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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

L-lysine sulphate

(FAD-2013-0045; CRL/130034)

European Union Reference Laboratory
Feed Additives

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-2013-0045 - CRL/130034**
Name of Product:	***L-lysine sulphate***
Active Agent (s):	**L-lysine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
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Report checked by: Date:	**Piotr Robouch (EURL-FA)** **16/05/2014**
Report approved by: Date:	**Christoph von Holst** **16/05/2014**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Articles 4(1) for *L-lysine sulphate*, under the category/functional group 3(c) 'nutritional additives'/'amino acids, their salts and analogues' according to Annex I of Regulation (EC) No 1831/2003. The *feed additive* is a solid yellow-brown and odourless granulate with a minimum purity of 55 %, expressed as *L-lysine* content. Specifically, authorisation is sought for the use for all animal species and categories. It is intended to be added directly to complete *feedingstuffs* and no minimum or maximum *feed additive* concentrations are suggested.

For the quantification of *L-lysine sulphate* in *feed additive* the Applicant submitted the ring-trial validated Community method designed for the analysis of amino acids in premixtures and *feedingstuffs*. The EURL identified instead and recommends for official control the ring-trial validated ISO method (EN ISO 17180:2013) based on Ion Exchange Chromatography coupled with post-column derivatisation and Ultraviolet or fluorescence detection (IEC-UV/FD). The following performance characteristics are reported: - a relative standard deviation for *repeatability* (RSD_r) ranging from 0.7 to 1.7 %; - a relative standard deviation for *reproducibility* (RSD_R) ranging from 1.5 to 2.5 %; and - a recovery rate (R_{Rec}) ranging from 97.8 to 100 %. For the identification of *sulphates*, the EURL recommends the generic European Pharmacopoeia monograph on *sulphates* (Eu.Ph. 01/2008:20301).

For the quantification of *L-lysine* in *feedingstuffs* the Applicant submitted the ring-trial validated Community method mentioned above based on IEC coupled with post-column derivatisation using amino acid analyser or high performance liquid chromatography equipped with ion exchange column. This method designed for the analysis of premixtures and *feedingstuffs* does not distinguish between the salts and the amino acid enantiomers. The following performance characteristics were reported for the quantification of total *lysine*: - RSD_r ranging from 2.1 to 3.5% and - RSD_R ranging from 3.0 to 13.1%. Based on the performance characteristics presented, the EURL recommends for official control, the Community method to quantify *lysine* in *feedingstuffs*.

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

L-lysine, *L-lysine sulphate*, nutritional additives, amino acids, *all animal species and categories*

1. BACKGROUND

In the current application authorisation is sought under Articles 4(1) (authorisation of a new feed additive) for *L-lysine sulphate*, under the category/functional group 3(c) 'nutritional additives'/'amino acids, their salts and analogues' according to Annex I of Regulation (EC) No 1831/2003 [1,2].

According to the Applicant the *feed additive* is produced through a direct fermentation process with a specific strain (*Escherichia coli K-12*) [3,4]. *L-lysine sulphate* is a solid yellow-brown and odourless granulate with a minimum purity of 55 %, expressed as *L-lysine* content [2,5].

Specifically, authorisation is sought for the use of *L-lysine sulphate* for all animal species and categories [1,2]. The *feed additive* is intended to be added directly to complete *feedingstuffs* and no minimum or maximum *L-lysine sulphate* concentrations are suggested [1,6].

Different forms of *L-lysine* are authorised as feed additive without any restrictions under Commission Directive 88/485/EEC [7].

Note: The EURL already evaluated the analytical methods in the frame of dossier FAD-2010-0067 (four forms of *lysine* – including *L-lysine sulphate* - produced from *Corynebacterium glutamicum*) and FAD-2013-0027 (three forms of *L-lysine* produced from *Escherichia coli K-12*) [8,9].

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 for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *L-lysine sulphate* 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 [10]

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

For the quantification of *L-lysine sulphate* in *feed additive* the Applicant submitted the ring-trial validated Community method [11]. This method designed only for the analysis of amino acids in premixtures and *feedingstuffs* does not distinguish between the salts and the amino acid enantiomers [12].

The EURL identified instead the recently published ring-trial validated ISO method (EN ISO 17180:2013 - "Animal feeding stuffs – Determination of *lysine*, methionine and threonine in commercial amino acid products and premixtures") [13]. This standard method is based on the experimental protocol described in the Community method for *lysine*. It does not distinguish between the salts and the amino acid enantiomers and it applies for products containing more than 10% of amino acid.

Free *lysine* is extracted with diluted hydrochloric acid and further diluted with sodium citrate buffer. After addition of norleucine as internal standard, the amino acids are separated by Ion Exchange Chromatography (IEC). Free *lysine* is quantified photometrically after post-column derivatisation with ninhydrine and UltraViolet (UV) detection at 570 nm or by fluorescence detection (FD) after post column reaction with ortho-phthaldialdehyde with detector set at excitation wavelenght 330 nm and emission 460 nm. The performance characteristics reported for the quantification of free *lysine* are listed in Table 1. Furthermore, recovery rates ranging from 97.5 to 100% were reported.

Based on the performance characteristics presented, the EURL recommends for official control the EN ISO 17180:2013 method, based on IEC-UV/FD to quantify free *lysine* in *feed additive*.

Furthermore, for the identification of the *sulphate* ion in the *feed additive*, the EURL recommends the European Pharmacopoeia monograph (Ph.Eur. 01/2008:20301) [14].

Table 1: Method performance characteristics obtained in the frame of ring-trial validation exercises for the quantification of (total) *lysine*.

Ring-Trial	Matrix	*lysine* g/kg	RSD_r %	RSD_R %
EN ISO 17180:2013 [13]	Feed Additive	459	0.8	2.3
	Premix 3	208	1.3	2.5
	Premix 4	168	1.3	2.3
	Premix 5	128	0.7	1.9
	Premix 6	123	1.7	2.1
	Premix 7	104	1.2	1.8
	Premix 8	102	1.2	1.5
	Premix 9	240	1.1	2.2
	Premix 10	233	0.8	1.8
	L-Lysine-HCl	760	0.9	1.8
Commission Regulation (EC) No 152/2009 [12]	Mixed pig feed	10	2.8	3.2
	Broiler compound	14	2.1	5.4
	Protein concentrate	48	2.4	3
	Premixture	98	2.1	6.7
EN ISO 13903:2005 [15]	Poultry meal	3.6	3.1	9.9
	Broiler finisher feed	3.5	3.5	9
	Broiler starter feed	1.4	2.4	9
	Corn	0.3	3.1	13.1
	Fishmeal	4.2	2.8	7.9

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

For the quantification of *L-lysine sulphate* in *feedingstuffs*, the Applicant submitted the ring-trial validated Community method mentioned above [11]. This method applies for the quantification of *free* (synthetic and natural) and of *total* (peptide-bound and free) amino acids, using an amino acid analyser or high performance liquid chromatography (HPLC) equipped with ion exchange column in premixtures and *feedingstuffs* [12].

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 IEC 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. *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 2.2. The amino acids are separated by IEC and determined by post column derivatisation with ninhydrin and photometric detection at 570 nm.

The Community 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 EN ISO 13903:2005 method [15]. The performance characteristics reported for the quantification of total *L-lysine* are listed in Table 1. Furthermore, the following limits of quantification were reported for *free lysine* and *total lysine*: 0.04 and 0.3 g/kg *feedingstuffs*, respectively [15].

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated Community method, based on IEC-UV to quantify *L-lysine* in *feedingstuffs*.

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 EN ISO 17180, based on ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-UV/FD), to quantify free *lysine* in the *feed additive*;
- the European Pharmacopoeia monograph (01/2008:20301) to characterise *sulphates* in the *feed additive*; and
- the ring-trial validated Community method, using ion exchange chromatography coupled with photometric detection (IEC-UV), to quantify *lysine* in *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the quantification of *lysine* in *feed additive*:

- ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-UV/FD) – EN ISO 17180

For the identification of *sulphate* in *feed additive*:

- European Pharmacopoeia Monograph 20301

For the quantification of *lysine* in *feedingstuffs*:

- ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-UV) – Commission Regulation (EC) No 152/2009

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *L-lysine sulphate* 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] *Application, Proposal of Registry Entry – Annex A

[2] *Application, Reference SANCO/G1: Forw. Appl. 1831/0040-2013

[3] *Technical dossier, Section II: 2.1.1 Name of the additive

[4] *Technical dossier, Section II: 2.3.1 Manufacturing process/active substance

[5] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition

[6] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition

[7] 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

[8] #FAD-2010-0067, Lysine, Ref. Ares(2011)301126 - 18/03/2011

[9] “FAD-2013-0027, L-Lysine, Ref. Ares(2014)1065471 - 04/04/2014

[10] 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

[11] *Technical dossier, Section II: 2.6 Method of analysis and reference samples

[12] 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

[13] Animal feeding stuffs – Determination of lysine, methionine and threonine in commercial amino acid products and premixtures; EN ISO 17180:2013

[14] European Pharmacopoeia Monograph 01/2008:20301 - Identification reactions of ions and functional groups – *sulphates*

[15] Animal feeding stuffs – Determination of amino acids content; EN ISO 13903:2005

*Refers to Dossier no: FAD-2013-0045

#https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2010-0067.pdf

“https://ec.europa.eu/jrc/sites/default/files/FinRep-FAD-2013-0027_L-lysine.pdf

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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- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Sachgebiet Futtermittel des Bayrischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)[1]

[1] Name and address according to Regulation (EC) No 885/2009: Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)