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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-2010-0053  
CRL/100064

Product Name: Chemically defined flavourings from  
Chemical Group 24 – Pyrazine derivatives

Active Substance(s): Twenty two chemically defined  
flavourings from Chemical Group 24

Rapporteur Laboratory: Community Reference Laboratory for  
Feed Additives (CRL-FA)

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Date: 10/12/2010

## EXECUTIVE SUMMARY

The *Chemically Defined Flavourings - Group 24 (Pyrazidine derivatives)*, in this application comprises 22 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 *CDG24* 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 *CDG24* 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 *CDG24* 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 *CDG24* 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 24, mixture of flavouring compounds, sensory additives, all species.

## 1. BACKGROUND

The *Chemically Defined Flavourings - Group 24 (CDG24)* 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 *CDG24* application contains 22 flavouring compounds (listed in Table 1) belonging to the group - described in Annex I of Commission Regulation (EC) No 1565/2000 [2] as - "*Pyrazine 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 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 24*, 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 *CDG24* (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;  $\pm 10\%$ ;  $\pm 20\%$ ) 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 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  $\mu\text{m}$  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 CDG24 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 CDG24 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 CDG24 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 CDG24 [4], and d-limonene

FL-no	CAS-no	EU Register name	RTL polar (min)	RTL non-polar (min)
14.005	15707-24-1	2,3-Diethylpyrazine	13.2	9.4
14.006	15707-23-0	2-Ethyl-3-methylpyrazine	11.7	7.1
14.015	34413-35-9	5,6,7,8-Tetrahydroquinoxaline	21.5	12.8
14.018	1124-11-4	2,3,5,6-Tetramethylpyrazine	13.9	9.4
14.019	14667-55-1	2,3,5-Trimethylpyrazine	11.9	7.1
14.020	123-32-0	2,5-Dimethylpyrazine	9.6	5.0
14.021	108-50-9	2,6-Dimethylpyrazine	9.8	5.0
14.022	13925-00-3	2-Ethylpyrazine	10.0	5.1
14.024	13925-07-0	2-Ethyl-3,5-dimethylpyrazine	12.9	9.2
14.025	63450-30-6	2,5 or 6-methoxy-3-methylpyrazine	10.5–12.0	6.6-6.8
14.027	109-08-0	2-Methylpyrazine	8.3	3.6
14.028	13708-12-8	5-Methylquinoxaline	27.6	15.9
14.032	22047-25-2	Acetylpyrazine	18.2	7.4
14.037	23747-48-0	5H-5-methyl-6,7-dihydrocyclopenta (b)pyrazine	17.9	10.8
14.043	24683-00-9	2-Isobutyl-3-methoxypyrazine	15.4–16.5	12.4–13.3
14.049	32974-92-8	2-Acetyl-3-ethylpyrazine	20	11.8
14.050	5910-89-4	2,3-Dimethylpyrazine	10	5.2
14.056	18138-04-0	Diethyl-5-methylpyrazine	14	11.8
14.062	24168-70-5	2-(sec-Butyl)-3-methoxypyrazine	15	12.3
14.100	55031-15-7	2-ethyl-3,(5or6)dimethylpyrazine	12.8-13.3	9.2–9.4
14.112	25680-58-4	2-Ethyl-3-methoxypyrazine	12.3	8.7
14.126	2847-30-5	2-Methoxy-3-methylpyrazine	10.5	6.6
01.045	5989-27-5	d-Limonene (standard)	6.70	8.33

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

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 22 *flavouring compounds* of the CDG24 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 22 *flavouring compounds* of the CDG24 in *feedingstuffs* and *water*.

##### ***Recommended text for the register entry (analytical method)***

For the identification of 22 *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 24 (CDG24)* 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. 1831/049-2010
  - [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-2010-0053  
# 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)
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- 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, Rennes (FR)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)