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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-2010-0117 CRL/100085
Product Name:	Chemically defined flavourings from Chemical Group 28 – Pyridine, pyrrole and quinoline derivatives
Active Substance(s):	Nine chemically defined flavourings from Chemical Group 28
Rapporteur Laboratory:	European Union Reference Laboratory for Feed Additives (EURL-FA)
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EXECUTIVE SUMMARY

The *Chemically Defined Flavourings - Group 28* (*Pyridine, pyrrole and quinoline derivatives*), in this application comprises nine 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* or in *water*.

For the identification of volatile chemically defined flavouring compounds *CDG 28* 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 28* 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 28* 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 28* 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.

For the identification of *piperine* in the *feed additive* the Applicant proposed a single laboratory validated and further verified method based on Gas Chromatography coupled to a Flame Ionization Detector (GC-FID). Based on the satisfactory performance characteristics presented, the EURL recommends for official control the single laboratory validated and further verified GC-FID method, submitted by the Applicant, for the qualitative identification of *piperine* in the *feed additive*.

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 28, mixture of flavouring compounds, sensory additives, all species.

1. BACKGROUND

The *Chemically Defined Flavourings - Group 28* (*CDG 28*) 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 28* application contains nine flavouring compounds (listed in Table 1) belonging to the group - described in Annex I of Commission Regulation (EC) No 1565/2000 [2] as – "*Pyridine, pyrrole and quinoline derivatives*".

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

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 28,* 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 28* (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 28* of interest (cf. Fig II.2-5 [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 *CDG 28* 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 28* 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.

Table 1. Retention Time Locked for the flavouring compounds of *CDG 28* [4], and d-limonene

FL-no	CAS-no	EU Register name	RTL polar (min)	RTL non-polar (min)
13.169	20662-84-4	Trimethyloxazole	6.5	4.1
14.004	83-34-1	3-Methylindole	38.6	18.4
14.007	120-72-9	Indole	37.6	15.6
14.038	1122-62-9	2-Acetylpyridine	17.8	7.8
14.047	1072-83-9	2-Acetylpyrrole	27.2	8.5
14.061	536-78-7	3-Ethylpyridine	11.4	6.1
14.064	123-75-1	Pyrrolidine	(*)	2.6
14.065	108-48-5	2,6-Dimethylpyridine	8.1	4.6
14.003	94-62-2	Piperine	Not detected	
01.045	5989-27-5	d-Limonene (standard)	6.7	8.3

FL-no: EU Flavour Number; RTL: Retention Time Locked
(*) Adsorbs on polar columns

For the identification of *piperine* in the *feed additive* the Applicant proposed a single laboratory validated and further verified method based on an extraction with acetone followed by analysis via Gas Chromatography coupled to a Flame Ionization Detector (GC-FID). Quantification is carried out using *piperine* as external standard and p-fluorovalerophenone as internal standard [12]. Satisfactory performance characteristics were reported in the frame of the validation [13] and verification [14] studies, obtained using samples containing a mixture of *piperine*, thymol and eugenol. The EURL assumes that the specificity of this method could be increased by substituting the flame ionization detection by mass spectrometry.

However, based on the performance characteristics presented, the EURL recommends for official control the single laboratory validated and further verified GC-FID method, submitted by the Applicant, for the qualitative identification of *piperine* in the *feed additive*.

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 eight *flavouring compounds* of the *CDG 28* in the *feed additive* of the individual (or mixture of) *flavouring compounds* of interest.

The EURL recommends for official control the single laboratory validated and further verified method submitted by the Applicant based on gas chromatography coupled to flame ionization detector, for the qualitative identification of *piperine* in the *feed additive*.

The Applicant provided no experimental data for *feedingstuffs* and *water*, therefore the EURL is unable to recommend a method for the identification of the nine *flavouring compounds* of the *CDG 28* in *feedingstuffs* and *water*.

Recommended text for the register entry (analytical method)

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

Gas-Chromatography Mass Spectrometry with Retention Time Locking (GC-MS-RTL)

For the identification of *piperine* in the *feed additive*:

Gas Chromatography coupled to a Flame Ionization Detector (GC-FID)

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 28* (*CDG 28*) 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. 1831/074-2010

[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

[12] *Technical dossier, Section II – Annex_II.9 Piperine analytical method

[13] *Technical dossier, Section II – Annex_II.10 Piperine method assay validation

[14] *Technical dossier, Section II – Annex_II.14 Piperine method assay verification

* Refers to Dossier No. FAD-2010-0117

\# 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)