namely buchu leaves oil, amyris oil, olibanum extract (wb), olibanum tincture, lime oil, neroli bigarade oil, 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] During the course of the assessment, this application was split and the present opinion covers only one out of the 20 initial preparations under application: olibanum extract from *Boswellia serrata* Roxb. ex Colebr.[5] for all animal species. During the assessment, the applicant requested a change in the species limiting the application for authorisation to dogs and horses.[6]

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 olibanum extract from *B. serrata*, when used under the proposed conditions of use (see Section 3.2.4).

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

1.2. Additional information

The additive under assessment, olibanum extract from *B. serrata* Roxb. ex Colebr., 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.

The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) issued an opinion on the substantiation of health claims related to various food(s)/food constituent(s), including *B. serrata*, claiming maintenance of joints, bone and muscles (EFSA NDA Panel, 2010).

'Indian frankincense (Olibanum indicum)' is described in a monograph of the European Pharmacopoeia 10.0 (PhEur, 2020). It is defined as the air-dried gum-resin exudate, obtained by incision in the stem or branches of *Boswellia serrata* Roxb. ex Colebr.

[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: *Boswellia serrata Roxb. ex Colebr.*, synonym
[6] Technical dossier/Supplementary information February 2019/SIn_reply_Olibanum extract.

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[7] in support of the authorisation request for the use of olibanum extract from *B. serrata* 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.

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.[8]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of olibanum extract from *B. serrata* is in line with the principles laid down in Regulation (EC) No 429/2008[9] and the relevant guidance documents: Guidance on safety assessment of botanicals and botanical preparations intended for use as ingredients in food supplements (EFSA Scientific Committee, 2009), Compendium of botanicals that have been reported to contain toxic, addictive, psychotropic or other substances of concern (EFSA, 2012), Guidance for the preparation of dossiers for sensory additives (EFSA FEEDAP Panel, 2012a), Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012b), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017a), Guidance on the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c), Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018), Guidance on the use of the benchmark dose approach in risk assessment (EFSA Scientific Committee, 2017), Guidance document on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals (EFSA Scientific Committee, 2019a), Statement on the genotoxicity assessment of chemical mixtures (EFSA Scientific Committee, 2019b), Guidance on the use of the threshold of toxicological concern approach in food safety assessment (EFSA Scientific Committee, 2019c), 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, 2021).[10]

3. Assessment

The additive under assessment, olibanum extract, is a dry extract of olibanum from *Boswellia serrata* Roxb. ex Colebr. It is intended for use as sensory additive (functional group: flavouring compounds) in feed for dogs and horses.

3.1. Origin and extraction

Boswellia serrata Roxb. ex Colebr. is a small- to medium-sized deciduous tree species belonging to the Burseraceae family. It is native to India but is now also found in North Africa, Somalia and parts of the Middle East. It is known primarily as a source of an oleoresin referred to as Indian olibanum or Indian frankincense. Traditionally, trees were tapped two or three times in a year and the milky resin collected, stored and allowed to air-dry, hardening to a transparent brown solid. The solid material was then reduced to a course powder and, in this form, widely used as an incense or in traditional (Ayurvedic) medicine.

[7] FEED dossier reference: FAD-2010-0322.
[8] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2010-0322-bdg08.pdf
[9] 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.
[10] https://www.efsa.europa.eu/sites/default/files/2021-05/general-approach-assessment-botanical-preparations-containing-genotoxic-carcinogenic-compounds.pdf

The additive produced by the applicant originates from oleoresin collected by tapping the trunk and larger branches of *B. serrata* trees growing wild in North-West India. [redacted][11]

3.2. Characterisation

3.2.1. Characterisation of the extract

The extract is an off-white to cream powder with a characteristic odour and taste. The additive is specified to contain ≥ 65% boswellic acids (total organic acids determined by titrimetry and expressed as boswellic acid equivalents), with 11-keto-β-boswellic acid (KBA) (2–5%) and 3-O-acetyl-11-keto β-boswellic acid (AKBA) (2–5%) selected as phytochemical markers.

Compliance with the proposed specifications was demonstrated in five batches of the additive. Total organic acids (determined by non-aqueous titration with potassium methoxide) accounted for 65.2–67.6% of the extract,[12] with 11-KBA and 3-AKBA representing, respectively, 2.53–3.86% and 2.35–3.57% of the extract (determined by high-performance liquid chromatography with ultraviolet detection, HPLC-UV).

The applicant provided a full characterisation of the five batches of the additive. Table 1 summarises the results of proximate analysis[14] and Table 2 the content of organic acids and essential oil.

Table 1: Proximate analysis of a dried extract of *Boswellia serrata* Roxb. ex Colebr. based on the analysis of five batches (mean and range). The results are expressed as % (w/w) of the extract

Constituent	Mean	Range
	% (w/w)	% (w/w)
Loss on drying	2.16	1.60–2.72
Ash	1.86	1.7–2.0
Total sugars	< 0.5	< 0.5–1.2
Protein	0.26	0.2–0.4
Fibre	0.54	0.5–0.7

With respect to the results presented in Table 2, the individual boswellic acids (**1a**-**1g**, according to the numbering of the compounds in Figure 1) were determined by HPLC-UV and the use of authentic standards,[13] and the essential oil content of the additive was determined as the volume collected by hydro-distillation.[15]

Table 2: Characterisation of the organic acids and essential oil fractions of a dried extract of *Boswellia serrata* Roxb. ex Colebr. based on the analysis of five batches (mean and range). The results are expressed as % (w/w) of the extract

Constituent	CAS No	Mean	Range
		% (w/w)	% (w/w)
Total organic acids[(a)]	–	66.0	65.2–67.6
Pentacyclic triterpene acids (total)[(b)]	–	40.3	37.9–43.4
11-keto-β-Boswellic acid (KBA, **1a***)	17019-92-0	2.85	2.53–3.86
3-O-Acetyl-11-keto-β-boswellic acid (AKBA, **1b***)	67416-61-9	2.67	2.35–3.57
α-Boswellic acid (αBA, **1c***)	471-66-9	7.52	7.24–7.74

[11] Technical dossier/Supplementary information February 2019/Annex_V_Olib_Extr_Production_CONFIDENTIAL.
[12] Technical dossier/Supplementary information February 2019/Annex_III_Olib_Ext_CoA.
[13] Technical dossier/Supplementary information February 2019/Annex_II_ Olib_Extr_HPLC_data.
[14] Technical dossier/Supplementary information February 2019/Annex_III_Olib_Extr_Weende_Microbial.
[15] Technical dossier/Supplementary information February 2019/Annex_IV_Olib_Ext_Ess Oil.

Constituent	CAS No	Mean	Range
		% (w/w)	% (w/w)
β-Boswellic acid (βBA, **1d***)	631-69-6	14.0	13.2–14.7
3-O-Acetyl-9,11-dehydro-β-boswellic acid (**1e***)	122651-20-1	2.07	0–3.77
3-O-Acetyl-α-boswellic acid (AαBA, **1f***)	89913-60-0	3.10	2.51–3.88
3-O-Acetyl-β-boswellic acid (AβBA, **1g***)	5968-70-7	8.17	6.98–9.84
'Other organic acids'[(c)]	–	25.7	22.1–29.1
Essential oil[(d)]	–	0.13	0.12–0.13

CAS No.: Chemical Abstracts Service number.
*: **1a**-**1g**: according to numbering of the compounds in Figure 1.
(a): Expressed as boswellic acid, determined by non-aqueous titration with potassium methoxide.
(b): Determined by HPLC-UV (at 205 and 230 nm) and quantified with the use of authentic standard.
(c): Determined by difference.
(d): Determined as the volume collected by hydro-distillation of 30 g of olibanum extract for 60 min.

The difference between total organic acids measured by titration and boswellic acids measured by HPLC is described as 'other organic acids'. These, according to the applicant, consist mainly of tirucallic acids, based on the findings reported by Sharma et al. (2016). According to these authors, in the material they examined, the boswellic acids comprised between 57% and 77% of all identified acids, and tirucallic acids between 27% and 43%.

The applicant was requested to further characterise the fraction described as 'other organic acids' in the additive under assessment.[16] Analyses by HPLC with high-resolution mass spectrometry (HPLC-HRMS) confirmed the presence of the seven boswellic acids included in Table 2 and identified three tirucallic derivatives (**2a**-**2c**, according to the numbering of the compounds in Figure 1): 3-keto tirucallic acid (**2a**) was the most abundant compound representing 80% of the tirucallic derivatives, followed by 3-O-acetyl-α-tirucallic acid (**2b**) and 3-O-acetyl-β-tirucallic acid (**2c**). In addition, several acids with the formula $C_{30}H_{48}O_2$ and $C_{32}H_{50}O_4$ were detected, of which most were present in small amounts. According to their elemental composition, these compounds are most likely to be triterpene acids related to boswellic and tirucallic acids (3-O-acetyl-α-boswellic acid = $C_{32}H_{50}O_4$). The relative proportions of boswellic acids, tirucallic acids and other triterpene acids identified by HPLC-HRMS were tentatively quantified by ultraviolet (UV) detection (at 210 nm) and expressed as area percent of the HPLC chromatogram (see Table 3). The differences with the results reported for boswellic acids in Table 2 are explained by the fact that the data in Table 2 are obtained by HPLC with authentic standards, whereas in Table 3, quantification is done by % areas.

The molecular structures of boswellic acids and tirucallic acids are shown in Figure 1.

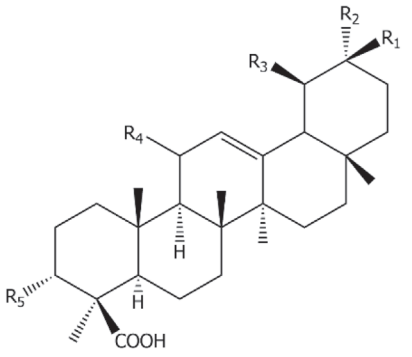
Table 3: Further characterisation of the fraction organic acids of a dried extract of *Boswellia serrata* Roxb. ex Colebr. by LC-HRMS/UV based on the analysis of five batches (mean and range). The content of each constituent is expressed as the area percent of the corresponding chromatographic peak (% HPLC area), assuming the sum of chromatographic areas of all detected peaks as 100%

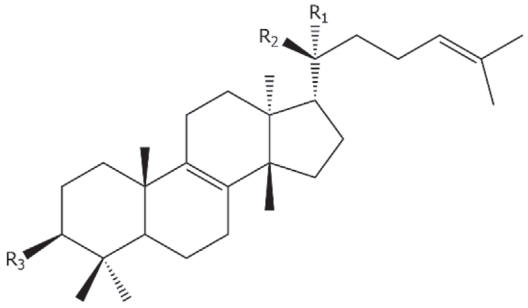
Constituent	CAS No	Mean	Range
		% LC area	% LC area
Sum of boswellic acids	–	32.9	29.7–38.9
11-keto-β-Boswellic acid (KBA, **1a**)	17019-92-0	2.7	2.2–4.1
3-O-Acetyl-11-keto-β-boswellic acid (AKBA, **1b**)	67416-61-9	2.5	1.6–3.2
α-Boswellic acid (αBA, **1c**)	471-66-9	5.9	5.1–7.4
β-Boswellic acid (βBA, **1d**)	631-69-6	12.4	11.3–13.5
3-O-Acetyl-9,11-dehydro-β-boswellic acid (**1e**)	122651-20-1	0.62	0.5–0.8
3-O-Acetyl-α-boswellic acid (AαBA, **1f**)	89913-60-0	2.4	2. 1–2.9
3-O-Acetyl-β-boswellic acid (AβBA, **1g**)	5968-70-7	6.5	5.8–7.0

[16] Technical dossier/Supplementary information September 2021/Annex IX_ Analysis of extracts.

Constituent	CAS No	Mean	Range
		% LC area	% LC area
Sum of tirucallic acids		38.6	31.9–42.0
3-Keto-tirucallic acid (**2a**)	–	31.2	24.2–34.8
3-O-Acetyl-α-tirucallic acid (**2b**)	–	4.6	4.3–5.3
3-O-Acetyl-β-tirucallic acid (**2c**)	–	2.7	2.4–2.9
Sum of $C_{30}H_{48}O_2$ (8 compounds)	–	18.6	17.9–19.4
Sum of $C_{32}H_{50}O_4$ (5 compounds)	–	9.9	9.3–11.0
Total acids		100	100

CAS no.: Chemical Abstracts Service number.





		R_1	R_2	R_3	R_4	R_5
1a	11-keto-β-Boswellic acid	H	CH_3	CH_3	=O	OH
1b	3-O-Acetyl-11-keto-β-boswellic acid	H	CH_3	CH_3	=O	OAc
1c	α-Boswellic acid	CH_3	CH_3	H	H_2	OH
1d	β-Boswellic acid	H	CH_3	CH_3	H_2	OH
1e	3-O-Acetyl-9,11-dehydro-β-boswellic acid	H	CH_3	CH_3	H	OAc
1f	3-O-Acetyl-α-boswellic acid	CH_3	CH_3	H	H_2	OAc
1g	3-O-Acetyl-β-boswellic acid	H	CH_3	CH_3	H_2	OAc

		R_1	R_2	R_3
2a	3-Keto-tirucallic acid	H	COOH	=O
2b	3-O-Acetyl-α-tirucallic acid	COOH	H	OAc
2c	3-O-Acetyl-β-tirucallic acid	H	COOH	OAc

Figure 1: Structural formula of the main components of olibanum extract: boswellic acids and tirucallic acids

The applicant performed further analyses to complete the characterisation of the additive. In particular, the low volatility compounds present in the additive were analysed by high temperature gas chromatography-mass spectrometry (GC-MS) analysis.[17] Several diterpenes and triterpenes were identified and quantified using incensol and βBA as external standards. Five diterpenes accounting together for 9–24% of the dry matter (DM) were found, three of which were identified as serratol (6.8–19%), cembrene (1.3–3.5%) and neocembrene A (0.17–0.72%). These diterpenes are characteristic for species of the *Boswellia* genus. For the triterpenes, heat degradation (loss of water, CO_2 or acetic acid) occurring during GC-MS analysis prevented their full separation and identification, and their quantification as individual compounds (in total 32–40% of DM, expressed as βBA). Therefore, quantification by HPLC is considered more reliable (see Table 2). A number of unknown triterpenes were also detected (4.8–11.2% of DM expressed as βBA).

Considering the analyses based on LC-HRMS and GC-MS together, the FEEDAP Panel concludes that the additive is fully characterised.

Substances of concern.

The applicant performed a literature search for the chemical composition of *B. serrata* and its preparations and the identity of any recognised substances of concern.[18] The occurrence of estragole in the oleo gum resin is reported in the EFSA Compendium (EFSA, 2012).[19] A number of publications identified the presence of estragole and methyleugenol (Hamm et al., 2005; Sharma et al., 2009a; Tisser and Young, 2014), which was confirmed by analytical data. The essential oils extracted from five

[17] Technical dossier/Supplementary information September 2021/Annex XI_ GC-MS_Boswellia Di- and Triperpenes.
[18] Technical dossier/Supplementary information February 2019/Literature_search_Olibanum_extract.
[19] Online version: https://www.efsa.europa.eu/en/data-report/compendium-botanicals

batches of the additive under assessment were analysed by GC-MS and the content of estragole (14.9% on average, range: 9.9–21.6%) and methyleugenol (5.6%, range: 2.9–7.7%) was determined.[20] Considering that the essential oil represents 0.12–0.13% of the additive under assessment, the presence of methyleugenol and estragole in the additive has been estimated to be on average 0.007% (max. 0.009%) and 0.019% (max. 0.028%), respectively.

3.2.2. Impurities

Data on chemical impurities were provided for three batches of olibanum extract. The concentrations of mercury were < 0.002 mg/kg in all batches, lead was in the range 0.038–0.040 mg/kg, cadmium 0.0015–0.0019 mg/kg and arsenic 0.015–0.025 mg/kg. In the same batches, aflatoxins B1, B2, G1 and G2 were below the limit of quantification and pesticides were not detected in a multiresidue analysis.Biphenyl (0.040–0.090 mg/kg) was detected in all three batches.[21]

The sum of dioxin-like polychlorinated biphenyls (PCBs) ranged between 0.022 and 0.0262 ng/kg. The sum of polychlorinated dibenzo-*p*-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) was in the range < 0.2–0.24 ng WHO PCDD/F-TEQ (World Health Organisation polychlorinated dibenzo-*p*-dioxin (PCDD) and polychlorinated dibenzofuran (PCDF) toxic equivalents)/kg.[22]

Analysis of microbial contamination of five batches of olibanum extract indicated that *Salmonella* spp. was absent in 25 g, *Escherichia coli* and *Enterobacteriaceae* were <10 colony forming unit (CFU)/g.[23]

The amounts of the undesirable substances detected do not raise safety concerns.

3.2.3. Shelf-life

The typical shelf-life of the additive is stated to be at least 12 months, when stored in tightly closed containers under standard conditions (in a cool, dry place protected from light).[24] However, no data supporting this statement were provided.

3.2.4. Conditions of use

Olibanum extract is intended to be added to feed for dogs and horses, without a withdrawal period. The maximum proposed use level is 590 mg/kg complete feed for dogs and 100 mg/kg complete feed for horses. The additive is not intended for use in water for drinking.

3.3. Safety

The assessment of safety is based on the use levels proposed by the applicant, i.e. 100 mg/kg complete feed for horses (corresponding to 66 mg total organic acids/kg feed, 40.3 mg total ($\alpha + \beta$) BAs/kg and 2.7 mg AKBA/kg feed) and 590 mg/kg complete feed for dogs (corresponding to 389 mg total organic acids/kg feed, 238 total ($\alpha + \beta$) BAs/kg and 15.8 mg AKBA/kg feed).

The additive under assessment mainly contains boswellic acids and tirucallic acids, the majority of which have been identified. The unidentified fraction of the additive is likely to consist of triterpene acids, related to boswellic and tirucallic acids.

The applicant provided a literature search on the absorption, distribution, metabolism and excretion (ADME)[25] and on the toxicology of boswellic acids, which is summarised in the following sections, and an additional search on the pharmacological effects of *Boswellia*.[26]

The presence of estragole and methyleugenol, two compounds with experimentally proven genotoxicity and carcinogenicity in rodents (as reviewed in EMA 2005; IARC, 2018; EMA, 2019), has been reported in the literature for essential oils of olibanum from *B. serrata* and has also been demonstrated for essential oil extracted from the additive (see Section 3.2.1). Information on the

[20] Technical dossier/Supplementary information January 2022.
[21] Technical dossier/Supplementary information February 2019/Annex_VII. Limit of quantification (LOQ) for mycotoxins: < 0.1 μg/kg for aflatoxins B1, B2, G1 and G2 and ochratoxin A; < 2 μg/kg for zearalenone, α- and β-zearalenone, HT-2 toxin and T-2 toxin and cytocalasin E; < 4 μg/kg for sterigmatocystin; < 5 μg/kg for nivalenol, fusarenon X, diacetoxyscirpenol, patulin, ergocryptin, ergometrin and ergosin; < 10 μg/kg for deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivlaneol, deoxynivalenol-3-glucoside, fumonisins B1, B2, B3, citrinin, ergocornin, ergocristin and ergotamine.
[22] Technical dossier/Supplementary information February 2019/Annex_VI.
[23] Technical dossier/Supplementary information February 2019/Annex_III.
[24] Technical dossier/Section II.
[25] Technical dossier/Supplementary information February 2019 and Supplementary information September 2021.
[26] Technical dossier/Supplementary information/September 2021.

absorption, distribution, metabolism and excretion and on the toxicology of estragole and methyleugenol is summarised in the following sections.

3.3.1. Absorption, distribution, metabolism and excretion

Studies with standardised B. serrata gum resin extracts

No ADME studies were available with the additive under assessment. The applicant submitted some publications retrieved from the literature on the ADME of standardised *B. serrata* extracts from the gum resin (BSE) and of some of the boswellic acids (BAs) present in the additive.[27]

The data available for absorption of the BAs point to poor absorption due to the high lipophilicity of the compounds that favours their retention in the cells and makes the passage through the intestinal barrier difficult. *In vitro* studies in Caco-2 cells demonstrated the low permeability of BAs, and their high cellular retention (Kruger et al., 2009 as referenced in Zhang et al., 2013). In this *in vitro* study, KBA and AKBA were classified as moderate and poorly permeable, respectively. Subsequently, Gerbeth et al. (2013), obtained greater permeability for several BAs after modifying the *in vitro* experimental conditions of the Caco-2 cell line model, to more closely mimic physiological *in vivo* conditions.

The recognised low absorption of BAs has driven the research to enhance their bioavailability.

In vivo, a comparative study was performed in the rat to evaluate the pharmacokinetic parameters of the principal BAs after oral administration of a standardised *B. serrata* gum resin extract at 240 mg/kg (3.97% KBA, 3.19% AKBA, 11.72% βBA, 8.99% AβBA, 5.60% αBA and 2.80% AαBA, total BA content of 36.23%) or a modified formulation of the extract containing lecithin to improve absorption (Hüsch et al., 2013). The oral administration of the formulated extract caused significantly higher plasma levels (up to sevenfold for KBA, and threefold for βBA as evaluated for the area under the plasma concentration time curve, AUC_{last}) compared to the non-formulated extract. For example, 8 h after administration of the extract, the plasma KBA concentration was 90 ng/mL and 335 ng/mL for the formulated extract.

The metabolism and distribution of BAs have been studied both *in vitro* and *in vivo*. Krüger et al. (2008) studied the metabolism of KBA and AKBA *in vitro*, in rat and human microsomes, as well as in rat hepatocytes. Subsequently, the data obtained *in vitro* were compared with data obtained *in vivo*, in rats. Analysis of the compounds and their metabolites was done by liquid chromatography tandem mass spectrometry (LC-MS/MS). In rat and human microsomes, KBA was rapidly metabolised. After 15-min incubation, 80% was converted to hydroxylated metabolites: three monohydroxylated, six dihydroxylated and two trihydroxylated metabolites. Less than 1% of the starting concentration remained after 120-min incubation. AKBA proved to be more stable than KBA, with 80% remaining unchanged after 120-min incubation, both in rat and human liver microsomes. In rat hepatocytes, the metabolism of KBA and AKBA was similar to that seen in microsomes. More than 80% of the initial KBA was metabolised after 30 min, while 80% of the starting AKBA concentration remained unchanged after 120 min. Only monohydroxylated metabolites of AKBA were formed *in vitro* and only 2% deacetylation of KBA occurred. For the *in vivo* study, rats were orally given a single dose of an oily suspension of KBA or AKBA at 12.5 mg/kg, corresponding to 240 mg/kg extract. Two hours after administration, the animals were killed, blood sampled and liver and brain removed for analysis. KBA was present in plasma, liver and brain and some hydroxylated metabolites in plasma and liver, but none in brain. A similar metabolic profile was identified *in vitro* and *in vivo* for KBA. *In vivo*, AKBA was detected in plasma, liver and brain, but hydroxylated metabolites were not detected. Overall, KBA was metabolised both *in vitro* and *in vivo* to several hydroxylated compounds, which were present in the liver of rats but not in brain. AKBA showed to be metabolised to a minor extent, both *in vitro* and *in vivo*. No conjugated derivatives were detected both *in vitro* and *in vivo* for both compounds and metabolites.

A similar study was carried out to evaluate the metabolic stability in human and rat liver microsomes of KBA, AKBA and other BAs present in the additive under assessment (αBA, βBA, AαBA and AβBA) followed by an *in vivo* metabolic study (Gerbeth et al., 2013). All the samples were analysed by LC-MS/MS. βBA was shown to undergo extensive phase I metabolism both in humans and in rat liver microsomes as was previously observed for KBA in the same models; αBA was extensively metabolised in human liver microsomes but not in rat liver microsomes; the acetylated AαBA and AβBA, like AKBA, were shown to be metabolically stable. This consistent metabolic stability of the acetylated BAs is attributed to the acetyl group at position 3. As for KBA and AKBA, the formation of

[27] Technical dossier/Supplementary information February 2019/Details on the search (databases, search terms and keywords, search strategy, selected articles and overview) available in the file: 2019-02-11_Literature_search_Olibanum_extract.

glucuronide derivatives of αBA, βBA, AαBA and AβBA did not occur *in vitro*. For the *in vivo* study, rats were orally given a single dose of a *B. serrata* gum resin extract (240 mg BSE/kg including 2.38 mg KBA, 1.91 mg AKBA, 3.36 mg αBA, 7.03 mg βBA, 1.68 mg AαBA and 5.4 mg AβBA). Blood was collected at several time points from 30 min up to 8 h, time of sacrifice of the animals. βBA, the compound present at the highest concentration in the extract, reached the highest plasma level at 8 h after administration followed by AβBA and αBA; the lowest levels were observed for KBA and AKBA (similar for both compounds). The peak plasma levels were attained from 4 to 8 h after administration of the extract, depending on the compound. Also in the brain, βBA was present at the highest level, 1,067 ng/g. The other BAs lacking the 11-keto group also reached quantifiable brain levels (mean values: from 12 to 485 ng/g).

Hüsch et al. (2013) studied more extensively the distribution of BAs in the rat after a single oral administration of a standardised *B. serrata* gum resin extract at 240 mg/kg (3.97% KBA, 3.19% AKBA, 11.72% βBA, 8.99% AβBA, 5.60% αBA and 2.80% AαBA). Compounds were quantified in several tissues and organs after 3 or 8 h administration of the extract. At 3 h, KBA, βBA and AβBA were not detected in brain and about 10 ng/g was found for AKBA, αBA and AαBA. At 8 h, the brain contents for some compounds were significantly higher (59 ng/g for AαBA, 102 ng/g for AβBA and 284 ng/g for αBA). Liver and kidney contents were the highest for all compounds at the two time points, with the acetylated boswellic acids AαBA and AβBA being at 8 h two to four times higher. In muscle, all compounds were quantified both at 3 and 8 h ranging the levels from 7 to 260 ng/g at 3 h and from 64 to 214 ng/g at 8 h.

In the study of Yin et al. (2012), mice were fed with αBA at 0.5 g/100 g feed for 4 or 8 weeks. Blood, liver, kidney, bladder, colon, brain and heart were taken for liquid chromatography (LC-MS) analysis of the compound. BA was not detected in blood after 4 weeks (limit o detection, LOD: 0.1 μg/mL) but was present at 0.61 μg/mL after 8 weeks. The compound was detected in all tissues analysed at both time points, with the highest level found in liver (5.2 and 11 μg/g at 4 and 8 weeks, respectively). In all samples, the BA levels were about twofold higher at 8 weeks of experiment as compared with 4 weeks, suggesting tissue accumulation.

There are several published studies reporting human plasma levels of some BAs after oral administration of the extract or of the individual compounds. Overall, the absorption of BAs in humans seems to be low, and depends on several factors, mainly the formulation administered (Abdel-Tawab et al., 2011).

No ADME data of BAs in dogs and horses were made available. Taking into account the *in vitro* and *in vivo* experimental data, the FEEDAP Panel considers that in these target species, a low and slow absorption of the BAs is expected, and an appreciable phase I metabolism of the non-acetylated and more hydrophilic compounds is expected to be excreted in urine. The compounds are widely distributed in the organism and have the potential to accumulate after repeated exposure.

Data about the pharmacokinetic behaviour of tirucallic acids are not available in the literature. As the chemical structure of tirucallic acids is similar to boswellic acids, it can be expected that the ADME is also similar. The lateral alkene chain in tirucallic acids is expected to be degraded or oxidised to an epoxide with subsequent hydrolysation.

There are no data available that would allow to assess whether the penta- and tetracyclic acids present in olibanum extract would result in residues in animal tissues.

Estragole and methyleugenol

Estragole is a lipophilic compound and as such readily and completely absorbed from the gastrointestinal tract. Phase I metabolism is catalysed by cytochromes P450 (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 estragole-2',3'-epoxide, which is hydrolysed to the corresponding diol with subsequent glucuronidation and excretion. Both metabolic pathways represent detoxification of estragole. The formation of genotoxic metabolites is initiated by oxidation of the side chain with formation of 1'-hydroxy-estragole. Sulfate conjugation of the hydroxyl group leads to 1'-sulfooxyestragole, which is highly unstable and breaks down to form a highly reactive carbonium ion, which can react covalently with DNA (as reviewed in EMA, 2019).

The metabolism of estragole was evaluated in experimental animals with special focus on the formation of its proximate metabolite, 1'-hydroxyestragole, and the influence of the dose administered on the quantity excreted in urine (Zangouras et al., 1981; Anthony et al., 1987). When ^{14}C-estragole (4-[^{14}C-methoxyl]-allylbenzene) was given in low doses to rodents, it was mainly excreted as $^{14}CO_2$ in

exhaled air as a result of demethylation and only a minor portion in urine in the form of several metabolites, resulting from hydroxylation in 1'-C and epoxidation at 2',3'-C followed by ring hydrolysis. In a single study found in two volunteers orally given 100 μg of methoxy-^{14}C-estragole, 1'-hydroxyestragole quantified in urine of both individuals was 0.2% and 0.4% of the dose; the majority of the dose was excreted in expired air as $^{14}CO_2$ in the first 8 h (Sangstar et al., 1987). Metabolites identified in urine indicate that estragole follows a similar biotransformation profile in rats, mice and humans. There are no studies in human volunteers with high doses of estragole, but in rats and in mice, it is consistently shown that as doses increase the urinary levels of 1'-estragole as glucuronide significantly increases.

The same metabolic pathways have been described for methyleugenol (as reviewed in EMA, 2005; IARC, 2018).

3.3.2. Toxicological studies

Toxicological (including genotoxicity) studies with the additive under assessment were not available. The literature search provided by the applicant[27] identified several studies which were performed with *B. serrata* extracts from the gum resin and were considered relevant for the present assessment and are described in the following sections.

3.3.2.1. Genotoxicity studies

Studies with standardised B. serrata gum resin extracts

The mutagenicity of a dry extract of *B. serrata* (composition not specified) was tested in Salmonella Typhimurium strains in the absence and presence of a metabolic activation system following the Organization for Economic Co-operation and Development (OECD) technical guidance (TG) 471 (Magesh et al., 2008). The results obtained did not show mutagenicity up to 5 mg/plate both in the presence and absence of S9. In a chromosomal aberration assay performed according to OECD TG 473 (1997 version), no significant alterations were reported up to 5 mg/plate when compared to the negative control, both in the presence and absence of metabolic activation (S9 mix).

Sharma et al. (2009b) assessed the genotoxicity of a *B. serrata* extract prepared from the gum resin by methanol extraction and acid precipitation, yielding a content of total acid 92–95% (by titrimetric method), which consisted of four boswellic acids (60–65%), 15–20% tirucallic acids and 13–18% other acids related to boswellic and tirucallic acids. The extract was administered by gavage to rats up to a maximum dose of 1,000 mg/kg body weight (bw) per day (corresponding to 600–650 mg BAs/kg and 150–200 mg/kg bw per day of tirucallic acids), the rats were subjected to a test battery that comprised a chromosome aberration test and a micronucleus test in bone marrow, a comet assay in peripheral blood and a sperm shape abnormality assay. The positive control cyclophosphamide induced a significant increase of DNA damage in the four cytogenetic assays confirming the sensitivity of the experimental systems. Exposure of the target tissues to the test compounds was not reported. No induction of any type of DNA damage was observed in the treated groups compared to the vehicle control animals.

Alluri et al. (2019) evaluated the genotoxic potential of the acidic and non-acidic fractions of *B. serrata* gum resin extracts, combined in a product, standardised to contain > 30% total BAs and ≥ 5% KBAs. The Ames test and the mouse bone marrow erythrocyte micronucleus assay were applied in accordance with OECD TGs 471 and 474. In bacteria, the product tested up to 5 mg/plate did not induce any increase in the number of revertant colonies. No increase in the frequency of micronuclei and no bone marrow cytotoxicity were reported in mice treated with 500, 1,000 and 2,000 mg/kg bw of the test item (two oral doses at an interval of 24 h, corresponding to > 150, 300 and 600 mg BAs/kg bw per day and ≥ 25, 50 and 100 mg KBAs/kg bw per day).

Lalithakumari et al. (2006) investigated, as part of their 90-day study in rats, DNA fragmentation patterns in liver of treated animals and did not observe any effects caused by the *B. serrata* gum resin extract containing 30% AKBA and 85% total acids.

Estragole and methyleugenol

Estragole and methyleugenol are compounds with experimentally proven genotoxicity and carcinogenicity in rodents (as reviewed in EMA, 2005, IARC, 2018; EMA, 2019).

3.3.2.2. Toxicology

Studies with standardised B. serrata gum resin extracts

A standardised proprietary *B. serrata* gum resin extract with a content of 20% AKBA was tested in several standard studies for its toxicological properties including a subacute (28-day) repeated dose oral toxicity study in rats performed according to OECD TG 407 (Krishnaraju et al., 2010). Male and female rats received daily oral doses of 0, 50, 250 or 2,500 mg/kg bw of the test item (corresponding to 0, 10, 50 or 500 mg AKBA/kg bw per day) for 28 consecutive days. No treatment-related effects were observed on haematology, clinical chemistry, gross pathology and histopathology. Based on the results, the authors of the study concluded that the no observed adverse effect level (NOAEL) for the test item in male and female Sprague-Dawley rats was at least 2,500 mg/kg bw per day (corresponding to 500 mg AKBA/kg bw per day), the highest dose tested.

No dose-related adverse effects were observed when another proprietary product standardised to contain > 30% total BAs and ≥ 5% KBAs, was tested in a repeated dose 28-day oral toxicity study in male and female Wistar rats at 0, 250, 500 or 1,000 mg/kg bw per day (corresponding to > 75, 150 or 300 mg BAs/kg bw per day and ≥ 12.5, 25 and 50 mg KBAs/kg bw per day) (Alluri et al., 2019). The authors of the study concluded that the NOAEL was at the highest dose tested of 1,000 mg/kg bw.

A standardised *B. serrata* gum resin extract containing 30% AKBA and 85% total acids was tested in a 90-day oral toxicity study in rats (Lalithakumari et al., 2006). Male and female rats received a diet containing 0%, 0.025%, 0.25% or 2.5% of the test material (corresponding to 75, 750 or 7,500 mg AKBA/kg feed). Weight gain was significantly lower in the high dose group. A dose-dependent decrease in food consumption was observed, although no difference was observed in the water intake. Significant differences in the weight of some organs were observed in the highest dose group as compared to the control group which did not correlate with significant abnormalities in histopathology or biochemistry in any of the groups. These differences in the highest dose group may have been caused by the decrease in body weight. The authors concluded that the administration of the test material to male and female Sprague-Dawley rats in the feed at levels up to 2.5% for 90 consecutive days did not induce any toxicologically significant effects as evidenced from body weight, selected organ weights as such and as percent of body weight and brain weight, feed and water intake, ocular health, hepatic DNA fragmentation, haematology and clinical chemistry and histopathological data. From the highest dose tested (2.5% of the test item) and based on the feed consumption data provided in the study (a rat of 275 g body weight consumes 16 g feed/day), the applicant derived an NOAEL of 1,456 mg/kg bw per day for the extract, which would correspond to an NOAEL of 1,238 mg/kg bw per day for total acids and 437 mg/kg bw per day for AKBA. The FEEDAP Panel notes that the study is poorly described and has major limitations, particularly the small numbers of animals tested and consequent lack of sensitivity of the study, which increase the uncertainty in the derivation of the NOAEL.

Singh et al. (1996) performed acute, subacute and chronic toxicity studies on boswellic acids in mice, rats and monkeys (not OECD compliant). The test material was a mixture of BAs daily prepared as a uniformly homogenised suspension of gum acacia (2% w/v) in normal saline. The content of BAs and the alcohol fraction in the *B. serrata* gum resin were 30% and 43%, respectively. The test material did not cause any mortality in rats and mice when administered orally and intraperitoneally in doses up to 2 g/kg. Daily oral administration of BAs in two doses (500 and 1,000 mg/kg) to rats and monkeys for 4 weeks revealed no significant changes in general behaviour, or clinical, haematological, biochemical[28] and pathological data (necropsy and histopathology on all animals). Other studies included a 6-month repeated oral dosing study in rats (250, 500 and 1,000 mg/kg bw per day), 6-month repeated oral dosing study in monkeys (*Macacca mulatta*) (125, 250 and 500 mg/kg bw per day), a teratogenicity study in rats (250, 500 and 1,000 mg/kg bw per day). This daily oral administration of BAs in three doses to rats and monkeys revealed no significant changes in general behaviour, or clinical, haematological, biochemical and pathological data, as well as no teratogenic effects.

A *B. serrata* gum resin extract was tested in a 90-day oral toxicity study performed according to OECD TG 408 (Singh et al., 2012). The test material, described as 'cream colour granules' which meets the characterisation of the unprocessed gum-resin (composition not provided), was administered by

[28] Haematology: bleeding time (BT), clotting time (CT), haemoglobin (Hb), total and differential leukocyte counts (TLC and DLC), red blood cell count (RBC); Biochemical parameters: blood sugar, blood urea, creatinine, bilirubin, serum oxalo-acetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), acid and alkaline phosphatase, and serum total protein tests; Urine: sugar, albumin, oxalates or phosphate crystals, epithelial cells and pus cells.

gavage for 90 days to rats at 0, 100, 500 and 1,000 mg/kg bw per day. The study's protocol comprised standard in life and post-mortem parameters to be investigated in such a study. Except effects on body weight in the highest dose group, no other adverse observations were observed. The study authors identified an NOAEL of the *B. serrata* preparation of 500 mg/kg bw per day based on the reduction in the body weight gain observed at the highest dose tested.

Estragole and methyleugenol

Estragole was included in the diet of female CD-1 mice at 0, 2.3 or 4.6 g/kg diet for 12 months. At least 50% of the animals in the exposed groups developed hepatic tumours by 18 months,[29] which were diagnosed as hepatomas type A (hepatocellular adenomas) or type B (hepatocellular adenocarcinomas) or mixed types A and B. The animals which were fed with the control diet did not show any hepatic tumour (Miller et al., 1983).

Van den Berg et al. (2011) performed an evaluation of the available evidence using the benchmark dose (BMD) approach and found that the application of dose-response modelling on the long-term chronic toxicity study (Miller et al., 1983) using hepatocellular carcinomas as a response, yielded a BMD lower confidence limit for a benchmark response of 10% ($BMDL_{10}$) of 3.3 mg/kg bw per day. However, the FEEDAP Panel notes that there is high uncertainty in derivation of a $BMDL_{10}$ for estragole from a carcinogenicity study in CD-1 mice. This strain of mice spontaneously develops a high incidence of hepatocellular adenomas and carcinomas, and the relevance of these tumours for human risk assessment is questionable. In addition, BMD modelling with only two dose levels is adding extra uncertainty in the derivation of the $BMDL_{10}$ value.

Miller et al. (1983) also investigated the possible carcinogenic activity of a variety of p-allylalkoxybenzenes in newborn male mice, injected intraperitoneally (i.p.) with nine different compounds at day 1, 8, 15 and 22 after birth. Among these, estragole, safrole and methyleugenol induced a significant number of hepatomas at 13 months, whereas anethol, elemicin, myristicin, dillapiole, parsley apiole and eugenol did not, under the limited conditions of the study.

In another experiment using the same treatment protocol, DNA was isolated from the liver of the treated mice and the occurrence and quantity of DNA adducts was investigated (Phillips et al., 1984). The highest amount of DNA adducts was observed with methyleugenol, estragole and safrole (73, 30 and 15 pmol/mg DNA, respectively). The yield of DNA adducts with myristicin, elemicin and dillapiole were 7.8, 2.7 and 1.2 pmol/mg DNA and the correspondent values for parsley apiol and anethol where below the limit of quantification (LOQ) of 1 pmol/mg DNA. No adducts at all were observed for eugenol. The incidence of DNA adducts correlated to the tumour incidence observed in the experiment by Miller et al. (1983). Two other studies on the induction of DNA adducts in liver of adult mice after i.p. injection of alkenylbenzenes (Randerath et al., 1984) and in human hepatoma cells in culture (Zhou et al., 2007) confirmed methyleugenol as the most potent derivative. The two *in vivo* studies resulted in the same order of potency (i.e. methyleugenol > safrole > estragole > elemicin > dillapiole). In the *in vitro* study, estragole was more potent than safrole.

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.[30] Higher incidences of kidney neoplasms, malignant mesothelioma, mammary gland fibroadenoma and subcutaneous fibroma and fibrosarcoma were observed in male rats only.[31] 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.

[29] Incidence of hepatomas in female mice (0/50, 25/50, 35/50).

[30] 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 hepatocholangiocarcinoma (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 hepatocholangiocarcinoma (0/50, 0/50, 0/50, 3/50, 13/17); glandular stomach (0/50, 1/50, 25/50, 34/50, 41/50)

[31] Males 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).

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 Scientific Committee, 2017) on the long-term chronic toxicity study (NTP, 2000) using hepatocellular carcinomas in male rats as a response, yielded a $BMDL_{10}$ of 22.2 mg/kg bw per day. Based on the above considerations on the relative potency of p-allylalkoxybenzenes, the FEEDAP Panel selects the $BMDL_{10}$ derived from the rat study with methyleugenol, with three test doses and derived applying model averaging, as reference point for the assessment group p-allylalkoxybenzenes.

3.3.2.3. Pharmacological side effects

The FEEDAP Panel notes that *B. serrata* at the proposed conditions of use as constituent in certain food products, e.g. food supplements, (900–1,200 mg of extract standardised to 60% boswellic acid daily or equivalent preparation) was claimed to support joint flexibility. On the basis of the data presented, the NDA Panel concluded that a cause and effect relationship has not been established between the consumption of this food constituent and the maintenance of normal joints (EFSA NDA Panel, 2010).

The applicant provided a literature search[32] covering studies investigating the pharmacological effects of *Boswellia*. The search identified 176 publications mainly aimed at investigating potential beneficial therapeutical effects of olibanum and its components and at elucidating the underlying mechanisms. These studies were not considered relevant for the assessment of olibanum extract as a feed flavouring additive.

Some of the publications submitted highlight the pro-inflammatory effects observed in certain *in vitro* test systems (e.g. Boden et al., 2001; Siemoneit et al., 2009) and possible drug interactions due to inhibitory activity on human cytochrome P450 enzymes (e.g. Frank and Unger, 2006). Whether these pro-inflammatory effects occur *in vivo* remains unknown.

3.3.3. Safety for the target species

No tolerance studies in dogs or horses have been provided by the applicant. A 90-day study performed in rats has been submitted (see Section 3.3.2.2), and from this study, an NOAEL of 1,238 mg total acids/kg bw per day has been derived.

Considering the limitations in the 90-day study and the possible pro-inflammatory effects, the FEEDAP Panel applied an uncertainty factor (UF) of 300 to the NOAEL to derive the safe daily dose for the target species and the maximum safe feed concentration of the extract following the EFSA Guidance on the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017c). Based on the average content of total organic acids of 66%, the calculated maximum safe concentrations of the additive in complete feed (88% dry matter, DM) are 330 and 276 mg/kg for dogs and horses, respectively, with respect to non-genotoxic endpoints.

Methyleugenol and estragole

The essential oil present in the additive was shown to contain on average 5.6% (maximum 7.7%) methyleugenol and 14.9% (maximum 21.6%) estragole. Considering that the essential oil represents 0.12–0.13% of the additive under assessment, the presence of methyleugenol and estragole in the additive has been estimated to on average 0.007% (max. 0.009%) and 0.019% (max. 0.028%), respectively.

For horses, at the maximum proposed use level of 100 mg extract/kg in feed, the concentrations of methyleugenol and estragole in the additive were calculated to be 9 and 28 μg/kg complete feed. The intake by the target animal at the maximum proposed use level of 100 mg/kg complete feed would be up to 0.205 μg methyleugenol/kg bw per day and 0.636 μg estragole/kg bw per day.

For dogs, at the safe concentration of 330 mg extract/kg feed, the concentrations of methyleugenol and estragole in the additive were calculated to be 30 and 92 μg/kg complete feed. The intake by the target animal (as μg/kg bw per day) would be 0.563 μg methyleugenol/kg bw per day and 1.75 μg estragole/kg bw per day.

Since methyleugenol and estragole share the same mode of action, they are allocated to the same assessment group (*p*-allylalkoxybenzenes) (EFSA Scientific Committee, 2019a) and an assessment of the combined exposure is performed. The margin of exposure (MOE) for each component is calculated

[32] Technical dossier/Supplementary information September 2021/Details on the search (databases, search terms and keywords, search strategy, evaluation) available in the file: 2021-09-30_Clarificatio_reply_BDG08_Olibanum_extract.

as the ratio of the reference point (the $BMDL_{10}$ of 22.2 mg methyleugenol/kg bw per day for both compounds, see Section 3.3.2.2) to the intake. The combined margin of exposure (MOET) is calculated for the assessment group as the reciprocal of the sum of the reciprocals of the MOE of the individual substances (Table 4).

Table 4: Compositional data, intake values (calculated for horses at 100 mg/kg and dogs at 330 mg/kg complete feed), reference points and margin of exposure (MOE) for methyleugenol and estragole and combined margin of exposure (MOET) for the assessment group p-allylalkoxybenzenes

Composition		Exposure		Hazard characterisation	Risk characterisation	
Assessment group	**Max conc. in the extract[(a)]**	**Max Feed conc.**	**Intake**	**$BMDL_{10}$**	**MOE**	**MOET[(b)]**
Constituent	**mg/kg**	**µg/kg**	**µg/kg bw per day**	**mg/kg bw per day**	–	–
Horses						
p-Allylalkoxybenzenes						
Methyleugenol	90	9	0.205	**22.2**	164,444	
Estragole	280	28	0.636	**22.2**	52,857	
MOET						40,000
Dogs						
p-Allylalkoxybenzenes						
Methyleugenol	90	30	0.563	**22.2**	39,467	
Estragole	280	92	1.750	**22.2**	12,686	
MOET						9,600

(a): The values of estragole and methyleugenol in the extract are estimated assuming that they are present in the extract at a concentration corresponding to the maximum analysed value (see Section 3.2.1) and assuming that the oil represents 0.12–0.13% of the additive.
(b): The margin of exposure (MOE) for each component is calculated as the ratio of the reference point ($BMDL_{10}$) to the intake. The combined margin of exposure (MOET) is calculated for the assessment group as the reciprocal of the sum of the reciprocals of the MOE of the individual substances.

When the estimated exposures for horses and dogs are compared to the $BMDL_{10}$ of 22.2 mg methyleugenol/kg bw per day (Suparmi et al., 2019), derived from rodent carcinogenicity studies (NTP, 2000; see Section 3.3.2.2), an MOET of 40,000 is calculated for horses and an MOET of 9,600 for dogs. The magnitude of these MOET values is indicative of a low concern for the target species.

3.3.3.1. Conclusions on safety for the target species

The additive is safe for horses at the maximum recommended level of 100 mg/kg complete feed. For dogs, the calculated safe concentration in feed is 330 mg/kg complete feed.

3.3.4. Safety for the consumer

Olibanum (*Boswellia carteri* Birdw. and other species of *Boswellia*) is added to a wide range of food categories for flavouring purposes (Burdock, 2009). Although individual consumption figures are not available, the Fenaroli's handbook of flavour ingredients reports use levels of olibanum and olibanum oil in food and beverages in the range of 1.0 mg/kg up to 11 mg/kg.

According to the literature, some retention of boswellic acids in the tissues of target animals is expected (see Section 3.3.1). No data on residues in products of animal origin (horse meat) were made available for any of the constituents of the extract. However, consultation of the EFSA Comprehensive European Food Consumption Database (FoodEx2)[33] indicated that horse meat is

[33] https://www.efsa.europa.eu/en/data-report/food-consumption-data#the-efsa-comprehensive-european-food-consumption-database

consumed in Europe in a restricted number of countries,[34] by a low percentage of consumers[35] and in low amounts.[36] Therefore, given the low frequency of consumption of horse meat, the limited absorption and the expected limited retention of boswellic acids in animal tissues (in the order of ng/g, see Section 3.3.1), the FEEDAP Panel considers that it is unlikely that the use of the additive would result in a relevant increase of the intake of the individual constituents by humans consuming products of animal origin (horse meat).

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

Consequently, based on the above and considering the low toxicity of boswellic acids (see Section 3.3.2.2), no safety concern would be expected for the consumer from the use of olibanum extract up to the highest safe use level in feed for horses.

3.3.5. Safety for the user

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

The applicant submitted three publications (Lalithakumari et al., 2006; Krishnaraju et al., 2010; Alluri et al., 2019) reporting the dermal and eye irritation of three proprietary *B. serrata* gum resin extracts containing 30% AKBA and 85% total acids, 20% AKBA or $\geq$ 5% AKBA. Since the three test items contain higher concentrations of total BAs and AKBA compared to the olibanum extract under assessment, they were relevant for the assessment of the additive. The test items were not irritating to the skin, and mildly or not irritating to the eye. The same conclusion is extended to the additive under assessment.

The additive under assessment should be considered as non-irritant to skin and eyes, but in the absence of data, no conclusion can be drawn on its potential to be a dermal and respiratory sensitiser.

When handling the additive, exposure of unprotected users to methyleugenol and estragole cannot be excluded. Therefore, to reduce the risk, the exposure of the users should be minimised.

3.3.6. Safety for the environment

B. serrata is not native to Europe. Therefore, the safety for the environment is assessed based on the individual components of the extract.

The additive under assessment mainly contains boswellic acids and tirucallic acids, which are pentacyclic and tetracyclic triterpenes. For boswellic acids, a low and slow absorption followed by phase I metabolism and excretion in urine is expected, particularly for the non-acetylated compounds. For tirucallic acids, the lateral alkene chain is expected to be oxidised to an epoxide with subsequent hydrolysis and conjugation. Because of the degradation of pentacyclic and tetracyclic triterpenes by equine metabolism and environmental processes, and considering the small environmental exposure of excreta from horses, the use of the additive in horse feed at the proposed conditions of use is regarded safe for the environment.

3.4. Efficacy

Olibanum (*Boswellia carteri* Birdw. and other species of *Boswellia*) and their extracts are listed in Fenaroli's Handbook of Flavour Ingredients (Burdock, 2009) and by FEMA with the reference number 2816 (olibanum oil).

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

4. Conclusions

Olibanum extract from *Boswellia serrata* Roxb. ex Colebr. may be produced from plants of different geographic origins and by various processes resulting in preparations with different composition and toxicological profiles. Thus, the following conclusions apply only to olibanum extract which is specified

[34] Belgium, Finland, France, Italy, Netherlands, Portugal, Slovenia, Spain and Sweden, according to the FoodEx2.
[35] 0.1–2.8% of all subjects are consumers of horse meat in countries where it is consumed, according to the FoodEx2.
[36] On average 0.01–1.94 g/day, less than 1 kg/year when considering all subjects, and 10.75–226.25 g/day when considering consumers only (95th percentile).

to contain ≥ 65% of boswellic acids, max. 0.009% methyleugenol and max. 0.028% estragole, and is produced by extraction from the oleoresin of *B. serrata*.

The additive under assessment is safe for horses at the maximum proposed use levels of 100 mg/kg of complete feedingstuffs. For dogs, the calculated safe concentration in feed is 330 mg/kg complete feed.

The additive is considered safe for consumers when used at the proposed conditions of use in horses.

The additive under assessment should be considered as non-irritant to skin and eyes, but in the absence of data, no conclusion can be drawn on its potential to be a dermal and respiratory sensitiser. When handling the additive, exposure of unprotected users to methyleugenol and estragole cannot be excluded. Therefore, to reduce the risk, the exposure of the users should be minimised.

The use of olibanum extract as a feed additive for horses is not expected to pose a risk for the environment.

Olibanum 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. Recommendations

The specification should ensure that the concentration of methyleugenol and estragole in the additive should be as low as possible and should not exceed 0.009% methyleugenol and 0.028% estragole.

6. Documentation 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*
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
14/02/2019	Reception of supplementary information from the applicant (partial submission: olibanum extract)- Applicant requested a change in the target species, limiting the application for authorisation to dogs and horses
12/03/2021	The application was split and a new EFSA-Q-2021-00145 was assigned to the preparation included in the present assessment
17/03/2021	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
02/04/2021	Partial withdrawal by applicant (EC was informed) for the following additive: olibanum tincture

Date	Event
30/09/2021	Reception of supplementary information from the applicant (partial submission: olibanum extract)
06/01/2022	Reception of supplementary information from the applicant (partial submission: olibanum extract). Scientific assessment re-started for the preparation included in the present assessment
27/01/2022	Opinion adopted by the FEEDAP Panel on olibanum extract. End of the Scientific assessment for the preparation included in the present assessment. The assessment of another preparation is still ongoing

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Abbreviations

AαBA	3-O-Acetyl-α-boswellic acid
AβBA	3-O-Acetyl-β-boswellic acid
ADME	Absorption, distribution, metabolism and excretion
AKBA	3-O-acetyl-11-keto β-boswellic acid
AUC	Area under the curve

Bas	Boswellic acids
BDG	Botanically defined group
BMD	benchmark dose
$BMDL_{10}$	lower confidence limit for a benchmark response of 10%
BSE	*Boswellia serrata* extract (of the gum resin)
Bw	body weight
CAS	Chemical Abstracts Service
CFU	Colony-forming unit
CYP450	cytochromes P450
DM	dry matter
EC	European Commission
EEIG	European economic interest grouping
EMA	European Medicines Agency
EURL	European Union Reference Laboratory
FEEDAP	EFSA Scientific Panel on Additives and Products or Substances used in Animal Feed
FEMA	Flavour Extract Manufacturers Association
FFAC	Feed Flavourings authorisation Consortium of (FEFANA) the EU Association of Specialty Feed Ingredients and their Mixtures
FoodEx2	EFSA Comprehensive European Food Consumption Database
GC–MS	gas chromatography–mass spectrometry
HPLC	High performance liquid chromatography
HRMS	high resolution mass spectrometry
IARC	International Agency for Research on Cancer
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	limit of detection
LOQ	Limit of quantification
KBA	11-keto-β-boswellic acid
MOE	margin of exposure
MOET	combined margin of exposure (total)
NDA	EFSA Panel on Dietetic Products, Nutrition and Allergies
NTP	National Toxicology Program
NOAEL	no observed adverse effect level
OECD	Organization for Economic Co-operation and Development
PCBs	polychlorobiphenyls
PCDD	polychlorinated dibenzo-*p*-dioxins
PCDF	polychlorinated dibenzofurans
SC	EFSA Scientific Committee
TEQ	Toxic equivalent
TG	Technical guidance
UF	uncertainty factor
UV	Ultraviolet
WHO	World Health Organization

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for buchu leaves oil, olibanum extract (wb), lime oil, petitgrain 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, petitgrain 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 *petitgrain 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 *petitgrain 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 *petitgrain 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.