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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 sulphate produced by fermentation with
Corynebacterium glutamicum KFCC11043
(FAD-2019-0015; CRL/180071)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2019-0015 - CRL/180071**
Name of Product:	***L-lysine sulphate produced by fermentation with Corynebacterium glutamicum KFCC11043***
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** **06/09/2019**
Report approved by: Date:	**Christoph von Holst** **06/09/2019**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *L-lysine sulphate produced by fermentation with Corynebacterium glutamicum KFCC11043*, 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 sulphate* contains a minimum of 55 % (w/w) of *L-lysine*.

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_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 generic European Pharmacopoeia monograph on sulphates (Ph. Eur. 20301) for the identification of *sulphate* in *L-lysine sulphate*.

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_r ranging from 2.1 to 2.8 % and RSD_R ranging from 3 to 6.7 %.

The Applicant did not provide any experimental data to determine *lysine* in *water*. Nevertheless, as concluded in previous EURL's amino acids reports, the IEC-VIS procedure described in the European Union method (or similar ones e.g. VDLUFA Method 4.11.6.) is considered fit for purpose for the determination of *lysine* in this matrix.

In the frame of this authorisation the EURL recommends for official control (i) the European Pharmacopoeia monograph (Ph. Eur. 01/2008:20301) for the identification of the sulphate ion

in L-lysine sulphate; (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 ring-trial validated Community 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) is not considered necessary.

KEYWORDS

L-lysine sulphate produced by fermentation with Corynebacterium glutamicum KFCC11043, 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 sulphate produced by fermentation with Corynebacterium glutamicum KFCC11043*, 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]. *Lysine* produced by fermentation with different microorganisms is already authorised as nutritional *feed additive* [3,4].

According to the Applicant, the pale-brown granulated powder *L-lysine sulphate* contains a minimum of 55 % (w/w) of *L-lysine* [1,5].

The *feed additive* is produced by fermentation with a genetically modified strain of Corynebacterium glutamicum [6]. The production strain is deposited in the "Korean Culture Center of Microorganism" (KCCM) with reference *Corynebacterium glutamicum KFCC11043* [6].

The *feed additive* is intended to be added directly into *feedingstuffs* or through *premixtures* and *water* for drinking [7,8]. However the Applicant did not propose any minimum or maximum content of *L-lysine sulphate* in *feedingstuffs* [1,7].

Note: The EURL has previously evaluated the analytical methods for the determination of *lysine* in the frame of several dossiers [9-18].

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 sulphate produced by fermentation with Corynebacterium glutamicum KFCC11043* 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 [19,20].

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" [16,21]. This standard method is based on the experimental protocol described in the EU method for analysis of free amino acids (including *lysine*) [22]. 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.

Table 1: Method performance characteristics obtained in the frame of ring-trial validation studies (EN ISO 17180:2013 [21], European Union method [22] and EN ISO 13903:2005 [23]) 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 %
[21]	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
[22]	Mixed pig feed	10	2.8	3.2
	Broiler compound	14	2.1	5.4
	Protein concentrate	48	2.4	3.0
	Premixture	98	2.1	6.7
[23]	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 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 [24,25]. 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 [16,22]. 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 provide any experimental data to determine *lysine* in *water* [19,24]. Nevertheless, as concluded in previous EURL's amino acids reports, 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 this matrix [16].

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)

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 generic European Pharmacopoeia monograph on sulphates (Ph. Eur. 01/2008:20301) for the identification of sulphate in *L-lysine sulphate* [26].

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 this authorisation the EURL recommends for official control (i) the European Pharmacopoeia monograph (Ph. Eur. 01/2008:20301) for the identification of the sulphate ion in L-lysine sulphate; (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 ring-trial validated Community 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 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 sulphate produced by fermentation with Corynebacterium glutamicum KFCC11043* 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. 1831/0024-2019 & Annex I – submission number 1550733653699-2374

[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] 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, 14.03.2017

[5] *Technical dossier, Section II: II.2.1.1. Chemical substances

[6] *Technical dossier, Section II: II.2.1.2. Micro-organisms

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

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

[9] 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

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

[11] 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

[12] 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

[13] 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

[14] 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

[15] 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

[16] 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

[17] 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

[18] 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

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

[20] *Technical dossier, Section II: Annex_II_06_01

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

[22] 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

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

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

[25] Bestimmung von Lysin, Methionin und Threonin in Aminosäurenhandelsprodukten und Vormischungen – 4.11.6, Methodenbuch III, 5. Erg. 2004, VDLUFA – Verlag, Darmstadt

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

*Refers to Dossier no: FAD-2019-0015

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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- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)