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EURL 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

Dossier related to:	FAD-2010-0067 EURL/ 100037
Name of Feed Additive:	Lysine as - Concentrated liquid lysine (base) - Lysine HCl, technical pure - L-lysine sulphate (solid) - L-lysine sulphate (liquid)
Active Agent (s):	Lysine
Rapporteur Laboratory:	European Union Reference Laboratory for Feed Additives (EURL-FA) Geel, Belgium
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Date:	18/03/2011
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Date:	18/03/2011

EXECUTIVE SUMMARY

In the current application authorisation is sought for four different forms of *Lysine* under Articles 4(1) (new use in feed) and 10(2) (re-evaluation of the already authorized feed additive – Council directive 70/524/EEC), under category 'nutritional additives' and functional group 3(c) 'amino acids, their salts and analogues' according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *Lysine* for all animal species and categories. The *feed additive* is proposed to be registered in four different forms: - *concentrated liquid Lysine (base)*; - *Lysine monohydrochloride (HCl) technically pure*; - *L-lysine sulphate solid form produced by fermentation with Corynebacterium glutamicum;* and - *Lysine sulphate liquid form produced by fermentation with Corynebacterium glutamicum*. The *feed additive* is intended to be mixed either in *premixtures* or to be added directly to complete *feedingstuffs* or *water*.

For the determination of *Lysine* in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method for amino acids (Commission Regulation (EC) No 152/2009). The method applies for the determination of *free* (synthetic and natural) and *total* (peptide-bound and free) amino acids, using High Performance Liquid Chromatography (HPLC) equipment. However, only performance characteristics for the determination of <u>total *Lysine*</u> were reported:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 2.1 to 2.8%;
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 3 to 6.7%.

Based on the performance characteristics presented, the EURL recommends for official control, the ring-trial validated Community method based on High Performance Liquid chromatography (HPLC) coupled with post-column derivatisation to determine <u>total *Lysine*</u> in *premixtures* and *feedingstuffs*.

The EURL identified two alternative ring-trial validated methods based on the <u>same</u> principle of the Community method (i.e. extraction of the sample with diluted hydrochloric acid and measuring the target analyte with HPLC coupled with post-column derivatisation system):

(i) the method developed by the “Association of Official Agricultural Chemists” "*Lysine*, Methionine and Threonine in Feed Grade Amino Acids and Premixes" (AOAC 999:13 - 2004); and

(ii) the similar method developed by the “Association of German Agricultural Analytical and Research Institutes” (VDLUFA, Germany – Method 4.11.6). Both methods are explicitly designed to determine <u>free *Lysine*</u> in *feed additive* and *premixtures* at amino acid contents higher than 100 g/kg.

The following performance characteristics were reported:

- RSD_r ranging from 0.5 to 3%; and
- RSD_R ranging from 1.5 to 4.3%

Based on the performance characteristics presented, the EURL recommends for official control, the abovementioned ring trial validated AOAC 999:13 and VDLUFA 4.11.6 methods based on High Performance Liquid chromatography (HPLC) coupled with post-column derivatisation to determine free *Lysine* in all the forms of the *feed additive* object of the current application.

The Applicant provided no experimental data for the identification of *Lysine* in *water*. Therefore, the EURL is neither able to evaluate nor recommend a method for the official control to determine *Lysine* 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) is not considered necessary.

KEYWORDS

Lysine, Concentrated liquid lysine (base), Lysine HCl technical pure, L-lysine sulphate (solid), L-lysine sulphate (liquid), nutritional additives, amino acids, all animal species and categories.

1. BACKGROUND

Lysine was already authorised as feed additive without any restrictions under Commission Directive 88/485/EEC [1]. In the current application authorisation is sought for four different forms of *Lysine* under Articles 4(1) (new use in feed) and 10(2) (re-evaluation of the already authorized feed additive – Council directive 70/524/EEC), category of 'nutritional additives' functional group 3(c) 'amino acids, their salts and analogues' according to Annex I of Regulation (EC) No 1831/2003 [2]. The following four forms of *Lysine* are submitted for authorisation: - *concentrated liquid L-lysine (base)*; - *L-lysine monohydrochloride (HCl) technically pure*; - *L-lysine sulphate solid form produced by fermentation with Corynebacterium glutamicum;* and - *Lysine sulphate liquid form produced by fermentation with Corynebacterium glutamicum*. According to the Applicant all these forms are produced through fermentation of non-toxicogenic, non-pathogenic species of Corynebacterium glutamicum, using sucrose, glucose, starch hydrolysates or molasses as carbon source and

ammonium sulphate and ammonia as nitrogen source [3]. The *concentrated liquid Lysine* is a dark brown aqueous solution of *Lysine* free base; *Lysine HCl* is a white to pale yellow or brownish crystalline powder and the solid form of *L-Lysine sulphate* is a light brown granulate or powder [4].

Specifically, authorisation is sought for the use of the *Lysine* for all animal species and categories. The *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs* or *water* as presented in Table 1 [5]. The Applicant suggested no minimum or maximum *Lysine* concentrations in *premixtures*, *feedingstuffs* and *water* [6].

Table 1: Proposed mode of use of the different forms of *Lysine* in *feed additive* (FA), *premixtures* (PM), *feedingstuffs* (FS) and *water* (W)

	FA	PM	FS	W
concentrated liquid Lysine (base)	X			
Lysine monohydrochloride (HCl) technically pure	X	X	X	X
L-lysine sulphate solid form produced by fermentation with Corynebacterium glutamicum	X		X	
Lysine sulphate liquid form produced by fermentation with Corynebacterium glutamicum	X	X	X	X

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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 (EFSA) for each application or group of applications. For this dossier, the methods of analysis submitted in connection with *Lysine*, 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 [7].

Description of the analytical methods for the determination of the active substances in feed additive, premixtures, feedingstuffs and water.

For the determination of *Lysine* (all relevant forms) in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method [8-10]. This method applies for the determination of *free* (synthetic and natural) and of *total* (peptide-bound and free) amino acids, using an amino acid analyzer or High Performance Liquid Chromatography (HPLC) equipment. The method does not distinguish between the salts and 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 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. *Lysine* can be determined in either oxidised or un-oxidised 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 2.2. The amino acids are separated by ion exchange chromatography and determined by post column derivatisation with ninhydrin and photometric detection at 570 nm or analysed by amino acid analyser.

This method was ring trial validated using four different matrices listed in Table 1. This method was further ring-trial validated by twenty-three laboratories, resulting in the ISO 13903:2005 method [11]. The performance characteristics reported for the determination of

total *Lysine* are listed in Table 2. Furthermore, a limit of quantification was derived for *free Lysine* and *total Lysine*: 0.035 and 0.3 g/kg *feedingstuffs,* respectively.

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated Community method, based on amino acid analyser or High Performance Liquid chromatography (HPLC) coupled with post-column derivatisation, to determine total *Lysine* in *premixtures* and *feedingstuffs*.

For the determination of the active substance in *L-lysine HCl technically pure* the Applicant proposes a tritrimetric method using perchloric acid as prescribed by the European Pharmacopeia [8]. Upon request of supplementary information by the EURL, for the determination of the *active substance* in all the other forms of *feed additive*, the Applicant proposed to apply the abovementioned described ring trial validated Community method designed for the analysis of *premixtures* and *feedingstuffs* [10]. However, the EURL identified two additional ring-trial validated methods based on the same principle described in the abovementioned Community method (i.e. extraction of the sample with diluted hydrochloric acid and measuring the target analyte with an amino acid analyser or HPLC coupled with post-column derivatisation system): (i) the method characterised by the "Association of Official Agricultural Chemists" (AOAC 999:13 - 2004 [12][13]) "*Lysine*, Methionine and Threonine in Feed Grade Amino Acids and Premixes"; and (ii) the method characterised by the "Association of German Agricultural Analytical and Research Institutes" (VDLUFA, Germany – Method 4.11.6 [14]). Both methods are designed for the determination of free *Lysine* in *feed additive* and *premixtures* at amino acid contents higher than 100 g/kg.

The *feed additive* and/or *premixtures* samples are dissolved in hydrochloric acid (0.1 mol/l) and diluted with sodium citrate buffer. The internal standard solution (Norleucine) is added and the amino acids are separated by ion exchange chromatography and determined by post column derivatisation with ninhydrin or by fluorescence detection after post-column reaction with *o*-phthaldialdehyde (OPA) or analysed by amino acid analyzer. The reported performance characteristics are listed in Table 2.

Based on the performance characteristics presented, the EURL recommends for official control, the two ring trial validated methods - AOAC 999:13 and VDLUFA 4.11.6 - based on amino acid analyser or High Performance Liquid chromatography (HPLC) coupled with post-column derivatisation, to determine all forms of *Lysine* in the *feed additive*.

The Applicant provided no experimental data for the determination of *Lysine* in *water.* Therefore the EURL is neither able to evaluate nor to recommend a method for official control to determine *Lysine* in *water*.

Table 2: Method performance characteristics obtained in the frame of four different ring-trial validation exercises based on similar methods for the determination of *Lysine* in *feed additive*, *premixtures* and *feedingstuffs*.
The performance characteristics reported refer to:
- *total Lysine* determination (peptide bound and free) for the Commission Regulation No 152/2009 & ISO 13903:2005
- *free Lysine* determination (synthetic and natural) for AOAC 999:13 & VDLUFA 4:11:6 (yellow highlight)

Intercomparison study	Matrix	*L-lysine* g/kg	RSD_r (%)	RSD_R (%)
Commission Regulation (EC) No 152/2009 [9] study carried out in 1990	Mixed pig feed	10	2.8	3.2
	Broiler compound	14	2.1	5.4
	Protein concentrate	48	2.4	3.0
	Premixture	98	2.1	6.7
ISO 13903:2005 [11] study carried out in 1994	Poultry meal	3.6	3.1	9.9
	Broiler finisher feed	3.5	3.5	9.0
	Broiler starter feed	1.4	2.4	9.0
	Corn	0.3	3.1	13.1
	Fishmeal	4.2	2.8	7.9
AOAC 999:13 [12] study carried out in 1996 [13]	Spray-dried L-lysine sulfate	459	0.8	2.3
	8 different Premixtures	102 - 240	0.7 - 1.7	1.5 - 2.5
	L-lysine-HCl	760	0.9	1.8
VDLUFA 4:11:6 [14] study carried out in 1997 and 1998	Spray-dried L-lysine sulfate	468	1.1	2.0
	4 different Premixtures	122 - 308	0.5 - 3.0	1.6 - 4.3

RSD_r, *RSD_R* - relative standard deviation for *repeatability* and *reproducibility*, respectively

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) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control:

- the ring-trial validated methods, characterised by AOAC and VDLUFA, using High-Performance Liquid Chromatography (HPLC) coupled to post column derivatisation and spectrophotometric or fluorescence detection, to determine *Lysine* in the *feed additive*.

- the ring trial validated Community method, using High-Performance Liquid Chromatography (HPLC) coupled to post column derivatisation and photometric detection, to determine total *Lysine* in *premixtures* and *feedingstuff*.

Recommended text for the register entry (analytical method)

For the determination of *Lysine* in *feed additive*:

- High-Performance Liquid Chromatography (HPLC)
  AOAC 999:13 - 2004 or VDLUFA, 4.11.6, Methodenbuch III

For the determination of total *Lysine* in *premixtures* and *feedingstuff*:

- High-Performance Liquid Chromatography (HPLC)
  Commission Regulation (EC) No 152/2009 (Annex III, F)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of the four forms of *Lysine* 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] Commission Directive 88/485/EEC of 26 July 1988 amending the Annex to Council Directive 82/471/EEC concerning certain products used in animal nutrition

[2] *Application/Ref:SANCO/D/2:Forw.Appl.1831/0052-2010
[3] *Technical dossier, Section II: 3.1 Active substance(s)/agent(s)
[4] *Technical dossier, Section II: 1.3 Qualitative and quantitative composition
[5] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
[6] *Application, (Annex A), FAD-2010-0067_Conditions of use_ *Lysine*
[7] 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
[8] *Technical dossier, Section II: 2.6.1 Method of analysis for the active substances
[9] 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
[10] * Supplementary information, "Lysine_feed add meth_AMAC"
[11] Animal feeding stuffs – Determination of amino acids content (EN ISO 13903:2005)
[12] AOAC Official Method 999:13 – Lysine, Methionine and Threonine in Feed Grade Amino Acids and Premixes
[13] Fontaine and Eudaimon, J. of AOAC Int. (2000), Vol. 83, No. 4, 771-783

[14] Bestimmung von Lysin, Methionin und Threonin in Aminosäurenhandelsprodukten und Vormischungen – 4.11.6, Methodenbuch III, 5. Erg. 2004, VDLUFA – Verlag, Darmstadt
*Refers to Dossier no: FAD-2010-0067

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was European Union Reference Laboratory for Feed Additives, IRMM, 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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

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