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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 produced by fermentation with
Corynebacterium glutamicum CGMCC 7.453
(FEED-2023-19953; CRL/230060)**



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in connection with the Application for Authorisation of a
Feed Additive according to Regulation (EC) No 1831/2003**

Dossier related to: **FEED-2023-19953 - CRL/230060**

Name of Product: ***L-lysine monohydrochloride produced by
fermentation with *Corynebacterium
glutamicum* CGMCC 7.453***

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

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

Report prepared by: **Zigmas Ezerskis**

Report checked by: **María José González de la Huebra**
Date: **21/03/2024**

Report approved by: **Ursula Vincent**
Date: **25/03/2024**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *L-lysine monohydrochloride produced by fermentation with a genetically modified Corynebacterium glutamicum* CGMCC 7.453, 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 *feed additive* contains a minimum of *L-lysine* content of 78.0 % (w/w, based on dry matter).

L-lysine monohydrochloride is intended to be added to *compound feed* through *premixtures* or directly into *compound feed* and *water*. However, the Applicant did not propose a minimum or maximum *L-lysine* content in *compound feed* or *water*.

In the frame of official control, the EURL recommends the Food Chemical Codex monograph "L-lysine monohydrochloride monograph" for the identification of *L-lysine monohydrochloride* in the *feed additive*.

For the determination of *lysine* in the *feed additive* and *premixtures* the Applicant proposed the ring-trial validated method EN ISO 17180 dedicated for the determination of free *lysine*, methionine and threonine in commercial amino acid products and *premixtures*. The method does not distinguish between the salts of amino acids and cannot differentiate between enantiomers. It is applicable for products containing more than 10 % of the amino acid. The following performance characteristics were reported for the determination of *lysine*: 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 %.

Based on the performance characteristics available, the EURL recommends for official control the EN ISO 17180 method based on IEC-VIS/FLD for the determination of *lysine* in the *feed additive* and *premixtures* (containing more than 10 % *lysine*).

For the determination of *lysine* in *compound feed* the Applicant proposed the ring-trial validated European Union (EU) method (Commission Regulation (EC) No 152/2009). The method is based on ion-exchange chromatography coupled to optical detection (IEC-VIS) and is dedicated for the analysis of free and total amino acids in *premixtures* and *compound feed*. The EU method was initially ring-trial validated using four different matrices and further validated in the second ring-trial, resulting in the EN ISO 13903 method. The following combined performance characteristics were reported for the determination of *lysine* in animal feedingstuffs: RSD_r ranging from 2.1 to 3.5 % and RSD_R ranging from 3.0 to 13.1 %. Furthermore, the following limits of determination (LOQ) were reported for free and total *lysine*: 0.035 and 0.3 g/kg feedingstuffs, respectively.

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated European Union (EU) method, based on IEC-VIS to determine *lysine* in *premixtures* and *compound feed*.

For the determination of *lysine* in *water* the Applicant did not propose any method. However, in the frame of the stability studies, the Applicant presented experimental data obtained for the determination of *lysine* in *water* when using the IEC-VIS procedure of the above-mentioned EU method. In addition, in the frame of the authorisation of several very similar lysine products the EURL recommended another procedure, namely IEC-VIS/FLD of the above-mentioned EN ISO 17180 method for the determination of lysine in *water*.

Based on the available information, the EURL recommends for official control the two procedures, namely IEC-VIS or IEC-VIS/FLD of the above-mentioned methods for the determination 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 produced by fermentation with a genetically modified Corynebacterium glutamicum CGMCC 7.453, nutritional additives, amino acids, all animal species.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for *L-lysine monohydrochloride produced by fermentation with a genetically modified Corynebacterium glutamicum* CGMCC 7.453, 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]. The authorisation is sought for all animal species [1,2]. *L-lysine monohydrochloride produced by fermentation* using other *Corynebacterium glutamicum* strains is already authorised as nutritional *feed additive* for all animal species under several Commission Implementing Regulations [3-8].

According to the Applicant, the *feed additive* contains a minimum of *L-lysine* content of 78.0 % (w/w, based on dry matter) [9].

L-lysine monohydrochloride is intended to be added to *compound feed* through *premixtures* or directly into *compound feed* and *water* [10]. However, the Applicant did not propose a minimum or maximum *L-lysine* content in *compound feed* or *water* [10].

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

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 produced by fermentation with a genetically modified Corynebacterium glutamicum* CGMCC 7.453 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 determination of *lysine* in the *feed additive* and *premixtures* the Applicant proposed [12] the ring-trial validated method EN ISO 17180 dedicated for the determination of free *lysine*, methionine and threonine in commercial amino acid products and *premixtures* [13]. The method does not distinguish between the salts of amino acids and cannot differentiate between enantiomers. It is applicable for products containing more than 10 % of the 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). The *lysine* is determined either after post-column derivatisation with ninhydrine and optical detection: (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 [13].

The performance characteristics reported for the determination of free *lysine* in commercial amino acid products and *premixtures* when using the EN ISO 17180 method are listed in Table 1.

Table 1: Method performance characteristics obtained in the frame of ring-trial validation studies (EN ISO 17180 [13], EU method [14] and EN ISO 13903 [15]) for the determination of *lysine* in feedingstuffs.

Ring-Trial	Matrix	<i>Lysine</i> content, g/kg	RSD _r , %	RSD _R , %
[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
[14, 15]	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
[15]	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.

Based on the performance characteristics available, the EURL recommends for official control the EN ISO 17180 method for the determination of free *lysine* in the *feed additive* and *premixtures* (containing more than 10 % *lysine*).

For the determination of *lysine* in *compound feed* the Applicant proposed [12] the ring-trial validated European Union (EU) method [14]. The EU method is designed for the determination of *free* (synthetic and natural) and of *total* (peptide-bound and free) amino acids in *premixtures* and *compound feed*, using an amino acid analyser or IEC coupled to post-column derivatisation and optical (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 optical 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 optical detection at 570 nm [14].

The EU method was initially ring-trial validated using four different matrices. This method was further ring-trial validated by 23 laboratories, resulting in the EN ISO 13903 method [15].

The performance characteristics reported for the determination of *lysine* in different animal feedingstuffs when using the EU method (or equivalent EN ISO 13903 method) are listed in Table 1. Furthermore, the following limits of determination (LOQ) were reported for free and total *lysine*: 0.035 and 0.3 g/kg feedingstuffs, respectively [15].

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated European Union (EU) method, based on IEC-VIS to determine *lysine* in *premixtures* and *compound feed*.

For the determination of *lysine* in *water* the Applicant did not propose any method [12]. However, in the frame of the stability studies, the Applicant presented experimental data obtained for the determination of *lysine* in *water* [16] when using the IEC-VIS procedure of the above-mentioned EU method. In addition, in the frame of the authorisation of several very similar lysine products [3-5,7,8], the EURL recommended another procedure, namely IEC-VIS/FLD of the above-mentioned EN ISO 17180 method for the determination of lysine in *water*.

Based on the available information, the EURL recommends for official control the two procedures, namely IEC-VIS or IEC-VIS/FLD of the above-mentioned methods 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)

An 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)

For the identification of *L-lysine monohydrochloride*, the Applicant proposed [17] an optical rotation described in a generic European Pharmacopeia monograph [18]. However, the EURL found the specific Food Chemical Codex (FCC) “L-lysine monohydrochloride monograph” [19], where different tests (including the test based on an optical rotation) are used for the identification of *L-lysine monohydrochloride*.

Therefore, the EURL recommends the Food Chemical Codex monograph 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) for the identification of *L-lysine monohydrochloride* in the *feed additive*; (ii) the ring-trial validated method EN ISO 17180 based on ion-exchange chromatography coupled to optical detection (IEC-VIS/FLD) to determine *lysine* in the *feed additive* and *premixtures*; (iii) the European Union (EU) method based on ion-exchange chromatography coupled to optical detection (IEC-VIS) for the determination of *lysine* in *premixtures* and *compound feed*; and (iv) the ion-exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS or IEC-VIS/FLD) for the determination 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 determination of *lysine* in the *feed additive*:

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

For the determination of *lysine* in *premixtures*:

- Ion-exchange chromatography coupled to post-column derivatisation and optical detection (IEC-VIS/FLD) – EN ISO 17180 or
- Ion-exchange chromatography coupled to post-column derivatisation and optical detection (IEC-VIS) – Commission Regulation (EC) No 152/2009 (Annex III, F)

For the determination of *lysine* in *compound feed*:

- Ion-exchange chromatography coupled to post-column derivatisation and optical detection (IEC-VIS) – Commission Regulation (EC) No 152/2009 (Annex III, F)

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

- Ion-exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS or 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 produced by fermentation with a genetically modified Corynebacterium glutamicum* CGMCC 7.453 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] *Forwarding of applications for authorisation of feed additives in accordance with Regulation (EC) No 1831/2003 – E-Submission Food Chain platform –
<https://webgate.ec.europa.eu/esfc/#/applications/50353>
<https://open.efsa.europa.eu/questions/EFSA-Q-2023-00723>
- [2] *Application – Annex 1
- [3] Commission Implementing Regulation (EU) 2023/1163 of 14 June 2023 concerning the authorisation of L-Lysine monohydrochloride and L-Lysine sulphate produced by *Corynebacterium glutamicum* CGMCC 17927 as feed additives for all animal species, OJ L 154, 15.6.2023
- [4] Commission Implementing Regulation (EU) 2022/1525 of 13 September 2022 concerning the authorisation of L-lysine monohydrochloride and L-lysine sulphate produced by fermentation with *Corynebacterium glutamicum* CGMCC 14498 as feed additives for all animal species, OJ L 237, 14.9.2022
- [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] 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
- [7] 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
- [8] 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, OJ L 307, 28.11.2019
- [9] *Technical dossier, Section II : II.1.3. Qualitative and quantitative composition
- [10] *Technical dossier, Section II: 2.5.1. Proposed mode of use in animal nutrition
- [11] EURL reports:
https://joint-research-centre.ec.europa.eu/publications/fad-2018-0012_en
https://joint-research-centre.ec.europa.eu/publications/fad-2018-0019_en
https://joint-research-centre.ec.europa.eu/publications/fad-2018-0028_en

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https://joint-research-centre.ec.europa.eu/publications/fad-2021-0075_en

- [12] *Technical dossier, Section II: II.6.1. Methods of analysis for the active substance
- [13] EN ISO 17180:2013 - Animal feeding stuffs – Determination of lysine, methionine and threonine in commercial amino acid products and premixtures
- [14] 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
- [15] EN ISO 13903:2005- Animal feeding stuffs – Determination of amino acids content
- [16] *Technical dossier, Section II: II.4.1. Stability
- [17] *Technical dossier, Section II: II.6.3. Methods of the analysis relating to the identity and characterisation of the additive
- [18] European Pharmacopoeia Monograph 07/2018:20207 – Optical rotation
- [19] Food Chemical Codex monograph "L-lysine monohydrochloride", FCC 7 (2010), p. 598
- *Refers to Dossier no: FEED-2023-19953

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)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
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-
- Wageningen Food Safety Research (WFSR)¹ (NL)
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 - Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)

¹ Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: RIKILT Wageningen UR, Wageningen.