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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**

**L-arginine** produced by fermentation with  
***Corynebacterium glutamicum* (KCCM10741P)**  
*(FAD-2017-0031; CRL/170023)*





**Evaluation Report on the Analytical Methods submitted  
in connection with the Application for Authorisation of a  
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Dossier related to: **FAD-2017-0031 - CRL/170023**

Name of Products: ***L-arginine produced by fermentation with  
Corynebacterium glutamicum  
KCCM10741P***

Active Agent (s): **L-arginine**

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

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Date: **17/10/2017**

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Date: **17/10/2017**

## EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-arginine* produced by fermentation with *Corynebacterium glutamicum* KCCM10741P under the category/functional group 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. *L-arginine* is already authorised as nutritional feed additive under Commission Regulation (EC) No 1139/2007 and Commission Implementing Regulation (EU) 2016/972.

For the quantification of *arginine* in *feed additive*, *premixtures*, *feedingstuffs* and *water* the Applicant submitted the ring-trial validated Community method. This method applies for the determination of free (synthetic and natural) and of total (peptide-bound and free) amino acids, using an amino acid analyzer or a high performance liquid chromatographer equipped with an Ion Exchange Column (IEC) and photometric detection (VIS). Intended for *premixtures* and *feedingstuffs*, it does not distinguish between the salts of amino acids and it cannot differentiate the amino acid enantiomers. The following performance characteristics were reported for the quantification of total *arginine* in *premixtures* and *feedingstuffs*: a relative standard deviation for repeatability (RSD<sub>r</sub>) ranging from 2.3 to 3.3 % and a relative standard deviation for reproducibility (RSD<sub>R</sub>) ranging from 7.2 to 9.7 %. The Applicant did not perform the validation and verification of the method for the quantification of *L-arginine* in *feed additive* and *water*, but the experimental data reported in the frame of the stability studies clearly demonstrate the applicability (extension of the scope) of the method to these matrices. From the experimental data submitted, the EURL estimated performance characteristics that are in agreement with those reported in the ring-trial validated Community method. Moreover, the EURL identified the "L-arginine monograph" of the Food Chemical Codex (FCC) for the characterisation of the *feed additive*.

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 *feed additive*, *premixtures*, *feedingstuffs* and *water* and the Food Chemical Codex monograph for the identification of *L-arginine* in 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.

## KEYWORDS

*L-arginine produced by fermentation with Corynebacterium glutamicum KCCM10741P, nutritional additives, amino acid, their salts and analogues, all animal species*

## 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* KCCM10741P under the category/functional group 3(c) 'nutritional additives'/amino acids, their salts and analogues', according to Annex I of Regulation (EC) No 1831/2003 [1,2].

Authorisation is sought for all animal species. *L-arginine* is already authorised as nutritional feed additive under Commission Regulation (EC) No 1139/2007 and Commission Implementing Regulation (EU) 2016/972 [3,4].

According to the Applicant, *L-arginine* is a white or pale brownish crystalline powder with a minimum purity of 98% [5]. The *feed additive* is produced by fermentation with *Corynebacterium glutamicum*. The production strain is deposited in the "Korean Culture Collection of Microorganisms" (KCCM) with deposition number KCCM10741P [6].

*L-arginine* is intended to be mixed either in *premixtures* or added directly to *feedingstuffs* or *water* for drinking [7]. However, the Applicant proposed no minimum or maximum *arginine* content in *feedingstuffs* [1].

Note: The EURL previously evaluated the analytical methods for the determination of *L-arginine* in the frame of dossiers: FAD-2006-0009, FAD-2014-0012 and FAD-2016-0037 [8-10].

## 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 produced by fermentation with Corynebacterium glutamicum KCCM10741P* 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 [11].

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***Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and water***

The EURL found the "*L-arginine* monograph" of the Food Chemical Codex (FCC) for the characterisation of *L-arginine* in the *feed additive*, where identification is based on infrared absorption in combination with the analysis of the optical rotation (ranging from +25.8° to +27.9°), while quantification is based on titration with perchloric acid (0.1 N) [12].

For the quantification of *arginine* in *feed additive, premixtures, feedingstuffs* and *water* the Applicant submitted the ring-trial validated Community method [13,14]. This method applies for the determination of free (synthetic and natural) and of total (peptide-bound and free) amino acids in *premixtures* and *feedingstuffs*, using an amino acid analyzer or a High Performance Liquid Chromatographer equipped with an Ion Exchange Column (IEC). This method 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). The procedure chosen for the determination of the total amino acids depends on the amino acids under investigation. *L-arginine* can be determined in either oxidised or non-oxidised samples. Oxidation is performed at 0 °C with a performic acid/phenol mixture. Excess of 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.

The Community method was further ring-trial validated by twenty-three laboratories for the determination of total *arginine* and resulted in the standard method EN ISO 13903:2005 [15].

While the Applicant did not perform any validation and verification studies for the quantification of total *arginine* in *feed additive* and *water*, the experimental data provided in the frame of the stability studies [16,17] clearly demonstrate the applicability of this method to these two additional matrices (extension of scope). From the experimental data reported, the EURL estimated relative standard deviations for *repeatability* (RSD<sub>r</sub>) and relative standard deviation for *intermediate precision* (RSD<sub>ip</sub>) (Table 1) [18] that are in agreement with those reported in EN ISO 13903:2005.

**Table 1:** Method performance characteristics reported in EN ISO 13903:2005 for the determination of total *arginine* compared to the performance characteristics as estimated by the EURL from feed additive and water stability tests [15,18].

Study	Matrix	<i>arginine content</i> (g/kg)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)	RSD <sub>ip</sub> (%)
EN ISO 13903:2005	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	7.2	
Stability	feed additive [16]	994.5	0.45		0.51
	water [17]	0.5 (g/l)	0.5		0.57

RSD<sub>r</sub>, RSD<sub>R</sub> & RSD<sub>ip</sub> - relative standard deviation for *repeatability, reproducibility & intermediate precision* respectively

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated Community method, based on IEC-VIS to quantify total *arginine* in *feed additive, premixtures, feedingstuffs* and *water*. Furthermore the EURL recommends the Food Chemical Codex monograph for the identification of *L-arginine* in 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 the Food Chemical Codex "L-arginine" monograph based on infrared absorption and optical rotation for the identification of *L-arginine* in the *feed additive*; and the ring-trial validated Community method, using ion exchange chromatography (IEC) coupled with post-column derivatisation and photometric detection (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 *arginine* in the *feed additive* and *water*:

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

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

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

## 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* KCCM10741P 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, Proposal of Registry Entry – Annex A
- [2] \*Application, Reference SANTE/E5: FORW. APPL. 1831-0023-2017
- [3] Commission Regulation (EC) No 1139/2007 of 1 October 2007 concerning the authorisation of L-arginine as a feed additive, O.J. L 256/11, 02.10.2007
- [4] Commission Implementing Regulation (EU) 2016/972 of 17 June 2016 concerning the authorisation of L-arginine produced by *Corynebacterium glutamicum* KCTC 10423BP as a feed additive for all animal species, O.J. L 161/18, 18.06.2016
- [5] \*Technical dossier, Section II: II.2 Characterisation of the active substance(s)/agent(s)
- [6] \*Technical dossier, Section II: Annex II 2 01
- [7] \*Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
- [8] FAD-2006-0009, L-arginine, Ref. D.08/FSQ/CVH/GS/D(2007)2255 - 31/01/2007  
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- [9] FAD-2014-0012, L-arginine produced by fermentation with *Corynebacterium glutamicum* (KCTC 10423BP), Ref. Ares(2014)4293375 - 19/12/2014  
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- [10] FAD-2016-0037, L-arginine produced by fermentation with *Corynebacterium glutamicum* KCCM80099, Ref. Ares(2016)6204211 - 31/10/2016  
[https://ec.europa.eu/jrc/sites/jrcsh/files/finrep\\_fad-2016-0037\\_arginine.pdf](https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2016-0037_arginine.pdf)
- [11] 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
- [12] Food Chemical Codex monograph "L-Arginine", FCC 7 (2010), p. 69
- [13] \*Technical dossier, Section II: 2.6.1 Methods of analysis and reference samples
- [14] 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 (Annex III, F)
- [15] EN ISO 13903:2005 - Animal feeding stuffs – Determination of amino acids content
- [16] \*Technical dossier, Section II: Annex II 4 01
- [17] \*Technical dossier, Section II: Annex II 4 06
- [18] FAD-2017-0031\_L-Arginine\_EURL\_Calc.xlsx

\*Refers to Dossier no: FAD-2017-0031



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## **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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- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
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