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European Union Reference Laboratory for Feed Additives

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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-2010-0097 CRL/100096
Product Name:	Chemically defined flavourings from Chemical Group 09 – Primary aliphatic saturated or unsaturated alcohols/ aldehydes/acids/acetals/esters with a second primary, secondary or tertiary oxygenated functional group including aliphatic lactones
Active Substance(s):	Thirty chemically defined flavourings from Chemical Group 09
Rapporteur Laboratory:	European Union Reference Laboratory for Feed Additives (EURL-FA)
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Report approved by: Date:	Christoph von Holst 18/03/2011

EXECUTIVE SUMMARY

The *Chemically Defined Flavourings - Group 09* (*Primary aliphatic saturated or unsaturated alcohols/aldehydes/acids/acetals/esters with a second primary, secondary or tertiary oxygenated functional group including aliphatic lactones*), in this application comprises thirty 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 99.5%.

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* or in *water*.

For the identification of volatile chemically defined flavouring compounds *CDG 09* 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 EURL. The Applicant provided the typical chromatogram for the *CDG 09* 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 *CDG 09* 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 *CDG 09* 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)

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 EURL 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 EURL evaluation report, available from the EURL website.

KEYWORDS
Chemically Defined Flavourings - Group 09, mixture of flavouring compounds, sensory additives, all species.

1. BACKGROUND

The *Chemically Defined Flavourings - Group 09* (*CDG 09*) 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 *CDG 09* application contains thirty flavouring compounds (listed in Table 1) belonging to the group - described in Annex I of Commission Regulation (EC) No 1565/2000 [2] as – "*Primary aliphatic saturated or unsaturated alcohols/aldehydes/acids/acetals/esters with a second primary, secondary or tertiary oxygenated functional group including aliphatic lactones*".

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 99.5% [3]. *Mixtures of flavouring* compounds are usually 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 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 *Chemically Defined Flavourings – Group 09,* 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 volatile chemically defined flavouring compounds *CDG 09* (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 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 [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. The Applicant provided the typical chromatogram for the *CDG 09* of interest (cf. Fig II.2-10 [4]).

The four acids (lactic, succinic, fumaric and 4-oxovaleric) of CDG 09 were analysed after the generation of the corresponding methyl chloroformate derivatives.

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 *CDG 09* 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 *CDG 09* in the *mixture of flavouring compounds.*

Table 1. Retention Time Locked for the flavouring compounds of *CDG 09* [4], and d-limonene

FL-no	CAS-no	EU Register name	RTL polar (min)	RTL non-polar (min)
07.001	78-98-8	2-Oxopropanal	5.87	1.83
08.004	598-82-3	Lactic acid (2-methoxycarbonyloxy-propionic acid methyl ester)	17.5*	8.4*
08.023	123-76-2	4-Oxovaleric acid (methyl 4-oxovalerate)	16.6*	7.1*
08.024	110-15-6	Succinic acid (dimethyl succinate)	17.2*	8.4*
08.025	110-17-8	Fumaric acid (cis-dimethyl fumarate) (tri-dimethyl fumarate)	16.2* 19.6*	8.3* 8.1*
09.402	141-97-9	Ethyl acetoacetate	13.45	5.48
09.433	97-64-3	Ethyl lactate	10.20	3.70
09.434	138-22-7	Butyl lactate	15.20	7.30
09.435	539-88-8	Ethyl 4-oxovalerate	17.56	8.30
09.444	123-25-1	Diethyl succinate	19.57	12.34
09.490	105-53-3	Diethyl malonate	16.75	8.70
09.491	7492-70-8	Butyl-O-butyryllactate	21.40	17.85
09.545	61931-81-5	Hex-3-enyl lactate	22.24	13.36
09.580	20279-51-0	Hexyl lactate	20.56	13.46
10.001	104-61-0	Nonano-1,4-lactone	28.92	17.50
10.002	104-67-6	Undecano-1,4-lactone	34.20	23.70
10.004	106-02-5	Pentadecano-1,15-lactone	34.15	30.90
10.006	96-48-0	Butyro-1,4-lactone	18.19	4.50
10.007	705-86-2	Decano-1,5-lactone	32.70	22.07
10.008	713-95-1	Dodecano-1,5-lactone	37.63	27.74
10.011	710-04-3	Undecano-1,5-lactone	35.38	25.04
10.013	108-29-2	Pentano-1,4-lactone	17.67	5.30
10.014	3301-94-8	Nonano-1,5-lactone	30.22	19.00
10.015	698-76-0	Octano-1,5-lactone	27.43	16.00
10.016	2721-22-4	Tetradecano-1,5-lactone	42.50	33.00
10.017	706-14-9	Decano-1,4-lactone	31.46	20.72
10.019	2305-05-7	Dodecano-1,4-lactone	36.82	26.60
10.020	105-21-5	Heptano-1,4-lactone	23.19	10.70
10.021	695-06-7	Hexano-1,4-lactone	20.60	7.80
10.022	104-50-7	Octano-1,4-lactone	26.11	14.12
01.045	5989-27-5	d-Limonene (standard)	6.70	8.33

FL-no: EU Flavour Number; RTL: Retention Time Locked

(*) Retention time of the *methyl chloroformate derivative* of the acid

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 (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 EURL 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 EURL recommends for official control the Agilent specific method submitted by the Applicant, for the identification of the thirty *flavouring compounds* of the *CDG 09* 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 EURL is unable to recommend a method for the identification of the thirty *flavouring compounds* of the *CDG 09* in *feedingstuffs* and *water*.

Recommended text for the register entry (analytical method)

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

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

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Chemically Defined Flavourings – Group 09* (*CDG 09*) 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. 1831/089-2010

[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-2010-0097

Refers to Dossier No. FAD-2009-0050

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:

- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (POL)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)
- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer, Speyer (DE)