EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers and Reference Materials (Geel)
Food and Feed Compliance

European Union Reference Laboratory
Feed Additives

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

Subject: Second amendment of the EURL evaluation report

Reference: FAD-2010-0218 Botanically defined flavourings from Group 06 - Laurales, Magnoliales, Piperales amended by JRC.F.5/CvH/MGH/AS/Ares(2022)4639609

In the period between the publication of the original EURL evaluation report [1] and the current date, eight flavouring compounds *(cassia bark extract, cinnamon bark oleoresin, laurel leaves extract/oleoresin, boldo extract, boldo tincture, mace oil, nutmeg oleoresin* and *kawakawa tincture)* were withdrawn from the grouped application FAD-2010-0218 *Botanically defined flavourings from Group 06 - Laurales, Magnoliales, Piperales* [2].

Upon request of DG SANTE, the EURL evaluated the new methods of analysis provided by the Applicant for three *feed additives* from the group, namely: *ylang ylang oil, camphor white oil* and *cinnamon tincture* and recently issued a partial amendment of the original EURL report [3].

Following an additional request from EFSA [4], the EURL evaluated in the frame of this second amendment the new supplementary information provided by the Applicant related to the methods of analysis proposed for other six *feed additives* so-called: *nutmeg oil, laurel leaves oil, pepper oil black, cinnamon oil, cassia oil* and *pepper oleoresin black* which belong to the same grouped application.

Hereafter is the amended report on the evaluation of the new methods of analysis submitted by the Applicant and proposed for official control of the following *feed additives: nutmeg oil, laurel leaves oil, pepper oil black, cinnamon oil, cassia oil* and *pepper oleoresin black.* The updated recommendations of this amendment replace the ones stated for these six *feed additives* in the original report issued by the EURL [1].

For *nutmeg oil, laurel leaves oil, pepper oil black, cinnamon oil* and *cassia oil* the Applicant proposed the quantification of their respective phytochemical markers, by gas chromatography coupled with flame ionisation detection (GC-FID), based on different available ISO standard methods.

Furthermore, the Applicant provided the analytical procedure with the specific operating conditions for the GC and applied it to the mentioned *feed additives* for the quantification of

their respective phytochemical markers. According to the 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 obtained chromatograms.

Nutmeg oil

According to the Applicant *nutmeg oil* is an essential oil obtained by distillation of the nuts of the plant species "*Myristica fragrans* Houtt" with a content of *pin-2(3)-ene* (phytochemical marker) ranging from 15 to 28 % (expressed as the relative individual peak area in the chromatogram) [5].

For the quantification of *pin-2(3)-ene* in *nutmeg oil* the Applicant proposed a gas chromatography coupled with flame ionisation detection (GC-FID) method based on the standard ISO 3215:1998 for "Oil of nutmeg, Indonesian type (*Myristica fragrans* Houtt.)" [6].

Furthermore, the description of the product and the range of *pin-2(3)-ene* stated in the ISO 3215 standard correspond to the range of the phytochemical marker as declared by the Applicant in the proposed consolidated specifications [5].

In addition, the Applicant presented a typical chromatogram of *nutmeg oil* demonstrating a good separation of the marker [6]. Moreover, the Applicant analysed the phytochemical marker (*pin-2(3)-ene*) in 5 different batches of *nutmeg oil* leading to a content ranging from 20.8 to 22.7 % [5], which is in line with the range specified in the ISO 3215 standard [6].

Given the performance characteristics and data currently available, the EURL recommends for official control the GC-FID method based on the ISO 3215 standard for the quantification of *pin-2(3)-ene* (phytochemical marker) in *nutmeg oil*.

Laurel leaves oil

According to the Applicant *laurel leaves oil* is an essential oil obtained by steam distillation of the leaves of *Laurus nobilis* L. with a content of *1,8-cineole* (phytochemical marker) ranging from 41 to 58 % (expressed as the relative individual peak area in the chromatogram) [7].

For the quantification of *1,8-cineole* in *laurel leaves 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" [8].

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

	1,8-cineole	
	Batch 1	*Batch 2*
Content, % (relative area)	52.6	50.9
[a]RSD_r, %	0.1	0.3
[a]RSD_{ip}, %	0.4	0.3

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively. [a)]Recalculated by EURL [9]

The Applicant verified the above mentioned method for the analysis of the phytochemical marker (*1,8-cineole)* following the "EURL–FA Validation and verification technical guide for Sensory feed Additives – flavouring compounds 2(b) from botanical origin" [10]. Table 1 shows a summary of the relevant performance characteristics obtained in the verification study. The precision values (relative standard deviations for *repeatability* and *intermediate precision*), recalculated by the EURL [10] from the verification study, ranged from 0.1 to 0.4 % for the determination of *1,8-cineole* in *laurel leaves oil* [8].

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* (phytochemical marker) in laurel leaves oil.

Pepper oil black

According to the Applicant *pepper oil black* is an essential oil obtained by super critical extraction (super critical extract) or by steam distillation (steam distilled) of the whole or broken unripe fruits of "*Piper nigrum* L" with a content of *beta-caryophyllene* (phytochemical marker) ranging from 8 to 30 % (super critical extract) and from 12 to 40 % (steam distilled) and expressed as the relative individual peak area in the chromatogram [11].

For the quantification of *beta-caryophyllene* in *pepper oil black* the Applicant proposed a gas chromatography coupled with flame ionisation detection (GC-FID) method based on the standard ISO 3061:2004 for "Oil of black pepper (*Piper nigrum* L.)" [12].

Furthermore, the description of the product and the range of *beta-caryophyllene* stated in the ISO 3061 standard corresponds to the range of the phytochemical marker as declared by the Applicant in the proposed specifications (steam distilled) [11].

In addition, the Applicant presented typical chromatograms of *pepper oil black* (super critical extract and steam distilled) demonstrating a good separation of the marker [12]. Moreover, the Applicant analysed the phytochemical marker (*beta-caryophyllene*) in 5 different batches of *pepper oil black* (super critical extract and steam distilled) leading to contents ranging from

11.5 to 15.6 % for the super critical extract and from 25.4 to 28.2 % for the steam distilled [11] being the latter within the range as specified in the ISO 3061 standard [12].

Given the performance characteristics and data currently available, the EURL recommends for official control the GC-FID method based on the ISO 3061 standard for the quantification of *beta-caryophyllene* (phytochemical marker) in *pepper oil black*.

Cinnamon oil

According to the Applicant, *cinnamon oil* is an essential oil obtained by distillation from bark (*cinnamon bark oil)* or from the leaves (*cinnamon leaf oil)* of the plant species "*Cinnamomum zeylanicum* Bl., C. verum J.S. Presl" being *cinnamaldehyde* the phytochemical marker in both products. The *cinnamon bark oil* has a content of *cinnamaldehyde* ranging from 55 to 75 % while for the *cinnamon leaf oil* the content of the phytochemical marker is below 3 % (expressed as the relative individual peak area in the chromatogram) [13].

For the quantification of *cinnamaldehyde* in both *cinnamon oils* the Applicant proposed a gas chromatography coupled with flame ionisation detection (GC-FID) method based on the standard ISO 3524:2003 for "Oil of cinnamon leaf, Sri Lanka type (*Cinnamomum zeylanicum Blume*)" [14]. Similar GC-FID methods are also described in the European Pharmacopoeia for the *cinnamon bark oil* (Eur. Pharm. 04/2011:1501) and for the *cinnamon leaf oil* (Eur. Pharm. 04/2008:1608) [14].

Furthermore, the description of the product and the range of *cinnamaldehyde* stated in the ISO standard and/or in the respective European Pharmacopoeia's monographs correspond to the range of the phytochemical marker as declared by the Applicant in the proposed specifications [13].

In addition, the Applicant presented typical chromatograms of *cinnamon bark oil* and of *cinnamon leaf oil* demonstrating a good separation of the phytochemical marker [14].

Moreover, the Applicant analysed *cinnamaldehyde* in 5 different batches of *cinnamon bark oil* and of *cinnamon leaf oil.* These analyses led to *cinnamaldehyde* contents ranging from 71.3 to 72.2 % (for the *cinnamon bark oil*) and from 1.0 to 1.2 % (for the *cinnamon leaf oil)* [13]. The obtained values are within the ranges as specified in the Eur. Pharm. monograph 04/2011:1501 (for the *cinnamon leaf oil)*, and within the ones specified in the ISO 3524 standard and the Eur. Pharm. monograph 01/2008:1608 (for the *cinnamon bark oil*) [14].

Given the data currently available, the EURL recommends for official control the GC-FID methods based on the ISO 3524 standard and on the Eur. Pharm. monographs 01/2008:1608 and 04/2011:1501 for the quantification of *cinnamaldehyde* (phytochemical marker) in *cinnamon oil.*

Cassia oil

According to the Applicant *cassia oil* is an essential oil obtained by distillation from leaves, stalks and twigs of the plant species "*Cinnamomum aromaticum* Nees (synonym: *Cinnamomum cassia* Nees ex Blume)" with a content of *(E)-cinnamaldehyde* (phytochemical marker) ranging from 70 to 89 % (expressed as the relative individual peak area in the chromatogram) [15].

For the quantification of *(E)-cinnamaldehyde* in *cassia oil* the Applicant proposed a gas chromatography mass spectrometry (GC-MS) method according to the generic ISO 11024:1998 standard for "Essential oils: General guidance on chromatographic profiles" [16]. Furthermore, the Applicant presented typical chromatograms of *cassia oil* using this GC-MS method thus demonstrating a good separation of the marker [17].

The Applicant provided the specific operating conditions in the method procedure [16] where, 1 µl of the diluted oil (1:10) is injected into the GC using split ratio 100:1. 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 [16].

Moreover, in the frame of the characterisation of the *feed additive,* the Applicant provided data for the quantification of *(E)-cinnamaldehyde* in eight different batches of *cassia oil* by using the mentioned GC-MS method. The reported content of the phytochemical marker *(E)-cinnamaldehyde* was ranging from 80.3 to 89.0 % [15].

Furthermore, the Applicant also analysed the phytochemical marker *(E)-cinnamaldehyde* in *cassia oil* using a gas chromatography coupled with flame ionisation detection (GC-FID) method in line with the standard method ISO 3216:1997 for "Oil of cassia, Chinese type [*Cinnamomum aromaticum* Nees, syn. *Cinnamomum cassia* Nees ex Blume]" [18]. The mentioned GC-FID method led to a *(E)-cinnamaldehyde* contents in *cassia oil* ranging from 76.9 to 87.4 % [18] which are within the range specified in the ISO 3216 standard [21], thus proving its applicability for the quantification of the phytochemical marker *(E)-cinnamaldehyde* in *cassia oil* [15].

Therefore, based on the evidences provided by the Applicant the EURL recommends for official control the GC-MS and the GC-FID methods mentioned above for the quantification of *(E)-cinnamaldehyde* (phytochemical marker) in *cassia oil.*

Pepper oleoresin black

According to the Applicant *pepper oleoresin black* is an oleoresin obtained by solvent extraction of dried unripe fruits of "*Piper nigrum* L" with a content of *piperine* (phytochemical marker) ranging from 20 to 50 % [19].

Table 2. Performance characteristics of the GC-FID method for the quantification of the phytochemical marker (*piperine*) in the *feed additive* (*pepper oleoresin black*) [20].

	piperine	
	Batch 1	*Batch 2*
Content, % (relative area)	38.4	38.6
[a]RSD_r, %	3.0	2.5
[a]RSD_{ip}, %	3.3	2.5

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively. [a)]Recalculated by EURL [21]

For the quantification of the *piperine* in *pepper oleoresin black* the Applicant proposed a high performance liquid chromatography coupled to photometric detection (HPLC-UV) method based on the ISO 11027:1993 standard for "Pepper and pepper oleoresins – Determination of piperine content – Method using high performance liquid chromatography" [20].

According to the procedure provided by the Applicant, the *pepper oleoresin black* (0.2 g) is mixed with methanol and stirred for 5 min. After centrifugation, an aliquot of the supernatant is further diluted with methanol and injected directly into the HPLC system. The phytochemical marker (*piperine*) is then separated by reversed-phase HPLC using a gradient elution. *Piperine* is quantified at 280 nm using an external standard curve [20].

The Applicant verified the method proposed for the analysis of the phytochemical marker (*piperine)* following the "EURL–FA Validation and verification technical guide for Sensory feed Additives – flavouring compounds 2(b) from botanical origin" [10]. Table 2 shows a summary of the relevant performance characteristics obtained from the verification study. The precision values, recalculated by the EURL [24] from the verification study, ranged from 2.5 to 3.3 % for the determination of *piperine* in *pepper oleoresin black* [20].

Based on the experimental evidences provided the EURL recommends for official control the HPLC-UV method based on the generic ISO 11027 standard for the quantification of *piperine* (phytochemical marker) in *pepper oleoresin black*.

Recommended text for the registry entry (analytical method)

For the determination of *pin-2(3)-ene* (phytochemical marker) in the *feed additive* (*nutmeg oil*); *1,8-cineole* (phytochemical marker) in the *feed additive* (*laurel leaves oil*); *beta-caryophyllene* (phytochemical marker) in the *feed additive* (*pepper oil black*) and *cinnamaldehyde* (phytochemical marker) in the *feed additive* (*cinnamon oil*):

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

For the determination of *(E)-cinnamaldehyde* (phytochemical marker) in the *feed additive* (*cassia oil*):

- gas chromatography coupled with flame ionisation detection (GC-FID) or with mass spectrometry detection (GC-MS)

For the determination of *piperine* (phytochemical marker) in the *feed additive* (*pepper oleoresin black)*:

- high performance liquid chromatography coupled to photometric detection (HPLC-UV)

References

[1] EURL Report FAD-2010-0218 Botanically defined flavourings from Group 06 - Laurales, Magnoliales, Piperales – JRC.D.5/SFB/CvH/RFO/mds/Ares(2013)2815290

[2] Annex_of_FFAC_WithdrawalsTransfers_Signed_2018-10-08 - Ares(2019)1299322

[3] Amendment to the EURL Report - JRC.F.5/CvH/MGH/AS/Ares(2022)4639609

[4] Request for amendment of the Evaluation Report on the Analytical Methods for the preparations included in the dossier FAD-2010-0218- Ares(2022)5137138

[5] Supplementary Information - 2020-06-24 SIn_reply_nutmeg_oil.pdf

[6] Supplementary Information - 2020_06_24_EURL_appendix_nutmeg_oil.pdf

[7] Supplementary Information - 2020-05-25-Laurel_leaves_oil_SIn_reply.pdf

[8] Supplementary Information - 2020_05_25_EURL_appendix_laurel_leaves_oil.pdf

[9] Supplementary information : «eurl-anova_laurel_leaves_oil.pdf»

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

[11] Supplementary Information - 2021-05-20-SIn_reply_pepper_oil_black.pdf

[12] Supplementary Information - 2021_05_20_EURL_appendix_pepper_oil_black.pdf

[13] Supplementary Information - 2020-10-01-SIn_reply_cinnamon_oil.pdf

[14] Supplementary Information - 2020-10-01_EURL_appendix_cinnamon_oil.pdf

[15] Supplementary Information - BDG-06-SIn-reply-Cassia oil.pdf

[16] Supplementary Information - Annex_I_SIn_reply_cassia_oil_GC_MS_method.pdf

[17] Supplementary Information _ Annex_II_SIn_reply_cassia_oil_batch_COA-chromatograms.pdf

[18] Supplementary Information - ISO 3216:1997 “Oil of cassia, Chinese type [Cinnamomum aromaticum Nees, syn. Cinnamomum cassia Nees ex Blume]”

[19] Supplementary Information - 2022-01-06_SIn_reply_pepper_oleoresin_black.pdf

[20] Supplementary Information - 2022-01-06_EURL_appendix_pepper_oleoresin_black.pdf

[21] Supplementary information : «eurl-anova_pepper_oleorisin_black.pdf»

Acknowledgements

The following National Reference Laboratories contributed to this report:

- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL) (PiWet National Veterinary Research Institute (NRL NVRI)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Wageningen Food Safety Research (WFSR)[1]
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)

- Prepared by María José González de la Huebra
- Reviewed and approved by Zigmas Ezerskis and Christoph von Holst (EURL-FA), respectively, Geel, 30/08/2022

[1] COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: RIKILT Wageningen UR, Wageningen.

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers and Reference Materials (Geel)
Food and Feed Compliance

European Union Reference Laboratory
Feed Additives

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

Subject: Amendment of the EURL evaluation report

Reference: FAD-2010-0218 Botanically defined flavourings from Group 06 - Laurales, Magnoliales, Piperales – JRC.D.5/SFB/CvH/RFO/mds/Ares(2013)2815290

Upon the request from DG SANTE, the EURL evaluated the new supplementary information provided by the Applicant and related to the methods of analysis proposed for three *feed additives* so-called: *ylang ylang oil, camphor white oil* and *cinnamon tincture*. These three *feed additives* belong to the group application FAD-2010-0218 *Botanically defined flavourings from Group 06 - Laurales, Magnoliales, Piperales*.

In the original report [1] the EURL evaluated and recommended analytical methods for eighteen flavouring compounds *(cassia oil, cassia bark extract, camphor oil, cinnamom oil, cinnamom bark oleoresin, cinnamom tincture, laurel leaves oil, laurel leaves extract/oleoresin, litsea berry oil, ylang-ylang oil, mace oil, nutmeg oil, nutmeg oleoresin, pepper oil, pepper oleoresin, kawakawa tincture, boldo extract and boldo tincture)* derived from different chemo-taxonomically related plants and belonging to the group "Laurales, Magnoliales, Piperales.

Following the priorities expressed by DG SANTE, 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 three out of the eighteen *feed additives* listed above namely *ylang ylang oil, camphor white oil* and *cinnamon tincture*.

Hereafter is the updated report on the evaluation of the new methods of analysis submitted by the Applicant and proposed for official control of *ylang ylang oil, camphor white oil* and *cinnamon tincture* and the revised version of the recommendations, that replace the ones stated for these three *feed additives* in the original report issued by the EURL [1].

Ylang ylang oil

According to the Applicant *ylang ylang oil* is an essential oil obtained by steam distillation of the flowers from *Cananga odorata* Hook. f. et Thomson forma *genuina* with a content of *beta-caryophyllene* (phytochemical marker) ranging from 4 to 17 % (expressed as the relative individual peak area in the chromatogram) [2].

For the quantification of the phytochemical marker *beta-caryophyllene* in *ylang ylang oil* the Applicant proposed a method based on gas chromatography coupled with flame ionisation detection (GC-FID) [3]. The method is based on the standard ISO 3063:2004 for "Oil of ylang-ylang [Cananga odorata (Lam.) Hook. f. et Thomson forma genuina]" [3].

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 [3]. Furthermore, the description of the product and the range of *beta-caryophyllene* stated in the above mentioned ISO 3063:2004 standard correspond to the range of the phytochemical marker as declared by the Applicant in the proposed consolidated specifications [2].

In addition, the Applicant presented a typical chromatogram of *ylang ylang oil* demonstrating a good separation of the marker [3]. Moreover, the Applicant analysed the phytochemical marker (*beta-caryophyllene*) in five different batches of *ylang ylang oil* leading to a content ranging from 7.2 to 7.8 % [2], which is within the range as specified in the above mentioned ISO 3063:2004 standard [3].

Given the performance characteristics and data currently available, the EURL recommends for official control the GC-FID method based on the ISO 3063 standard for the quantification of *beta-caryophyllene* (phytochemical marker) in *ylang ylang oil*.

Camphor white oil

According to the Applicant *camphor white oil* is an essential oil obtained by steam distillation of the whole plant of *Cinnamomum camph*ora L. with a content of *1,8-cineole* (phytochemical marker) ranging from 27 to 43 % (expressed as the relative individual peak area in the chromatogram) [4].

For the quantification of the phytochemical marker *1,8-cineole* in *camphor white oil* the Applicant proposed a method based on gas chromatography coupled with flame ionisation detection (GC-FID) [5]. The proposed method is based on a generic ISO 11024:1998 standard for "Essential oils: General guidance on chromatographic profiles" [5].

Table 1. Performance characteristics of the GC-FID method for the quantification of the phytochemical marker (*1,8-cineole*) in the *feed additive* (*camphor white oil*) [5].

	1,8-cineole	
	Batch 1	*Batch 2*
Content, % (relative area)	39.6	39.5
[a]RSD_r, %	0.04	0.16
[a]RSD_{ip}, %	0.57	0.27

RSD_r and RSD_{ip}: relative standard deviations for *repeatability* and *intermediate precision*, respectively.
[a)]Recalculated by EURL [6]

According to the procedure, 1 µl of the *camphor white oil* is injected directly into the GC at a split ratio of 1:100. 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. Specific operating conditions for the GC are provided by the Applicant [5]. The Applicant verified the above mentioned method for the analysis of the phytochemical marker (*1,8-cineole)* following the "EURL–FA Validation and verification technical guide for Sensory feed Additives – flavouring compounds 2(b) from botanical origin" [7]. Table 1 shows a summary of the relevant performance characteristics obtained in the verification study. The precision values, recalculated by the EURL [6] from the verification carried out by the Applicant, ranged from 0.04 to 0.57 % for the determination of *1,8-cineole* in *camphor white oil* [5].

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* (phytochemical marker) in *camphor white oil.*

Cinnamon tincture

According to the Applicant, *cinnamon tincture* is a hydro alcoholic extract obtained by maceration of the bark of *Cinnamomum verum* J.Presl*. (syn. C. zeylanicum)* containing total polyphenols, total flavonoids and cinnamaldehyde as major constituents [8].

Contrary to the other two *feed additives* subject of this amendment, for *cinnamon tincture* the Applicant did not provide a method for the determination of a phytochemical marker. Instead, the Applicant proposed to characterise the *feed additive* (*cinnamon tincture*) by the determination of dry matter, ash content, total polyphenols, total flavonoids and cinnamaldehyde [9].

For the determination of dry matter and ash content in the *feed additive* the Applicant proposed the use of gravimetric methods [9].

For the determination of total polyphenols and total flavonoids the Applicant proposed spectrophotometry methods based on the relevant European Pharmacopoeia monographs [10-11].

For the determination of cinnamaldehyde the Applicant proposed a high performance thin layer chromatography (HPTLC) method [12]. This method is based on the one described in the European Pharmacopoeia monograph for Cinnamon [11].

The Applicant has provided the result of the analysis of five different batches of the *feed additive* (*cinnamon tincture*) characterised by applying the methods mentioned above. These analyses led to average values of 0.92 % for the dry matter; 11.3 % for the ash content; 0.34 % of total polyphenols; 0.0008 % of total flavonoids and 0.0008 % of cinnamaldehyde [8, 13-14].

However, according to the Applicant the use of the HPTLC profile of the hexanic extract as a fingerprint of the *feed additive* is considered a more reliable way to ensure the absence of adulteration and thus preferred than the analysis of individual phytomarkers at an established range [8].

For the characterisation of the *feed additive* the EURL recommends for official control the above mentioned methods based on gravimetry, spectrophotometry and high performance thin layer chromatography (HPTLC).

Furthermore, the Applicant did not provide experimental data or an analytical method for the determination of *ylang ylang oil*, *camphor white oil* and *cinnamon tincture* in premixtures and feedingstuffs, as the unambiguous determination of these *feed additives* added to the mentioned matrices is not achievable experimentally.

Recommended text for the registry entry (analytical method)

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

- gas chromatography coupled with flame ionisation detection (GC-FID) (based on ISO 3063)

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

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

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

- gravimetry for the determination of dry matter and ash content;
- spectrophotometry for the determination of *total polyphenols* and *total flavonoids* content;
- high performance thin-layer chromatography (HPTLC) for the determination of *cinnamaldehyde* content.

References

[1] EURL Report FAD-2010-0218 Botanically defined flavourings from Group 06 - Laurales, Magnoliales, Piperales – JRC.D.5/SFB/CvH/RFO/mds/Ares(2013)2815290

[2] Supplementary Information – 2021-01-07_SIn_reply_ylang_ylang_oil.pdf

[3] Supplementary Information – 2021-01-07_EURL_appendix_ylang-ylang_oil.pdf

[4] Supplementary Information – 2021-04-08_SIn_reply_camphor_oil_white.pdf

[5] Supplementary Information – 2021-04-08-EURL_appendix_SIn_reply_camphor_oil_white.pdf

[6] Supplementary information : «eurl-anova_camphor_white_oil.pdf»

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

[8] Supplementary Information FAD-2010-0218_Cinnamon tincture_SIn_Feb20- Section II_Identity - Section II_Identity.pdf

[9] Supplementary Information FAD-2010-0218_Cinnamon tincture_SIn_Feb20- Section II_Identity -Annex_II_2_Methods of analysis

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

[11] European Pharmacopoiea monograph 04/2011:0387 Cinnamon (cinnamomi cortex)

[12] Supplementary Information FAD-2010-0218_Cinnamon tincture_SIn_Feb20-Annex_II_12_HPTLC Association method for fingerprint.pdf

[13] Supplementary Information FAD-2010-0218_Cinnamon tincture_SIn_Feb20- Section II_Identity - Annex_II_3_Results of analysis.pdf

[14] Supplementary Information FAD-2010-0218_Cinnamon tincture_SIn_Feb20-Annex_II_11_Detailed report of cinnamaldehyde quantification.pdf

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- Prepared by María José González de la Huebra
- Reviewed and approved by Zigmas Ezerskis and Christoph von Holst (EURL-FA), respectively, Geel, 23/06/2022

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Institute for Reference Materials and Measurements (Geel)
Standards for Food Bioscience
European Union Reference Laboratory for Feed Additive - Authorisation

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

EURL 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 06
FAD-2010-0218 - CRL/100188

European Union Reference Laboratory
Feed Additives

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

Dossier related to:	**FAD-2010-0218** **CRL/100188**
Feed additive:	**Botanically defined flavourings from Group 06 - Laurales, Magnoliales, Piperales**
Active Substance(s):	**Eighteen compounds from botanically defined flavourings Group 06**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
Report prepared by:	**Rebeca Fernandez Orozco and Roberto Molteni (EURL-FA)**
Report revised by: Date:	**PR (EURL-FA)** **27/06/2013**
Report approved by: Date:	**Christoph von Holst (EURL-FA)** **28/06/2013**

EXECUTIVE SUMMARY

The *Botanically Defined Flavourings – Group 6* BDG 06 (*Laurales, Magnoiales, Piperales*) is an application comprising *eighteen flavouring compounds* (*) for which authorisation as *feed additive* 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 volatile phytochemical markers in the *feed additive*, 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 06* of interest. In order to demonstrate the transferability of the proposed analytical method (relevant for the method verification), the Applicant 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 06* 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 06* in the *mixture of flavouring compounds.*

For the qualitative identification of non-volatile phytochemical markers (*boldine*, *kavain* and *piperine*) in *mixture of flavouring compounds*, the Applicant submitted High-Performance Liquid Chromatography methods with UV detection (HPLC-UV), together with the ISO 11027 standard method for the determination of piperine.

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) *flavouring compounds* of interest (*) the GC-MS-RTL and HPLC-UV methods submitted by the Applicant.

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 *active substance(s)* of interest (*) 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 06, mixture of flavouring products, sensory additives, all animal species and categories

1. BACKGROUND

In the current application 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 the *Botanically Defined flavourings - Group 06* (*BDG 06*), a grouped application for which authorisation as *feed additive* 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 [1]. Authorisation is sought for the use of the *feed additive* for all species and categories [2].

The *BDG 06* application includes *eighteen flavouring compounds* (listed in Table 1) derived from different chemo-taxonomically related plants and belonging to the group "*Laurales, Magnoliales, Piperales*". According to European Pharmacopeia [3] these *feed additives* include oils, distillates, oleoresins, solvent based and water based extracts, concentrates, tinctures, absolutes and other preparation types.

The *flavouring compounds* of interest are intended to be incorporated only into *feedingstuffs* or drinking *water* [4]. The Applicant suggested no minimum or maximum levels for the different flavouring compounds [2], but normal contents of *flavouring compounds* in *feedingstuffs* range 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 06*, and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

Identification /Characterisation of the feed additive

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, mycotoxins, dioxins and PAHs) 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

The Applicant identified for each of the *flavouring compounds* of interest one or several phytochemical markers and the respective characteristic concentration ranges [4] (listed in Table 1).

For the identification of seven volatile phytochemical markers (cf. Table 2) 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 maintains 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 2) [8]. These libraries were provided to the EURL.

Table 1. The phytochemical markers and the analytical methods [4] used for the determination of the *flavouring compounds* (BDG06)

Botanical origin	*Flavouring compound*	CAS-no	phytochemical marker	& Ranger (%)	Method
Cinnamomum aromaticum	Cassia oil	8007-80-5	Cinnamaldehyde	60 – 90	GC-MS-RTL
			Eugenol	70 - 80	
	Cassia bark extract	84961-46-6	Cinnamaldehyde	3 - 8	
Cinnamomum camphora	Camphor oil	8008-51-3	1,8-Cineole	15 – 40	
Cinnamomum zeylanicum, Cinnamomum verum	Cinnamom oil	8015-91-6	Cinnamaldehyde	0.1 - 80	
	Cinnamom bark oleoresin	84961-46-6	Cinnamaldehyde	65 – 75	
	Cinnamom tincture	n.a.	Cinnamaldehyde	55 - 75	
Laurus nobilis	Laurel leaves oil	8002-41-3	1,8-Cineole	35 - 55	
	Laurel leaves extract/oleoresin	84603-73-6	1,8-Cineole	50 – 65	
Litsea cubeba	Litsea berry oil	68855-99-2	Neral	25 - 35	
Cananga odorata	Ylang-ylang oil	8006-81-3	β-Caryophyllene	30 - 40	
Myristica fragrans	Mace oil	8007-12-3	Myristicin	5 – 15	
			Pin-2(3)-ene	15 - 30	
	Nutmeg oil	8008-45-5	Myristicin	5 – 15	
			Pin-2(3)-ene	15 - 45	
	Nutmeg oleoresin	84082-68-8	Myristicin	5 – 15	
			Pin-2(3)-ene	15 – 30	
Piper nigrum	Pepper oil	8006-82-4	β-Caryophyllene	5 - 30	
	Pepper oleoresin	84929-41-9	β-Caryophyllene	5 - 30	
			Piperine	15 – 55	HPLC-3
Piper methysticum	Kawakawa tincture	n.a.	Kavain	0.05 - 0.15	HPLC-2
Peumus boldus	Boldo extract	n.a.	Boldine	0.1 - 0.2	HPLC-1
	Boldo tincture	n.a.	Boldine	0.0005 - 0.005	

GC-MS-RTL: Gas Chromatography-Mass Spectrometry-Retention Time Locked; **HPLC**: High Performance Liquid Chromatography; **n.a.** not available.
Neral: 3,7-Dimethylocta-2,6-dienal; **Boldine**: 1,10-Dimethoxy-2,9-dihydroxyaporphine; **Kavain**: 5,6-Dihydro-4-methoxy-6-(2-phenylethenyl)-2H-Pyran-2-one

At first a GC-MS system suitability check is performed using an equal-weight mixture of Linalool, Acetophenone, Benzyl Acetate, Benzyl Alcohol and 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 [9].

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.

For the analysis of solid flavouring premixtures, the extraction is carried out using either the Soxhlet Extraction system or the Accelerated Solvent Extractor Dionex ASE 200 [6]. The extract is evaporated at vacuum to 50 mL. The solution is filtered on a 0.45 μm nylon filter and injected in the GC-MS [6] at constant "nominal" inlet pressure. Liquid samples of volatile phytochemical markers are diluted (1:1) with acetone and injected in the GC-MS [6] at constant "nominal" inlet pressure. The Applicant provided the typical chromatogram for the *BDG 06* of interest (cf. Fig II.2-4 [4]).

In order to demonstrate the transferability of the proposed analytical method (relevant for the method verification), the Applicant 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 06* 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 06* in the *mixture of flavouring compounds.*

Based on the satisfactory experimental evidence provided, the EURL recommends for official control the GC-MS-RTL (Agilent specific) method submitted by the Applicant for the qualitative identification in the *feed additive* of the individual (or mixture of) volatile phytochemical markers (cf. Table 2), related to the *flavouring compounds* of interest listed in Table 1.

Table 2. GC-MS-RTL for volatile phytochemical markers of *BDG 06* [4] and d-limonene

FL-no	CAS-no	EU Register name	RTL polar (min)	RTL non-polar (min)
03.001	470-82-6	1,8-Cineole	7.00	8.19
01.007	87-44-5	beta-Caryophyllene	17.64	20.40
05.014	104-55-2	Cinnamaldehyde	29.10	14.50
04.003	97-53-0	Eugenol	31.80	17.90
n.a.	607-91-0	Myristicin	34.28	22.70
05.170	106-26-3	Neral	19.70	14.12
01.004	80-56-8	Pin-2(3)-ene	3.82	6.00
01.045	5989-27-5	d-Limonene (standard)	6.70	8.33

FL-no: EU Flavour Number;
GC-MS-RTL: Gas Chromatography-Mass Spectrometry-Retention Time Locked;
n.a.: not available.

For the identification of the non-volatile phytochemical markers (*boldine* and *kavain*), the Applicant submitted the Reversed Phase High-Performance Liquid Chromatography method with UV detection (RP-HPLC-UV) at 245 and 220 nm for *boldine* [11] and *kavain* [12], respectively. Extracts are analysed by HPLC. A gradient elution with three solvents (acetonitrile:water; acetonitrile; water) is used for the identification of *boldine* [11]. While an isocratic elution with a mixture of 0.1% phosphoric acid, isopropyl alcohol and acetonitrile is used for the identification of *kavain*, [12].

For the qualitative identification of the *peper oleoresin* flavouring compound (Table 1), the Applicant identified two phytomarkers and submitted two identification methods: - the RTL-GC-MS method mentioned above for the identification of *β-caryophyllene,* and the the ISO 11027 standard method [13] for the determination of *piperine*. The oleoresin samples are diluted in ethanol and piperine is determined by HPLC-UV at 343 nm using external calibration.

In order to demonstrate the transferability of the proposed HPLC methods, the Applicant tested for each of the non-volatile flavouring compounds of interest two model: - a liquid premixture (containing from 5 to 20 % of flavouring compound and 80% ethanol or water); - a solid premixture (containing 20% of flavouring compound, 20% of silicic acid and 60% of calcium carbonate as carriers), and subsequently analysed using the HPLC method investigated. All substances were properly identified and determined qualitatively [11,14,15,16]. The Applicant concluded that the proposed analytical methods are suitable to determine qualitatively the presence of the non-volatile phytochemical markers from *BDG 06* in the *mixture of flavouring compounds.*

Based on the satisfactory experimental evidence provided, the EURL recommends for official control the three HPLC methods submitted by the Applicant for the qualitative identification in the *feed additive* of the phytochemical markers (boldine, kavain and piperine) related to the individual (or mixture of) non-volatile *flavouring compounds* of interest (listed in Table 1).

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 phytochemical markers (cf. Table 2,3), which are used to trace the *feed additive* 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

In the frame of this authorisation the EURL recommends for official control:

- the Agilent specific method submitted by the Applicant, based on gas-chromatography mass-spectrometry coupled to Retention Time Locking (RTL-GC-MS for the qualitative identification in the *feed additive* of seven volatile phytochemical markers of the individual (or mixture of) *flavouring compounds*;
- Reversed Phase High-Performance Liquid Chromatography with UV detection (RP-HPLC-UV) for the qualitative identification in the *feed additive* of the non-volatile phytochemical markers (*boldine* and *kavain*) of the individual (or mixture of) *flavouring compounds*;
- HPLC-UV method described in the ISO 11027 standard for the identification in the *feed additive* of the non-volatile phytochemical marker (*piperine*) of the *flavouring compound (pepper oleoresin).*

The Applicant did not provide any experimental method or data for the identification of phytochemical markers in *feedingstuffs* and *water*. Therefore the EURL cannot evaluate nor recommend any method for official control to identify phytochemical markers in *feedingstuffs* or *water*.

Recommended text for the register entry (analytical method)

For the identification of seven volatile phytochemical markers in mixture of *flavouring compounds*:

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

For the determination of the phytochemical markers *boldine* and *kavain* in mixture of *flavouring compounds*:

High-Performance Liquid Chromatograph with UV detection for *boldine* and *kavain* (HPLC-UV)

For the determination of the phytochemical marker *piperine* in mixture of *flavouring compounds*:

High-Performance Liquid Chromatograph with UV detection (HPLC-UV) - ISO 11027

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 06* (*BDG 06*) 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. 1831/00114-2010

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

[3] *Technical dossier, Section II – Annex-II-5

[4] *Technical dossier, Section II: Identity, characterisation and conditions of use of the additive; Methods of analysis

[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] *Technical dossier, Section II – Annex-II-06 Methods assay

[10] *Supplementary Information - SIN-2011 - GCMS_CDG_B06\ Annex_V_GCMS_Premixture.pdf

[11] *Supplementary Information – SIN2011 – HPLC_B06-1 – Annex II HPLC B06-1
[12] *Supplementary Information – SIN2011 – HPLC_B06-2 - – Annex_II_HPLC_B06-2
[13] *Supplementary Information – SIN2012 – Annex_I_ISO Method 11027
[14] *Supplementary Information – SIN2011 – HPLC_B06-1 - Annex_IV_HPLC_B06-1
[15] *Supplementary Information – SIN2011 – HPLC_B06-2 - – Annex_III_HPLC_B06-2
[16] *Supplementary Information – SIN2012 – Annex_II_HPLC_B06-4

* Refers to Dossier No. FAD-2010-0218

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

The following National Reference Laboratories contributed to this report:

- Fødevarestyrelsen, Ringsted (DK)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen, Jena (DE)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, Labore Landwirtschaft, Leipzig (DE)