Ref. Ares(2021)7641323 - 10/12/2021

EUROPEAN COMMISSION
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Directorate F - Health, Consumers and Reference Materials (Geel)
Food and Feed Compliance

European Union Reference Laboratory
Feed Additives

JRC F.5/CvH/MGH/AS/Ares

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, concentrated liquid L-lysine and **concentrated liquid L-lysine monohydrochloride**
produced by fermentation with *Escherichia coli* NITE BP-02917
(FAD-2021-0075; CRL/200081)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2021-0075 - CRL/200081**
Name of Product:	***L-lysine monohydrochloride, concentrated liquid L-lysine produced and concentrated liquid L-lysine monohydrochloride produced by fermentation with Escherichia coli* NITE BP-02917**
Active Agent (s):	**L-lysine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**María José González de la Huebra**
Report checked by: Date:	**Stefano Bellorini** **09/12/2021**
Report approved by: Date:	**Christoph von Holst** **09/12/2021**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4 for *L-lysine monohydrochloride*, *concentrated liquid L-lysine* and *concentrated líquid L-lysine monohydrochloride* produced *by* fermentation with *Escherichia coli* NITE BP-02917, under the categories/functional groups 3(c) 'nutritional additives'/'amino acids, their salts and analogues' and 2(b) 'sensory additives'/flavouring compounds' according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for all animal species.

According to the Applicant, *L-lysine monohydrochloride* has a minimum purity (mass fraction on dry matter basis) of 99 % and a minimum *L-lysine* content of 78 % while the *concentrated liquid L-lysine* and the *concentrated líquid L-lysine monohydrochloride* have a minimum *L-lysine* content of 50 % and 22.4 %, respectively.

When used as nutritional additive the solid form of the *feed additive* (*L-lysine monohydrochloride*) is intended to be added directly into *feedingstuffs* or through *premixtures* while the liquid forms (*concentrated liquid L-lysine* and *concentrated líquid L-lysine monohydrochloride*) are intended to be added directly into *feedingstuffs.* When used as sensory additive the three *L-lysine* forms are intended to be added directly into *feedingstuffs* and *water* for drinking.

The Applicant did not propose any minimum or maximum content of L-lysine in *feedingstuffs* when used as nutritional additive, however a recommended inclusion level of 25 mg/kg *feedingstuffs* was proposed by the Applicant when used as sensory additive.

For the quantification of *lysine* in the *feed additive* the Applicant proposed 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 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 *lysine* in *premixtures*, *feedingstuffs* and *water* the Applicant proposed the ring-trial validated European Union method (Commission Regulation (EC) No 152/2009) based on IEC coupled with optical 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 3.0 to 6.7 %. Furthermore, as concluded in previous amino acids reports, the EURL also considers the IEC-VIS/FLD procedure described above as fit-for-purpose for the determination of *lysine* in *water*.

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 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 European Union method based on IEC-VIS for the quantification of *lysine* in *premixtures* and *feedingstuffs*; (for *feedingstuffs* only when used as nutritional additive) and (iv) the ion-exchange chromatography methods coupled with post-column derivatisation and optical detection (IEC-VIS/FLD) or coupled with post-column derivatisation and optical detection (IEC-VIS) for the quantification of *lysine* in *water* (only when used as nutritional 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.

KEYWORDS

L-lysine monohydrochloride, concentrated liquid L-lysine and *concentrated liquid L-lysine monohydrochloride* produced by fermentation with *Escherichia coli* NITE BP-02917, nutritional additives, sensory additives, amino acids their salts and analogues, flavouring compounds, 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*, *concentrated liquid L-lysine* and *concentrated líquid L-lysine monohydrochloride* produced *by* fermentation with *Escherichia coli* NITE BP-02917, under the categories/functional groups 3(c) 'nutritional additives'/'amino acids, their salts and analogues', and 2(b) 'sensory additives'/flavouring compounds' according to Annex I of Regulation (EC) No 1831/2003 [1]. The authorisation is sought for all animal species [1,2]. Other *L-lysine feed additives* produced by fermentation with different *Escherichia Coli* strains are already authorised as nutritional additives by Commission Regulations (EC) No 2021/1964 and (EC) No 2019/1964 [3, 4]. However, none of the three L-lysine forms are currently authorised as sensory additives.

According to the Applicant, *L-lysine monohydrochloride* has a minimum purity (mass fraction on dry matter basis) of 99 % and a minimum *L-lysine* content of 78 % while the *concentrated liquid L-lysine* and the *concentrated líquid L-lysine monohydrochloride* have a minimum *L-lysine* content of 50 % and 22.4 %, respectively [5].

The *feed additive*s are produced by fermentation with an *Escherichia Coli* strain deposited under accession number NITE BP-02917.

When used as nutritional additive the solid form of the *feed additive* (*L-lysine monohydrochloride*) is intended to be added directly into *feedingstuffs* or through *premixtures* while the liquid forms (*concentrated liquid L-lysine* and *concentrated líquid L-lysine monohydrochloride*) are intended to be added directly into *feedingstuffs.* When used as sensory additive the three *L-lysine* forms are intended to be added directly into *feedingstuffs* and *water* for drinking [6].

The Applicant did not propose any minimum or maximum content of *L-lysine* in *feedingstuffs* when used as nutritional additive, however a recommended inclusion level of 25 mg/kg *feedingstuffs* was proposed by the Applicant when used as sensory additive [6].

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

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*, *concentrated liquid L-lysine* and *concentrated liquid L-lysine monohydrochloride* 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 proposed 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" [8]. This

standard method is based on the experimental protocol described in the EU method for the analysis of free amino acids (including *lysine*) [8]. 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 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. 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 *lysine* in *premixtures*, *feedingstuffs* and *water* the Applicant suggested using the European Union (EU) method dedicated for the determination of amino acids in feed [9]. This method was 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 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 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 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 optical detection at 570 nm

The EU 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 [10]. 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 [10].

In the frame of stability studies the Applicant provided experimental data of the applicability of this method for the determination of *lysine* in *water* (ranging from 0.5 to 5.0 g L-lysine/1000 g water) for the three products [11].

However, the EURL is unable to recommend the European Union method for the official control of these products in *feedingstuffs* and in *water* when intended to be used as sensory additive/flavouring compound, due to the fact that the recommended inclusion level in *feedingstuffs* when used as flavouring compound is very low (e.g. 25 mg/kg) which is below the limit of quantification reported for *lysine* [10].

Table 1: Method performance characteristics obtained in the frame of ring-trial validation studies (EN ISO 17180:2013 [8], European Union method [9] and EN ISO 13903:2005 [10]) for the determination of total *lysine* in the *feed additive*, *premixtures* and *feedingstuffs*.

Ring-Trial	Matrix	*lysine* content g/kg	RSD_r %	RSD_R %
[8]	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]	Mixed pig feed	10	2.8	3.2
	Broiler compound	14	2.1	5.4
	Protein concentrate	48	2.4	3.0
	Premix	98	2.1	6.7
[10]	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

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated EU method based on IEC-VIS to quantify *lysine* in *premixtures feedingstuffs* and *water* (for *feedingstuffs* and *water* only when used as nutritional additive). Furthermore, as concluded in previous amino acids reports [7], the EURL also considers the IEC-VIS/FLD procedure described above 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)

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)

The EURL found the Food Chemical Codex (FCC) "L-lysine monohydrochloride monograph", where different tests (including optical rotation) are used for the identification of *L-lysine monohydrochloride* in the *feed additive* [11] and thus recommends the Food Chemical Codex monograph for the identification of *L-lysine monohydrochloride.*

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 17180:2013 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 European Union method based on IEC-VIS for the quantification of *lysine* in *premixtures* and *feedingstuffs* (for *feedingstuffs* only when used as nutritional additive); 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 *wate*r (for *water* only when used as nutritional additive).

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* (for feedingstuffs only when used as nutritional additive):

- 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 (*only when used as nutritional additive):

- 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, concentrated liquid L-lysine* and *concentrated liquid L-lysine monohydrochloride* produced by fermentation with *Escherichia coli* NITE BP-02917 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. 1831/0060-2021

[2] *Application, Annex I – Submission number 1616273949716-2926

[3] Commision 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

[4] Commision Implementing Regulation (EU) 2021/669 of 23 April 2021concerning the the authorisation of technically pure L-lysine monohydrochloride and liquid L-lysine base produced by *Corynebacterium casei* KCCM 80190 or *Corynebacterium glutamicum* KCCM 80216 or *Corynebacterium glutamicum* KCTC 12307BP as feed additives for all animal species

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

[6] *Technical dossier, Section II: II.5.1. Proposed mode of use in animal nutrition

[7] EURL Evaluation Reports: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2013-0045_l-lysine%20sulphate.pdf

https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0052_lysine.pdf
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https://ec.europa.eu/jrc/sites/default/files/finrep_fad-2020-0067-lyshcl-lys-sulphate.pdf
https://ec.europa.eu/jrc/sites/default/files/finrep-fad-2020-00820085-lysine-sulfate.pdf

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

[9] 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

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

[11] *Technical dossier, Section II: Annex II_62

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

*Refers to Dossier no: FAD-2021-0075

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

The following National Reference Laboratories contributed to this report:

- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 - Labore Landwirtschaft, Nossen (DE)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural**.** Generalitat de Catalunya, Cabrils (ES)

- Wageningen Food Safety Research[1] (WFSR) (NL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- Ruokavirasto, Helsinki[2] (FI)

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

[2] Name and address according to COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: Elintarviketurvallisuusvirasto/Livsmedelssäkerhetsverket (Evira), Helsinki/Helsingfors.