



D08/FSQ/CVH/GS/D(2007)2255

CRL Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to: **EFSA-Q-2006-031**
FAD-2006-0009

Name of Additive: **L-arginine**

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

Rapporteur Laboratory: **Community Reference Laboratory for Feed Additives (CRL-FA), Geel, Belgium**

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13/12/06

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Date: **31/01/07**

EXECUTIVE SUMMARY

In the current application authorisation is sought for L-arginine under the category 'nutritional additives', functional group 'amino acids, their salts and analogues', according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought to use L-arginine for supplementing feed for all animal species. The product is a crystalline powder with a minimum content of 80 % L-arginine. The feed additive is intended to be included into feedingstuffs at a final concentration up to 5-6 % of total L-arginine, depending on the concentration of L-arginine already present in the feed components.

For the determination of the active substance (L-arginine) in the *feed additive* the applicant proposes a titrimetric method using perchloric acid. Since the feed additive contains L-arginine in its base form, the CRL considers the more appropriate titrimetric method using hydrochloric acid as prescribed by the Ph.Eur.. The applicant also provides a High Performance Liquid Chromatography (HPLC) method which is specific for the analyte, but without specifying related validation data. For official control purposes, the CRL recommends validated methods based on the same technique, such as the method 4.11.6 of the Association of German Agricultural Analytical and Research Institutes (VDLUFA) [Methodenbuch III, 5. Erg. 2004, VDLUFA – Verlag, Darmstadt] and the similar AOAC Method 999.13 [Fontaine and Eudaimon, J. of AOAC Int., Vol. 83, No. 4, 2000]. These methods have been validated for the quantitative determination of three free (non protein bound) amino acids (lysine, methionine and threonine) in feed grade amino acid commercial products and premixtures with more than 10 % individual amino acid content, and can be applied also for the determination of L-arginine.

The applicant does not describe whether the additive is intended to be directly incorporated into feedingstuffs or through *premixtures*. However, for the determination of L-arginine in *premixtures* for official controls, the same above mentioned Official or validated methods are recommended by the CRL.

For the determination of the active substance (L-arginine) in *feedingstuffs* the applicant proposes the official Community and fully ring-trial validated method for determination of amino acids [Commission Directive 98/64/EC]. The method is applicable for both the determination of free (synthetic and natural) and the determination of total (peptide-bound and free) amino acids, using an amino acid analyser or HPLC equipment with post column derivatisation with ninhydrin and photometric detection at 570 nm. The same method is adopted by ISO and described in the ISO standard 13903:2005 [Animal feedingstuffs – determination of amino acids content - ISO 13903:2005], which additionally reports the results from a second intercomparison study performed on different premixtures and feeds [Llames & Fontaine, J. of AOAC Int., Vol. 77, No. 6, 1994]. Performance characteristics for the target analyte (L-arginine) include the relative repeatability standard deviation (RSD_r) ranging from 2.34 to

3.31 % and relative reproducibility standard deviation (RSD_R) ranging from 7.18 to 9.66 %, depending on the matrix. The method does not distinguish between the salts of amino acids, nor differentiates between D and L forms of amino acids. The method is considered suitable for official controls.

Further testing or validation by the CRL is not considered necessary.

KEYWORDS

L-arginine, nutritional additive, amino acid

1. BACKGROUND

L-arginine is a feed additive for which authorisation is sought under the category 'nutritional additives', functional group 'amino acids, their salts and analogues', according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1]. According to the applicant it contains minimum 80 % L-arginine [1] as active substance produced by a strain of *Corynebacterium glutamicum* derived from *C. glutamicum* (ATCC-13870) by conventional mutation [2]. The fermentation medium consists of glucose syrup, ammonium sulphate, corn steep liquor, phosphoric acid, magnesium sulphate [3].

The intended use (*cf.* EFSA-Q-2006-031) of the current application is for all animal species, by inclusion of the product into feedingstuffs at a concentration depending on the concentration of L-arginine already present in the feed components, obtaining a final concentration not exceeding 5-6 % of total L-arginine in feedingstuffs [4].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 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 tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the methods of analysis submitted in connection with L-arginine, *cf.* EFSA-Q-2006-031, and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

Description of the method for the determination of the active substance in the feed additive and in feedingstuffs

For the determination of L-arginine in the *feed additive* the applicant proposed a titrimetric method using perchloric acid [5]. A similar method is prescribed by the European Pharmacopoeia (Ph. Eur.) for the assay of arginine hydrochloride [6]. Since the feed additive contains L-arginine in its base form, the CRL considers the more appropriate method prescribed by the Ph.Eur. which uses hydrochloric acid for the titration [7]. However, titration

procedures used for the assay of pure amino acids are unspecific and the accuracy of the results can be affected by impurities.

The applicant also provided a high performance liquid chromatography (HPLC) method used to analyse L-arginine for process control purposes [8], [9] and which is specific for this analyte. Related validation data have not been submitted, thus the CRL can not evaluate the suitability of the method.

Nevertheless, specific validated methods based on the same technique are available, such as the method 4.11.6 of the Association of German Agricultural Analytical and Research Institutes (VDLUFA) [10] and the similar AOAC Method 999.13 [11]. These methods are applicable for the quantitative determination of free (non protein bound) amino acids in feed grade amino acid commercial products and premixtures with more than 10 % individual amino acid content. The methods have been validated for the determination of lysine, methionine and threonine, but can be easily applied also for the determination of arginine. Therefore, these methods are recommended by the CRL for official control purposes.

For the determination of L-arginine in *feedingstuffs* the applicant proposed the official Community and fully ring-trial validated method for determination of amino acids [12].

The method is applicable for both the determination of *free* (synthetic and natural) and the determination of *total* (peptide-bound and free) amino acids, using an amino acid analyser or HPLC equipment. The *free* amino acids are extracted with diluted hydrochloric acid. Coextracted nitrogenous macromolecules are precipitated with sulfosalicylic acid. The solution is filtered and adjusted to pH 2.20. The amino acids are separated by ion exchange chromatography and determined by post column derivatisation with ninhydrin and photometric detection at 570 nm. The procedure chosen for the determination of the *total* amino acids depends on the amino acids under investigation. The target analyte (L-arginine) can be determined in either oxidised or unoxidised samples. Oxidation is required when measuring simultaneously cyst(e)ine and methionine and the oxidation procedure is performed at 0 °C with a performic acid/phenol mixture. Excess oxidation reagent is decomposed with sodium disulphite. The oxidised or unoxidised sample is hydrolysed with hydrochloric acid (6 mol/L) for 23 hours. The hydrolysate is adjusted to pH 2.20. Amino acids are separated by ion exchange chromatography and determined by post column derivatisation with ninhydrin and photometric detection at 570 nm. The same method is adopted by ISO and described in the ISO standard 13903:2005 [13], which reports the results from a second intercomparison study involving twenty-three laboratories applying the procedure for total amino acid to five different matrixes (broiler finisher feed, broiler starter feed, corn, fishmeal and poultry meal) [14]. Performance characteristics for the target analyte (L-arginine) included the relative repeatability standard deviation (RSD_r) ranging between 2.34 and 3.31 % and relative reproducibility standard deviation (RSD_R) ranging between 7.18

and 9.66 %, depending on the matrix. The method does not distinguish between the salts of amino acids, nor differentiates between D and L forms of amino acids.

The method is considered suitable for official controls for the determination of free and total L-arginine in *feedingstuffs*.

Identification/Characterisation of the feed additive

The product is a white or almost white crystalline powder with a minimum content of 80 % L-arginine and a maximum content of 18 % water [15].

Qualitative and quantitative composition of inorganic impurities in the additive

For the determination of heavy metals (Pb, Cd and Hg) and arsenic (As), the applicant proposed the methods described in the United States Pharmacopoeia and in the Japan Pharmacopoeia [5].

For official controls various standard methods based on atomic absorption spectrophotometry (AAS), inductively coupled plasma mass spectrometry (ICP-MS) and cold vapour atomic fluorescence spectrophotometry (CV-AFS) routinely applied by official control authorities are available and recommended by the CRL.

4. CONCLUSIONS AND RECOMMENDATIONS

For the determination of the active substance (L-arginine) in the *feed additive* the applicant proposed a titrimetric method using perchloric acid. Since the feed additive contains L-arginine in its base form, the CRL considers the titrimetric method using hydrochloric acid as prescribed by the Ph.Eur. as more appropriate technique. The applicant also provided an HPLC method which is specific for the analyte without providing the related validation data. For official control purposes, the CRL recommends specific validated methods based on the same technique, such as the VDLUFA method 4.11.6 and the similar AOAC Method 999.13. These methods have been validated for the quantitative determination of three free (non protein bound) amino acids (lysine, methionine and threonine) in feed grade amino acid commercial products and premixtures with more than 10 % individual amino acid content, and can be easily applied also for the determination of arginine.

For the determination of the active substance (L-arginine) in *feedingstuffs* the official Community and fully ring-trial validated method for determination of amino acids is proposed by the applicant. The same method is described in the ISO standard 13903:2005 which additionally reports results from a second intercomparison study. Although not explicitly

mentioned, the performance characteristics were obtained for the determination of *total* L-arginine. Nevertheless, the method is considered suitable for official controls and the CRL recommends using the same performance characteristics both for the determination of total and free L-arginine in *feedingstuffs*.

The applicant does not describe whether the additive is intended to be directly incorporated into feedingstuffs or through *premixtures*. However, for the determination of L-arginine in *premixtures* for official controls, the same above mentioned official or ring-trial validated methods are recommended by the CRL.

For the determination of heavy metals (Pb, Cd and Hg) and arsenic (As), the applicant proposed the methods described in the United States Pharmacopoeia and in the Japan Pharmacopoeia. For official controls various standard methods based on atomic absorption spectrophotometry (AAS), inductively coupled plasma mass spectrometry (ICP-MS) and cold vapour atomic fluorescence spectrophotometry (CV-AFS) routinely applied by official control authorities are available and recommended by the CRL.

Further testing or validation by the CRL is not considered necessary.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

Community method for the determination of aminoacids (Commission Directive 98/64/EC amending Directive 71/393/EEC).

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of L-arginine have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

6. REFERENCES

- [1] Annex III, Proposal of Register entry
- [2] Main dossier , Section I, 1
- [3] Main dossier, Section I, 2
- [4] Main dossier, Section I, 4
- [5] Main dossier, Appendix D
- [6] European Pharmacopoeia 5th Edition, monograph 01/2005:0805 (Arginine hydrochloride)

- [7] European Pharmacopoeia 5th Edition, monograph 01/2005:0806 (Arginine)
- [8] Supplementary dossier July 2006, Section I, 5
- [9] Supplementary dossier July 2006, Appendix 10
- [10] Bestimmung von Lysin, Methionin und Threonin in Aminosäurehandelsprodukten und Vormischungen – 4.11.6, Methodenbuch III, 5. Erg. 2004, VDLUFA – Verlag, Darmstadt
- [11] Fontaine and Eudaimon, J. of AOAC Int., Vol. 83, No. 4, 2000
- [12] Commission Directive 98/64/EC of 3 September 1998 establishing Community methods of analysis for the determination of amino-acids, crude oils and fats, and olaquinox in feedingstuffs and amending Directive 71/393/EEC
- [13] Animal feedingstuffs – determination of amino acids content (ISO 13903:2005)
- [14] Llames & Fontaine, J. of AOAC Int., Vol. 77, No. 6, 1994
- [15] Supplementary dossier, Section I, 3

7. RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additives (CRL-FA), Geel, Belgium.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Central Institute for Supervising and Testing in Agriculture, Czech Republic.
- Plantedirektoratets Laboratorium, Denmark.
- Service Commun des Laboratoires du MINEFI, France.
- Thüringer Landesanstalt für Landwirtschaft, Germany.
- Sachsische Landesanstalt für Landwirtschaft, Germany.
- Landwirtschaftliche Untersuchungs- und Forschungsanstalt Speyer, Germany.
- National Research Institute of Animal Production in Krakow, Poland.
- Laboratori Agroalimentari, Department d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya, Spain.
- The Laboratory of the Government Chemist, United Kingdom.