JRC.F.5/CvH/ZE/AS/Ares

Subject: Addendum to the EURL evaluation report

Reference: FAD-2020-0084 – Rosemary extract (JRC F.5/CvH/ZE/AS/Ares(2021)3404906)

Upon the request from DG SANTE [1], the EURL evaluated the supplementary information provided [2-8] in the frame of the dossier FAD-2020-0084 for the determination of *carnosic acid* as an active substance of *rosemary extract* in *compound feed*.

For the quantification of *carnosic acid* in *compound feed* the Applicant submitted a single-laboratory validated and further verified method based on gas chromatography with flame ionisation detection (GC-FID) [2].

Following this method, *carnosic acid* is extracted from the sample of feed (chicken fat used as feed material or pet kibble) with acetonitrile containing biphenyl as an internal standard. The extract is centrifuged and an aliquot of the supernatant is derivatised with N,O-bis-(trimethylsilyl)trifluoroacetamide before the chromatographic analysis. The quantification is performed by a standard calibration curve built using standard solutions of *carnosic acid* and biphenyl (an internal standard) treated in the same way as the target sample [2].

The performance characteristics of the GC-FID method reported by the Applicant and re-calculated by the EURL [3] in the frame of the validation and verification studies [4-8] for the quantification of *carnosic acid* in chicken fat and pet kibble are presented in Table 1.

Table 1. The performance characteristics reported by the Applicant and re-calculated by the EURL [3] in the frame of the validation and the verification studies [4-8] for the quantification of *carnosic acid* in chicken fat and pet kibble.

	Chicken Fat		Kibble	
	Validation	Verification	Validation	Verification
Measured mass fraction, mg/kg	522 – 529	252	35.1 – 35.7	12.9
RSD_r, %	2.4 – 2.6	1.4	2.2	5.0
RSD_{ip}, %	2.2 – 3.7	2.9	2.2 – 7.6	5.3
R_{Rec}, %	95	84	75	99
Reference	[3,4,8]	[3,5,6,8]	[3,4,8]	[3,5,7,8]

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

In addition, the Applicant reported a limit of quantification (LOQ) of 13.5 mg *carnosic acid* / kg of chicken fat and a LOQ of 5 mg *carnosic acid* / kg of kibble [4].

Based on the performance characteristics available the EURL recommends for official control the single-laboratory validated and verified GC-FID method for the quantification of *carnosic acid* in *compound feed*.

Recommended text for the registry entry (analytical method) (replacing the previous recommendations)

For the quantification of *carnosic acid* in the *feed additive*:

- High performance liquid chromatography with spectrophotometric detection (HPLC-UV)

For the quantification of *carnosic acid* in *compound feed*:

- Gas chromatography with flame ionisation detection (GC-FID)

References

[1] Supplementary Information – Request for SIN ARES(2022)983374

[2] Supplementary Information – Annex I KNCLS-WWM-007 Carnosic Acid by GC issue 2

[3] Supplementary Information – Calculation performance characteristics by EURL

[4] Supplementary Information – Annex II - Method Validation Quantification of Carnosic Acid by GC

[5] Supplementary Information – Annex III(a) - EURLFA-technical_guide_for_validation_and_verification_v2014_(003)

[6] Supplementary Information – Annex III(b) - Verification report chicken fat

[7] Supplementary Information – Annex III(c) - Verification report pet food kibble

[8] 2023-01-30_FAD-2020-0084-0072-2020-Method verification

Acknowledgements

The following National Reference Laboratories contributed to this addendum:

- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)

- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)

Addendum

- Prepared by Zigmas Ezerskis

- Reviewed and approved by María José González de la Huebra and Christoph von Holst (EURL-FA), respectively, Geel, 14/03/2023

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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

Rosemary extract
(FAD-2020-0084; CRL/190072)

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-2020-0084 - CRL/190072**
Name of Product:	***Rosemary extract***
Active Agent (s):	**Carnosic acid**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**Zigmas Ezerskis**
Report checked by: Date:	**María José González de la Huebra** **21/05/2021**
Report approved by: Date:	**Christoph von Holst** **21/05/2021**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *rosemary extract* under the category / functional group 1(b) "technological additives / antioxidants", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, the authorisation is sought for cats and dogs.

The *feed additive* (*rosemary extract*) is a liquid or solid preparation of natural origin, based on an extract from dried leaves of *Rosmarinus officinalis L*. According to the Applicant, the *feed additive* contains *carnosic acid* as an active substance with a minimum content of 9 % (w/w). Additionally, the *feed additive* contains 1 to 2 % (w/w) of *carnosol* as a minor component with antioxidative properties. The *feed additive* is intended to be used directly into *feedingstuffs* or through *premixtures* with no proposed minimum or maximum levels in *feedingstuffs*. However, the Applicant suggested inclusion levels of 500 mg *rosemary extract* / kg *feedingstuffs*.

For the quantification of the active substance *carnosic acid* in the *feed additive* the Applicant submitted a single-laboratory validated and further verified method based on high performance liquid chromatography (HPLC) with spectrophotometric (UV) detection at 280 nm.

The following performance characteristics of the HPLC-UV method have been obtained in the frame of the validation and verification studies for the quantification of *carnosic acid* in the *feed additive*: a relative standard deviation for *repeatability* (RSD_r) ranging from 0.8 to 2.1 %; a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 1.3 to 2.5 %; and a *recovery* rate (R_{Rec}) ranging from 96 to 112 %.

Based on the available performance characteristics the EURL recommends the single-laboratory validated and further verified HPLC-UV method for official control for the quantification of *carnosic acid* in the *feed additive*.

In addition, the *feed additive* was further characterised by the Applicant applying the above mentioned HPLC-UV method for the analysis of another minor component having antioxidative properties, namely *carnosol*. Based on the acceptable performance profile the EURL considers the HPLC-UV method as suitable for an additional characterisation of the *feed additive*.

For the quantification of *carnosic acid* in *premixtures* and *feedingstuffs* the Applicant referred to a single-laboratory validated method based on gas chromatography with flame ionisation detection (GC-FID) after derivatisation with N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), which was submitted by the same Applicant and evaluated in the frame of the previous dossier FAD-2004-0003.

Acceptable recovery and precision values were obtained in the frame of the validation studies for the quantification of *carnosic acid* in *premixtures* and *feedingstuffs*, but the Applicant did not present verification studies of the above mentioned GC-FID method for the quantification of *carnosic acid* in *premixtures* and *feedingstuffs*.

Furthermore, the Applicant did not submit any method or data for the quantification of *rosemary extract* in *premixtures* and *feedingstuffs* as the accurate quantification of *rosemary extract* added to *premixtures* and *feedingstuffs* is not achievable experimentally.

Based on the available information, the EURL is not able to recommend any method for official control for the quantification of *carnosic acid* or *rosemary extract* 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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

Carnosic acid, *carnosol, rosemary extract*, technological additives, antioxidants, cats and dogs.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for *rosemary extract* under the category / functional group 1(b) "technological additives / antioxidants", according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1,2]. Specifically, the authorisation is sought for cats and dogs [2].

The *feed additive* (*rosemary extract*) is a liquid or solid preparation of natural origin, based on an extract from dried leaves of *Rosmarinus officinalis L*. [3]. According to the Applicant, the *feed additive* contains *carnosic acid* as an active substance with a minimum content of 9 % (w/w). Additionally, the *feed additive* contains 1 to 2 % (w/w) of *carnosol* as a minor component with antioxidative properties [4].

The *feed additive* is intended to be used directly into *feedingstuffs* or through *premixtures* with no proposed minimum or maximum levels in *feedingstuffs* [5]. However, the Applicant suggested inclusion levels of 500 mg *rosemary extract* / kg *feedingstuffs* [6].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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 *Rosemary extract* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the quantification of the active substance *carnosic acid* in the *feed additive* the Applicant submitted a single-laboratory validated and further verified method based on high performance liquid chromatography (HPLC) with spectrophotometric (UV) detection at 280 nm [7].

Following this method, the liquid (50 to 100 µl) or solid (6 to 10 mg) samples of the *feed additive* are mixed with 1 ml of acetone (for liquid preparations) or 1 ml of acetonitrile (for solid preparations) and sonicated for 1 min. The obtained solutions are centrifuged at 10000 *g* for 5 min and aliquots of the supernatants are further analysed by HPLC-UV at 280 nm. The quantification of *carnosic acid* in the target samples is performed by an external calibration curve prepared from standard solutions of *carnosic acid* [7].

The performance characteristics of the HPLC-UV method obtained from the validation and verification studies for the quantification of *carnosic acid* in the *feed additive* are presented in Table 1.

Based on the available performance characteristics the EURL recommends the single-laboratory validated and further verified HPLC-UV method for official control for the quantification of *carnosic acid* in the *feed additive*.

Table 1 Performance characteristics of the HPLC-UV method obtained from the validation [8] and verification [9] studies for the quantification of *carnosic acid* in the *feed additive*

	Feed additive	
	Validation	**Verification**
Carnosic acid, % (w/w)	10.0 – 10.4	
RSD_r, %	0.8 – 1.3	1.4 – 2.1
RSD_{ip}, %	1.3	1.4 – 2.5
R_{Rec}, %	96	107 – 112
Reference	[8,9]	[9]

RSD_r and RSD_{ip}: a relative standard deviations for *repeatability* and *intermediate precision*, respectively; R_{Rec}: a *recovery* rate.

For the quantification of *carnosic acid* in *premixtures* and *feedingstuffs* the Applicant referred [10] to a single-laboratory validated method based on gas chromatography with flame ionisation detection (GC-FID), which was submitted by the same Applicant in the frame of the previous dossier FAD-2004-0003 [11,12].

Following this method, *carnosic acid* is extracted from the sample (5 g) with acetonitrile. The extract is centrifuged and an aliquot of the supernatant is derivatised with N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) for 10 min before the chromatographic analysis. The quantification is perfomed by an internal standard calibration curve built using standard solutions of *carnosic acid* treated in the same way as the target samples, and with biphenyl as an internal standard [11,12].

The following performance characteristics of the GC-FID method were obtained from the validation studies for the quantification of *carnosic acid* at the mass fraction of 13.8 mg/kg *feedingstuffs*: a relative standard deviation for *repeatability* (RSD_r) of 2.4 % and a *recovery* rate (R_{Rec}) of 105 %. In addition, a limit of quantification (LOQ) of 5.4 mg *carnosic acid* / kg *feedingstuffs* was established [12]. Furthermore, in the previous evaluation report of FAD-2004-0003 [13], the EURL admitted an acceptable recovery and precision values obtained in the frame of the validation studies for the quantification of the *carnosic acid* in *premixtures*.

However, the Applicant did not present verification studies of the above mentioned GC-FID method for the quantification of *carnosic acid* in *premixtures* and *feedingstuffs*.

Furthermore, the Applicant did not submit any method or data for the quantification of *rosemary extract* in *premixtures* and *feedingstuffs* as the accurate quantification of *rosemary extract* added to *premixtures* and *feedingstuffs* is not achievable experimentally.

Based on the available information, the EURL is not able to recommend any method for official control for the quantification of *carnosic acid* or *rosemary extract* in *premixtures* and *feedingstuffs*.

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

An evaluation of corresponding methods of analysis is not relevant for the present application.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

In addition, the *feed additive* was further characterised by the Applicant applying the above mentioned HPLC-UV method for the analysis of another minor component having antioxidative properties, namely *carnosol*. The following performance characteristics were obtained in the frame of the validation studies: RSD_r and RSD_{ip} ranging from 1.8 to 3.8 % and R_{Rec} ranging from 95 to 103 % [8].

Based on this performance profile the EURL considers the HPLC-UV method as suitable for the additional characterisation of the *feed additive*.

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, as last amended by Regulation (EU) 2015/1761) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control a single-laboratory validated and further verified HPLC-UV method for the quantification of *carnosic acid* in the *feed additive*. In addition, the EURL considers the HPLC-UV method as suitable for the analysis of *carnosol* in the frame of the characterisation of the *feed additive*.

Based on the available information, the EURL is not able to recommend any method for official control for the quantification of *carnosic acid* or *rosemary extract* in *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the quantification of *carnosic acid* in the *feed additive*:

– High performance liquid chromatography with spectrophotometric detection (HPLC-UV)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Rosemary extract* 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 SANTE_E5_FWD. APPL. 1831-0072-2020

[2] *Application, Annex 1 – submission number 1575979457226-2496

[3] *Technical dossier, Section II: 2.1.1. Name of the additive

[4] *Technical dossier, Section II: 2.1.3. Qualitative and Quantitative composition

[5] *Technical dossier, Section II: 2.5.1. Proposed mode of use in animal nutrition

[6] *Technical dossier, Section II: 2. Introduction

[7] *Technical dossier, Section II – Annex_II_6_1

[8] *Technical dossier, Section II – Annex_II_6_2

[9] *Technical dossier, Section II – Annex_II_6_3

[10] *Technical dossier, Section II: 2.6.1. Methods of analysis for the active substance

[11] +Technical dossier, Section II – NCLS_A-28_report

[12] +Technical dossier, Section II – Gamble_GC_for_CA_white_paper

[13] EURL evaluation report: FAD-2004-0003 – Rosemary extract liquid
https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2004-0003.pdf

*Refers to Dossier no: FAD-2020-0084
+Refers to Dossier no: FAD-2004-0003

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, 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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)