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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
produced by ***Corynebacterium glutamicum* CGMCC 7.266**
(*FAD-2018-0019; CRL/180020*)



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Dossier related to: **FAD-2018-0019 - CRL/180020**

Name of Product: ***L-lysine monohydrochloride and L-lysine sulphate produced by Corynebacterium glutamicum CGMCC 7.266***

Active Agent: **L-lysine**

Rapporteur Laboratory: **European Union Reference Laboratory for Feed Additives (EURL-FA)
JRC Geel, Belgium**

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Date: **03/10/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-lysine monohydrochloride* and *L-lysine sulphate* produced by *Corynebacterium glutamicum* CGMCC 7.266, 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 *L-lysine monohydrochloride* has a minimum purity (mass fraction) of 98.5 % (minimum of 78 % of *L-lysine*) while *L-lysine sulphate* contains a minimum of 55 % of *L-lysine*.

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* and the generic European Pharmacopoeia monograph on sulphates (Ph. Eur. 20301) for the identification of *sulphate* in *L-lysine sulphate*.

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 (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 3.0 to 6.7 %. In the frame of the stability studies the Applicant presented experimental data obtained analysing *lysine* in *water* with the Community method and demonstrated its applicability for the determination of *lysine* 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 European Pharmacopoeia monograph (Ph. Eur. 01/2008:20301) for the identification of the *sulphate* ion in *L-lysine sulphate*; (iii) 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*); (iv) the ring-trial validated Community method based on IEC-VIS for the quantification of *lysine* in *premixtures*, *feedingstuffs* and *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

L-lysine monohydrochloride and L-lysine sulphate produced by Corynebacterium glutamicum CGMCC 7.266, 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 L-lysine sulphate produced by Corynebacterium glutamicum CGMCC 7.266*, 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* [3,4].

According to the Applicant, the light yellow granulated powder *L-lysine monohydrochloride* has a minimum purity (mass fraction) of 98.5 % (minimum of 78 % of *L-lysine*) while the light yellow or brown granulated powder *L-lysine sulphate* contains a minimum of 55 % of *L-lysine* [1,5-7].

The *feed additive* is produced by fermentation with a non-genetically modified strain of *Corynebacterium glutamicum* [8]. The production strain is deposited in the "China General Microbiological Culture Collection Center" (CGMCC) with reference *Corynebacterium glutamicum CGMCC 7.266* [9].

The two forms of the *feed additive* are intended to be added directly into *feedingstuffs* or through *premixtures* and *water* for drinking [10]. However the Applicant did not propose any minimum or maximum content of *L-lysine* in *feedingstuffs* [1,10].

Note: The EURL has previously evaluated the analytical methods for the determination of *lysine* in the frame of several dossiers [11-16].

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 produced by Corynebacterium glutamicum CGMCC 7.266* 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"[17,18]. This standard method is based on the experimental protocol described in the Community method for *lysine* [19]. 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 (HPLC) with an Ion Exchange Column (IEC). Free *lysine* is quantified either after post-column derivatisation with ninhydrine and Visible (VIS) detection set 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.

For the quantification of *L-lysine* in *premixtures, feedingstuffs* and *water* the Applicant submitted the ring-trial validated Community method mentioned above [17,19]. 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 VIS 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.

Table 1: Method performance characteristics obtained in the frame of ring-trial validation studies (EN ISO 17180:2013 [18], Community method [19] and EN ISO 13903:2005 [20]) for the determination of total *L-lysine* in the *feed additive*, *premixtures* and *feedingstuffs*.

Ring-Trial	Matrix	<i>lysine</i> content g/kg	RSD _r %	RSD _R %
[18]	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
[19]	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
[20]	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

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

Furthermore, the following limits of quantification were reported for *free lysine* and *total lysine*: 0.04 and 0.3 g/kg *feedingstuffs*, respectively.

Based on the performance characteristics available, the EURL recommends for official control: i) the ring-trial validated method EN ISO 17180:2013 based on IEC-VIS/FLD for the quantification of *free lysine* in the *feed additive* and *premixtures* (containing more than 10 % *lysine*); and ii) 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 for the determination of *lysine* in *water*. However, in the frame of the stability studies, the Applicant applied the Community method and demonstrated its applicability for the determination of *lysine* in *water* [21,22]. Hence the EURL recommends for official control the Community method based on IEC-VIS to quantify *lysine* in *water*.

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)

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 following two monographs: (i) the "L-lysine monohydrochloride monograph" of the Food Chemical Codex (FCC) where identification is based on infrared absorption; and [23] (ii) the generic European Pharmacopoeia monograph on sulphates (Ph. Eur. 01/2008:20301) for the identification of sulphate in *L-lysine sulphate* [24].

The EURL recommends for official control 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*.

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 European Pharmacopoeia monograph (Ph. Eur. 01/2008:20301) for the identification of the sulphate ion in L-lysine sulphate; (iii) 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*); (iv) the ring-trial validated Community method based on IEC-VIS for the quantification of *lysine* in *premixtures*, *feedingstuffs* and *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 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 photometric detection (IEC-VIS)

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* produced by *Corynebacterium glutamicum* CGMCC 7.266 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. 1831/0025-2018 & Annex I – submission number 1522925430816-2198
- [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] Commission Implementing Regulation (EU) 2017/439 of 13 March 2017 concerning the authorisation of L-lysine sulphate produced by *Escherichia coli* as a feed additive for all animal species, O.J. L 67/70, 14.03.2017

- [5] *Technical dossier, Section II: 1.3. Qualitative and quantitative composition
- [6] *Technical dossier, Section II: Annex 2.5.2.a
- [7] *Technical dossier, Section II: Annex 2.5.2.b
- [8] *Technical dossier, Section II: 2.2.2.2 Micro-organisms
- [9] *Technical dossier, Section II: Annex 2.2.1.2.a
- [10] *Technical dossier, Section II: 2.5.1. Proposed mode of use in animal nutrition
- [11] 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>
- [12] FAD-2010-0067, Lysine, Ref. Ares(2011)301126 - 18/03/2011
<https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2010-0067.pdf>
- [13] 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
- [14] 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
- [15] 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
- [16] 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
- [17] *Technical dossier, Section II: 2.6.1 Methods of analysis for the active substance
- [18] EN ISO 17180:2013 - Animal feeding stuffs – Determination of lysine, methionine and threonine in commercial amino acid products and premixtures
- [19] 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
- [20] EN ISO 13903:2005- Animal feeding stuffs – Determination of amino acids content
- [21] *Technical dossier, Section II: Annex 2.1.3.a
- [22] *Technical dossier, Section II: Annex 2.1.3.b
- [23] Food Chemical Codex monograph "L-lysine monohydrochloride", FCC 7 (2010), p.598
- [24] European Pharmacopoeia Monograph 01/2008:20301 - Identification reactions of ions and functional groups – sulphates

*Refers to Dossier no: FAD-2018-0019

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.

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