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Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Botanically defined flavourings group BDG 08 - Sapindales

(FAD-2010-0322; CRL/100211)

European Union Reference Laboratory
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Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	**FAD-2010-0322 - CRL/100211**
Name of Product:	***buchu leaves oil, olibanum extract (wb), lime oil, petigrain bigarade oil, bitter orange extract of the whole fruit, lemon oil expressed, lemon oil distilled (residual fraction), lemon oil distilled (volatile fraction), orange oil cold pressed, orange terpenless (concentrated 4 times), orange terpenless (concentrated 10 times), orange terpenless (folded), orange terpenes, mandarin oil and quebracho extract (wb) from botanically defined flavourings Group (BDG 08) - Sapindales***
Phytochemical marker(s):	**d-limonene, d,l-isomenthone, 11-keto-β-boswellic acid, 3-O-acetyl-11-keto-β-boswellic acid, linalyl acetate, linalool, naringin, tannins**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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EXECUTIVE SUMMARY

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

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

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

For the quantification of the phytochemical marker *d-limonene* in *lime oil* the Applicant submitted a GC-FID method based on the corresponding standard ISO 3519:2005 for the characterisation of the "oil of lime distilled, Mexican type (Citrus aurantifolia [Christm.]

[1] The original application included further additives that have been withdrawn: cashew oil, neroli bigarade oil, petitgrain bigarade absolute, mandarin terpenes, grapefruit oil expressed, grapefruit extract (sb), grapefruit extract and olibanum tincture (SANTE/E5/AR/bvp/ddg2.E.5(2019) - Ref. Ares(2019)1299322 - 26/02/2019).

Swingle)". The quantification is performed using the normalisation approach for the estimation of the area percentage of individual components. The Applicant presented a chromatogram and the specific analytical procedure for the analysis of *d-limonene* in *lime oil*.

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

For the quantification of the phytochemical marker *naringin* in *bitter orange extract of the whole fruit* the Applicant submitted a single-laboratory validated and further verified method based on HPLC-UV (284 nm). The method has been developed for the determination of total flavonoids (including *naringin* alone) in a mixture of citrus flavonoids. The quantification of *naringin* is performed using the normalisation approach for the estimation of the area percentage of individual components. The Applicant provided validation and verification studies demonstrating the applicability of the method for the analysis of pure *naringin*. Furthermore, *naringin* has been satisfactory quantified in the *feed additive* by the proposed method in 5 different lots of *bitter orange extract of the whole fruit*.

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

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

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

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

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

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

compounds in low chromed hide powder described in ISO 14088:2020 for the quantification of *tannins* in *quebracho extract (wb).*

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

KEYWORDS

buchu leaves oil, olibanum extract (wb), lime oil, petigrain bigarade oil, bitter orange extract of the whole fruit, lemon oil expressed, lemon oil distilled (residual fraction), lemon oil distilled (volatile fraction), orange oil cold pressed, orange terpenless (concentrated 4 times), orange terpenless (concentrated 10 times), orange terpenless (folded), orange terpenes, mandarin oil and quebracho extract (wb) from botanically defined flavourings group 08 (BDG 08), Sapindales, sensory additives, flavouring compounds, all animal species.

1. BACKGROUND

In the current grouped application an authorisation is sought under Articles 4(1) (new use in water) and 10(2) (re-evaluation of additives already authorised under the provisions of the Council Directive 70/524/EEC) for *buchu leaves oil, olibanum extract (wb), lime oil, petigrain bigarade oil, bitter orange extract of the whole fruit, lemon oil expressed, lemon oil distilled (residual fraction), lemon oil distilled (volatile fraction), orange oil cold pressed, orange terpenless (concentrated 4 times), orange terpenless (concentrated 10 times), orange terpenless (folded), orange terpenes, mandarin oil and quebracho extract (wb)* from *botanically defined flavourings group 08 (BDG 08),* 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 [1,2].

The several flavouring preparations, which have a natural origin (botanically defined) and are derived from plant species belonging to the botanical order "*Sapindales"* are described in the current dossier [3]. The original application included additional additives which have been withdrawn[2] [4]. For each preparation the Applicant indicated the corresponding phytochemical marker(s) and their amount [5-16]. The proposed specifications of the additives are presented in Table 1. 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 [17]. However, the Applicant suggested the typical maximum inclusion level of the *feed additives* of 25 mg/kg *feedingstuffs* [17].

[2] Here the list of the withdrawn preparations: cashew oil, neroli bigarade oil, petitgrain bigarade absolute, mandarin terpenes, grapefruit oil expressed, grapefruit extract (sb), grapefruit extract and olibanum tincture.

Table 1. Specifications for additives belonging to botanically defined group BDG 08 [5]

Additive	Plant species	Description	Marker	% (*)
buchu leaves oil	Agothosma betulina	light to brownish yellow liquid	d-limonene	19 - 26 [6]
			d,l-isomenthone	19 – 27 [6]
olibanum extract (wb)	Boswellia serrata Roxb. Ex Colebr.	off-white to creamish powder	11-keto-β-boswellic acid [7]	2 – 5 (**) [7]
			3-O-acetyl-11-keto-β-boswellic acid [7]	2 – 5 (**) [7]
lime oil	Citrus aurantiifolia (Christm.) Swingle	pale yellow clear liquid	d-limonene	40 – 60 [5]
petigrain bigarade oil	Citrus aurantium L.	yellow liquid	linalyl acetate	40 – 72 [8]
			linalool	10 – 32 [8]
bitter orange extract of the whole fruit	Citrus aurantium L. Var myrtifolia	light brown hygroscopic powder	naringin	20 – 30 [9]
lemon oil expressed	Citrus limon (L.) Burm.	yellow to green clear liquid	d-limonene	60 – 73 [10]
lemon oil distilled residual fraction [11]	Citrus limon (L.) Burm.	green to brown yellow clear liquid [11]	d-limonene	51 - 63 [11]
lemon oil distilled volatile fraction [11]	Citrus limon (L.) Burm.	green to brown yellow clear liquid [11]	d-limonene	66 - 78 [11]
orange oil cold pressed	Citrus sinensis (L.) Pers.= C. aurantium L. var. Dulcis	orange to orange brown liquid	d-limonene	93 - 97 [12]
orange terpenless concentrated 4 times [13]	Citrus sinensis (L.) Pers. = C. aurantium L. var. Dulcis	orange clear liquid [13]	d-limonene	89 - 96 [13]
orange terpenless concentrated 10 times [13]	Citrus sinensis (L.) Pers. = C. aurantium L. var. Dulcis	red brown clear liquid [13]	d-limonene	79 - 89 [13]
orange terpenless folded [13]	Citrus sinensis (L.) Pers. = C. aurantium L. var. Dulcis	orange to dark orange clear liquid [13]	d-limonene	85 - 95 [13]
orange terpenes	Citrus sinensis (L.) Pers. = C. aurantium L. var. Dulcis	pale yellow to yellow orange clear liquid	d-limonene	93 - 97.5 [14]
mandarin oil	Citrus reticulata Blanco	green/greenish brown transparent liquid	d-limonene	65 - 80 [15]
quebracho extract (wb)	Schinopsis Balansae	reddish brown powder	tannins	≥ 70 (**) [16]

(*) The content of phytochemical markers is expressed in terms of: relative %, i.e. chromatographic signal fraction using the total signal of all constituents; or (**) absolute %, i.e. mass fraction, using a standard substance

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *Botanically defined flavourings group BDG 08 - Sapindales* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

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 proposed a method based on gas chromatography coupled with flame ionisation detection (GC-FID). The method is based on the generic standard ISO 11024 on general guidance on chromatographic profiles for essential oils [6,13,18].

According to the procedure, 1 µl of the *buchu leaves oil* and the *orange terpenless (concentrated 10 times)* oil is injected directly into the GC at a split ratio of 1:100. The quantification is performed by FID using the normalisation approach for the estimation of the area percentage of individual components. Specific operating conditions for the GC are provided by the Applicant [19-20]. The Applicant verified the above mentioned method for the analysis of the phytochemical markers following the "EURL–FA Validation and verification technical guide for Sensory feed Additives – flavouring compounds 2(b) from botanical origin" [21]. The EURL recalculated the performance characteristics obtained. Precision values corresponding to 1.8 and 4.4 %, respectively, were derived for *d-limonene* and *d,l-isomenthone* in *buchu leaves oil*, and 3.0 % for *d-limonene* in *orange terpenless (concentrated 10 times)* oil [22].

Based on the experimental evidences provided the EURL recommends for official control the GC-FID method based on the generic ISO 11024 standard 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.

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 based on high performance liquid chromatography (HPLC) with spectrophotometric (UV) detection described in the European Pharmacopeia monograph for Indian Frankincense (Olibanum indicum) [7,23,24].

1 g of the *feed additive* is dissolved in 90 ml of methanol, shaken and sonicated for 10 min. The solution is adjusted up to 100 ml with methanol and centrifuged for 5 min. 1 ml of the clear solution is further diluted up to 10 ml with a mixture of mobile phases A (phosphoric acid:water 0.1:99.9 v:v) and B (phosphoric acid:acetonitrile 0.1:99.9 v:v) (16:64, v:v) and 20 µl are injected into the HPLC. Reference solutions containing the two phytochemical markers are properly diluted at first in methanol and afterwards in a mixture of mobile phases A and B (16:64, v:v) and injected into the HPLC. The markers are detected by spectrophotometric (UV) detection at 250 nm. The quantification of *11-keto-β-boswellic acid* and *3-O-acetyl-11-keto-β-boswellic acid* is performed by mean of specific expressions and is indicated as percentage content (absolute value). The Applicant provided results for the analysis of 5 batches of *olibanum extract* using slightly modifying the above described method. The results obtained are within the proposed specifications [25].

Based on the experimental evidences available the EURL recommends for official control the HPLC-UV method described in the European Pharmacopeia monograph for Indian Frankincense (Olibanum indicum) for the quantification of the phytochemical markers: *11-keto-β-boswellic acid* and *3-O-acetyl-11-keto-β-boswellic acid* in the *feed additive* (*olibanum extract*).

For the quantification of the phytochemical marker *d-limonene* in ***lime oil*** the Applicant proposed a method based on GC-FID. The method is based on the international standard ISO 3519:2005 for the characterisation of the "oil of lime distilled, Mexican type (Citrus aurantifolia [Christm.] Swingle)" [5,26,27]. Furthermore, the description of the product and the range of *D-limonene* indicated in the above mentioned ISO 3519:2005 standard corresponds to the range of the phytochemical marker as proposed by the Applicant [5].

According to the procedure, 1 µl of the *lime oil* is injected directly into the GC at a split ratio of 1:100. The quantification is performed by FID 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 the phytochemical marker *d-limonene* in *lime oil* [26].

The EURL recommends for official control the GC-FID method based on the ISO 3519:2005 standard for the quantification of the phytochemical marker: *d-limonene* in *lime oil*.

For the quantification of the phytochemical markers *linalyl acetate* and *linalool* in ***petigrain bigarade oil*** the Applicant proposed a method based on GC-FID. The method is based on the standard ISO 8901:2003 for "Oil of bitter orange petitgrain, cultivated (Citrus aurantium L.)" [8,28,29]. Furthermore, the description of the product indicated in the above mentioned ISO 8901:2003 standard and the range of *linalyl acetate* and *linalool* correspond to the range of the phytochemical markers as proposed by the Applicant [8].

According to the procedure, 1 µl of the *petigrain bigarade oil* is diluted 1:10 in acetone and injected into the GC at a split ratio of 1:100. The quantification is performed by FID 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 the phytochemical markers *linalyl acetate* and *linalool* in *petigrain bigarade oil* [25].

The EURL recommends for official control the GC-FID method based on the ISO 8901:2003 standard for the quantification of the phytochemical markers *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 coupled to spectrophotometric detection (HPLC-UV) [9,30-33]. The method has been developed for the determination of total flavonoids (including *naringin* alone) in a mixture of citrus flavonoids.

40 mg of sample are weighed in a 20 ml flask and diluted to volume with dimethyl sulfoxide . In parallel, a reference solution is prepared by weighing 30 mg of *naringin* in a 100 ml flask and diluted to volume with dimethyl sulfoxide. Both solutions are homogenised. 10 µl of the solutions are injected into the HPLC system and the marker is detected by spectrophotometry (UV) at 284 nm. The quantification of the various flavonoids is performed using the normalisation approach for the estimation of the area percentage of individual components. The content of naringin in the sample is determined using an external standard. The Applicant provided validation and verification studies with satisfactory performance characteristics thus demonstrating the applicability of the method for the determination of pure *naringin* [32-33]. In addition, diluted samples of *bitter orange extract of the whole fruit* spiked with a *naringin* standard have been analysed by HPLC-UV to demonstrate qualitatively the presence of *naringin* in the extracts and in flavouring premixtures [34]. Furthermore, in the frame of the batch-to-batch variation studies, the Applicant successfully analysed 5 different lots of *bitter orange extract of the whole fruit*. The results obtained for *naringin* (22.8-25.5 %) are in the range of the one proposed by the Applicant (20-30 %) [35].

Therefore, even if the method has not been directly validated/verified for the matrix of interest, the EURL considers the method fit-for-purpose and recommends for official control this HPLC-UV method for the quantification of the phytochemical marker *naringin* in *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 proposed a method based on GC-FID. The method is based on the standard ISO 855:2003 for "Oil of lemon (Citrus limon (L.) Burm. f.), obtained by expression" [10,11,36-38]. Furthermore, the description of the product indicated in the above mentioned ISO 855:2003 standard and the range of *D-limonene* correspond to the range of the phytochemical marker as proposed by the Applicant [10,11].

According to the procedure, 1 µl of the *lemon oil* is diluted 1:10 in acetone and injected into the GC at a split ratio of 1:100. The quantification is performed by FID using the normalisation approach for the estimation of the area percentage of individual components. For the various types of *lemon oil*, the Applicant presented a chromatogram and the specific analytical procedure for the analysis of the phytochemical marker *d-limonene* [36,37].

The EURL recommends for official control the GC-FID method based on ISO 855:2003 for the quantification of the phytochemical marker *d-limonene* in *lemon oil expressed*, *lemon oil distilled (residual fraction)* and *lemon oil distilled (volatile fraction).*

For the quantification of the phytochemical marker *d-limonene* in ***orange oil cold pressed***, ***orange terpenless (concentrated 4 times)*** oil, ***orange terpenless (folded)*** oil and ***orange terpenes*** oil the Applicant proposed a method based on GC-FID. The method is based on the standard ISO 3140:2019 for "Essential oil of sweet orange expressed (Citrus sinensis (L.))" [12-14,39-42]. The product is characterised via GC-FID. Furthermore, the description of the products indicated in the above mentioned ISO 3140:2019 standard and the range of *D-limonene* correspond to the range of the phytochemical marker as proposed by the Applicant [12-14].

According to the procedure, 1 µl of the products is directly injected into the GC at a split ratio of 1:100 (for *orange terpenless (folded)* oil the sample is diluted 1:10 in acetone). The quantification is performed by FID using the normalisation approach for the estimation of the area percentage of individual components. For the various types of *orange oil*, the Applicant presented a chromatogram and the specific analytical procedure for the analysis of the phytochemical marker *d-limonene* [39-41].

The EURL recommends for official control the GC-FID method based on ISO 3140:2019 for the quantification of the phytochemical marker *d-limonene* in in *orange oil cold pressed*,

orange terpenless (concentrated 4 times) oil, *orange terpenless (folded) oil* and *orange terpenes* oil.

For the quantification of the phytochemical marker *d-limonene* in ***mandarin oil*** the Applicant proposed a method based on GC-FID. The method is based on the standard ISO 3528:2012 for "Essential oil of mandarin, Italian type (Citrus reticulate Blanco)" [15,43,44]. Furthermore, the description of the product indicated in the above mentioned ISO 3528:2012 standard and the range of *d-limonene* correspond to the range of the phytochemical marker as proposed by the Applicant [15].

According to the procedure, 1 µl of the *mandarin oil* is directly injected into the GC at a split ratio of 1:100. The quantification is performed by FID 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 phytochemical marker *d-limonene* in *mandarin oil* [43].

The EURL recommends for official control the GC-FID method based on ISO 3528:2012 for the quantification of the phytochemical marker *d-limonene* in *mandarin oil.*

For the quantification of the phytochemical marker *tannins* in ***quebracho extract (wb)*** the Applicant submitted the method of ISO/DIS 14088/IUC 32 (currently issued as ISO 14088:2020) "Leather - Chemical tests - Quantitative analysis of tanning agents by filter method" [16,45-47]. The standard describes a method suitable for the determination of tanning agents in all vegetable tanning products. It is based on indirect gravimetric analysis of tanning agents with fixing of the absorbent compounds in low chromed hide powder.

According to the information in Annex A of the standard, an appropriate amount of heterogeneous product with particles not larger than 300 µg is placed into a 1000 ml flask. 800 ml of distilled water at 60 to 80 °C are added. The solution is shaken to fully dissolve the tanning agents and left to cool down in a water bath at (20 ± 2) °C. Distilled water is added in order to have 1000 ml of solution. If the tanning content in the solution goes beyond the limits the analysis should be repeated with a suitable sample size. A "Procter bell" containing hide powder is placed in a beaker to be filled with the solution. When the hide powder is completely soaked the solution is siphoned. The flow of the solution is adjusted to 8 to 10 drops / min of the de-tanned solution. A total of 90 ml is collected in 120 ± 10 min. The first 30 ml of the filtrate should be disposed of, while the next 60 ml should be collected in a dry 100 ml glass measuring cylinder to determine the non-tanning agents. 50 ml of the filtered solution are transferred into a previously calibrated silver dish and placed on the water bath waiting for complete evaporation. The dish is placed in a thermostatic heater at 105 ± 2 °C for about 18 ± 2 h. Finally the dish is placed in a silica gel dryer and weighed after 15 min. The quantification of *tannins* in *quebracho extract (wb)* is obtained from the difference among the

content of soluble solids and non-tanning solids and indicated as percentage content (absolute value).

The Applicant provided results for the analysis of *tannins* in 3 batches of *quebracho extract (wb)*. The results obtained are within the proposed specifications [48].

The EURL recommends for official control the ISO 14088:2020 method based on indirect gravimetric analysis of tanning agents with fixing of the absorbent compounds in low chromed hide powder for the quantification of the phytochemical marker *tannins* in *quebracho extract (wb).*

Due to the intrinsic nature of the BDG 08, the accurate quantification of the *feed additives* in *premixtures* and *feedingstuffs* is not achievable experimentally. Furthermore, the Applicant did not provide any experimental data to determine the *feed additives* in *water* [49]. Therefore, the EURL cannot evaluate or recommend any method for official control to quantify the *feed additives* in *premixtures*, *feedingstuffs* and *water*.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

The evaluation of corresponding methods of analysis is not relevant for the present application.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

The Applicant presented for the full characterisation of volatile chemically defined substances in essential oils or botanical extracts: an in-house developed GC-MS method for *buchu leaves oil, lime oil, petigrain bigarade oil, lemon oil expressed, lemon oil distilled (residual fraction), lemon oil distilled (volatile fraction), orange oil cold pressed, orange terpenless (concentrated 4 times), orange terpenless (concentrated 10 times), orange terpenless (folded), orange terpenes* and *mandarin oil* [5-16,50], the tests described in the European Pharmacopeia monograph "Indian Frankincense (Olibanum indicum)" for *olibanum extract (wb)* [7,24], the single-laboratory validated and further verified method based on HPLC-UV for *bitter orange extract of the whole fruit* [9,31], and several tests including the method described in ISO 14088, the determination of total ash, lipid and protein content, measurements of selected ions by ion chromatography (IC), and inductively coupled plasma-mass spectrometry (ICP-MS) for *quebracho extract (wb)* [16,47].

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.

4. CONCLUSIONS AND RECOMMENDATIONS

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

Recommended text for the register entry (analytical method)

For the quantification of *d-limonene* and *d,l-isomenthone* (phytochemical markers) in the *feed additive* (*buchu leaves oil*):

- gas chromatography coupled with flame ionisation detection (GC-FID) (based on ISO 11024)

For the quantification of *11-keto-β-boswellic acid* and *3-O-acetyl-11-keto-β-boswellic acid* (phytochemical markers) in the *feed additive* (*olibanum extract (wb)*):

- high performance liquid chromatography (HPLC) with spectrophotometric (UV) detection - European Pharmacopeia monograph "Indian Frankincense (Olibanum indicum)"

For the quantification of *d-limonene* (phytochemical marker) in the *feed additive* (*lime oil*):

- gas chromatography coupled with flame ionisation detection (GC-FID) (based on ISO 3519)

For the quantification of *linalyl acetate* and *linalool* (phytochemical markers) in the *feed additive* (*petigrain bigarade oil*):

- gas chromatography coupled with flame ionisation detection (GC-FID) (based on ISO 8901)

For the quantification of *naringin* (phytochemical marker) in the *feed additive* (*bitter orange extract of the whole fruit*):

- high performance liquid chromatography (HPLC) with spectrophotometric (UV) detection

For the quantification of *d-limonene* (phytochemical marker) in the *feed additives* (*lemon oil expressed, lemon oil distilled (residual fraction)* and *lemon oil distilled (volatile fraction)*):

- gas chromatography coupled with flame ionisation detection (GC-FID) (based on ISO 855)

For the quantification of *d-limonene* (phytochemical marker) in the *feed additives* (*orange oil cold pressed, orange terpenless (concentrated 4 times)* oil, *orange terpenless (folded)* oil and *orange terpenes* oil):

- gas chromatography coupled with flame ionisation detection (GC-FID) (based on ISO 3140)

For the quantification of *d-limonene* (phytochemical marker) in the *feed additive* (*orange terpenless (concentrated 10 times)*):

- gas chromatography coupled with flame ionisation detection (GC-FID) (based on ISO 11024)

For the quantification of *d-limonene* (phytochemical marker) in the *feed additive* (*mandarin oil*):

- gas chromatography coupled with flame ionisation detection (GC-FID) (based on ISO 3528)

For the quantification of *tannins* (phytochemical marker) in the *feed additive* (*quebracho extract (wb)*):

- indirect gravimetric analysis of tanning agents with fixing of the absorbent compounds in low chromed hide powder – ISO 14088

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Botanically defined flavourings group BDG 08 - Sapindales* have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

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7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, Geel, Belgium. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761.

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- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)

[3] Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: RIKILT Wageningen UR, Wageningen (NL).