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Community Reference Laboratory for Feed Additives Authorisation

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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:	EFSA-Q-2005-230
Name of Additive:	VitaLys® Liquid VitaLys® Dry
Active Subtance(s):	L-lysine
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additives Authorisations (CRL-FAA), Geel, Belgium
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EXECUTIVE SUMMARY

In the current application authorisation is sought for VitaLys® 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 VitaLys® in its two forms, VitaLys® Liquid and VitaLys® Dry, for all animal species. VitaLys® Liquid is a brown liquid lysine concentrate with a minimum content of 25 % L-lysine and a maximum water content of 40 %. VitaLys® Dry is a light brown, powder lysine concentrate with a minimum content of 44 % L-lysine and a maximum content of 5 % moisture. The feed additive is intended to be mixed into premixtures, mineral mixed feedingstuffs, complementary or complete feedingstuffs at a final concentration range depending on both the nutritional requirements of the animals and the concentration of L-lysine in the feed materials contained in the complete feedingstuffs.

For the determination of the active substance (L-lysine) in the *feed additive*, in *premixtures* and in *feedingstuffs* the official Community and fully ring-trial validated method for determination of amino acids [Commission Directive 98/64/EC] is proposed by the applicant. 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 analyzer or an High Performance Liquid Chromatography (HPLC) equipment with post column derivatisation with ninhydrin and photometric detection at 570 nm. The method's performance characteristics related to the target analyte include a relative repeatability standard deviation (RSD_r) ranging between 2.1 and 2.8 % and a relative reproducibility standard deviation (RSD_R) ranging between 3.0 and 6.7 %, depending on the matrix.
The same method is described in the ISO standard 13903:2005 [Animal feedingstuffs – determination of amino acids content - ISO 13903:2005], which reports also the results from a second intercomparison study [Llames & Fontaine, J. of AOAC Int., Vol. 77, No. 6, 1994]. Performance characteristics for the target analyte (L-lysine) included the relative repeatability standard deviation (RSD_r) ranging between 2.37 and 3.46 % and relative reproducibility standard deviation (RSD_R) ranging between 7.94 and 13.08 %, depending on the matrix. The reported limits of quantification (LOQ) are 0.3 g/kg for total lysine and 0.035 g/kg for free lysine. The method is considered suitable for official control purposes for the determination of total and free L-lysine in *feedingstuffs* and in *premixtures*.

However, for official control purposes regarding the determination of free L-lysine in the *feed additive* the CRL recommends a fully ring trial validated method which is very similar to the above mentioned Community method and which is explicitly designed for the determination of *free* amino acids in feed additives. The method has been published in the method collection of the "Association of German Agricultural Analytical and Research Institutes" (VDLUFA, Germany) [VDLUFA Methodenbuch III, 1993 2. Erg., Aminosaeuren 4.11.1]. The reported RSD_R of the method for the determination of free L-lysine in a feed additive matrix was 1.95 %.
Neither method distinguishes between the salts of amino acids, nor differentiates between D and L forms of amino acids.

For the identification/characterisation of the producer micro-organism the applicant proposed a molecular method for the detection of *Corynebacterium glutamicum* (DSM 14764) based on colony hybridization developed by the Flemming Jørgensen Biotechnological Institute (Denmark). The method is considered suitable for the intended purpose.

For the determination of heavy metals (As, Pb and Cd), mycotoxins (aflatoxin B1, B2, G1 and G2) and dioxins (PCDD/PCDF) the applicant proposed standard methods which are considered suitable for official control purposes. Internal methods are proposed by the applicant for other mycotoxins (ochratoxin, zearalenone, T2, deoxynivalenol (DON)). For official control purposes fully ring trial validated methods that are available at the CRL are recommended.

Methods issued by the Nordic Committee on Food Analysis are proposed by the applicant for microbiological quality of the final products, which the CRL considers suitable for the intended purpose. However, for official control purposes the CRL recommends corresponding ISO/CEN standard methods.

Further testing or validation is not considered necessary.

KEYWORDS

VitaLys® Dry, VitaLys® Liquid, L-Lysine sulphate, nutritional additive, amino acid

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1. BACKGROUND

VitaLys® 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]. It contains L-lysine (as sulphate salt) as active substance produced with *Corynebacterium glutamicum* (DSM 14764). The substrate consists of syrup, molasses, fermented grass juice from the green crop drying industry, grain, starch products and hydrolysates hereof [2]. The producer micro-organism strain is deposited at the "Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh (DSMZ)".

VitaLys® is presented in two different forms:

- VitaLys® Liquid
- VitaLys® Dry

A similar product is already authorised as feed additive for all animal species (Commission Directive 88/485/EEC) produced using a different substrate which consists of syrup, molasses, cereals, starch products and their hydrolysates.

The intended use (*cf.* EFSA-Q-2005-230) of the current application is for all animal species, by mixing the feed additive into premixtures, mineral mixed feedingstuffs, complementary or complete feedingstuffs [3] at a final concentration range depending on both the nutritional requirements of the animals and the concentration of L-lysine in the used feed materials contained in the complete feedingstuffs (*cf.* proposed Registry Entry)

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 suitability of the methods of analysis and validation studies submitted in connection with VitaLys®, *cf.* EFSA-Q-2005-230, was evaluated.

3. EVALUATION

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

For the determination of L-lysine in the *feed additive,* in *premixtures* and in *feedingstuffs the* official Community and fully ring-trial validated method for determination of amino acids [4] has been proposed by the applicant [5].

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 analyzer 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-lysine) can be determined in either oxidised or unoxidised samples. Oxidation 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. The amino acids are separated by ion exchange chromatography and determined by post column derivatisation with ninhydrin and photometric detection at 570 nm. The method has been validated in an international intercomparison study using four different matrixes, which were mixed pig feed, broiler compound feed, protein concentrate and premixture. Performance characteristics for the target

analyte, include a relative repeatability standard deviation (RSD_r) ranging between 2.1 and 2.8 %, depending on the matrix and a relative reproducibility standard deviation (RSD_R) ranging between 3.0 and 6.7 %, depending on the matrix.

The same method is described in the ISO standard 13903:2005 [6], which reports also 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) [7]. Performance characteristics for the target analyte (L-lysine) include a RSD_r ranging between 2.37 and 3.46 %, depending on the matrix and a RSD_R ranging between 7.94 and 13.08 %, depending on the matrix. The reported limits of quantification (LOQ) are 0.3 g/kg for total lysine and 0.035 g/kg for free lysine.

The method is considered suitable for official control purposes for the determination of total and free L-lysine in *feedingstuffs* and for total L-lysine in *premixtures*.

For official control regarding the determination of *free* L-lysine in the *feed additive* the CRL recommends a fully ring trial validated method conducted at relevant concentration of the active substance in four premixtures and one commercial product. This method follows the sample principle than the official Community method for the determination of free L-lysine, i.e. extraction of the sample with diluted hydrochloric acid and measuring the target analyte with an aminoacid analyser of HPLC after post-column derivatisation. The method and the results from the related interlaboratory study are presented in the method collection of the "Association of German Agricultural Analytical and Research Institutes" (VDLUFA, Germany) [8]. The reported values for the RSD_R was 1.95 % for a L-lysine commercial product.

Identification/Characterisation of the feed additive

VitaLys® Liquid is a brown liquid lysine concentrate with a minimum content of 25 % L-lysine, 60 % dry matter and a maximum water content of 40 %.

VitaLys® Dry is a light brown, powder lysine concentrate with a minimum content of 44 % L-lysine, 95 % dry matter and a maximum content of 5 % moisture.

VitaLys® is produced with *Corynebacterium glutamicum* (DSM 14764).

Identification of the producer micro-organism

For the detection and identification of the producer micro-organism during the manufacturing process the applicant proposed a molecular method for detection of *Corynebacterium*

glutamicum (DSM 14764) based on colony hybridization developed by the Flemming Jørgensen Biotechnological Institute (Denmark) [9].

The micro-organisms are grown (2 days at 30 °C) on well dried plates using lysine maintenance medium (LM). Single colonies are inoculated into a microtitre plate containing LM broth and incubated overnight with shaking at 30 °C. The bacteria are then transferred by vacuum filtration from the liquid culture to a nylon filter using a dot-blot manifold. The DNA is released by lysis of the cells by treatment with lysozyme, then fixed on the filter and treated with proteinase K. A DNA probe is produced by standard Polymerase Chain Reaction (PCR) reaction using *lysE* genes and is labelled with digoxigenin. Hybridization is carried out overnight at 65 °C. The digoxigenin labelled probe is treated with an antibody (anti-digoxigenin) linked to alkaline phosphatase. The complex is detected by a colour reaction.

The method is considered suitable for the purpose of identification of the producer micro-organism.

Absence of the producer micro-organism in the final product

Both VitaLys® Liquid and VitaLys® Dry are tested for absence of the producer micro-organism [10]. Samples taken from the final products are sprayed on LM plates. After incubation (2 days at 30 °C), any colonies are identified by the same hybridization method described above, which is considered appropriate for the intended purpose.

Determination of moisture content

For the determination of moisture content in the feed additive the official Community method for determination of moisture [11] has been proposed by the applicant. The method is considered suitable for the intended purpose.

Qualitative and quantitative composition of any impurities in the additive

For the determination of heavy metals (As, Pb and Cd), mycotoxins (aflatoxin B1, B2, G1 and G2) and dioxins (PCDD/PCDF) the applicant proposed standard methods which are applied by an external accredited laboratory for performing the analysis [12]. These methods are considered suitable for official control purposes.

The same laboratory applies internal methods for the determination of other mycotoxins (ochratoxin, zearalenone, T2, DON) [12]. For official control purposes fully ring trial validated methods that are available at the CRL are recommended.

Microbiological quality of the final products was ensured by investigating *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella*, moulds and yeasts, *Enterobacteriaceae* and aerobic micro-organisms. The applicant proposed the methods issued by the Nordic Committee on Food Analysis [10]. The methods are considered suitable for the intended purpose. However, for official control purposes the corresponding ISO/CEN standards are recommended by the CRL.

4. CONCLUSIONS AND RECOMMENDATIONS

For the determination of the active substance (L-lysine) in the *feed additive*, in *premixtures* and in *feedingstuffs* the official Community and fully ring-trial validated method for determination of amino acids [4] is proposed by the applicant [5]. The same method is described in the ISO standard 13903:2005 [6] which additionally reports results from a second intercomparison study [7]. The method is considered suitable for official control purposes for the determination of total and free L-lysine in *feedingstuffs* and in *premixtures*. Neither the Commission Directive nor the ISO standard explicitly indicate, whether the performance characteristics were obtained for the determination of the *free* L-lysine or for the determination of *total* L-lysine. Therefore the CRL recommends using the same performance characteristics for both methods.

For official control purposes regarding the determination of free L-lysine in the *feed additive* the CRL recommends a fully ring trial validated method from the method collection of the "Association of German Agricultural Analytical and Research Institutes" (VDLUFA, Germany) [8].

Identification/characterisation of the producer micro-organism is performed by detection of *Corynebacterium glutamicum* (DSM 14764) by colony hybridization [9]. The method is considered suitable for the intended purpose.

For the determination of moisture content in the feed additive the official Community method for determination of moisture [11] has been proposed by the applicant.

For the determination of heavy metals (As, Pb and Cd), mycotoxins (aflatoxin B1, B2, G1 and G2) and dioxins (PCDD/PCDF) the applicant proposed standard methods [12] which are

considered suitable for official control purposes. Internal methods are proposed by the applicant for other mycotoxins (ochratoxin, zearalenone, T2, DON) [12]. For official control purposes fully ring trial validated methods that are available at the CRL are recommended.

Methods issued by the Nordic Committee on Food Analysis are proposed by the applicant for microbiological quality of the final products [10]. These methods are considered suitable for the intended purpose by the CRL. However, the corresponding ISO/CEN standards are recommended for official control purposes.

5. DOCUMENTATION AND SAMPLES PROVIDED TO CRL

In accordance with the requirements of Regulation (EC) No 1831/2003, samples of VitaLys® Dry and VitaLys® Liquid have been sent to the Community Reference Laboratory for Feed Additives Authorisation.

The dossier has been made available to the CRL by EFSA.

6. REFERENCES

[1] Reference SANCO/D/1 Forw. Appl. 1831/021-2005

[2] Annex III Proposal of Register entry

[3] Section I, Subject 4, Item 4.3

[4] 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

[5] Section I, Subject 5

[6] Animal feedingstuffs – determination of amino acids content (ISO 13903:2005)

[7] Llames & Fontaine, J. of AOAC Int., Vol. 77, No. 6, 1994

[8] VDLUFA Methodenbuch III, 1993 2. Erg., Aminosauren 4.11.1

[9] Appendix, Section I, Item 2.2, Annex No. 3

[10] Appendix, Section I, Subject 3, Item 3.2.7

[11] Second Commission Directive 71/393/EEC of 18 November 1971 establishing Community methods of analysis for the official control of feedingstuffs

[12] Additional information submitted by the applicant (e-mail of 23 th March, 2006)

7. RAPPORTEUR LABORATORY

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