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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 fermentation with
Corynebacterium glutamicum CGMCC 17927
(FAD-2021-0051; CRL/210031)**



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Dossier related to: **FAD-2021-0051 - CRL/210031**

Name of Product: ***L-lysine monohydrochloride and
L-lysine sulphate produced by
fermentation with *Corynebacterium
glutamicum* CGMCC 17927***

Active Agent (s): **L-lysine**

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

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Date: **21/02/2022**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *L-lysine monohydrochloride* and *L-lysine sulphate* produced by fermentation with *Corynebacterium glutamicum* CGMCC 17927, 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 authorisation is sought for all animal species.

According to the Applicant, the minimum *L-lysine* content for *L-lysine monohydrochloride* is 78.8 % (w/w) while the content of *L-lysine* for *L-lysine sulphate* is 55.0 % (w/w).

The two forms of the *feed additive* are intended to be added directly into *feedingstuffs* or through complementary feed, *premixtures* and *water* without any recommended inclusion levels. Furthermore, no minimum or maximum contents have been proposed by the Applicant.

For the quantification of *lysine* in the *feed additive*, *premixtures*, *feedingstuffs* and *water* the Applicant proposed the ring-trial validated method EN ISO 13903:2005. This method is equivalent to the ring-trial validated European Union method (Commission Regulation (EC) No 152/2009) based on ion-exchange chromatography (IEC) coupled with post-column derivatisation and optical (visible) (VIS) detection. 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 %.

However, the EURL is aware of the ring-trial validated method EN ISO 17180:2013 based on ion-exchange chromatography (IEC) coupled with post-column derivatisation and optical (visible or fluorescence) detection (IEC-VIS/FLD). It applies for products containing more than 10 % of amino acid and, as the previous method, does not distinguish between the salts of amino acids and cannot differentiate between enantiomers. 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 %. Furthermore, 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 (Ph. Eur. 20301) for the identification of sulphate ions in the *feed additive*.

Based on the performance characteristics available, the EURL recommends for official control (i) the "L-lysine monohydrochloride monograph" of the Food Chemical Codex (FCC) 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 ions in the *feed additive (L-lysine sulphate)*; (iii) the ring-trial validated method EN ISO 17180:2013 based on ion-exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS/FLD) to quantify free *lysine* in the *feed additives* and *premixtures* (containing more than 10 % lysine); (iv) the European Union method based on ion-exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS) for the quantification of *lysine* in *premixtures* and *feedingstuffs*; and (v) the ion-exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS/FLD) or coupled with post-column derivatisation and optical detection (IEC-VIS) methods for the quantification of *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 *L-lysine sulphate* produced by fermentation with *Corynebacterium glutamicum* CGMCC 17927, nutritional additives, amino acids, all animal species

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *L-lysine monohydrochloride* and *L-lysine sulphate* produced by fermentation with *Corynebacterium glutamicum* CGMCC 17927, 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 authorisation is sought for all animal species [1]. These two forms of *L-lysine* produced by fermentation with different *Corynebacterium glutamicum* strains are currently authorised as *feed additives* under several Commission Implementing Regulations [2-5].

According to the Applicant, *L-lysine monohydrochloride* has a minimum *L-lysine* content (mass fraction on dry matter basis) of 78.8 % while *L-lysine sulphate* has a minimum *L-lysine* content (mass fraction on dry matter basis) of 55.0 % [6].

The two forms of the *feed additive* are intended to be added directly into *feedingstuffs* or through complementary feed, *premixtures* and *water* without any recommended inclusion levels. Furthermore, no minimum or maximum contents have been proposed by the Applicant [7].

Note: The EURL has previously evaluated the analytical methods for the determination of *lysine* in the frame of several dossiers [8].

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

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, premixtures, feedingstuffs* and *water* the Applicant suggested using the EN ISO 13903:2005 method [9]. This method is an additional ring-trial validation (by 23 laboratories) of the European Union (EU) method dedicated for the determination of amino acids in feed [10] and designed for the quantification of free (synthetic and natural) and of total (peptide-bound and free) amino acids in *premixtures* and *feedingstuffs*, using an amino acid analyser or ion-exchange chromatography (IEC) coupled with post-column derivatisation and optical (visible) (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 h. 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 [10].

The EU method was originally ring-trial validated using four different matrices listed in Table 1. This method was further ring-trial validated resulting in the EN ISO 13903:2005 method [9] proposed by the Applicant in the frame of this application. 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 [9].

In the frame of stability studies the Applicant provided experimental data of the applicability of the proposed method for the determination of *lysine* in *water* for both products [11].

Furthermore, for the quantification of *lysine* in the *feed additive* the EURL is aware of 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" [12]. This standard method is based on the experimental protocol described in the EU method for the analysis of free amino acids (including *lysine*) [10]. 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.

Table 1: Method performance characteristics obtained in the frame of ring-trial validation studies (EN ISO 17180:2013 [12], European Union method [10] and EN ISO 13903:2005 [9]) for the determination of total *lysine* in the *feed additive*, *premixtures* and *feedingstuffs*.

Ring-Trial	Matrix	<i>lysine</i> content g/kg	RSD _r %	RSD _R %
[12]	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
[9, 10]	Mixed pig feed	9.6	2.8	3.2
	Broiler compound	13.9	2.1	5.4
	Protein concentrate	47.7	2.4	3.0
	Premix	98.0	2.1	6.7
[9]	Poultry meal	36.3	3.1	9.9
	Broiler finisher feed	10.7	3.5	9.0
	Broiler starter feed	13.5	2.4	9.0
	Corn	2.6	3.1	13.1
	Fishmeal	42.2	2.8	7.9

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

Free *lysine* is extracted with diluted hydrochloric acid and further diluted with a 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 [12]. 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*) and the ring-trial validated EU method based on IEC-VIS to quantify *lysine* in *premixtures*, *feedingstuffs* and *water*. Furthermore, as concluded in previous amino acids reports [8], the EURL also considers the IEC-VIS/FLD procedure described above [12] as fit-for-purpose for the determination of *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)

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 Food Chemical Codex (FCC) "L-lysine monohydrochloride monograph", where different tests (including the test based on an optical rotation) are used for the identification of *L-lysine monohydrochloride* in the *feed additive* [13] and thus recommends the Food Chemical Codex monograph for the identification of *L-lysine monohydrochloride*.

While for the identification of *L-lysine sulphate* in the *feed additive* a specific method is not available, the EURL found the generic European Pharmacopoeia monograph (Ph. Eur. 01/2008:20301) for the identification of the sulphate ions in *L-lysine sulphate* [14]. The EURL recommends for official control the European Pharmacopoeia monograph for the identification of the sulphate ions 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) 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 ions in the *feed additive (L-lysine sulphate)*; (iii) the ring-trial validated method EN ISO 17180:2013 based on ion-exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS/FLD) to quantify free *lysine* in the *feed additives* and *premixtures* (containing more than 10 % lysine); (iv) the European Union method based on ion-exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS) for the quantification of *lysine* in *premixtures* and *feedingstuffs*; and (v) the ion-exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS/FLD) or coupled with post-column derivatisation and optical detection (IEC-VIS) methods for the quantification of *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 (L-lysine sulphate)*:

- European Pharmacopoeia monograph 20301

For the quantification of *lysine* in the *feed additives 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 optical 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); or
- ion-exchange chromatography coupled with post-column derivatisation and optical 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 fermentation with

Corynebacterium glutamicum CGMCC 17927 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, Reference SANTE/E5: FORW. APPL. 1831-0041-2021 & Annex I – submission number 1616532569105-2946
- [2] Commission Implementing Regulation (EU) 2019/1964 of 26 November 2019 concerning the authorisation of L-lysine base, liquid, L-lysine monohydrochloride, liquid, L-lysine monohydrochloride, technically pure, and L-lysine sulphate as feed additives for all animal species O.J. L 307, 28.11.2019
- [3] Commission Implementing Regulation (EU) 2020/1798 of 30 November 2020 concerning the authorisation of L-lysine monohydrochloride produced by *Corynebacterium glutamicum* DSM 32932 and L-lysine sulphate produced by *Corynebacterium glutamicum* KFCC 11043 as feed additives for all animal species O.J. L 402, 01.12.2020
- [4] Commission Implementing Regulation (EU) 2020/997 of 9 July 2020 concerning the authorisation of L-lysine base, liquid, L-lysine sulphate and L-lysine monohydrochloride, technically pure, as feed additives for all animal species O.J. L 221, 10.07.2020
- [5] Commission Implementing Regulation (EU) 2021/2095 of 9 November 2021 concerning the authorisation of L-lysine base, L-lysine monohydrochloride and L-lysine sulphate as feed additives for all animal species O.J. L 427, 30.11.2021
- [6] *Technical dossier, Section II: II.2.1.3. Qualitative and quantitative composition
- [7] *Technical dossier, Section II: II.5.1. Proposed mode of use in animal nutrition
- [8] EURL Evaluation Reports:
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2018-0019_l-lysinehcl_sulphate.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2018-0028_l-lysine.pdf
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<https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2020-00820085-lysine-sulfate.pdf>
- [9] EN ISO 13903:2005- Animal feeding stuffs – Determination of amino acids content
- [10] 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
- [11] *Technical dossier, Section II: Annex 2.1.3a & Annex 2.1.3b
- [12] EN ISO 17180:2013 - Animal feeding stuffs – Determination of lysine, methionine and threonine in commercial amino acid products and premixtures

- [13] Food Chemical Codex monograph "L-lysine monohydrochloride", FCC 7 (2010), p.598
- [14] European Pharmacopoeia Monograph 01/2008:20301 - Identification reactions of ions and functional groups – sulphates

*Refers to Dossier no: FAD-2021-0051

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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- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
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- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
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