EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F – Health and Food
Food and Feed Compliance

European Union Reference Laboratory
Feed Additives

JRC.F.5/UV/MGH/AS/Ares

Subject: Second amendment of the EURL evaluation report

Reference: EURL Report FAD-2010-0219 Botanically defined flavourings from BDG07 - Geraniales, Myrtales, Poales – JRC.D.5/SFB/CvH/RFO/mds/Ares (2014)120564

In the original report [1] the EURL evaluated and recommended analytical methods for eighteen flavouring compounds, namely: *geranium oil*, *geranium rose oil*, *eucalyptus oil*, *eucalyptus tincture*, *clove oil*, *clove tincture*, *broom teatree oil*, *purple loosetrife tincture*, *tea tree oil*, *melaleuca cajuputi oil*, *niaouli oil*, *allspice oil*, *bay oil*, *pomegranate bark extract*, *bambusa tincture*, *citronella oil*, *lemongrass oil* and *vetiveria oil* derived from different chemo-taxonomically related plants and belonging to the group "Geraniales, Myrtales, Poales".

After the publication of the original EURL evaluation report [1], six flavouring compounds, namely *geranium oil*, *broom teatree oil*, *allspice oil*, *bay oil*, *bambusa tincture* and *vetiveria oil* were withdrawn from the grouped application FAD-2010-0219 (*Botanically defined flavourings from BDG07 - Geraniales*, *Myrtales*, *Poales*) [2-3].

Following the priorities expressed by EFSA, the EURL recently issued a partial amendment of the mentioned report for four *feed additives*, namely *geranium rose oil, eucalyptus oil, lemongrass oil* and *clove oil* belonging to the group "Geraniales, Myrtales, Poales" [4]. The amendment aimed also at harmonising the original evaluation with the recent reports issued for this type of *feed additives*.

Upon a new EFSA request [5], in this second amendment the EURL will focus on the evaluation of the suitability of the new methods submitted by the Applicant for official control of another five *feed additives* of this group, namely *clove tincture*, *eucalyptus tincture*, *citronella oil*, *tea tree oil* and *melaleuca cajuputi oil.*

Hereafter, is the updated report on the evaluation of the new methods of analysis submitted by the Applicant and proposed for official control of *clove tincture*, *eucalyptus tincture*, *citronella oil*, *tea tree oil* and *melaleuca cajuputi oil*. It also includes the revised version of the recommendations, that replace the ones stated for these five *feed additives* in the original report issued by the EURL [1].

Clove tincture

According to the Applicant, *clove tincture* is a hydro alcoholic extract obtained of the flower bud or claw of *Syzygium aromaticum* (otherwise called "Clove") containing a mixture of chemical components that are naturally present in clove and contribute to the sensory effect of the product in animal feed, being *eugenol* a marker substance of *clove extracts.* Additionally, the Applicant identified four substances that can be considered of concern in clove extracts namely *eugenol*, estragole, eugenol acetate and methyleugenol [6].

The Applicant determined dry matter, ash content, carrier and macronutrients contents in the *feed additive* (*clove tincture*) by classical methods. Additionally, the Applicant also provided methods for the determination of total polyphenols, total flavonoids and the following terpenic compounds: *eugenol*, estragole, methyleugenol and eugenol acetate [6].

For the determination of the total polyphenols and total flavonoids the Applicant proposed the use of spectrophotometric methods described in the European Pharmacopoeia [7] while for the terpenic compounds the Applicant proposed a chromatographic method (GC-FID) according to the European Pharmacopoeia [8].

The Applicant has provided the result of the analysis of five different batches of the *feed additive* (*clove tincture*) characterised by applying the methods mentioned above. These analyses led to average values of 0.50 % total polyphenols (expressed as gallic acid equivalents) and 0.037 % total flavonoids (expressed as quercetin equivalents). Regarding the terpenic compounds, an average value of 0.0038 % for *eugenol*, less than 0.000017 % for eugenol acetate, 0.00008 % for estragol and 0.00019 % for methyleugenol were reported by the Applicant [6].

However, according to the Applicant the use of the HPTLC profile of the hexane extract as a fingerprint of the *feed additive* is considered a more reliable way to ensure the absence of adulteration and thus preferred one than the analysis of individual phytomarkers at an established range. The Applicant applied the HPTLC method to five different batches of the *feed additive* (*clove tincture*) reporting an average gallic acid content of 0.87 % [6].

Eucalyptus tincture

According to the Applicant, *eucalyptus tincture* is a hydro alcoholic extract obtained of the leaves of *Eucalypus globulus* containing a mixture of chemicals components that are naturally present in *Eucalypus globulus* and contribute to the sensory effect of the product in animal feed being *eucalyptol* (*1,8 cineole*) a marker substance of *eucalyptus tincture* and a substance of concern in eucalyptus extracts [9].

The Applicant determined dry matter, ash content, carrier and macronutrients contents in the *feed additive* (*clove tincture*) by classical methods. Additionally, the Applicant also provided methods for the determination of total polyphenols, total flavonoids and *1,8-cineole* (*eucalyptol*) [9].

For the determination of the total polyphenols and the total flavonoids the Applicant proposed the use of spectrophotometric method described in the European Pharmacopoeia [7] while for *1,8-cineole* (*eucalyptol*) the Applicant proposed an chromatographic method (GC-FID) according to European Pharmacopoeia [8].

The Applicant has provided the result of the analysis of five different batches of the *feed additive* (*eucalyptus tincture*) characterised by applying the methods mentioned above. These analyses led to average values of 0.45 % total polyphenols (expressed as gallic acid equivalents) and 0.028 % total flavonoids (as quercetin equivalents). For the *1,8-cineole* (*eucalyptol*) an average value of 0.0028 % was reported [9].

However, according to the Applicant the use of the HPTLC profile of the hexane extract as a fingerprint of the *feed additive* is considered a more reliable way to ensure the absence of adulteration and thus preferred one than the analysis of individual phytomarkers at an established range. The Applicant applied the HPTLC method to five different batches of the *feed additive* (*eucalyptus tincture*) reporting an average gallic acid of 0.27 % [9].

Citronella oil

According to the Applicant, *citronella oil* (winterianus and nardus types) are essential oils obtained by steam distillation of the fresh or partly dried aerial parts from *Cymbopogon winterianus* Jowitt ex Bor (for the winterianus type) or from *Cymbopogon nardus* (L.) Rendle (for the nardus type) with a content of *citronellal* (phytochemical marker) expressed as the relative individual peak area in the chromatogram, ranging from 30 to 45 % (for the winterianus type) and from 3 to 6 % (for the winterianus type) [10].

For the quantification of the phytochemical marker *citronellal* in the *citronella oil* (winterianus and nardus types) the Applicant proposed two methods based on gas chromatography coupled with flame ionisation detection (GC-FID) [11]. For the *citronella oil* (winterianus type) the proposed method is based on the standard ISO 3848:2016 for "Essential oil of citronella, Java type" while for the *citronella oil* (nardus type) is based on the standard ISO 3849:2003 for "Oil of citronella, Sri Lanka type [*Cymbopogon nardus* (L.) W. Watson var.*lenabatu* Stapf.] [10-11].

According to the specific analytical procedure, 1 µl of the oil is injected into the GC using split ratio 100:1. The eluted compounds are detected by FID and the quantification is

performed using the normalisation approach for the estimation of the area percentage of individual components (including also the phytochemical marker) in the chromatogram [11]. Furthermore, the description of the products (winterianus and nardus types) and the ranges of *citronellal* content stated in the respective standards ISO 3848:2016 and ISO 3849:2003, respectively, are similar or equal to the content ranges of the phytochemical marker as declared by the Applicant in the proposed consolidated specifications [10].

In addition, the Applicant presented typical chromatograms of *citronella oil* (winterianus and nardus types) demonstrating a good separation of the marker [11]. Moreover, the Applicant analysed the phytochemical marker (*citronellal*) in eight batches of *citronella oil* (winterianus type) and in four batches of *citronella oil* (nardus type) leading to an average contents of 35.1 % (winterianus type) and of 4.6 % (nardus type) [10], which are within the ranges specified in the ISO 3848:2016 and ISO 3849:2003 standards, respectively [10].

Given the performance characteristics and data currently available, the EURL recommends for official control the GC-FID methods based on i) the ISO 3848 standard for the quantification of *citronellal* (phytochemical marker) in *citronella oil* (winterianus type) and ii) the ISO 3849 standard for the quantification of *citronellal* (phytochemical marker) in *citronella oil* (nardus type).

Tea tree oil

According to the Applicant, *tea tree oil* is an essential oil obtained by steam distillation of the leaves and terminal branchlets of *Melaleuca alternifolia* Cheel with a content of *4-terpineol* (phytochemical marker) ranging from 30 to 48 % (expressed as the relative individual peak area in the chromatogram) [12].

For the quantification of the phytochemical marker *4-terpineol* in the *tea tree oil* the Applicant proposed a method based on gas chromatography coupled with flame ionisation detection (GC-FID) [12]. The method is based on the standard ISO 4730:2017 for "Essential oil of *Melaleuca*, terpinen-4-ol type (Tea Tree oil)" [12-13].

According to the specific analytical procedure, 1 µl of the oil is injected into the GC using a split ratio 40:1. The eluted compounds are detected by FID and the quantification is performed using the normalisation approach for the estimation of the area percentage of individual components (including also the phytochemical marker) in the chromatogram [13]. Furthermore, the description of the product and the range of *4-terpineol* content stated in the above mentioned ISO 4730:2017 standard are similar to the range of the phytochemical marker content as declared by the Applicant in the proposed consolidated specifications [12].

In addition, the Applicant presented a typical chromatogram of *tea tree oil* demonstrating a good separation of the marker [13]. Moreover, the Applicant analysed the phytochemical marker (*4-terpineol*) in seven batches of *tea tree oil* leading to an average content of 39.4 % [12], which is within the ranges as specified in the ISO 4730:2017 standard [13].

Given the performance characteristics and data currently available, the EURL recommends for official control the GC-FID method based on the ISO 4730 standard for the quantification of *4-terpineol* (phytochemical marker) in *tea tree oil.*

Melaleuca cajuputi oil

According to the Applicant, *melaleuca cajuputi oil* is an essential oil obtained by steam distillation of the leaves from the plant species *Melaleuca cajuputi* Powell and/or *Melaleuca leucadendra* (L.) with a content of *1,8-cineole* (phytochemical marker) ranging from 50 to 70 % (expressed as the relative individual peak area in the chromatogram) [14].

For the quantification of *1,8-cineole* (phytochemical marker) in *melaleuca cajuputi oil* the Applicant proposed a gas chromatography coupled with flame ionisation detection (GC-FID) method based on a generic ISO 11024:1998 standard for "Essential oils: General guidance on chromatographic profiles" [14-15].

The Applicant verified the above mentioned method for the analysis of *1,8-cineole* (phytochemical marker) following the "EURL–FA Validation and verification technical guide for Sensory feed Additives – flavouring compounds 2(b) from botanical origin" [16]. Table 1 shows a summary of the relevant performance characteristics obtained in the verification study.

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 *1,8-cineole* in *melaleuca cajuputi oil.*

Table 1. Performance characteristics of the GC-FID method for the quantification of the phytochemical marker *1,8-cineole* in the *feed additive* (*melaleuca cajuputi oil*) [15].

	melaleuca cajuputi oil	
	Batch 1	**Batch 2**
Content, % (relative area)	51.9	55.5
[a]RSDr, %	0.11	0.13
[a]RSDip, %	0.22	0.13

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and for *intermediate precision*, respectively.
[a)]Re-calculated by EURL [17].

The *feed additives* subject of this second amendment have a natural origin (botanically defined) and are derived from plant species belonging to the botanical order "Geraniales, Myrtales, Poales". Consequently, due to their intrinsic nature, the accurate quantification of the *feed additives* in *premixtures* and *compound feed* is not achievable experimentally.

Furthermore, the Applicant did not provide any experimental data to determine the *feed additives* in *water*. Therefore, the EURL cannot evaluate or recommend any method for official control to quantify *clove tincture*, *eucalyptus tincture*, *citronella oil*, *tea tree oil* and *melaleuca cajuputi oil* in *premixtures*, *compound feed* and *water.*

Recommended text for the registry entry (analytical method)

For the characterisation of the *feed additive* (*clove tincture*):

- spectrophotometry for the determination of total polyphenols and total flavonoids
- gas chromatography coupled with flame ionisation detection (GC-FID) for the determination of *eugenol*, estragole, methyleugenol and eugenol acetate
- high performance thin-layer chromatography (HPTLC) for the determination of gallic acid

For the characterisation of the *feed additive* (*eucalyptus tincture*):

- spectrophotometry for the determination of total polyphenols and total flavonoids
- gas chromatography coupled with flame ionisation detection (GC-FID) for the determination of 1,8-cineole (eucalyptol)
- high performance thin-layer chromatography (HPTLC) for the determination of gallic acid

For the determination of *citronellal* (phytochemical marker) in the *feed additive* (*citronella oil* (winterianus type)):

- gas chromatography coupled with flame ionisation detection (GC-FID) (ISO 3848)

For the determination of *citronellal* (phytochemical marker) in the *feed additive* (*citronella oil*) (nardus type)):

- gas chromatography coupled with flame ionisation detection (GC-FID) (ISO 3849)

For the determination of *4-terpineol* (phytochemical marker) in the *feed additive* (*tea tree oil*):

- gas chromatography coupled with flame ionisation detection (GC-FID) (ISO 4730)

For the determination of *1,8-cineole* (phytochemical marker) in the *feed additive* (*melaleuca cajuputi oil*):

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

References

[1] EURL Report FAD-2010-0219 Botanically defined flavourings from BDG07 - Geraniales, Myrtales, Poales – JRC.D.5/SFB/CvH/RFO/mds/Ares (2014)120564

[2] Supplementary Information: EC_Partial_withdrawal_of_applications_for_various_Botanically_Defined_-_from_BDG01_to_BDG20_270219.pdf

[3] Supplementary Information – 0116-2010 Partial withdrawal bambusa & allspice.pdf

[4] Amendment of the EURL Report FAD-2010-0219 Botanically defined flavourings from BDG07 - Geraniales, Myrtales, Poales – JRC.F.5/CvH/MGH/AS/Ares(2023)3823305

[5] Request of second amendment of the Evaluation Report on the Analytical Methods for five preparations included in the dossier FAD-2010-0219 - Ares(2024)1072031

[6] Supplementary Information 20221206_FAD-2010-0219_SIn_Clove tincture_Original Submission and Correction_ Section II_Identity-2.pfd &-Annex_II_2_Methods of analysis & Annex_1_Complementary information.pdf &Annex_II_3_Results of analysis.pdf

[7] European Pharmacopoiea, Chapter 2.8.14 Determination of tannins in herbal drugs

[8] European Pharmacopoiea 10.0

[9] Supplementary Information 0220930_FAD-2010-0219_SIn_Eucalyptus tincture_ Section II & Annex_1_Complementary information.pdf

[10] Supplementary Information 2023-06-16-BDG-07-SIn-reply-citronella_oil.pdf

[11] Supplementary Information EURL_appendix_citronella_oil.pdf

[12] Supplementary Information 20231013_BDG07-SIn-reply-tea_tree_oil.pdf

[13] Supplementary Information EURL_appendix_tea_tree_oil.pdf

[14] Supplementary Information – 2023-04-14-BDG-07-SIn-reply-melaleuca cajuputi oil.pdf

[15] Supplementary Information – EURL_appendix_melaleuca_cajuputi_oil.pdf

[16] EURL–FA Validation and verification technical guide for Sensory feed Additives – flavouring compounds 2(b) from botanical origin

[17] Supplementary Information – eurl-anova- melaleuca_cajuputi_oil.pdf

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

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In the original report [1] the EURL evaluated and recommended analytical methods for eighteen flavouring compounds, namely: *geranium oil, geranium rose oil, eucalyptus oil, eucalyptus tincture, clove oil, clove tincture, broom teatree oil, purple loosetrife tincture, tea tree oil, melaleuca cajuputi oil, niaouli oil, allspice oil, bay oil, pomegranate bark extract, bambusa tincture, citronella oil, lemongrass oil and vetiveria oil* derived from different chemo-taxonomically related plants and belonging to the group "Geraniales, Myrtales, Poales".

Since the publication of the original EURL evaluation report [1], six flavouring compounds named *geranium oil, broom teatree oil, allspice oil, bay oil, bambusa tincture* and *vetiveria oil* were withdrawn from the grouped application FAD-2010-0219 *Botanically defined flavourings from BDG07 - Geraniales, Myrtales, Poales* [2-3].

Following the priorities expressed by EFSA, in this amendment, aimed to harmonise the original evaluation with the more recent reports issued for this type of *feed additives,* the EURL will focus exclusively on the evaluation of the suitability of the new analytical methods submitted by the Applicant for official control of four of the remaining twelve *feed additives* namely *geranium rose oil, eucalyptus oil, lemongrass oil* and *clove oil*.

Hereafter is the updated report on the evaluation of the new methods of analysis submitted by the Applicant and proposed for official control of *geranium rose oil, eucalyptus oil, lemongrass oil* and *clove oil* and the revised version of the recommendations, that replace the ones stated for these four *feed additives* in the original report issued by the EURL [1].

Geranium rose oil

According to the Applicant *geranium rose oil* is an essential oil obtained by steam distillation of the herbaceous parts of the plant species *Pelargonium* spp. with a content of *citronellol*

(phytochemical marker) ranging from 25 to 36 % (expressed as the relative individual peak area in the chromatogram) [5].

For the quantification of the phytochemical marker *citronellol* in *geranium rose oil* the Applicant proposed a method based on gas chromatography coupled with flame ionisation detection (GC-FID) [6]. The method is based on the standard ISO 4731:2012 for "Essential oil of geranium (*Pelargonium* × ssp.)" [5-6].

According to the specific analytical procedure, 1 µl of the oil is injected into the GC using split ratio 100:1. The eluted compounds are detected by FID and the quantification is performed using the normalisation approach for the estimation of the area percentage of individual components (including also the phytochemical marker) in the chromatogram [6]. Furthermore, the description of the product from North Africa and the range of *citronellol* stated in the above mentioned ISO 4731:2012 standard corresponds to the range of the phytochemical marker as declared by the Applicant in the proposed consolidated specifications [6].

In addition, the Applicant presented a typical chromatogram of *geranium rose oil* demonstrating a good separation of the marker [6]. Moreover, the Applicant analysed the phytochemical marker (*citronellol*) in five batches of *geranium rose oil* leading to an average content of 32.3 % [6], which is within the range as specified in the above mentioned ISO 4731:2012 standard for the product from North Africa origin [7].

Given the performance characteristics and data currently available, the EURL recommends for official control the GC-FID method based on the ISO 4731 standard for the quantification of *citronellol* (phytochemical marker) in *geranium rose oil*.

Eucalyptus oil

According to the Applicant *eucalyptus oil* is an essential oil obtained by steam distillation of the leaves and twigs of *Eucalypus globulus* Labill. with a content of *1,8-cineole* (phytochemical marker) higher than 70 % (expressed as the relative individual peak area in the chromatogram) [8].

For the quantification of the phytochemical marker *1,8-cineole* in *eucalyptus oil* the Applicant proposed a method based on gas chromatography coupled with flame ionisation detection (GC-FID) [8]. The method is based on the standard ISO 700:2002 for "Crude or rectified oils of Eucalyptus globulus (*Eucalyptus globulus* Labill.)" [7-8].

According to the specific analytical procedure, 1 µl of the oil is injected into the GC using split ratio 100:1. The eluted compounds are detected by FID and the quantification is performed using the normalisation approach for the estimation of the area percentage of

individual components (including also the phytochemical marker) in the chromatogram [8]. Furthermore, the description of the product and the range of *1,8-cineole* stated by the above mentioned ISO 700:2002 standard correspond to the content of the phytochemical marker as declared by the Applicant in the proposed consolidated specifications [7].

In addition, the Applicant presented a typical chromatogram of *eucalyptus oil* demonstrating a sufficient separation of the marker [8]. Moreover, the Applicant analysed the phytochemical marker (*1,8-cineole*) in two different batches of *eucalyptus oil* leading to an averaged content of 81.0 % [8], which is within the range as specified in the above mentioned ISO 700:2002 standard [8].

Given the performance characteristics and data currently available, the EURL recommends for official control the GC-FID method based on the ISO 700 standard for the quantification of *1,8-cineole* (phytochemical marker) in *eucalyptus oil*.

Lemongrass oil

According to the Applicant *lemongrass oil* is an essential oil obtained by steam distillation of the aerial parts of *Cymbopogon flexuosus* (Nees ex Steud.) Will. Watson with contents of the phytochemical markers i.e. *neral* and *trans-3,7-dimethylocta-2,6-dienal (geranial),* ranging from 25 to 35 % and from 35 to 47 %, respectively (expressed as the relative individual peak area in the chromatogram) [9].

For the quantification of the phytochemical markers *neral* and *geranial* in *lemongrass oil* the Applicant proposed a method based on gas chromatography coupled with flame ionisation detection (GC-FID) [10]. The method is based on the standard ISO 4718:2004 for "Oil of lemongrass [*Cymbopogon flexuosus* (Nees ex Steudel) J.F. Watson]" [9-10].

According to the specific analytical procedure, 1 µl of the oil is injected into the GC using split ratio 100:1. The eluted compounds are detected by FID and the quantification is performed using the normalisation approach for the estimation of the area percentage of individual components (including also the phytochemical markers) in the chromatogram [10]. Furthermore, the description of the product and the ranges of *neral* and *geranial* stated in the above mentioned ISO 4718:2004 standard correspond to the ranges of the phytochemical markers as declared by the Applicant in the proposed consolidated specifications [9].

In addition, the Applicant presented a typical chromatogram of *lemongrass oil* demonstrating a good separation of the markers [10]. Moreover, the Applicant analysed the phytochemical markers i.e. *neral* and *geranial*, in two different batches of *lemongrass oil* leading to an averaged content of 31.4 % for *neral* and of 41.4 % for *geranial* [10], which are within their respective ranges as specified in the above mentioned ISO 4718:2004 standard [3].

Given the performance characteristics and data currently available, the EURL recommends for official control the GC-FID method based on the ISO 4718 standard for the quantification of *neral* and *trans-3,7-dimethylocta-2,6-dienal (geranial)* (phytochemical markers) in *lemongrass oil*.

Clove oil

The Applicant requested the authorisation of three types of clove oils namely *clove leaf oil* (eugenol type), *clove bud oil* and *clove leaf oil* (beta-caryophyllene type).

According to the Applicant *clove leaf oil* (eugenol type) is an essential oil obtained by steam distillation of the leaves from *Syzygium aromaticum* (L.) Merr. & L.M.Perry with a content of *eugenol* (phytochemical marker), expressed as the relative individual peak area in the chromatogram, ranging from 80 to 95 %. C*love bud oil* is an essential oil obtained by steam distillation of the dried flower buds from *Syzygium aromaticum* (L.) Merr. & L.M.Perry with a content of *eugenol* (phytochemical marker) expressed as the relative individual peak area in the chromatogram, ranging from 67-85 % [11].

For the quantification of the phytochemical marker *eugenol* in *clove leaf oil* (eugenol type) and in c*love bud oil* the Applicant proposed methods based on gas chromatography coupled with flame ionisation detection (GC-FID) [12]. The proposed methods for *clove leaf oil* (eugenol type) is based on the standard ISO 3141:1997 ("Oil of clove leaves [Syzygium aromaticum (L.) Merr. et Perry, syn. Eugenia caryophyllus (Sprengel) Bullock et S. Harrison]") while the one proposed for c*love bud oil* is based on the standard ISO 3142:1997 ("Oil of clove buds [Syzygium aromaticum (L.) Merr. et Perry, syn. Eugenia caryophyllus (Sprengel) Bullock et S. Harrison]") [11-12].

According to the specific analytical procedure submitted for both oils, 1 µl of the oil is injected into the GC using split ratio 100:1. The eluted compounds are detected by FID and the quantification is performed using the normalisation approach for the estimation of the area percentage of individual components (including also the phytochemical markers) in the chromatogram [12]. Furthermore, the description of the product and the ranges of *eugenol* stated in the above mentioned ISO 3141:1997 standard are similar to the ranges of the phytochemical markers as declared by the Applicant in the proposed consolidated specifications [11].

In addition, the Applicant presented a typical chromatograms of *clove leaf oil (eugenol type)* and of c*love bud oil* demonstrating a good separation of the markers [12]. Moreover, the Applicant analysed the phytochemical marker i.e. *eugenol*, in two batches of *clove leaf oil (eugenol type)* and in five batches of *clove bud oil* leading to averaged *eugenol* contents of

82.6 % and of 82.7 %, respectively [12], which are within *eugenol* ranges as specified in the above mentioned ISO 3141:1997 and ISO 3142:1997 standards [12].

Given the performance characteristics and data currently available, the EURL recommends for official control the GC-FID methods based on the ISO 3141 and on the ISO 3142 standards for the quantification of *eugenol* (phytochemical markers) in *clove leaf oil* (eugenol type) and in c*love bud oil,* respectively.

Additionally, the Applicant described the *clove leaf oil* (beta-caryophyllene type) as an essential oil obtained by steam distillation of *clove leaf oil* (eugenol type) with a content of *beta-caryophyllene* (phytochemical marker), expressed as the relative individual peak area in the chromatogram, ranging from 70 to 90 % [11].

For the quantification of *beta-caryophyllene* (phytochemical marker) in *clove leaf oil* (beta-caryophyllene type) the Applicant proposed a gas chromatography coupled with flame ionisation detection (GC-FID) method based on a generic ISO 11024:1998 standard for "Essential oils: General guidance on chromatographic profiles" [11-12].

The Applicant verified the above mentioned method for the analysis of *beta-caryophyllene* (phytochemical marker) following the "EURL–FA Validation and verification technical guide for Sensory feed Additives – flavouring compounds 2(b) from botanical origin" [13]. Table 1 shows a summary of the relevant performance characteristics obtained in the verification study.

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 *beta-caryophyllene* in *clove leaf oil* (beta-caryophyllene type).

Table 1. Performance characteristics of the GC-FID method for the quantification of the phytochemical marker *beta-caryophyllene* in the *feed additive* (*clove leaf oil* (beta-caryophyllene type)) [14].

	beta-caryophyllene	
	Batch 1	**Batch 2**
Content, % (relative area)	82.0	82.1
[a]RSDr, %	0.20	0.04
[a]RSDip, %	0.20	0.10

RSD_r and RSD_{ip}: relative standard deviations for *repeatability and* for *intermediate precision, respectively.*
[a)]Recalculated by EURL [14].

The *feed additives* subject of this amendment have a natural origin (botanically defined) and are derived from plant species belonging to the botanical order "Geraniales, Myrtales, Poales". Consequently, due to their intrinsic nature, the accurate quantification of the *feed additives* in *premixtures* and *compound feed* is not achievable experimentally.

Furthermore, the Applicant did not provide any experimental data to determine the *feed additives* in *water*. Therefore, the EURL cannot evaluate or recommend any method for official control to quantify *geranium rose oil, eucalyptus oil, lemongrass oil* and *clove oil* in *premixtures*, *compound feed* and *water.*

Recommended text for the registry entry (analytical method)

For the determination of *citronellol* (phytochemical marker) in the *feed additive* (*geranium* rose oil):

- Gas chromatography coupled with flame ionisation detection (GC-FID) (ISO 4731)

For the determination of *1,8-cineole* (phytochemical marker) in the *feed additive* (*eucalyptus oil*):

- Gas chromatography coupled with flame ionisation detection (GC-FID) (ISO 700)

For the determination of *neral* and *trans-3,7-dimethylocta-2,6-dienal (geranial),* (phytochemical markers) in the *feed additive* (*lemongrass oil*):

- Gas chromatography coupled with flame ionisation detection (GC-FID) (ISO 4718)

For the determination of *eugenol* (phytochemical marker) in the *feed additive* (*clove leaf oil* (eugenol type)):

- Gas chromatography coupled with flame ionisation detection (GC-FID) (ISO 3141)

For the determination of *eugenol* (phytochemical marker) in the *feed additive* (c*love bud oil*):

- Gas chromatography coupled with flame ionisation detection (GC-FID) (ISO 3142)

For the determination of *beta-caryophyllene* (phytochemical marker) in the *feed additive* (*clove leaf oil* (beta-caryophyllene type)):

- Gas chromatography coupled with flame ionisation detection (GC-FID) (ISO 11024)

References

[1] EURL Report FAD-2010-0219 Botanically defined flavourings from BDG07 - Geraniales, Myrtales, Poales – JRC.D.5/SFB/CvH/RFO/mds/Ares (2014)120564

[2] Supplementary Information: EC_Partial_withdrawal_of_applications_for_various_Botanically_Defined_-_from_BDG01_to_BDG20_270219.pdf

[3] Supplementary Information – 0116-2010 Partial withdrawal bambusa & allspice.pdf

[4] Request of partial amendment of the Evaluation Report on the Analytical Methods for four preparations included in the dossier FAD-2010-0219 - Ares(2023)3146918

[5] Supplementary Information – SIn_reply_Geranium_rose_oil.pdf

[6] Supplementary Information – 2022-05-09-EURL_appendix_geranium_rose_oil.pdf & 2022-04-13_Geranium-rose-oil_data-from-applicant.xlsx

[7] Supplementary Information – 2022-11-29_BDG-07-SIn-reply-Eucalyptus oil-1.pdf

[8] Supplementary Information – EURL_appendix_eucalyptus_oil.pdf

[9] Supplementary Information – 20230131-BDG-07-SIn-reply-lemongrass oil.doc

[10] Supplementary Information – EURL_appendix_lemongrass_oil.pdf,

[11] Supplementary Information – 2023-03-21-SIn_reply_Clove_oil.doc,

[12] Supplementary Information – 2023-03-09_EURL_appendix_clove_oil.pdf; K10 clove bud oil.xlsx, L11 clove bud oil.xlsx &J9 clove leaf oil eugenol type.xlsx

[13] EURL–FA Validation and verification technical guide for Sensory feed Additives – flavouring compounds 2(b) from botanical origin

[14] Supplementary Information – eurl-anova-clove leaf oil_beta-caryophyllene type.pdf

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The following National Reference Laboratories contributed to this report:

- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
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- Państwowy Instytut Weterynaryjny, Pulawy (PL)

- Prepared by María José González de la Huebra
- Reviewed and approved by Zigmas Ezerskis and Christoph von Holst (EURL-FA), respectively, Geel, 31/05/2023

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

EUROPEAN COMMISSION
DIRECTORATE GENERAL
JOINT RESEARCH CENTRE
Directorate D - Institute for Reference Materials and Measurements
European Union Reference Laboratory for Feed Additives

JRC.D.5/SFB/CvH/RFO/mds/Ares

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 Group 07

FAD-2010-0219 - CRL/100210

European Union Reference Laboratory
Feed Additives

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-2010-0219** **CRL/100210**
Product Name:	**Botanically defined flavourings from BDG07 - Geraniales, Myrtales, Poales**
Active Substance(s):	**Eighteen compounds from botanically defined flavourings BDG07**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)**
Report prepared by:	**Rebeca Fernandez Orozco**
Report revised by: Date:	**Piotr Robouch (EURL-FA)** **14/01/2014**
Report approved by: Date:	**Christoph von Holst** **16/01/2014**

EXECUTIVE SUMMARY

The *Botanically Defined Flavourings – Group 7* BDG 07 (*Geraniales, Myrtales, Poales*) is an application comprising *eighteen flavouring compounds* (*) for which authorisation as *feed additives* is sought under the category/functional group 2(b) "sensory additives"/"flavouring compounds", according to the classification system of Annex I of Regulation (EC) No 1831/2003. In the current application submitted according to Articles 4(1) and 10(2) of Regulation (EC) No 1831/2003, the authorisation for all species and categories is requested. *Mixtures of flavouring compounds* are intended to be incorporated only into *feedingstuffs* or drinking *water*. The Applicant suggested no minimum or maximum levels for the different flavouring compounds, but normal contents of *flavouring compounds* in *feedingstuffs* range up to from 0.1 to 100 mg/kg.

For the identification of eleven volatile phytochemical markers in the *feed additives,* the Applicant submitted a qualitative multi-analyte gas-chromatography mass-spectrometry (GC-MS) method, using Retention Time Locking (RTL), which allows a close match of retention times on GC-MS. By making an adjustment to the inlet pressure, the retention times can be closely matched to those of a reference chromatogram. It is then possible to screen samples for the presence of target compounds using a mass spectral database of RTL spectra. The Applicant provided the typical chromatogram for the *BDG 07* of interest. In order to demonstrate the transferability of the proposed analytical method, a second independent laboratory tested two model premixtures of twenty chemically defined flavourings representing the whole spectrum of compounds in use as feed flavourings with respect to their volatility and polarity. All twenty substances were extracted either from a liquid premixture or a solid premixture, and subsequently analysed using the same GC/MS method. All twenty model substances were properly identified. Since the volatile phytochemical markers of *BDG 07* are within the volatility and polarity range of the model mixture tested, the Applicant concluded that the proposed analytical method is suitable to determine qualitatively the presence of the volatile phytochemical markers from *BDG 07* in the *mixture of flavouring compounds.*

For the qualitative identification of the three non-volatile phytochemical markers: (1) *Punicalagin* in *Pomegranate bark extract*, (2) *Pyrogallol* in *Purple loosetrife tincture*, and (3) *L-Tyrosine* in *Bambusa tincture*, the Applicant submitted the following three methods: (I) a single laboratory validated and further verified method using a Reversed Phase High-Performance Liquid Chromatography (RP-HPLC) with UV detection at 260 nm; (II) the European Pharmacopoeia method (Ph. Eur. 7.0 01/2008:1537), based on a colorimetric assay of tannins expressed as pyrogallol; and (III) the European Pharmacopeia method (Ph. Eur. 6.6 01/2010:20256), based on ion-exchange chromatography with post column ninhydrin derivatisation. The third method is similar to the one described in the Community method (Commission Regulation (EC) No 152/2009 Annex III, F) and already evaluated by the

EURL in the frame of the L-Tyrosine dossier (FAD-2010-0260). Based on the information available the EURL recommends for official control the first two methods mentioned above together with the Community method for the qualitative identification of the three non-volatile phytochemical markers.

As no experimental data were provided by the Applicant for the identification of the *active substance(s)* in *feedingstuffs* and *water*, no methods could be evaluated. Therefore the EURL is unable to recommend a method for the official control to identify the *flavouring compound(s)* of interest (cf. Table 1) in *feedingstuffs* or *water*.

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) is not considered necessary.

(*) Full list provided in EURL evaluation report, available from the EURL website.

KEYWORDS

Botanically Defined Flavourings - Group 07, mixture of flavouring products, sensory additives, all species.

1. BACKGROUND

The *Botanically Defined Flavourings - Group 07* (*BDG 07*) is a grouped application of eighteen feed additives for which authorisation is sought under the category "sensory additives", functional group 2(b) "flavouring compounds" [1], according to the classification system of Annex I of Regulation (EC) No 1831/2003.

In the current application submitted according to Article 4(1) (two new additives to be added in the Register, ie. *Carophyl Clover tincture* and *Lemongrass oil*; and new use in water) and Article 10(2) (re-evaluation of additives already authorised under Directive 70/524/EC) of Regulation (EC) No 1831/2003, the authorisation for all species and categories is requested [1].

The *BDG 07* application contains *eighteen flavouring compounds* derived from different chemo-taxonomically related plants (listed in Table 1), belonging to the group "*Geraniales, Myrtales, Poales*". *Botanically defined flavourings* used as *feed additives* include oils, distillates, oleoresins, solvent based and water based extracts, concentrates, tinctures, absolutes and other preparation types, according to British and European Pharmacopeia [3].

Flavouring compounds are intended to be incorporated only into *feedingstuffs* or drinking *water*. The Applicant suggested no minimum or maximum levels for the different flavouring

compounds [2], but normal contents of *flavouring compounds* in *feedingstuffs* range up to from 0.1 to 100 mg/kg [4].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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 07*, and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

Qualitative and quantitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. arsenic, cadmium, lead, mercury, and dioxins) are available from the respective European Union Reference Laboratories [5].

Description of the analytical methods for the determination of the active substance in feed additive, premixtures and feedingstuffs

For the identification of eleven volatile phytochemical markers of *BDG 07* (cf. Table 1) in the *feed additive,* the Applicant submitted a qualitative multi-analyte gas-chromatography mass-spectrometry (GC-MS) [6] method, using Retention Time Locking (RTL) [7] methodology for which a patent is owned by Agilent Technology [8]. The Applicant does not mention about similar RTL systems from companies other than Agilent.

RTL allows a close match of retention times on Agilent GC-MS. By making an adjustment to the inlet pressure, the retention times can be closely matched to those of a reference chromatogram. It is then possible to screen samples for the presence of target compounds using a mass spectral database. The Applicant maintained phytochemical markers database/libraries (for the retention times and for MS spectra) containing data for more than four hundred phytochemical markers (including those listed in Table 1) [8]. These libraries were provided to the EURL.

At first a GC-MS system suitability check is performed using an equal-weight mixture of Linalool, Acetophenone, Benzyl Acetate, Benzyl Alcohol, Hydroxycitronellal. The obtained characteristics of the chromatogram - related to quantitative compositions, peak shapes and elution order - should be comparable with those of the reference chromatogram [8].

Retention times of d-limonene are measured at five inlet pressures (normal; ±10%; ±20%) to construct the calibration curve "retention time" *vs.* "inlet pressure". The "nominal" inlet pressure is then interpolated using the Agilent GC-RTL software and the retention time of d-limonene of the "reference" chromatogram (8.3 or 6.7 min for non-polar or polar columns, respectively). This "nominal" inlet pressure is finally used when analysing the samples of interest with an Agilent GC-MS. The retention times of the peaks detected in the chromatograms are compared to those of the reference chromatogram to identify the various compounds detected, using the phytochemical markers database/libraries.

Upon request by the EURL, the Applicant provided the experimental evidence showing that for each *flavouring compounds* in Table 1 the volatile phytochemical markers can be identified [9].

For the analysis of solid flavouring premixtures, the extraction is carried out using either the Soxhlet Extraction system or Pressurised Liquid Extraction [6]. The resulting extracts are injected in the GC-MS [6] at constant "nominal" inlet pressure. The Applicant provided the typical chromatogram for the *BDG 07* of interest (cf. Fig II.2-5 [9]).

In order to demonstrate the transferability of the proposed analytical method, a second independent laboratory tested two model premixtures of twenty chemically defined flavourings representing the whole spectrum of compounds in use as feed flavourings with respect to their volatility and polarity. All twenty substances were extracted either from a liquid premixture (containing 1 % of each flavouring compound and 80% of sunflower oil as liquid carrier) or a solid premixture (containing 1% of each flavouring compound, 20% of silicic acid and 60% of calcium carbonate as carriers), and subsequently analysed using the same GC/MS method described for identification and assay. All twenty model substances were determined qualitatively [10]. Since the volatile phytochemical markers of *BDG 07* are within the volatility and polarity range of the model mixture tested, the Applicant concluded that the proposed analytical method is suitable to determine qualitatively the presence of the volatile phytochemical markers from *BDG 07* in the *mixture of flavouring compounds*.

Based on the satisfactory experimental evidence provided, the EURL recommends for official control for the qualitative identification in the *feed additive* of the individual (or mixture of) volatile phytochemical markers, related to the *flavouring compounds* of interest (listed in Table 1) the GC-MS-RTL (Agilent specific) method submitted by the Applicant.

Table 1. Phytochemical markers and related *flavouring compounds* in *BDG 07* [4].

Flavouring compound	EU Register name phytochemical marker	CAS nr	RTL polar (min)	RTL non-polar (min)	Analytical method
Eucalyptus oil	1,8-Cineole	470-82-6	7	8.2	GC-MS-RTL
Eucalyptus tincture					
Melaleuca cajuputi oil					
Niaouli oil					
Lemongrass oil	3,7-Dimethylocta-2,6-dienal	141-27-5	19.7 – 21.0	14.1 – 15.0	
Tea tree oil	4-Terpinenol	562-74-3	17.7	12.5	
Broom teatree oil	Leptospermone	567-75-9	51	26	
Clove oil	beta-Caryophyllene	87-44-5	17.64	20.4	
Lemongrass oil	Citral	5392-40-5	19.7 – 21.0	14.1 – 15.0	
Citronella oil	Citronellal	106-23-0	14	11.6	
Geranium oil	Citronellol	106-22-9	21.9	14.1	
Geranium rose oil					
Clove oil	Eugenol	97-53-0	31.8	17.9	
Clove tincture					
Allspice oil					
Bay oil					
Geranium oil	Geraniol	106-24-1	24.1	14.8	
Vetiveria oil	Vetiverol	89-88-3	39.9	29.9	
Pomegranate bark extract	Punicalagin isomers	65995-63-3			HPLC
Purple loosetrife tincture	Pyrogallol	87-66-1			TLC
Bambusa tincture	L-Tyrosine	60-18-4			AAM

GC-MS-RTL: Gas Chromatography-Mass Spectrometry-Retention Time Locked;
HPLC: High Performance Liquid Chromatography;
TLC: Thin Layer Chromatography;
AAM: Amino acid Analyser of Marker

For the identification of the phytochemical marker *Punicalagin* in *Pomegranate bark extract*, the Applicant submitted a single laboratory validated and further verified method using a Reversed Phase High-Performance Liquid Chromatography (RP-HPLC) with UV detection at 260 nm [11,12]. Based on the satisfactory experimental evidence provided, the EURL recommends this method for official control.

For the identification of the phytochemical marker *Pyrogallol* in *Purple loosetrife tincture*, the Applicant submitted the internationally recognised European Pharmacopoeia method [13], based on a colorimetric assay of tannins expressed as pyrogallol. Even though no performance characteristics are provided, the EURL recommends the European Pharmacopeia method (Ph. Eur. 7.0 01/2008:1537) for official control.

For the identification of the phytochemical marker *L-Tyrosine* in *Bambusa tincture*, the Applicant submitted the European Pharmacopeia method [14], based on ion-exchange chromatography with post column ninhydrin derivatisation. This method is similar to the one described in the Community method (Commission Regulation (EC) No 152/2009 Annex III, F). The EURL evaluated already this method in the frame of the L-Tyrosine dossier (FAD-2010-0260) and recommended it for official control.

As no experimental data were provided by the Applicant for the identification of the *active substance(s)* in *feedingstuffs* and *water*, no methods could be evaluated. Therefore the EURL is unable to recommend a method for the official control to identify the *flavouring compound(s)* of interest (cf. Table 1) in *feedingstuffs* or *water*.

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) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

The EURL recommends for official control:

- the Agilent specific method submitted by the Applicant, for the identification of the eleven volatile phytochemical markers of the *BDG 07* in the *feed additive* of the individual (or mixture of) *flavouring compounds* of interest;
- the validated and further verified RP-HPLC method for the qualitative identification of the phytochemical marker *Punicalagin* in *Pomegranate bark extract*.

— the European Pharmacopoeia (Ph. Eur. 7.0 method 01/2008:1537), for the qualitative identification of the phytochemical marker *Pyrogallol* in *Purple loosetrife tincture.*

— Ion exchange chromatography method with post-column derivatisation and photometric detection (Commission Regulation (EC) No 152/2009), for the qualitative identification of the phytochemical marker *L-Tyrosine* in *Bambusa tincture.*

The Applicant provided no experimental data for *feedingstuffs* and *water*, therefore the EURL is unable to recommend a method for the official control to identify the *flavouring compound(s)* of interest in *feedingstuffs* or *water*.

Recommended text for the register entry (analytical method)

For the identification of eleven volatile phytochemical markers of *BDG 07* in individual (or mixture of) *flavouring compounds*:

Gas-chromatography mass-spectrometry with retention time locking (GC-MS-RTL)

For the determination of the phytochemical marker *Punicalagin* in *Pomegranate bark extract.*

Reversed Phase High-Performance Liquid Chromatograph (RP-HPLC)

For the determination of the phytochemical marker *Pyrogallol* in *Purple loosetrife tincture*:

colorimetric assay of tannins expressed as pyrogallol - European Pharmacopeia 7.0, method 01/2008:1537

For the determination of the phytochemical marker *L-Tyrosine* in *Bambusa tincture*:

ion-exchange chromatography with post column derivatisation and photometric detection – Commission Regulation (EC) No 152/2009

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 07* (*BDG 07*) 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

[1] *Application, Reference SANCO/D/2 Forw. Appl. 1831/00116-2010

[2] *Application, Proposal for Register Entry – Annex A

[3] *Technical dossier, Section II – Annex II 05

[4] *Technical dossier, Section II – Sect_II_Identity: 2.1. Identity of the additives - 2.5. Conditions of use of the additive – 2.6. Method of analysis and reference samples

[5] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories

[6] *Technical dossier, Section II – Annex_II_7_GCMS-CDG
"GC/MS method for the identification and assay of feed flavourings"

[7] #Technical dossier, Section II – Annex_II_07_RTL Lock

[8] #Technical dossier, Section II – Annex_II_06_Flavour RTL

[9] *Supplementary information Annex_ I_ GCMS_ Introduction Matrices BDG7
GC/MS method for the identification of botanical flauvorings

[10] *Supplementary information. Annex_ V_ GCMS_Premixture
GC/MS method for the identification of botanical flauvorings in premixtures

[11] *Supplementary information. Annex_I_HPLC_B07-2_Description_Matrix
Analytical method for the determination of the marker punicalagin (description, SOP) and evidence on the applicability for the determination of the marker in the matrix 'extract'

[12] *Supplementary information. Annex_III_HPLC_B07-2_Premixture
Report on the applicability of the analytical method for the identification of the marker punicalagin in solid premixture

[13] *Supplementary information. Annex_I_TLC_B07-3_Ph-Eur_Loosetrife_2008-1537
European Pharmacopoeia 7.0/ Loosetrifi (Lythri herba) 01/2008:1537

[14] *Supplementary information. Annex_I_AAM_B07-1_Ph-Eur_Amino Acid analysis_2010-20256
Analytical method for the determination of amino acids; European Pharmacopoeia 6.6/ 2.2.56 Amino Acid Analysis 01/2010:20256

* Refers to Dossier No. FAD-2010-0219

Refers to Dossier No. FAD-2009-0050 (i.e. CDG 25)

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was European Union Reference Laboratory for Feed Additives, IRMM, 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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

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