



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE
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



**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: **PR (EURL-FA)**
Date: **27/06/2013**

Report approved by: **Christoph von Holst (EURL-FA)**
Date: **28/06/2013**

EXECUTIVE SUMMARY

The *Botanically Defined Flavourings – Group 6* BDG 06 (*Laurales, Magnoliales, 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
<i>Cinnamomum aromaticum</i>	Cassia oil	8007-80-5	Cinnamaldehyde	60 – 90	GC-MS-RTL
			Eugenol	70 - 80	
	Cassia bark extract	84961-46-6	Cinnamaldehyde	3 - 8	
<i>Cinnamomum camphora</i>	Camphor oil	8008-51-3	1,8-Cineole	15 – 40	
<i>Cinnamomum zeylanicum</i> , <i>Cinnamomum verum</i>	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	
<i>Laurus nobilis</i>	Laurel leaves oil	8002-41-3	1,8-Cineole	35 - 55	
	Laurel leaves extract/oleoresin	84603-73-6	1,8-Cineole	50 – 65	
<i>Litsea cubeba</i>	Litsea berry oil	68855-99-2	Neral	25 - 35	
<i>Cananga odorata</i>	Ylang-ylang oil	8006-81-3	β -Caryophyllene	30 - 40	
<i>Myristica fragrans</i>	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	
<i>Piper nigrum</i>	Pepper oil	8006-82-4	β -Caryophyllene	5 - 30	
	Pepper oleoresin	84929-41-9	β -Caryophyllene	5 - 30	
			Piperine	15 – 55	HPLC-3
<i>Piper methysticum</i>	Kawakawa tincture	n.a.	Kavain	0.05 - 0.15	HPLC-2
<i>Peumus boldus</i>	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; $\pm 10\%$; $\pm 20\%$) 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)