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JOINT RESEARCH CENTRE
Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives

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CRL 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-2010-0028 CRL/100026
Product Name:	Chemically defined flavourings from Chemical Group 23 – benzyl- alcohols, aldehydes, acids, esters and acetals
Active Substance(s):	Thirty six chemically defined flavourings from Chemical Group 23
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA)
Report prepared by:	Piotr Robouch (CRL-FA)
Report revised by: Date:	Roberto Molteni (CRL-FA) 22/09/2010
Report approved by: Date:	Christoph von Holst 30/09/2010

EXECUTIVE SUMMARY

The *Chemically Defined Flavourings - Group 23* (*CDG23 - benzyl- alcohols, aldehydes, acids, esters and acetals)*, in this application comprises 36 substances, for which authorisation as feed additives is sought under the category "sensory additives", functional group 2(b) "flavouring compounds", according to the classification system of Annex I of Regulation (EC) No 1831/2003.

In the current application submitted according to Article 4(1) and Article 10 (2) of Regulation (EC) No 1831/2003, the authorisation for all species and categories is requested. The flavouring compounds of interest have a purity ranging from 95% to 99%.

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 in *feedingstuffs*.

For the identification of volatile chemically defined flavouring compounds *CDG23* 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 maintained two FLAVOR2 databases/libraries (for retention times and for MS spectra) containing data for more than 409 flavouring compounds. These libraries were provided to the CRL. The Applicant provided the typical chromatogram for the *CDG23* of interest.

In order to demonstrate the transferability of the proposed analytical method (relevant for the method verification), the Applicant prepared a model mixture of flavouring compounds on a solid carrier to be identified by two independent expert laboratories. This mixture contained twenty chemically defined flavourings belonging to twenty different chemical groups to represent the whole spectrum of compounds in use as feed flavourings with respect to their volatility and polarity. Both laboratories properly identified all the flavouring compounds in all the formulations. Since the substances of *CDG23* 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 substances from *CDG23* in the *mixture of flavouring compounds.*

Based on the satisfactory experimental evidence provided, the CRL recommends for official control for the qualitative identification in the *feed additive* of the individual (or mixture of)

flavouring compounds of interest listed in Table 1 (*) the GC-MS-RTL (Agilent specific) method 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 CRL is unable to recommend a method for the official control to identify the *active substance(s)* of interest listed in 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 CRL evaluation report, available from the CRL website.

KEYWORDS
Chemically Defined Flavourings - Group 23, mixture of flavouring compounds, sensory additives, all species.

1. BACKGROUND

The *Chemically Defined Flavourings - Group 23* (*CDG23*) is a grouped application for which authorisation as feed additive 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. The *CDG23* application contains 36 flavouring compounds (listed in Table 1) belonging to the group - described in Annex I of Commission Regulation (EC) No 1565/2000 [2] as - " *benzyl- alcohols, aldehydes, acids, esters and acetals* ".

In the current application submitted according to Article 4(1) (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 flavouring compounds of interest are produced by different routes of manufacturing, providing a purity ranging from 95% to 99% [3]. *Mixtures of flavouring* compounds are usual prepared as liquid (diluted in an appropriate solvent, such as propane-1,2-diol) or solid (with an inorganic carrier, such as silicic acid + calcium carbonate) formulations.

Mixtures of flavouring compounds 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 [3], but normal contents of single 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 Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL 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 *Chemically Defined Flavourings – Group 23,* 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 Community 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 volatile chemically defined flavouring compounds *CDG23* (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 two FLAVOR2 database/libraries (for the retention times and for MS spectra) containing data for more than 409 flavouring compounds (including those listed in Table 1) [8]. These libraries were provided to the CRL.

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 [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 FLAVOR2 screener database. Further confirmation is performed using the FLAVOR2 mass spectral library [8].

Two sample preparation protocols are described. Solid samples of *mixture of flavouring compounds* are extracted with the Soxhlet or with the Accelerated Solvent Extractor (80%/20% hexane/acetone mixture). 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 *mixture of flavouring compounds* are diluted (1:1) with acetone and injected in the GC-MS [6] at constant "nominal" inlet pressure. All compound (except *gallic acid*) were analysed applying the analytical method described above. *Gallic acid* was analysed separately after derivatisation as the methyl chloroformate (MCF) derivative. The Applicant provided the typical chromatogram for the *CDG23* of interest (cf. Fig II.2-10 [4]).

In order to demonstrate the transferability of the proposed analytical method (relevant for the method verification), the Applicant prepared a model mixture of flavouring compounds on a solid carrier (containing silicic acid and calcium carbonate) to be identified by two independent expert laboratories. This mixture contained twenty chemically defined flavourings belonging to twenty different chemical groups to represent the whole spectrum of compounds in use as feed flavourings with respect to their volatility and polarity. Both laboratories properly identified all the flavouring compounds in all the formulations [10, 11]. Since the substances of *CDG23* 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 substances from *CDG23* in the *mixture of flavouring compounds.*

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

Table 1. Retention Time Locked for the flavouring compounds of *CDG23* [4], and d-limonene

FL-no	CAS-no	EU Register name	RTL polar (min)	RTL non-polar (min)
02.010	100-51-6	Benzyl alcohol	24.80	8.23
02.039	536-60-7	4-Isopropylbenzyl alcohol	30.54	15.92
02.128	105-13-5	p-Anisyl alcohol	34.40	15.45
05.013	100-52-7	Benzaldehyde	15.40	6.16
05.015	123-11-5	4-Methoxybenzaldehyde	29.20	14.40
05.016	120-57-0	Piperonal	33.70	16.90
05.017	120-14-9	Veratraldehyde	37.10	21.05
05.018	121-33-5	Vanillin	40.40	18.89
05.022	122-03-2	4-Isopropylbenzaldehyde	22.42	14.12
05.029	104-87-0	p-Tolualdehyde	18.86	9.24
05.055	90-02-8	Salicylaldehyde	19.98	8.09
05.129	135-02-4	2-Methoxy-benzaldehyde	27.45	14.08
08.021	65-85-0	Benzoic acid	50.80	12.98
08.080	149-91-7	Gallic acid (#)	no elution	31.70
09.014	140-11-4	Benzyl acetate	20.90	11.65
09.019	104-21-2	p-Anisyl acetate	31.55	19.54
09.051	103-37-7	Benzyl butyrate	24.75	17.45
09.077	104-57-4	Benzyl formate	19.79	9.07
09.132	122-63-4	Benzyl propionate	22.81	14.64
09.316	6938-45-0	Benzyl hexanoate	29.67	23.52
09.426	103-28-6	Benzyl isobutyrate	22.40	16.05
09.458	103-38-8	Benzyl isovalerate	25.17	19.07
09.581	6259-76-3	Hexyl salicylate	32.90	27.30
09.705	102-16-9	Benzyl phenyl-acetate	42.83	30.22
09.725	93-58-3	Methyl benzoate	18.00	9.67
09.726	93-89-0	Ethyl benzoate	19.37	12.02
09.727	120-51-4	Benzyl benzoate	41.60	29.20
09.738	103-41-3	Benzyl cinnamate	57.00	37.00
09.748	118-61-6	Ethyl salicylate	23.28	15.19
09.749	119-36-8	Methyl salicylate	22.50	12.79
09.750	87-19-4	Isobutyl salicylate	26.04	19.93
09.751	87-20-7	Isopentyl salicylate	28.90	23.30
09.752	118-58-1	Benzyl salicylate	44.65	31.90
09.755	94-46-2	Isopentyl benzoate	25.96	20.45
09.757	120-50-3	Isobutyl benzoate	22.83	17.11
09.762	2050-08-0	Pentyl salicylate	30.45	24.45
01.045	5989-27-5	d-Limonene (standard)	6.70	8.33

FL-no: EU Flavour Number; RTL: Retention Time Locked
(#) methyl chloroformate derivative of gallic acid elution peak

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 CRL is unable to recommend a method for the official control to identify the *active substance(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 CRL recommends for official control the Agilent specific method submitted by the Applicant, for the identification of the 36 *flavouring compounds* of the *CDG23* in the *feed additive* of the individual (or mixture of) *flavouring compounds* of interest.

The Applicant provided no experimental data for *feedingstuffs* and *water*, therefore the CRL is unable to recommend a method for the identification of the 36 *flavouring compounds* of the *CDG23* in *feedingstuffs* and *water*.

Recommended text for the register entry (analytical method)

For the identification of 36 *flavouring compounds* in mixtures of flavourings:

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

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Chemically Defined Flavourings – Group 23* (*CDG23*) have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] *Application, Reference SANCO/D/2 Forw. Appl. 1831/020-2010

[2] Commission Regulation (EC) No 1565/2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96 of the European Parliament and of the Council

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

[4] *Technical dossier, Section II – Sect_II_Identity.pdf: 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_05_FFAC 2008 GCMS method.pdf "GC/MS method for the identification and assay of feed flavourings"

[7] *Technical dossier, Section II – Annex_II_07_RTL Lock.pdf

[8] *Technical dossier, Section II – Annex_II_06_Flavour RTL.pdf

[9] *Technical dossier, Section II – Annex_II_04_Methods assay.pdf

[10] #Supplementary Information – Analytical report Pancosma.pdf

[11] #Supplementary Information – Analytical report Phytosynthese.pdf

* Refers to Dossier No. FAD-2010-0028

Refers to Dossier No. FAD-2009-0050

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was Community 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:

- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer, Speyer (DE)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Renes (FR)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)