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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:	FAD-2009-0001 CRL/070024
Name of Additive:	L-isoleucine
Active Subtance(s):	L-isoleucine
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives (CRL-FA), Geel, Belgium
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EXECUTIVE SUMMARY

In the current application authorisation is sought for L-isoleucine 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-isoleucine for supplementing feed for all animal species. The product is a crystalline powder with a minimum content of 92 % L-isoleucine. The feed additive is intended to be included into premixtures and feedingstuffs at a final concentration depending on the concentration of L-isoleucine already present in the feed components and on the nutritional requirements of the different animal species.

For the determination of the active substance (L-isoleucine) in the *feed additive*, *premixtures*, and *feedingstuffs* the applicant proposes the official Community and fully ring-trial validated method for determination of amino acids [Commission Regulation (EC) No 152/2009]. The method is applicable for both the determination of free (synthetic and natural) and the determination of total (peptide-bound and free) amino acids including L-isoleucine, using an amino acid analyser or High Pressure Liquid Chromatography (HPLC) combined with post-column derivatisation using ninhydrin as derivatisation agent 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], 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-isoleucine) include the relative standard deviation for repeatability (RSD_r) ranging from 2.00 to 5.38 % and relative standard deviation for reproducibility (RSD_R) ranging from 6.84 to 14.62 %, depending on the matrix. The method is suitable for official controls for the determination of free and total L-isoleucine in *feedingstuffs*. Although performance characteristics for the *feed additive* itself and, for *premixtures* are not available, the method can be considered suitable also for official control of active substance in these matrices. It is not suitable to differentiate between the salts or D- and L-forms of amino acids, or between naturally occuring and added L-isoleucine.

Alternatively, validated methods based on the same techniques, 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] can complement the official Community method for the determination of L-isoleucine in the *feed additive* and in *premixtures* and therefore are considered suitable for official control purposes in the frame of the authorisation.

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

KEYWORDS

L-isoleucine, nutritional additive, amino acid

1. BACKGROUND

L-isoleucine is a product for which authorisation as feed additive 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 the product contains a minimum of 92 % L-isoleucine [2] as active substance produced by fermentation using the genetically modified strain AG3149 derived from *E. Coli K-12* [3], deposited in a Japanese international recognised culture collection as FERM ABP-10641 [4]. The fermentation medium includes cereal starch hydrolysates, ammonium sulphate, ammonia, amino nitrogen provided by hydrolysates of soybean flakes/meal or amino acids, mineral salts and vitamins, antifoaming agent [5].

The intended use of the current application is for all animal species, by inclusion of the product into premixtures and feedingstuffs at a concentration depending on the concentration of L-isoleucine already present in the feed components and on the nutritional requirements of the different animal species [2].

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-isoleucine (FAD-2009-0001) 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

The product is a white crystalline powder with a minimum content of 92 % L-isoleucine and a maximum content of 1.5 % water [2].

Methods of analysis of the active substance in the feed additive, premixtures, and feedingstuffs

For the determination of L-isoleucine in the *feed additive*, in *premixtures* and in *feedingstuffs* the applicant proposed the official Community and fully ring-trial validated method for determination of amino acids [6].

The method is applicable for both the determination of *free* (synthetic and natural) and the determination of *total* (peptide-bound and free) amino acids (including L-isoleucine), using an amino acid analyser or HPLC. 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 2.2. The amino acids are separated by ion exchange chromatography and determined by post-column derivatisation with ninhydrin and photometric detection at 570 nm. Two different procedures – one of them involving an oxidation step, can be applied for the determination of *total* amino acids, depending on the amino acids under investigation. Oxidation is required when measuring simultaneously cyst(e)ine and methionine and is performed at 0 °C in a performic acid/phenol mixture, whilst the target analyte (L-isoleucine) can be determined in either oxidised or unoxidised samples. 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 2.2. 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 [7],

which reports the results from a second intercomparison study involving twenty-three laboratories applying the procedure for total amino acid to five different matrices (broiler finisher feed, broiler starter feed, corn, fishmeal and poultry meal) [8]. Performance characteristics for the target analyte (L-isoleucine) included the relative standard deviation for repeatability (RSD_r) ranging between 2.00 and 5.38 % and relative standard deviation for reproducibility (RSD_R) ranging between 6.84 and 14.62 %, depending on the matrix. The method does not distinguish between the salts of amino acids, between D- and L-forms of amino acids, nor between naturally occurring or added L-isoleucine. The method is suitable for official controls for the determination of free and total L-isoleucine in *feedingstuffs.* Although performance characteristics for the *feed additive* itself and, for *premixtures* are not available, the method can be considered suitable also for official control of active substance in these matrices.

In addition, the CRL notes that specific validated methods based on the same techniques are available, such as the method 4.11.6 of the Association of German Agricultural Analytical and Research Institutes (VDLUFA) [9] and the similar AOAC Method 999.13 [10]. 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 isoleucine. These methods can complement the official Community method for the determination of L-isoleucine in the *feed additive* and in *premixtures* and therefore are considered suitable for official control purposes in the frame of the authorisation.

4. CONCLUSIONS AND RECOMMENDATIONS

For the determination of the active substance (L-isoleucine) 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-isoleucine. The method is considered suitable for official control and can be considered suitable also for official control of active substance in the *feed additive,* and in *premixtures*.

Alternatively, specific validated methods based on the same techniques, such as the VDLUFA method 4.11.6 and the similar AOAC Method 999.13. can complement the official Community method for the determination of L-isoleucine in the *feed additive* and in *premixtures* for official control purposes.

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 Regulation (EC) No 152/2009).

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of L-isoleucine 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] Regulation (EC) No 1831/2003
[2] *Annex A, Proposal for Register entry
[3] *Technical dossier, Section 2.2.1
[4] *Technical dossier, Section 2.2.1.2.B.5
[5] *Technical dossier, Section 2.2.5
[6] 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, p. 1
[7] Animal feedingstuffs – determination of amino acids content (ISO 13903:2005)
[8] Llames & Fontaine, J. of AOAC Int. (1994), Vol. 77, No. 6, 1362–1402
[9] Bestimmung von Lysin, Methionin und Threonin in Aminosäurenhandelsprodukten und Vormischungen – 4.11.6, Methodenbuch III, 5. Erg. 2004, VDLUFA – Verlag, Darmstadt
[10] Fontaine and Eudaimon, J. of AOAC Int. (2000), Vol. 83, No. 4, 771-783

* Refers to Dossier No: FAD-2009-0001

7. RAPPORTEUR LABORATORY

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