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Institute for Reference Materials and Measurements
**Community Reference Laboratory for Feed Additives**

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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

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| Dossier related to: | EFSA-Q-2007-103<br>FAD-2007-0015 |
| Name of Additive: | L-valine Feed Grade |
| Active Subtance(s): | L-valine |
| 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-valine Feed Grade 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-valine Feed Grade for supplementing feed for all animal species. The product is a crystalline powder with a minimum content of 95 % L-valine. The feed additive is intended to be included into feedingstuffs at a final concentration up to 2500 to 3000 mg of total L-valine/ kg complete feedingstuffs, depending on the concentration of L-valine already present in the feed components.

For the determination of the active substance (L-valine) 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 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 High Pressure Liquid Chromatography (HPLC) equipment combined with post column derivatisation using 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], 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-valine) include the relative standard deviation for repeatability ($RSD_r$) ranging from 1.71 to 3.80 % and relative standard deviation for reproducibility ($RSD_R$) ranging from 8.83 to 16.05 %, depending on the matrix. The method is considered suitable for official controls. It is not suitable to differentiate between the salts or D- and L-forms of amino acids, or between naturally occuring and added L-valine.

Alternatively, for the determination of the active substance (L-valine) in the *feed additive* and *premixtures* for official controls, the CRL considers validated methods based on the same technique suitable, 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) at an individual concentration of more than 10 % in feed grade amino acid commercial products. The methods are therefore also considered applicable for the quantification of L-valine.
Further testing or validation by the CRL is not considered necessary.

## KEYWORDS

L-valine, nutritional additive, amino acid

## 1. BACKGROUND

L-valine 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 a minimum of 95 % L-valine [2] as active substance produced by fermentation using the genetically modified strain AG314 derived from *E. Coli K-12* [3]. The fermentation medium includes cereal starch hydrolysates, ammonium sulphate, ammonia, amino nitrogen provided by hydrolysates of soybean flakes/meal, mineral salts and vitamins, antifoaming agent [4].

The intended use (*cf.* EFSA-Q-2007-103) 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-valine already present in the feed components, obtaining a final level not exceeding 2500 to 3000 mg of total L-valine / kg complete feedingstuffs [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-valine, *cf.* EFSA-Q-2007-103 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 95 % L-valine and a maximum content of 1 % water [2], [5].

*Qualitative and quantitative composition of impurities in the additive*

Analysis for potential presence of undesirable substances/contaminants and microbial quality were performed on batches from the pilot scale production using standardised or internal methods [6], [7].

For official controls various standard methods routinely applied by official control authorities are available and recommended by the CRL.

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

For the determination of L-valine 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 [8].

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 2.2. 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-valine) 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 [9], 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) [10]. Performance characteristics for the target analyte (L-valine) included the relative standard deviation for repeatability ($RSD_r$) ranging between 1.71 and 3.80 % and relative standard deviation for reproducibility ($RSD_R$) ranging between 8.83 and 16.05 %, 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-valine. The method is considered suitable for official controls for the determination of free and total L-valine in the *feed additive*, in *premixtures* and in *feedingstuffs*.

In addition, the CRL notes that 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) [11] 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 valine. Therefore, these methods are also considered suitable for the determination of L-valine in the *feed additive* and in *premixtures* for official control purposes in the frame of the authorisation.

## 4. CONCLUSIONS AND RECOMMENDATIONS

For the determination of the active substance (L-valine) in the *feed additive, premixtures,* and *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-valine. Nevertheless, the same performance characteristics can be used both for the determination of total and free L-valine in *feedingstuffs*.

The method is considered suitable for official controls.

Alternatively, for the determination of L-valine in the *feed additive* and in *premixtures* 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.

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-valine 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 III, Proposal of Register entry
[3] Technical dossier, Section 2.2.2.2
[4] Technical dossier, Section 2.2.5
[5] Technical dossier, Section 2.2.5.3
[6] Technical dossier, Section 2.2.3.3
[7] Technical dossier, Section 2.5.1
[8] 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 olaquindox in feedingstuffs and amending Directive 71/393/EEC
[9] Animal feedingstuffs – determination of amino acids content (ISO 13903:2005)
[10] Llames & Fontaine, J. of AOAC Int. (1994), Vol. 77, No. 6, 1362–1402
[11] Bestimmung von Lysin, Methionin und Threonin in Aminosäurenhandelsprodukten und Vormischungen – 4.11.6, Methodenbuch III, 5. Erg. 2004, VDLUFA – Verlag, Darmstadt
[12] Fontaine and Eudaimon, J. of AOAC Int. (2000), Vol. 83, No. 4, 771-783

## 7. RAPPORTEUR LABORATORY

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