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

**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**

L-arginine
produced by fermentation with
Corynebacterium glutamicum KCCM80182
(*FAD-2018-0045; CRL/180034*)



**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-2018-0045- CRL/180034**

Name of Product: ***L-arginine
produced by fermentation with
Corynebacterium glutamicum
KCCM80182***

Active Agent: **L-arginine**

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
JRC Geel, Belgium**

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Date: **08/02/2019**

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Date: **08/02/2019**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-arginine produced by fermentation with Corynebacterium glutamicum KCCM80182*, under the category/functional groups 2(b) 'sensory additives/flavouring compounds' and 3(c) 'nutritional additives'/amino acids, their salts and analogues' according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for all animal species. According to the Applicant *L-arginine* has a minimum purity (mass fraction) of 98 %. The *feed additive* is intended to be added directly into *feedingstuffs* or through *premixtures* and *water* for drinking. However the Applicant did not propose any minimum or maximum content of *L-arginine* in *feedingstuffs*.

For the quantification of *L-arginine* in the *feed additive* the Applicant submitted an in-house validated analytical method based on reversed phase high performance liquid chromatography coupled with ultraviolet detection (HPLC-UV). The Applicant did not present a verification study and therefore the EURL cannot recommend the method for official control purposes.

For the quantification of the *L-arginine* content in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009) based on ion exchange chromatography coupled with photometric detection (IEC-VIS). This method, designed for the analysis of amino acids in *premixtures* and *feedingstuffs*, does not distinguish between the salts and the amino acid enantiomers. The Community method was further ring-trial validated by twenty-three laboratories for the determination of total *arginine* in feed and resulted in the equivalent standard method EN ISO 13903:2005. The following performance characteristics were reported for the quantification of total *arginine*: RSD_r ranging from 2.3 to 3.3 % and RSD_R ranging from 7.2 to 9.7 %.

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated Community method based on IEC-VIS to quantify *arginine* in *premixtures* and *feedingstuffs*.

The Applicant provided no experimental data to determine *arginine* in *water*. Nevertheless, as concluded in the previous EURL reports and specified in the corresponding legislation, the EURL recommends the Community method for official control for the quantification of *arginine* in the *feed additive* and *water*.

In addition, the EURL identified the "L-arginine monograph" of the Food Chemical Codex (FCC) for the identification 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) is not considered necessary.

KEYWORDS

L-arginine produced by fermentation with Corynebacterium glutamicum KCCM80182, nutritional additives, amino acids, sensory additives, flavouring compounds, all animal species and categories

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *L-arginine produced by fermentation with Corynebacterium glutamicum KCCM80182*, under the category/functional groups 2(b) 'sensory additives/flavouring compounds' and 3(c) 'nutritional additives'/amino acids, their salts and analogues' according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for all animal species [1-3].

L-arginine is already authorised as sensory additive produced by "chemical synthesis or protein hydrolysis" and as nutritional additive produced by fermentation with *Corynebacterium glutamicum* [4-7].

According to the Applicant, the white crystalline powder *L-arginine* has a minimum purity (mass fraction) of 98 % [1,3,8]. The *feed additive* is produced by fermentation with a genetically modified strain of *Corynebacterium glutamicum*. The production strain is deposited in the "Korean Culture Center of Microorganisms" (KCCM) under accession number KCCM 80182 [9].

The *feed additive* is intended to be added directly into *feedingstuffs* or through *premixtures* and *water* for drinking [10]. However the Applicant did not propose any minimum or maximum content of *L-arginine* in *feedingstuffs* [1].

Note: The EURL has previously evaluated the analytical methods for the determination of *L-arginine* in the frame of several dossiers including, as sensory feed additive, within the Chemical Defined flavouring Group 34 [11-16].

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 *L-arginine* 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 *L-arginine* in the *feed additive* the Applicant submitted an in-house validated analytical method based on reversed phase high performance liquid chromatography (HPLC) and ultraviolet detection (UV) [17,18].

The feed additive is diluted in distilled water and an aliquot is directly injected into the HPLC system equipped with a C18 column. The analyte is eluted via potassium phosphate buffer. *L-arginine* is detected with a photodiode array detector (PDA) established with external standard at 210 nm and quantified via a calibration curve. In the frame of the validation study, the Applicant reported relative standard deviations for repeatability (RSD_r) and intermediate precision (RSD_{ip}) below 1 %, and a recovery rate (R_{rec}) of 100 % [17,18]. However, the Applicant did not present a verification study or any additional test performed by a second independent laboratory applying the above mentioned method. Therefore, the EURL cannot conclude on the fitness-for-purpose of the method for official control purposes.

For the quantification of the *L-arginine* content in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method [19]. This method applies for the determination of free (synthetic and natural) and of total (peptide-bound and free) amino acids (including arginine), using an amino acid analyzer or HPLC equipment provided with an ion exchange column. The method is intended for *premixtures* and *feedingstuffs*, it does not distinguish between the salts of amino acids and it cannot differentiate the amino acid enantiomers.

The free amino acids are extracted with diluted hydrochloric acid. Co-extracted nitrogenous macromolecules are precipitated with sulfosalicylic acid and removed by filtration. The solution is filtered and adjusted to pH 2.2. The amino acids are separated by IEC and free *arginine* is determined after post-column derivatisation with ninhydrin by spectrophotometric detection at 570 nm (visible – VIS).

Table 1: Method performance characteristics reported in EN ISO 13903:2005 for the determination of total *arginine* [20]

Matrix	arginine content (g/kg)	RSD _r (%)	RSD _R (%)
poultry meal	43.5	3.3	9.7
broiler finisher feed	12.8	2.3	8.6
broiler starter feed	15.7	2.7	8.2
corn	4	3.3	9.5
fishmeal	34	3.0	7.2

RSD_r and RSD_R - relative standard deviation for *repeatability* and *reproducibility*, respectively.

The procedure chosen for the determination of the total amino acids depends on the amino acids under investigation. *Arginine* can be determined in either oxidised or non-oxidised samples. Oxidation is performed at 0 °C with a performic acid/phenol mixture. The excess of the oxidation reagent is decomposed with sodium disulfite. The oxidised or non-oxidised sample is hydrolysed with hydrochloric acid (6 mol/l) containing 1 g phenol/l for 23 hours. The hydrolysate is adjusted to pH 2.2. The amino acids are separated by IEC and total *arginine* is determined by post-column derivatisation with ninhydrin and photometric detection at 570 nm. Even if within the scope of the Community method, validation data for the determination of *arginine* in *premixtures* and *feedingstuffs* are not presented.

The Community method was further ring-trial validated by twenty-three laboratories for the determination of total *arginine* in feed and resulted in the equivalent standard method EN ISO 13903:2005 [20]. The reported performance characteristics are listed in Table 1.

The Applicant provided no experimental data to determine *arginine* in *water* [17]. Nevertheless, as concluded in the previous EURL reports and specified in the corresponding legislation (even if the determination of *arginine* in the *feed additive* and *water* is not explicitly stated in the scope of the Community method), the EURL recommends the Community method for official control for the quantification of *arginine* in the *feed additive* and *water* [6,7,13,14].

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated Community method based on IEC-VIS to quantify *arginine* in the *feed additive*, *premixtures*, *feedingstuffs* and *water*.

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)

For the identification of the *feed additive* the EURL recommends the "L-arginine monograph" of the Food Chemical Codex (FCC) where a test based on infrared absorption is described [21].

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 (i) the "*L-arginine*" monograph of the Food Chemical Codex (FCC) based on infrared absorption for the identification of *L-arginine* in the *feed additive* and (ii) the ring-trial validated Community method based on ion exchange chromatography coupled to visible detection (IEC-VIS) for the quantification of *arginine* in the *feed additive*, *premixtures*, *feedingstuffs* and *water*.

Recommended text for the register entry (analytical method)

For the identification of *L-arginine* in the *feed additive*:

- Food Chemical Codex "L-arginine monograph"

For the quantification of *L-arginine* in the *feed additive* and *water*:

- ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS)

For the quantification of *L-arginine* in *premixtures* and *feedingstuffs*:

- ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS), Commission Regulation (EC) No 152/2009 (Annex III, F)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *L-arginine produced by fermentation with Corynebacterium glutamicum KCCM80182* 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

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- [2] *Application, Reference SANTE/E5: Forw. Appl. 1831/0052-2018
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- [4] Commission Implementing Regulation (EC) No 2018/249 of 15 February 2018 concerning the authorisation of concerning the authorisation of taurine, beta-alanine, L-alanine, L-arginine, L-aspartic acid, L-histidine, D,L-isoleucine, L-leucine, L-phenylalanine, L-proline, D,L-serine, L-tyrosine, L-methionine, L-valine, L-cysteine, glycine, monosodium glutamate and L-glutamic acid as feed additives for all animal species and L-cysteine hydrochloride monohydrate for all species except cats and dogs, O.J. L 53/134, 23.02.2018
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- [8] *Technical dossier, Section II: II.2.2.1. Chemical substances
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- [18] *Technical dossier, Section II: Annex Annex_II_6_01 CJ L- His Method validation report
- [19] Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed, O.J. L 54, 26.02.2009
- [20] EN ISO 13903:2005- Animal feeding stuffs – Determination of amino acids content
- [21] Food Chemical Codex monograph "L-arginine", FCC 7 (2010) p.69

*Refers to Dossier no: FAD-2018-0045

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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