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EURL 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-2010-0093 EURL/ 100067
Name of Feed Additive:	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:	Stefano Bellorini (EURL-FA)
Report checked by: Date:	Piotr Robouch (EURL-FA) 16/02/2011
Report approved by: Date:	Christoph von Holst 16/02/2011

EXECUTIVE SUMMARY

In the current application authorisation is sought for *Crina® Poultry Plus* under Articles 4(1), category of 'zootechnical additives' functional group 4(d) 'other zootechnical additives' according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *Crina® Poultry Plus* for chicken for fattening. The active substances in the *feed additive* are a preparation of *benzoic acid* and three essential oil compounds (*thymol, eugenol* and *piperine*). The feed additive, as proposed by the Applicant, is intended to be mixed to complete *feedingstuffs* at a dose ranging from 300 to 450 mg, which is equivalent to 250 to 375 mg *benzoic acid* per kg *feedingstuffs*.

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

* For the *feed additive*:

- a standard deviation for *repeatability* (RSD_r) ranging from 0.5 to 1%;
- a standard deviation for *intermediate precision* (RSD_{ip}) ranging from 0.7 to 1.3%, and
- a *recovery* rate (R_{Rec}) ranging from 99.7 to 101%.

* For *premixtures*:

- RSD_r ranging from 0.5 to 4.5%;
- RSD_{ip} ranging from 1.8 to 5.4%, and
- 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 *feed additive* and *premixtures*.

For the determination of the *benzoic acid* in *feedingstuff* 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:

- RSD_r ranging from 1.6 to 3.3%;
- RSD_{ip} ranging from 1.9 to 3.9%, and
- R_{Rec} ranging from 94.5 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 determination 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 Ionization 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 determine *thymol, eugenol* and *piperine* in the *feed additive*.

For the determination of *Crina® Poultry Plus* in *premixtures* and *feedingstuffs* the Applicant proposed to identify only *benzoic acid* as appropriate marker substance for the recognition of the whole *feed additive*. No experimental data were provided by the Applicant for the identification 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 identify *thymol, eugenol* and *piperine* in *premixtures* and *feedingstuffs*.

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, Chicken for fattening.

1. BACKGROUND

In the current application authorisation is sought for a feed additive *Crina® Poultry Plus* under Articles 4(1) (Authorisation of a feed additive or a new use of a feed additive), under the category of 'zootechnical additives' functional group 4(d), 'other zootechnical additives' according to Annex I of Regulation (EC) No 1831/2003 [1]. Specifically, authorisation is sought for the use of *Crina® Poultry Plus* for chickens for fattening. The active substances proposed to be registered are a preparation of *benzoic acid* and three essential oil compounds having a minimum content (in % w/w) of :

- 80% *benzoic acid*
- 1% *thymol*
- 0.5% *eugenol*
- 0.05% *piperine*

The formulation is implemented with processing aids ranging from 13 to 18% (main carriers are silicic acid, diatomaceous earth as mineral part and monopropylene glycol, soy oil as organic) [2], [3]. According to the Applicant *Crina® Poultry Plus* is a light brown, free flowing, granulated powder with an average particle size of 1.2 mm and a low dusting potential [4]. The *feed additive*, as proposed by the Applicant, is intended to be mixed to complete *feedingstuffs* at a dose ranging from 300 to 450 mg kg^{-1}[5]. Upon request of the EURL, the Applicant stated that the abovementioned doses refer to the *feed additive CRINA® Poultry Plus* as a whole (sum of active substances + technological additives). Therefore the abovementioned conditions of use correspond to *benzoic acid* content in complete *feedingstuffs* ranging from 250 to 375 mg kg^{-1}[6].

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 [7].

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

For the determination of the *benzoic acid* in the *feed additive*, the Applicant proposed a single laboratory validated and further verified method [8] based on an extraction with aqueous solution of 50 mM sodium hydroxide followed by analysis via 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 detected at a wavelength of 272 nm. Quantification is carried out using *benzoic acid* as external standard.

For the determination of the *benzoic acid* in *premixtures*, the Applicant proposed a single laboratory validated and further verified method [9] 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 [10], [11] and verification studies [12], [13] 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 *feed additive* and *premixtures*.

For the determination of the *benzoic acid* in *feedingstuffs*, the Applicant proposed a single laboratory validated and further verified method [14] based on an extraction with 50 mM sodium hydroxide, Solid Phase Extraction (SPE) purification and derivatization followed by analysis by Gas Chromatography Isotope Dilution Mass Spectrometry (GC-IDMS). 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 [15] and verification studies [16] are presented in Table 1. Furthermore the Applicant reported a limit of detection (LOD) and quantification (LOQ) of 30 and 85 mg/kg, respectively.

Table 1: Method performance characteristics 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 [8]		RP-HPLC-UV [9]		GC-MS [14]	
	validation [10]	verification [12]	validation [11]	verification [13]	validation [15]	verification [16]
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} (%)	99.7 - 99.8	101	96.0 - 99.9	105	94.5 - 101	96.0

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

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 determination of *thymol, eugenol* and *piperine* in the *feed additive*, the Applicant proposed a single laboratory validated and further verified method [17] 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 the abovementioned essential oil compounds as external standards and p-fluorovalerophenone as internal standard. The performance characteristics derived from the validation [18] and verification studies [19], [20], [21] are presented in Table 2. Furthermore, the Applicant provided a LOD ranging from 3 to 4 mg/l for *thymol, eugenol* and *piperine.*

Table 2: Method performance characteristics for the determination of *thymol, eugenol* and *piperine* in the *feed additive* using GC-FID [17].

Active substance	Thymol		Eugenol		Piperine	
	validation [18]	verification [19]	validation [18]	verification [20]	validation [18]	verification [21]
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} (%)	99.6 - 102	97.4	101 - 104	98.2	98.0 - 105	94.0

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

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 determine *thymol, eugenol* and *piperine* in the *feed additive*.

For the determination of *Crina® Poultry Plus* in *premixtures* and *feedingstuffs* the Applicant proposed to use *benzoic acid* as a marker substance for the recognition of the whole *feed additive* [22]. As justification the Applicant considers that *benzoic acid* is the major constituant into the formulation. No experimental data were provided by the Applicant for the identification 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 identify *thymol, eugenol* and *piperine* in *premixtures* and *feedingstuffs*.

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

Recommended text for the register entry (analytical method)

For the determination *benzoic acid* in *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 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 *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/D/2:Forw.Appl.1831/0068-2010
[2] *Application, (Annex A), FAD-2010-0093_DescriptAdd_ *Crina® Poultry Plus*
[3] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition
[4] *Technical dossier, Section II: 2.1.5 Physical state of each form of the product
[5] *Application, (Annex A), FAD-2010-0093_Conditions of use_ *Crina® Poultry Plus*
[6] *Supplementary information, "FAD-2010-0093-Answer.msg"
[7] 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
[8] *Technical dossier, Section II, Annex 2 24
[9] *Technical dossier, Section II, Annex 2 32
[10] *Technical dossier, Section II, Annex 2 25
[11] *Technical dossier, Section II, Annex 2 33
[12] *Technical dossier, Section II, Annex 2 26
[13] *Technical dossier, Section II, Annex 2 34
[14] *Technical dossier, Section II, Annex 2 35
[15] *Technical dossier, Section II, Annex 2 36
[16] *Technical dossier, Section II, Annex 2 37
[17] *Technical dossier, Section II, Annex 2 27
[18] *Technical dossier, Section II, Annex 2 28
[19] *Technical dossier, Section II, Annex 2 29
[20] *Technical dossier, Section II, Annex 2 30
[21] *Technical dossier, Section II, Annex 2 31
[22] *Technical dossier, Section II: 2.6.1 Method of analysis for the active substances
*Refers to Dossier no: FAD-2010-0093

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:

- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer, Speyer (DE)
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)