



EUROPEAN COMMISSION

JOINT RESEARCH CENTRE  
Directorate F – Health, Consumers and Reference Materials  
**European Union Reference Laboratory for Feed Additives**

JRC F.5/CvH/SB/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 and concentrated liquid L-lysine**  
produced by fermentation with *Corynebacterium casei* KCCM80190  
(*FAD-2019-0014; CRL/180053*)





**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-2019-0014 - CRL/180053**

Name of Product: ***L-lysine monohydrochloride and  
concentrated liquid L-lysine produced by  
fermentation with Corynebacterium casei  
KCCM80190***

Active Agent: **L-lysine**

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

Report prepared by: **Stefano Bellorini**

Report checked by: **Zigmas Ezerskis**  
Date: **06/09/2019**

Report approved by: **Christoph von Holst**  
Date: **06/09/2019**

## EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-lysine monohydrochloride and concentrated liquid L-lysine produced by fermentation with Corynebacterium Casei KCCM80190*, 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 the *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 a slightly modified protocol of the European Union method dedicated for the determination of amino acids in feed. However, the EURL previously evaluated *lysine* dossiers and recommended for the quantification of *lysine* in the *feed additives* and *premixtures* (containing more than 10 % *lysine*) 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 ( $RSD_t$ ) 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* and *feedingstuffs* the Applicant suggested using the ring-trial validated VDLUFA 4.11.6 method. However, the EURL previously evaluated *lysine* dossiers and recommended for the quantification of *lysine* in *premixtures* and *feedingstuffs* the ring-trial validated European Union 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*:  $RSD_t$  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 analysing *lysine* in *water* with a slightly modified protocol of the VDLUFA 4.11.6 method 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 European Union method based on IEC-VIS for the quantification of *lysine* in *premixtures* and *feedingstuffs*; and (iv) the slightly modified VDLUFA 4.11.6 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 fermentation with Corynebacterium Casei KCCM80190*, 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 fermentation with Corynebacterium Casei KCCM80190*, 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 pale brownish crystalline powdered *L-lysine monohydrochloride* has a minimum purity (mass fraction) of 98.5 % (minimum of 78 % of *L-lysine*), while the *concentrated* dark brown *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 casei*. The production strain is deposited in the "Korean Centre of Microorganisms" (KCCM) under accession number *KCCM80190*.

The two forms of the *feed additive* are intended to be added directly into *feedingstuffs* (or through *premixtures*) and *water* for drinking [5]. 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 [7-16].

## 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 *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 a slightly modified protocol of the European Union (EU) method dedicated for the determination of amino acids in feed [17,18].

However, the EURL previously evaluated and recommended for the quantification of *lysine* in the *feed additives* and *premixtures* (containing more than 10 % *lysine*) 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" [19]. This standard method is based on the experimental protocol described in the EU method for analysis of free amino acids (including *lysine*) [20]. 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 *lysine* in *premixtures* and *feedingstuffs* the Applicant suggested using the ring-trial validated VDLUFA 4.11.6 method dedicated for the determination of free *lysine*, methionine and threonine in the products of amino acids and *premixtures* containing more than 10 % of free amino acid [21,22]. However, the EURL previously evaluated and recommended for the quantification of *L-lysine* in *premixtures* and *feedingstuffs* the mentioned above ring-trial validated EU method [20]. 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 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 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 [23]. 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 [23].

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* and *feedingstuffs*.

The Applicant did not perform any validation/verification studies to demonstrate the suitability of the EU method for the determination of *lysine* in *water* [22]. However, in the frame of the stability studies, the Applicant presented experimental data obtained analysing *lysine* in *water* [5,24]. The tests were carried out using a slightly modified protocol of the above mentioned VDLUFA 4.11.6 method [21]. This method, equivalent to EN ISO 17180:2013, is based on IEC coupled with VIS or FLD [19]. 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 [19], European Union method [20] and EN ISO 13903:2005 [23]) for the determination of total *L-lysine* in the *feed additive*, *premixtures* and *feedingstuffs*.

Ring-Trial	Matrix	<i>lysine</i> content g/kg	RSD <sub>r</sub> %	RSD <sub>R</sub> %
[19]	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
[20]	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
[23]	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<sub>r</sub>, RSD<sub>R</sub> - 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 [25].

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 European Union method based on IEC-VIS for the quantification of *lysine* in *premixtures* and *feedingstuffs*; and (iv) the slightly modified VDLUFA 4.11.6 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 fermentation with Corynebacterium casei KCCM80190* 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. 1831/0025-2019 & Annex I – submission number 1550673514730-2370
- [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: II.2.1.1. Chemical substances
- [5] \*Technical dossier, Section II: II.4.1.3. Stability of the additive used in water
- [6] \*Technical dossier, Section II: II.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](https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2013-0027_L-lysine.pdf)
- [10] 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](https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2013-0045_l-lysine%20sulphate.pdf)
- [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](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](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  
[https://ec.europa.eu/jrc/sites/jrcsh/files/finrep\\_fad-2018-0012\\_l-lysine.pdf](https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2018-0012_l-lysine.pdf)

- 
- [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 [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-2018-0019_l-lysinehcl_sulphate.pdf)
- [15] FAD-2018-0028, L-lysine monohydrochloride and concentrated liquid L-lysine produced by *Corynebacterium glutamicum* KCCM10227 Ref. Ares(2018)5200878 - 10/10/2018 [https://ec.europa.eu/jrc/sites/jrcsh/files/finrep\\_fad-2018-0028\\_l-lysine.pdf](https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2018-0028_l-lysine.pdf)
- [16] FAD-2018-0037, L-lysine monohydrochloride and concentrated liquid L-lysine produced by *Corynebacterium glutamicum* NRRL-B-67535 Ref. Ares(2018)6419577 - 13/12/2018 [https://ec.europa.eu/jrc/sites/jrcsh/files/finrep\\_fad-2018-0037-lysine.pdf](https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2018-0037-lysine.pdf)
- [17] \*Technical dossier, Section II: II.6.1. Methods of analysis for the active substance
- [18] \*Technical dossier, Section II – Annex\_II\_06\_01
- [19] EN ISO 17180:2013 - Animal feeding stuffs – Determination of lysine, methionine and threonine in commercial amino acid products and premixtures
- [20] 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
- [21] Bestimmung von Lysin, Methionin und Threonin in Aminosäurehandelsprodukten und Vormischungen – 4.11.6, Methodenbuch III, 5. Erg. 2004, VDLUFA – Verlag, Darmstadt
- [22] \*Technical dossier, Section II: II.6.3. Methods of the analysis related to the identity and characterisation of the additive
- [23] EN ISO 13903:2005- Animal feeding stuffs – Determination of amino acids content
- [24] \*Technical dossier, Section II – Annex\_II\_4\_03
- [25] Food Chemical Codex monograph "L-lysine monohydrochloride", FCC 7 (2010), p.598
- \*Refers to Dossier no: FAD-2019-0014

## **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)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 — Labore Landwirtschaft, Nossen (DE)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)