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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, concentrated liquid L-lysine and **L-lysine sulphate** produced by Corynebacterium glutamicum KCCM80183
(FAD-2019-0016; CRL/180035)

L-lysine monohydrochloride produced by Corynebacterium glutamicum DSM32932
(FAD-2019-0028; CRL/190017)

European Union Reference Laboratory
Feed Additives

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

Dossiers related to:	**FAD-2019-0016 - CRL/180035** **FAD-2019-0028 – CRL/190017**
Name of Product:	***L-lysine monohydrochloride, concentrated liquid L-lysine, L-lysine sulphate produced by Corynebacterium glutamicum KCCM80183; and L-lysine monohydrochloride produced by Corynebacterium glutamicum DSM32932***
Active Agent:	**L-lysine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Zigmas Ezerskis** **11/11/2019**
Report approved by: Date:	**Christoph von Holst** **12/11/2019**

EXECUTIVE SUMMARY

In the current applications authorisation is sought under Article 4(1) for *L-lysine monohydrochloride*, *concentrated liquid L-lysine* and *l-lysine sulphate produced by Corynebacterium glutamicum KCCM80183* and *L-lysine monohydrochloride produced by Corynebacterium glutamicum DSM32932*, 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 Applicants, *L-lysine monohydrochloride* contains a minimum (mass fraction) of 78 % of *L-lysine* as active substance, while the *concentrated liquid L-lysine* and the *L-lysine sulphate* contain a minimum of 50 and 55 % of *L-lysine*, respectively.

The different forms of the *feed additive* are intended to be added directly into *feedingstuffs* or through *premixtures*. *L-lysine monohydrochloride*, *concentrated liquid L-lysine* and *l-lysine sulphate produced by Corynebacterium glutamicum KCCM80183* can also be included in *water* for drinking. However the Applicants did not propose any minimum or maximum content of *L-lysine* in *feedingstuffs*.

For the quantification of *lysine* in the *feed additive*, the Applicants submitted the European Union (EU) method dedicated for the determination of amino acids in *premixtures* and *feedingstuffs*. However, for the quantification of *lysine* in the *feed additive* the EURL previously evaluated and recommended 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 %.

For the quantification of *L-lysine* in *premixtures* and *feedingstuffs* one Applicant submitted the ring-trial validated European Union method (Commission Regulation (EC) No 152/2009) based on IEC coupled with photometric detection (IEC-VIS), which was previously recommended by the EURL. 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 to 6.7 %.

The different forms of *Lysine* produced by *Corynebacterium glutamicum KCCM80183* can also be included in *water* for drinking. However, the corresponding Applicant did not provide any experimental data to determine *lysine* in *water*. Nevertheless, as concluded in previous

amino acids reports of the EURL, the IEC-VIS procedure described in the European Union method is considered fit-for-purpose for the determination of *lysine* in *water*.

In addition, the EURL found 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 ion in *L-lysine sulphate*.

In the frame of these authorisations 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*); and (iv) the European Union 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), as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

L-lysine monohydrochloride, concentrated liquid L-lysine and l-lysine sulphate produced by Corynebacterium glutamicum KCCM80183, L-lysine monohydrochloride produced by Corynebacterium glutamicum DSM32932, nutritional additives, amino acids, all animal species and categories

1. BACKGROUND

In the current applications authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *L-lysine monohydrochloride, concentrated liquid L-lysine* and *l-lysine sulphate produced by Corynebacterium glutamicum KCCM80183* and *L-lysine monohydrochloride produced by Corynebacterium glutamicum DSM32932*, 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 [1-4]. The above mentioned forms of *L-lysine* are already authorised as *feed additive* under Commission Directive 88/485/EEC of 26 July 1988 (code 3.2.2, 3.2.3, 3.2.4

and 3.2.5) and for *L-lysine sulphate* (produced by Escherichia coli, code 3c323) under Commission Implementing Regulation (EU) 2017/439 of 13 March 2017 [5,6].

According to the Applicants, the dry crystalline powdered *L-lysine monohydrochloride* contains a minimum (mass fraction) of 78 % of *L-lysine* as active substance, while the dark brown *concentrated liquid L-lysine* and the pale brown granulated *L-lysine sulphate* contain a minimum of 50 and 55 % of *L-lysine*, respectively [7,8].

The different forms of the *feed additive* are produced by fermentation with two genetically modified strains of *Corynebacterium glutamicum* [9]. The production strain related to FAD-2019-0016 is deposited at the "Korean Centre of Microorganisms" (KCCM) with the reference *Corynebacterium glutamicum KCCM80183*, while the production strain relevant to FAD-2019-0028 is deposited at the "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (DSMZ) in Germany under deposition number *Corynebacterium glutamicum DSM 32932* [9].

The different forms of the *feed additive* are intended to be added directly into *feedingstuffs* or through *premixtures* [10,11]. *L-lysine monohydrochloride*, *concentrated liquid L-lysine* and *L-lysine sulphate produced by Corynebacterium glutamicum KCCM80183* can also be included in *water* for drinking [12]. However, the Applicants did not propose any minimum or maximum content of *L-lysine* in *feedingstuffs* [1,2,10,11].

Note: The EURL has previously evaluated the analytical methods for the determination of *lysine* in the frame of several dossiers [13-24].

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 these particular dossiers, the methods of analysis submitted in connection with *L-lysine monohydrochloride*, *concentrated liquid L-lysine, 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*, the Applicants submitted the European Union (EU) method dedicated for the determination of amino acids in feed and/or a slightly modified protocol of this method [25-28].

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" [29]. This standard method is based on the experimental protocol described in the European Union method for the analysis of free amino acids (including *lysine*) [27]. 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 different forms of *feed additive* and *premixtures* (containing more than 10 % *lysine*).

For the quantification of *lysine* in *premixtures* and *feedingstuffs* one Applicant (FAD-2019-0016) suggested 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 [30-31]. However, as also suggested by the second Applicant (FAD-2019-0028), the EURL previously evaluated and recommended for the quantification of *L-lysine* in *premixtures* and *feedingstuffs* the mentioned above ring-trial validated EU method [26-27]. This method was designed for the quantification of free (synthetic and natural) and 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.

Table 1: Method performance characteristics obtained in the frame of ring-trial validation studies (EN ISO 17180:2013 [29], European Union method [27] and EN ISO 13903:2005 [32]) for the determination of total *L-lysine* in the *feed additive*, *premixtures* and *feedingstuffs*.

Ring-Trial	Matrix	*lysine* content g/kg	RSD_r %	RSD_R %
[29]	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
[27]	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
[32]	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_r, RSD_R - relative standard deviation for *repeatability* and *reproducibility,* respectively

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 [32]. 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 [32].

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated European Union method, based on IEC-VIS to quantify *lysine* in *premixtures* and *feedingstuffs*.

The products presented by Applicant FAD-2019-0016 can also be included in water for drinking [12]. However, the Applicant did not provide any experimental data to determine *lysine* in *water* [25]. Nevertheless, as concluded in previous amino acids reports of the EURL, even if the determination of *lysine* in *water* is not explicitly stated in the scope of the European Union method (or similar ones e.g. VDLUFA Method 4.11.6.), the IEC-VIS procedure described above is considered fit-for-purpose for the determination of *lysine* in *water* [24].

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 [33]. The EURL recommends the Food Chemical Codex for the identification of *L-lysine monohydrochloride* in the *feed additive*.

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 ion in *L-lysine sulphate* [34]. The EURL recommends for official control 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 these authorisations 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*); and (iv) the European Union 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), as described in Commission Regulation (EC) No 152/2009 (Annex III, F)

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 *l-lysine sulphate produced by Corynebacterium glutamicum KCCM80183* and *L-lysine monohydrochloride produced by Corynebacterium glutamicum DSM32932* have been sent to the European Union Reference Laboratory for Feed Additives. The dossiers have been made available to the EURL by EFSA.

6. REFERENCES

[1] *Application, Proposal of Registry Entry – Annex A

[2] ˣApplication, Proposal of Registry Entry – Annex A

[3] *Application, Reference SANTE/E5: Forw. Appl. 1831/0023-2019 & Annex I – submission number 1550734032082-2376

[4] ˣApplication, Reference SANTE/E5: Forw. Appl. 1831/0036-2019 & Annex I – submission number 1555610235381-2404

[5] 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

[6] 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, 14.3.2017

[7] *Technical dossier, Section II: II.1.3. Qualitative and quantitative composition

[8] ˣTechnical dossier, Section II: 2.2 Characterisation of the active substance(s)/agent(s)

[9] ˣTechnical dossier, Section II: 2.2.1.2. Micro-organisms

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

[11] ˣTechnical dossier, Section II: 2.5.1. Proposed mode of use in animal nutrition

[12] *Technical dossier, Section II: II.4.1.3 Stability of the additive used in water

[13] 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

[14] FAD-2010-0067, Lysine, Ref. Ares(2011)301126 - 18/03/2011 https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep- FAD-2010-0067.pdf

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[16] 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

[17] 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

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[20] 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

[21] 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

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[23] FAD-2019-0014, L-lysine monohydrochloride and concentrated liquid L-lysine produced by fermentation with Corynebacterium casei KCCM80190 Ref. Ares(2019)5620408 - 06/09/2019 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2019-0014-lysine.pdf

[24] FAD-2019-0015, L-lysine sulphate produced by fermentation with Corynebacterium glutamicum KFCC11043 Ref. Ares(2019)5620442 - 06/09/2019 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2019-0015_l-lysine_sulphate.pdf

[25] *Technical dossier, Section II: II.6.1. Methods of analysis for the active substance

[26] ˣTechnical dossier, Section II: 2.6.1. Methods of analysis for the active substance

[27] 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

[28] *Technical dossier, Section II – Annex_II_06_01

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

[30] *Technical dossier, Section II: II.6.3. Methods of the analysis relatin to the identity and characterisation of the additive

[31] VDLUFA MB III 4.11.6 Bestimmung von Lysin, Methionin und Threonin in Aminosäurenhandelsprodukten und Vormischungen

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

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

[34] European Pharmacopoeia Monograph 01/2008:20301 - Identification reactions of ions and functional groups – sulphates

*Refers to Dossier no: FAD-2019-0016
ˣRefers to Dossier no: FAD-2019-0028

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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