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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 monohydrochloride and L-lysine sulphate
from Corynebacterium glutamicum CCTCC M 2015595
(FAD-2016-0052; CRL/160025)

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-2016-0052 - CRL/160025**
Feed Additive:	***L-lysine monohydrochloride*** *and* ***L-lysine sulphate*** *from Corynebacterium glutamicum CCTCC M 2015595*
Active Agent (s):	**L-lysine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**Stefano Bellorini**
Report checked by: Date:	**Piotr Robouch (EURL-FA)** **27/02/2017**
Report approved by: Date:	**Christoph von Holst** **28/02/2017**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-lysine monohydrochloride and L-lysine sulphate from Corynebacterium glutamicum CCTCC M 2015595*, 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. Authorisation is sought for all animal species. *L-lysine* is already authorised as *feed additive* under Commission Directive 88/485/EEC.

For the quantification of *L-lysine monohydrochloride* and *L-lysine sulphate* in *feed additive* the Applicant submitted 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 %. In addition, the EURL identified the "L-lysine monohydrochloride monograph" of the Food Chemical Codex (FCC) for the characterisation of *L-lysine monohydrochloride* in the *feed additive* and the generic European Pharmacopoeia monograph on sulphates (Ph. Eur. 20301) for the identification of *sulphates* in *L-lysine sulphate*.

For the quantification of *L-lysine monohydrochloride* and *L-lysine sulphate* in *premixtures*, *feedingstuffs* and *water* the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009) based on IEC coupled with post-column derivatisation using an amino acid analyser or high performance liquid chromatography equipped with ion exchange column and photometric detection (UV). This method, designed only 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 %. Since the Applicant provided no experimental data to determine *L-lysine* in *water*, the EURL cannot evaluate nor recommend a method for the official control to determine *L-lysine* in *water*. Based on the performance characteristics presented, the EURL recommends for official control the four standard methods mentioned above for the identification or quantification of *lysine* in the *feed additive*, *premixture* and/or *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 monohydrochloride and L-lysine sulphate from Corynebacterium glutamicum CCTCC M 2015595, nutritional additives, amino acids, their salts and analogues, all animal species

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *L-lysine monohydrochloride* and *L-lysine sulphate from Corynebacterium glutamicum CCTCC M 2015595*, 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. Authorisation is sought for all animal species [1-2]. *L-lysine* is already authorised as feed additive under 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 [3].

According to the Applicant, the white or light brown powdered *L-lysine monohydrochloride* [4] and the brown granulated *L-lysine sulphate* [5] have a minimum purity (mass fraction) of 98.5 % and 73.0 %, respectively. Furthermore, the minimum content (mass fraction) of the *L-Lysine (active substance)* is 78.8 % and 55.0 %, respectively [1,6].

The *feed additive* is produced by fermentation with a not genetically modified strain of Corynebacterium glutamicum [7]. The production strain is deposited in the "Chinese Centre for Type Culture Collection Centre" (CCTCC) with reference Corynebacterium glutamicum CCTCC M 2015595 [2,7,8].

L-lysine monohydrochloride and L-lysine sulphate are intended to be mixed in *premixtures* or added directly to *feedingstuffs* or *water* for drinking [9]. However the Applicant did not propose any minimum or maximum content in *feedingstuffs* [1,9].

Note: The EURL has previously evaluated the analytical methods in the frame of four dossiers [10-13].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EU) 2015/1761, 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 monohydrochloride and 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 [14].

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

For the quantification of *lysine* in the *feed additive* the Applicant submitted the ring-trial validated method EN ISO 17180:2013 - "Animal feeding stuffs – Determination of *lysine*, methionine and threonine in commercial amino acid products and premixtures" [15,16]. This standard method is based on the experimental protocol described in the Community method for *lysine* [17]. It does not distinguish between the salts of amino acids and it cannot differentiate between enantiomers. 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 high performance liquid chromatography equipped with ion exchange column (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 a detector excitation wavelength at 330 nm and emission at 460 nm. The performance characteristics reported for the quantification of free *lysine* are listed in Table 1. Furthermore, the Applicant reported recovery rates ranging from 97.5 to 100 %.

In addition, the EURL found the following two monographs: (i) the "L-lysine monohydrochloride monograph" of the Food Chemical Codex (FCC) for the characterisation of *L-lysine monohydrochloride* in the *feed additive*, where identification is based on infrared absorption and quantification on titration with perchloric acid [18]; (ii) the generic European Pharmacopoeia monograph on sulphates (Ph. Eur. 01/2008:20301) for the identification of sulphate in L-lysine sulphate [19].

Based on the performance characteristics available, the EURL recommends for official control the EN ISO 17180:2013 method for the quantification of free lysine in the *feed additive* and *premixtures* (containing more than 10 % *lysine*), the Food Chemical Codex for the identification of *L-lysine monohydrochloride* in the *feed additive*, and the European Pharmacopoeia monograph for the identification of the sulphate ion in *L-lysine sulphate.*

For the quantification of *L-lysine* in *premixtures*, *feedingstuffs* and *water* the Applicant submitted the ring-trial validated Community method mentioned above [15,17]. This method can only be applied in *premixtures* and *feedingstuffs* for the quantification of *free* (synthetic and natural) and of *total* (peptide-bound and free) amino acids, using an amino acid analyser or IEC coupled with post-column derivatisation and UV detection. It does not distinguish between the salts of amino acids and it cannot differentiate between 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 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 of 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 [20]. 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.

However, the Applicant provided no experimental data to demonstrate the applicability of the Community method for the determination of *L-lysine* in *water* [15]. Therefore the EURL cannot evaluate nor recommend a method for the official control to determine *L-lysine* in *water*.

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 *premixtures* and *feedingstuffs*.

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

Ring-Trial	Matrix	*lysine* content g/kg	RSD_r %	RSD_R %
EN ISO 17180:2013 [16]	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 [17]	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 [20]	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

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 (i) the "L-lysine monohydrochloride monograph" of the Food Chemical Codex (FCC) based on infrared absorption and titration for the identification of *L-lysine monohydrochloride* in the *feed additive*; (ii) the European Pharmacopoeia monograph (Ph. Eur. 01/2008:20301) for the identification of the *sulphate* ion in *L-lysine sulphate*; (iii) the ring-trial validated EN ISO 17180:2013 based on ion exchange chromatography coupled to ultraviolet or fluorescence detection (IEC-UV/FD) to quantify free *lysine* in *feed additive* and *premixtures* (containing more than 10 % *lysine*); and (iv) the Community method based on IEC-UV for the quantification of *lysine* in *premixtures* and *feedingstuffs*.

Since the Applicant provided no experimental data for the determination of *L-lysine* in *water*, the EURL cannot evaluate nor recommend a method for the official control to determine *L-lysine* in *water*.

Recommended text for the register entry (analytical method)

For the identification of *L-lysine monohydrochloride* in the *feed additive*:

- Food Chemical Codex " L-lysine monohydrochloride monograph"

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

- European Pharmacopoeia monograph 20301

For the quantification of *lysine* in *feed additive* and *premixtures* containing more than 10 % *lysine*:

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

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

- ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-UV) – 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 "*L-lysine monohydrochloride and 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 SANTE/E5: Forw. Appl. 1831-0040-2016

[3] 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, O.J. L 239 , 30/08/1988 P. 0036 – 0039

[4] *Technical dossier, Section II: Annex 2.1.3.c CoA 5 batches Lys HCl.pdf

[5] *Technical dossier, Section II: Annex 2.1.3.d CoA 5 batches Lys sulphate.pdf

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

[7] *Technical dossier, Section II: 2.2.1.2 Micro-organism

[8] *Technical dossier, Section II: Annex 2.2.1.2.b Qingdao strain deposition.pdf

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

[10] FAD-2005-0022, Vitalys Liquid and Dry, Ref. D.08/FSQ/CVH/GS/(2006) D11012 https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2005-0022.pdf

[11] FAD-2010-0067, Lysine, Ref. Ares(2011)301126 - 18/03/2011 https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2010-0067.pdf

[12] FAD-2013-0027, L-lysine, Ref. Ares(2014)1065471 - 04/04/2014 https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2013-0027_L-lysine.pdf

[13] FAD-2013-0045, L-lysine sulphate, Ref. Ares(2014)1617815 - 19/05/2014 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2013-0045_l-lysine%20sulphate.pdf

[14] 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

[15] *Technical dossier, Section II: II.6.1 Methods of analysis for the active substance

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

[17] 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 (Annex III, F)

[18] Food Chemical Codex monograph "*L-lysine monohydrochloride*", FCC 7 (2010), p.598

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

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

*Refers to Dossier no: FAD-2016-0052

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation is the European Union Reference Laboratory for Feed Additives, JRC, 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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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- RIKILT Wageningen UR, Wageningen (NL)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 — Labore Landwirtschaft, Nossen (DE)
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