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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  
**Escherichia coli (NITE BP-02186)**  
*(FAD-2017-0028; CRL/170009)*



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Dossier related to: **FAD-2017-0028 - CRL/170009**

Name of Products: ***L-arginine produced by fermentation with  
Escherichia coli (NITE BP-02186)***

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 *Escherichia coli* NITE BP-02186, under the category/functional group 3(c) 'nutritional additives'/amino acids, their salts and analogues' and 2(b) 'sensory additives'/flavouring compounds', 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 characterisation of the *feed additive*, the EURL identified the "L-arginine monograph" of the Food Chemical Codex (FCC).

For the quantification of *arginine* in the *feed additive* and *premixtures*, the Applicant submitted a validated and further verified method derived from the ring trial validated method described in EN ISO 17180:2013. The method is based on ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS). It is specifically intended for lysine, methionine and threonine in commercial *feed additives* and *premixtures*. The method does not distinguish between the salts of amino acids and it cannot differentiate between enantiomers. The corresponding method performance characteristics, recalculated by the EURL from the experimental data provided, are in agreement with those reported in the standard thus demonstrating the applicability of the slightly modified protocol to the quantification of *arginine* in the *feed additive* and *premixtures*.

For the quantification of *L-arginine* in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009). The method was further ring-trial validated by CEN resulting in EN ISO 13903:2005. The method is based on ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS). This method does not distinguish between the salts and the amino acid enantiomers. The following performance characteristics were reported for the quantification of total *arginine*: 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 %.

For the quantification of *L-arginine* in *water* the Applicant suggested a procedure based on the standard EN ISO 13903:2005. The Applicant did not perform a validation and verification study, but provided, in the frame of stability studies, the detailed experimental protocol used supported by experimental data. From the data reported the EURL calculated the following performance characteristics: RSD<sub>r</sub> ranging from 0.6 to 0.8 % and relative standard deviation for intermediate precision (RSD<sub>ip</sub>) ranging from 0.6 to 1.0 %. The performance characteristics obtained are in agreement with those stated in the EN ISO 13903:2005 standard thus

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demonstrating the applicability (extension of the scope) of the slightly modified protocol to the quantification of *arginine* in *water*.

Based on the performance characteristics available, the EURL recommends for official control: (i) the "*L-arginine* monograph" of the Food Chemical Codex (FCC) for the identification of *L-arginine* in the *feed additive*; (ii) the validated and further verified IEC-VIS analytical method for the quantification of *arginine* in the *feed additive*; (iii) the Community method based on IEC-VIS for the quantification of *arginine* in *premixtures* and *feedingstuffs*; and (iv) the IEC-VIS analytical method to quantify *arginine* in *water*.

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 Escherichia coli NITE BP-02186, nutritional additives, amino acid, their salts and analogues, sensory additives, flavouring compounds, 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 *Escherichia coli* NITE BP-02186, under the category/functional group 3(c) 'nutritional additives'/'amino acids, their salts and analogues' and 2(b) 'sensory additives'/'flavouring compounds', according to Annex I of Regulation (EC) No 1831/2003 [1,2].

The present 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 to pale yellow crystalline powder with a minimum purity of 98 % [5,6]. The *feed additive* is produced by fermentation with a genetically modified strain derived from *Escherichia coli* K-12 [7]. The production strain is registered in the "National Institute of Technology and Evaluation" (NITE) with deposition number NITE BP-02186.

*L-arginine* is intended to be mixed either in *premixtures* or added directly to *feedingstuffs* or *water* for drinking [8]. As sensory additive, it is normally added as constituent of flavouring *premixtures* [8]. However, the Applicant proposed no minimum or maximum *L-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 [9-11].

## 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 Escherichia coli* NITE BP-02186 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 [12].

##### ***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) [13].

Furthermore, the Applicant reported the "*Arginine*" monograph published in the European Pharmacopoeia where an assay method based on titration is described to quantify the *feed additive* [14-15]. However, the Applicant clearly stated that the method based on High Performance Liquid Chromatography (HPLC) is more practical both for the industry and for official control purposes. Therefore, for the quantification of *arginine* in the *feed additive* and *premixtures*, the Applicant submitted a validated and further verified method derived from the ring trial validated EN ISO 17180:2013 [14,16,17]. This standard method, specifically intended for lysine, methionine and threonine in commercial *feed additives* and *premixtures*, is based on the experimental protocol described in the Community method for amino acids [18]. None of these methods distinguish between the salts of amino acids and they cannot differentiate between enantiomers.

Free *arginine* is extracted with diluted hydrochloric acid and further diluted with sodium citrate buffer. After addition of norleucine as internal standard, the amino acid is separated by HPLC equipped with Ion Exchange Column (IEC). Free *arginine* is quantified photometrically after post-column derivatisation with ninhydrine and visible (VIS) detection at 570 nm.

The method performance characteristics presented in Table 1 were recalculated by the EURL [19]. They are in agreement with those reported in the EN ISO 17180:2013 standard thus demonstrating the applicability (extension of the scope) of the slightly modified protocol to the quantification of *arginine* in the *feed additive* and *premixtures* [16,19].

**Table 1:** Comparison of the method performance characteristics as reported in EN ISO 17180:2013 for the determination of lysine, methionine and threonine in *feed additive* and *premixtures* [16] and the ones recalculated by the EURL, for *arginine* [19].

Matrix	<i>amino acid</i>	<i>content (g/kg)</i>	$RSD_r$ (%)	$RSD_{ip}$ (%)
Feed Additive	Lysine [16]	459-760	0.8-0.9	1.8-2.3
	Methionine [16]	933	0.9	1.5
	Threonine [16]	955	1.2	2.2
	Arginine [19]	min. 965	0.9-1.4	0.9-2.6
Premixtures	Lysine [16]	102-240	0.7-1.7	1.5-2.5
	Methionine [16]	89-320	0.5-1.6	1.5-2.6
	Threonine [16]	82-221	0.7-1.4	1.9-2.3
	Arginine [19]	30	2.9	2.9

$RSD_r$  and  $RSD_{ip}$  - relative standard deviation for *repeatability* and *intermediate precision*, respectively

Based on the performance characteristics available, the EURL recommends for official control the validated and further verified IEC-VIS analytical method based on the EN ISO 17180:2013 standard for the quantification of *arginine* in the *feed additive*. Furthermore the EURL recommends the Food Chemical Codex monograph for the identification of *L-arginine* in the *feed additive*.

For the quantification of *L-arginine* in *feedingstuffs* the Applicant submitted the ring-trial validated Community method [14,18]. 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 HPLC equipped with an IE column. 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 (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.



**Table 2:** Method performance characteristics reported in EN ISO 13903:2005 for the determination of total *arginine* [20].

Matrix	<i>arginine</i> content (g/kg)	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
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<sub>r</sub> and RSD<sub>R</sub> - relative standard deviation for *repeatability* and *reproducibility*, respectively

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 [20]. The reported performance characteristics are listed in Table 2.

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*.

For the quantification of *L-arginine* in *water* the Applicant submitted a procedure based on the standard EN ISO 13903:2005 (equivalent to the Community method) [14,20].

*Arginine* is extracted with diluted hydrochloric acid. Norvaline is added as internal standard and the extract is further diluted with lithium citrate buffer. The amino acid is determined via IEC coupled to post-column derivatisation with ninhydrin and photometric detection at 570 nm. Quantification is obtained by comparing the peak areas of *arginine* and norvaline from the sample extracts with those obtained by the analysis of the calibration solution.

The Applicant did not perform any validation and verification studies, but provided in the frame of stability studies a detailed experimental protocol supported by experimental data [21]. From the data reported, the EURL calculated a relative standard deviation for *repeatability* (RSD<sub>r</sub>) and a relative standard deviation for *intermediate precision* (RSD<sub>ip</sub>), respectively, ranging from 0.6 to 0.8 % and from 0.6 to 1.0 % [19]. The performance characteristics obtained are in good agreement with those reported in the EN ISO 13903:2005 standard thus demonstrating the applicability (extension of the scope) of the slightly modified protocol to the quantification of *arginine* in *water* [20].

Based on the performance characteristics calculated, the EURL recommends for official control the IEC-VIS analytical method based on the EN ISO 13903:2005 standard (equivalent to the Community method) to quantify *arginine* in *water*.

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 in combination with optical rotation and titration for the identification of *L-arginine* in the *feed additive*; (ii) the validated and further verified IEC-VIS analytical method based on the EN ISO 17180:2013 standard for the quantification of *arginine* in the *feed additive*; (iii) the Community method based on IEC-VIS for the quantification of *arginine* in *premixtures* and *feedingstuffs*; and (iv) the IEC-VIS analytical method based on the EN ISO 13903:2005 standard to quantify *arginine* in *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 *Escherichia coli* NITE BP-02186 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. 1831-0019-2017
  - [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: 2.1.2 Proposal for classification
  - [6] \*Technical dossier, Section II: 2.1.5 Physical state of each form of the product
  - [7] \*Technical dossier, Section II: 2.3 Manufacturing process, including any processing procedures
  - [8] \*Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
  - [9] FAD-2006-0009, L-arginine, Ref. D.08/FSQ/CVH/GS/D(2007)2255 - 31/01/2007  
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  - [10] FAD-2014-0012, L-arginine produced by fermentation with *Corynebacterium glutamicum* (KCTC 10423BP), Ref. Ares(2014)4293375 - 19/12/2014  
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  - [11] 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)
  - [12] 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
  - [13] Food Chemical Codex monograph "L-Arginine", FCC 7 (2010), p. 69
  - [14] \*Technical dossier, Section II: 2.6.1 Methods of analysis and reference samples
  - [15] European Pharmacopoeia 8.2 Monograph 07/2014:0806 – Arginine
  - [16] EN ISO 17180:2013 - Animal feeding stuffs – Determination of lysine, methionine and threonine in commercial amino acid products and premixtures
  - [17] \*Technical dossier, Section II: Annex II\_30
  - [18] 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)
  - [19] FAD-2017-0028\_EURL\_Calculations.xlsx
  - [20] EN ISO 13903:2005 - Animal feeding stuffs – Determination of amino acids content
  - [21] \*Technical dossier, Section II: Annex II\_33
- \*Refers to Dossier no: FAD-2017-0028

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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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