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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 **concentrated liquid L-lysine**
produced by Corynebacterium glutamicum NRRL-B-67535
(FAD-2018-0037; CRL/180024)

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-2018-0037 - CRL/180024**
Name of Product:	***L-lysine monohydrochloride and concentrated liquid L-lysine produced by Corynebacterium glutamicum NRRL-B-67535***
Active Agent:	**L-lysine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Stefano Bellorini** **12/12/2018**
Report approved by: Date:	**Christoph von Holst** **13/12/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-lysine monohydrochloride* and *concentrated liquid L-lysine produced by Corynebacterium glutamicum NRRL-B-67535*, 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.

According to the Applicant, the dry crystalline powdered *L-lysine monohydrochloride* has a minimum purity (mass fraction) of 98.5 % (minimum of 78.5 % of *L-lysine*) and *concentrated liquid L-lysine* contains a minimum of 50 % of *L-lysine*.

The two forms of the *feed additive* are intended to be added directly into *feedingstuffs* (or through *premixtures*) and *water* for drinking. However the Applicant did not propose any minimum or maximum content of *L-lysine* in *feedingstuffs* or *water*.

For the quantification of *lysine* in the *feed additive* the Applicant submitted the ring-trial validated method EN ISO 17180:2013 based on ion exchange chromatography coupled to visible or fluorescence detection (IEC-VIS/FLD). This standard method 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. The following performance characteristics are reported: a relative standard deviation for repeatability (RSD_r) ranging from 0.7 to 1.7 % and a relative standard deviation for reproducibility (RSD_R) ranging from 1.5 to 2.5 %. In addition, the EURL identified the "L-lysine monohydrochloride monograph" of the Food Chemical Codex (FCC) for the identification of *L-lysine monohydrochloride* in the *feed additive*.

For the quantification of *L-lysine* 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 photometric detection (IEC-VIS). 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. The following performance characteristics were reported for the quantification of total *lysine*: RSD_r ranging from 2.1 to 2.8 % and RSD_R ranging from 3 to 6.7 %.

In the frame of the stability studies the Applicant presented experimental data obtained by analysing *lysine* in *water* with the slightly modified AOAC official method 999.13 based on IEC-VIS/FLD. The results presented are considered sufficient to demonstrate the suitability of the procedure for the analysis of the amino acid in *water*.

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 for the identification of *L-lysine monohydrochloride* in the *feed additive*; (ii) the ring-trial validated method EN ISO 17180:2013 based on IEC-VIS/FLD to quantify free *lysine* in the *feed additive* and *premixtures* (containing more than 10 % *lysine*); (iii) the Community method based on IEC-VIS for the quantification of *lysine* in *premixtures* and *feedingstuffs*; and (iv) the modified AOAC method based on IEC-VIS/FLD to quantify *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), as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

L-lysine monohydrochloride and *concentrated liquid L-lysine produced by Corynebacterium glutamicum NRRL-B-67535*, nutritional additives, amino acids, all animal species and categories

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *L-lysine monohydrochloride* and *concentrated liquid L-lysine produced by Corynebacterium glutamicum NRRL-B-67535*, 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]. The two forms of *L-lysine* are already authorised as *feed additives* 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 (code 3.2.2. 3.2.3 and 3.2.4) [3].

According to the Applicant, the dry crystalline powdered *L-lysine monohydrochloride* has a minimum purity (mass fraction) of 98.5 % [4] (minimum of 78.5 % of *L-lysine* [1]) and *concentrated liquid L-lysine* contains a minimum of 50 % of *L-lysine* [1,4].

The *feed additive* is produced by fermentation with a genetically modified strain of *Corynebacterium glutamicum* [5]. The production strain is deposited in the "Agricultural Research Culture Collection" (NRRL) with the reference *Corynebacterium glutamicum NRRL-B-67535* [5].

The two forms of the *feed additive* are intended to be added directly into *feedingstuffs* (or through *premixtures*) and *water* for drinking [6]. However the Applicant did not propose any minimum or maximum content of *L-lysine* in *feedingstuffs* or *water* [1,6].

Note: The EURL has previously evaluated the analytical methods for the determination of *lysine* in the frame of several dossiers [7-15].

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 *concentrated liquid L-lysine* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

3. EVALUATION

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

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" [16,17]. This standard method is based on the experimental protocol described in the Community method for the analysis of free amino acids (including *lysine*) [18]. It does not distinguish between the salts of amino acids and 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 (HPLC) with an Ion Exchange Column (IEC). Free *lysine* is quantified either after post-column derivatisation with ninhydrine and Visible (VIS) detection at 440 nm and 570 nm or by fluorescence detection (FLD) 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.

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*).

For the quantification of *L-lysine* in *premixtures, feedingstuffs* and *water* the Applicant submitted the ring-trial validated Community method mentioned above [16,18]. This method was designed for the quantification of free (synthetic and natural) and total (peptide-bound and free) amino acids in *premixtures* and *feedingstuffs*, using an amino acid analyser or IEC coupled with post-column derivatisation and VIS detection. It does not distinguish between the salts of amino acids and 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. *Lysine* can be determined in either oxidised or non-oxidised samples. Oxidation is performed at 0 °C with a performic acid/phenol mixture. The excess of oxidation reagent is decomposed with sodium disulfite. The oxidised or non-oxidised 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 [19]. The performance characteristics reported for the quantification of total *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 [19].

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated Community method, based on IEC-VIS to quantify *lysine* in *premixtures* and *feedingstuffs*.

The Applicant did not perform any validation/verification studies to demonstrate the suitability of the Community method [18] for the determination of *lysine* in *water*. However, in the frame of the stability studies, the Applicant presented experimental data obtained by analysing *lysine* in *water* [20,21]. The tests were carried out using a slightly modified protocol of the ring trial validated AOAC Official Method 999.13 designed for the analysis of *lysine* in feed additives and premixtures [22]. This method, equivalent to EN ISO 17180:2013, is based on IEC coupled with VIS or FLD [17]. The results presented are considered sufficient to demonstrate the suitability of the procedure for the analysis of the amino acid in *water*. Hence, the EURL recommends this method for official control.

Table 1: Method performance characteristics obtained in the frame of ring-trial validation studies (EN ISO 17180:2013 [17], Community method [18] and EN ISO 13903:2005 [19]) for the determination of total *L-lysine* in the *feed additive*, *premixtures* and *feedingstuffs*.

Ring-Trial	Matrix	*lysine* content g/kg	RSD_r %	RSD_R %
[17]	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
[18]	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
[19]	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

Methods of analysis for the determination of the residues of the additive in food (section 2.6.2 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

The evaluation of corresponding methods of analysis is not relevant for the present application.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

The EURL found the "L-lysine monohydrochloride monograph" of the Food Chemical Codex (FCC) where identification is based on infrared absorption [23].

The EURL recommends the Food Chemical Codex for the identification of *L-lysine monohydrochloride* in the *feed additive*.

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, as last amended by Regulation (EU) 2015/1761) 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 for the identification of *L-lysine monohydrochloride* in the *feed additive*; (ii) the ring-trial validated method EN ISO 17180:2013 based on ion exchange chromatography coupled to visible or fluorescence detection (IEC-VIS/FLD) to quantify free *lysine* in the *feed additive* and *premixtures* (containing more than 10 % *lysine*); (iii) the Community method based on IEC-VIS for the quantification of *lysine* in *premixtures* and *feedingstuffs*; and (iv) the modified AOAC Official Method 999.13 method based on IEC-VIS/FLD to quantify *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 quantification of *lysine* in the *feed additive* and *premixtures* (containing more than 10 % *lysine*):

- ion exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS/FLD) – EN ISO 17180

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

- ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS), Commission Regulation (EC) No 152/2009 (Annex III, F)

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

- ion exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS/FLD)

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 *concentrated liquid L-lysine produced by Corynebacterium glutamicum NRRL-B-67535* 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. 1831/0041-2018 & Annex I – submission number 1527694207546-2224

[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 239/36, 30.08.1988

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

[5] *Technical dossier, Section II: 2.2.1.2. Micro-organisms

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

[7] 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

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

[9] 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

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[11] FAD-2016-0052, L-lysine monohydrochloride and L-lysine sulphate from Corynebacterium glutamicum CCTCC M 2015595, Ref. Ares(2017)1063023 - 28/02/2017 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0052_lysine.pdf

[12] FAD-2017-0024, L-lysine monohydrochloride and concentrated liquid L-lysine produced by Corynebacterium glutamicum NRRL-50775 Ref. Ares(2017)5262025 - 27/10/2017 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2017-0024_lysine.pdf

[13] FAD-2018-0012, L-lysine monohydrochloride and concentrated liquid L-lysine produced by Corynebacterium glutamicum NRRL B-67439 Ref. Ares(2018)4141251 - 07/08/2018

[14] FAD-2018-0019, L-lysine monohydrochloride and L-lysine sulphate produced by Corynebacterium glutamicum CGMCC 7.266 Ref. Ares(2018)5066361 - 03/10/2018

[15] FAD-2018-0028, L-lysine monohydrochloride and concentrated liquid L-lysine produced by Corynebacterium glutamicum KCCM10227 Ref. Ares(2018)5200878 - 10/10/2018

[16] *Technical dossier, Section II: 2.6.1. Methods of analysis for the active substance

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

[18] 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

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

[20] *Technical dossier, Section II – Annex 2.4.1h

[21] *Technical dossier, Section II – Annex 2.6.3 II_4_01

[22] AOAC Official Method 999.13 – Lysine, Methionine and Threonine in Feed Grade Amino Acids and Premixes

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

*Refers to Dossier no: FAD-2018-0037

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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