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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

Crina® Poultry Plus

(FAD-2013-0052; CRL/130026

European Union Reference Laboratory
Feed Additives

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

Dossier related to:	**FAD-2013-0052 - CRL/130026**
Name of Product:	**Crina® Poultry Plus**
Active Agent (s):	**Benzoic Acid, Thymol, Eugenol and Piperine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
Report prepared by:	**Zigmas Ezerskis**
Report checked by: Date:	**Piotr Robouch (EURL-FA)** **11/06/2014**
Report approved by: Date:	**Christoph von Holst** **17/06/2014**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for *Crina® Poultry Plus* under category/functional group 4(d) 'Zootechnical additives' / 'other zootechnical additives' according to the classification system of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of the *feed additive* for chicken for fattening, chicken reared for laying, minor poultry species (for fattening and reared for laying).

Crina® Poultry Plus is a light brown, free flowing powder preparation consisting of the four main active substances: a minimum of 80% of *benzoic acid*; 1% of *thymol*; 0.5% of *eugenol*; and 0.05% of *piperine*. Additionally, the *feed additive* contains silicic acid, diatomaceous earth, monopropylene glycol and soy oil as carriers.

The *feed additive* is to be used in *premixtures* and *feedingstuffs* with a proposed maximum limit of 300 mg/kg complete *feedingstuffs*, which correspond to maximum *benzoic acid* content of 240 mg/kg complete *feedingstuffs*.

For the determination of *benzoic acid* in the *feed additive* and *premixtures* the Applicant proposed a single laboratory validated and further verified method, based on Reverse Phase High-Performance Liquid Chromatography coupled to an ultraviolet detector (RP-HPLC-UV). The following performance characteristics were reported in the frame of validation and verification studies: - a standard deviation for *repeatability* (RSD_r) ranging from 0.5 to 4.5%; - a standard deviation for *intermediate precision* (RSD_{ip}) ranging from 0.7 to 5.4%; and - a *recovery* rate (R_{Rec}) ranging from 96 to 105%.

Based on the performance characteristics presented the EURL recommends for official control the single laboratory validated and further verified RP-HPLC-UV method, submitted by the Applicant, to determine *benzoic acid* in the *feed additive* and *premixtures*.

For the determination of *Crina® Poultry Plus* in *premixtures* and *feedingstuffs* the Applicant suggested to use *benzoic acid* - the major constituent of the *feed additive* as a marker. For the determination of *benzoic acid* in *feedingstuffs* the Applicant proposed a single laboratory validated and further verified method, based on Gas Chromatography Isotope Dilution Mass Spectrometry (GC-IDMS). The following performance characteristics were reported in the frame of validation and verification studies: - RSD_r ranging from 1.6 to 3.3%; - RSD_{ip} ranging from 1.9 to 3.9%, and - R_{Rec} ranging from 95 to 101%.

Based on the performance characteristics presented the EURL recommends for official control the single laboratory validated and further verified GC-IDMS method, submitted by the Applicant, to determine *benzoic acid* in *feedingstuffs*.

For the quantification of *thymol, eugenol* and *piperine* in the *feed additive* the Applicant proposed a single laboratory validated and further verified method based on Gas Chromatography coupled to a Flame Ionisation Detector (GC-FID). The following performance characteristics were reported: - RSD_r ranging from 1.5 to 4.6%; - RSD_{ip} ranging from 1.7 to 5.6%, and - R_{Rec} ranging from 94 to 105%.

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, to quantify *thymol, eugenol* and *piperine* in the *feed additive*.

No experimental data were provided by the Applicant for the quantification of the *thymol, eugenol* and *piperine* in *premixtures* and *feedingstuffs*. Therefore, the EURL is not able to evaluate nor recommend a method for the official control to quantify *thymol, eugenol* and *piperine* in *premixtures* and *feedingstuffs*, containing *Crina® Poultry Plus*.

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.

KEYWORDS

Crina® Poultry Plus, *benzoic acid*, *thymol*, *eugenol*, *piperine*, zootechnical additives, other zootechnical additives, chicken for fattening, chicken reared for laying, minor poultry species for fattening and minor poultry species reared for laying

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new *feed additive*) for *Crina® Poultry Plus* under category/functional group 4(d) 'Zootechnical additives' / 'other zootechnical additives' according to the classification system of Regulation (EC) No 1831/2003 [1]. Specifically, authorisation is sought for the use of the *feed additive* for chicken for fattening, chicken reared for laying, minor poultry species (for fattening and reared for laying) [1,2].

Crina® Poultry Plus is a light brown, free flowing powder preparation consisting of the four main active substances: a minimum of 80% of *benzoic acid*; 1% of *thymol*; 0.5% of *eugenol*; and 0.05% of *piperine*. Additionally, the preparation includes a maximum of total 0.6% of *benzyl salicylate*, *iso-amyl salicylate* and *trans-anethole* [2,3]. Additionally, the *feed additive* contains silicic acid, diatomaceous earth, monopropylene glycol and soy oil as the main carriers [3].

The *feed additive* is to be used in *premixtures* and *feedingstuffs* with proposed maximum limit of 300 mg/kg complete *feedingstuffs* [2], which correspond to maximum *benzoic acid* content of 240 mg/kg complete *feedingstuffs*.

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 (EFSA) for each application or group of applications. For these dossiers, the methods of analysis submitted in connection with *Crina® Poultry Plus*, 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, aflatoxin B1 and dioxins) are available from the respective European Union Reference Laboratories [4].

Description of the analytical methods for the determination of the active substances in feed additive, premixtures and feedingstuffs

For the determination of *benzoic acid* in the *feed additive*, the Applicant proposed a single laboratory validated and further verified method [5] based on an extraction with 50 mM sodium hydroxide aqueous solution followed by analysis using Reverse Phase High-Performance Liquid Chromatography coupled to an Ultraviolet detector (RP-HPLC-UV), applying a gradient method with 20 mM ammonium acetate and methanol as mobile phase and detection at a wavelength of 272 nm. Quantification is carried out using *benzoic acid* as external standard.

For the determination of *benzoic acid* in *premixtures*, the Applicant proposed a single laboratory validated and further verified method [6] similar to the abovementioned RP-HPLC-UV method. The following modifications were implemented in the sample treatment

protocol: extraction with methanol/water 70:30 (v/v) followed by analysis applying a gradient method with 10 mM ammonium acetate and methanol as mobile phase. The performance characteristics derived from the validation [7],8] and verification studies [9] are presented in Table 1.

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified RP-HPLC-UV methods, submitted by the Applicant, to determine *benzoic acid* in the *feed additive* and *premixtures*.

For the determination of *Crina® Poultry Plus* in *feedingstuffs* the Applicant suggested to use *benzoic acid* as a marker [11], and submitted another single laboratory validated and further verified method based on an extraction with 50 mM sodium hydroxide, Solid Phase Extraction (SPE) purification and derivatisation followed by analysis using Gas Chromatography Isotope Dilution Mass Spectrometry (GC-IDMS) [11]. Quantification is carried out using benzoic acid as external standard and benzoic-d_5 acid as internal standard. The performance characteristics derived from the validation [13] and verification studies [14] are presented in Table 1. Furthermore, the Applicant reported limits of detection (LOD) and quantification (LOQ) of 30 and 85 mg/kg *feedingstuffs*, respectively.

Based on the performance characteristics presented, the EURL recommends for official control, the single laboratory validated and further verified GC-IDMS method, submitted by the Applicant, to determine *benzoic acid* in *feedingstuffs*.

Table 1: The performance characteristics of the single laboratory validated and verified methods for the determination of *benzoic acid* in the *feed additive* (FA), *premixtures* (PM) and *feedingstuffs* (FS).

Active substance	Benzoic acid					
Matrix	FA		PM		FS	
Method	RP-HPLC-UV [5]		RP-HPLC-UV [6]		GC-MS [11]	
Reference	validation [7]	verification [9]	validation [8]	verification [10]	validation [13]	verification [14]
RSD_r (%)	0.6 - 1.0	0.5	0.5 - 2.4	4.5	1.6 - 3.3	2.4
RSD_{ip} (%)	1.0 - 1.3	0.7	1.8 - 3.2	5.4	1.9 - 3.9	2.9
R_{rec} (%)	100	101	96.0 - 100	105	95 - 101	96

RSD_r and RSD_{ip} - relative standard deviation for *repeatability* and *intermediate precision*, respectively; R_{rec} - recovery rate.

Table 2: The performance characteristics of the single laboratory validated and verified GC-FID method for the determination of *thymol, eugenol* and *piperine* in the *feed additive* [15].

Active substance	Thymol		Eugenol		Piperine	
Reference	validation [16]	verification [17]	validation [16]	verification [18]	validation [16]	verification [19]
RSD_r (%)	2.5 - 3.9	1.5	2.4 - 3.6	1.5	3.5 - 4.6	4.1
RSD_{ip} (%)	3.4 - 4.5	1.8	3.9 - 4.9	1.7	4.9 - 5.6	4.9
R_{rec} (%)	100 - 102	97	101 - 104	98	98 - 105	94

RSD_r and RSD_{ip} - relative standard deviation for *repeatability* and *intermediate precision*, respectively; R_{rec} - recovery rate.

For the quantification of *thymol, eugenol* and *piperine* in the *feed additive*, the Applicant proposed a single laboratory validated and further verified method [15] based on an extraction with acetone followed by analysis using Gas Chromatography coupled to a Flame Ionisation Detector (GC-FID). Quantification is carried out using the abovementioned essential oil compounds as external standards and p-fluorovalerophenone as internal standard. The performance characteristics derived from the validation [16] and verification studies [17] are presented in Table 2. Furthermore, the Applicant provided a LOD ranging from 3 to 4 mg/l for *thymol, eugenol* and *piperine.*

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, to quantify *thymol, eugenol* and *piperine* in the *feed additive*.

No experimental data were provided by the Applicant for the quantification of the *thymol, eugenol* and *piperine* in *premixtures* and *feedingstuffs*. Therefore, the EURL is not able to evaluate nor recommend a method for the official control to quantify *thymol, eugenol* and *piperine* in *premixtures* and *feedingstuffs*, containing *Crina® Poultry Plus*.

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 single laboratory validated and further verified methods submitted by the Applicant using:

- Reverse Phase High-Performance Liquid Chromatography coupled to UV detector (RP-HPLC-UV) to determine *benzoic acid* in the *feed additive* and *premixtures*;
- Gas Chromatography Isotope Dilution Mass Spectrometry (GC-IDMS) to determine *benzoic acid* in *feedingstuffs*; and
- Gas Chromatography coupled to Flame Ionisation Detector (GC-FID) to determine *thymol, eugenol* and *piperine* in the *feed additive*.

No experimental data were provided by the Applicant for the quantification of the *thymol, eugenol* and *piperine* in *premixtures* and *feedingstuffs*. Therefore, the EURL is not able to evaluate nor recommend a method for the official control to quantify *thymol, eugenol* and *piperine* in *premixtures* and *feedingstuffs*, containing *Crina® Poultry Plus*.

Recommended text for the register entry (analytical method)

For the determination *benzoic acid* in the *feed additive* and *premixtures*:

- Reverse Phase High-Performance Liquid Chromatography coupled to an Ultraviolet detector (RP-HPLC-UV)

For the determination *benzoic acid* in *feedingstuffs*:

- Gas Chromatography Isotope Dilution Mass Spectrometry (GC-IDMS)

For the determination of *thymol, eugenol* and *piperine* in the *feed additive*:

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

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Crina® Poultry Plus* 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, Ref. SANCO/G/1: Forw. Appl. 1831/(0047)-2013
[2] *Application, Proposal for Register Entry - Annex A
[3] *Technical dossier, Section II: Identity, characterisation and conditions of use of the additive; Methods of analysis
[4] 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
[5] *Technical dossier, Section II, Annex_II_32
[6] *Technical dossier, Section II, Annex_II_40
[7] *Technical dossier, Section II, Annex_II_33
[8] *Technical dossier, Section II, Annex_II_41
[9] *Technical dossier, Section II, Annex_II_34
[10] *Technical dossier, Section II, Annex_II_42
[11] *Technical dossier, Section II: 2.6.1. Methods of analysis for the active substance
[12] *Technical dossier, Section II, Annex_II_43
[13] *Technical dossier, Section II, Annex_II_44
[14] *Technical dossier, Section II, Annex_II_45
[15] *Technical dossier, Section II, Annex_II_35
[16] *Technical dossier, Section II, Annex_II_36
[17] *Technical dossier, Section II, Annex_II_37
[18] *Technical dossier, Section II, Annex_II_38
[19] *Technical dossier, Section II, Annex_II_39

*Refers to Dossier no: FAD-2013-0052

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:

- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Fødevarestyrelsen, Ringsted (DK)[1]
- Sachgebiet Futtermittel des Bayrischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)[2]
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Laboratorio Arbitral Agroalimentario, Ministerio de Agricultura, Alimentación y Medio Ambiente, Madrid (ES)[3]
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen, Jena (DE)

[1] Official name and address according to Regulation (EC) No 885/2009: Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby

[2] Name and address according to Regulation (EC) No 885/2009: Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim

[3] Name and address according to Regulation (EC) No 885/2009: Laboratorio Arbitral Agroalimentario, Ministerio de Agricultura, Pesca y Alimentación, Madrid