EUROPEAN COMMISSION
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European Union Reference Laboratory
Feed Additives

JRC F.5/CvH/ZE/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 sulphate produced by fermentation with
***Corynebacterium glutamicum* KCCM 80227**
(FAD-2020-0082; CRL/200024)

L-lysine sulphate produced by fermentation with
***Escherichia coli* CGMCC 7.398**
(FAD-2020-0085; CRL/200073)

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Dossier related to:	**FAD-2020-0082 - CRL/200024** **FAD-2020-0085 – CRL/200073**
Name of Product:	***L-lysine sulphate produced by fermentation with Corynebacterium glutamicum KCCM 80227; and L-lysine sulphate produced by fermentation with Escherichia coli CGMCC 7.398***
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: Date:	**María José González de la Huebra** **14/04/2021**
Report approved by: Date:	**Christoph von Holst** **14/04/2021**

EXECUTIVE SUMMARY

In the current applications authorisations are sought under Article 4(1) for *L-lysine sulphate* produced by fermentation with *Corynebacterium glutamicum* KCCM 80227 and *L-lysine sulphate* produced by fermentation with *Escherichia coli* CGMCC 7.398, 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 authorisations are sought for all animal species.

According to the Applicants, the *feed additives* contain a minimum of 52 % (w/w) of *L-lysine* in *L-lysine sulphate* produced by fermentation with *Corynebacterium glutamicum* KCCM 80227 and a minimum of 55 % (w/w) of *L-lysine* in *L-lysine sulphate* produced by fermentation with *Escherichia coli* CGMCC 7.398.

The *feed additives* are intended to be added directly into *feedingstuffs* or through *premixtures*. However, the Applicants did not propose any minimum or maximum content of *L-lysine sulphate* in *feedingstuffs*.

For the identification of the sulphate in *L-lysine sulphate* the EURL found the European Pharmacopoeia monograph (Ph. Eur. 01/2008:20301).

For the quantification of *lysine* in the *feed additive* and *premixtures* the Applicants proposed the ring-trial validated method EN ISO 17180:2013 based on ion-exchange chromatography (IEC) coupled with optical (visible – VIS or fluorescence – FLD) detection which is dedicated for the determination of lysine, methionine and threonine in commercial amino acid products and *premixtures* containing more than 10 % of amino acid. The method does not distinguish between the amino acids and their salts, or between different salts of the same amino acids, and it cannot differentiate between enantiomers. The following performance characteristics were reported in the frame of the ring-trial validation studies for the quantification of free *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 %.

For the quantification of *lysine* in *feedingstuffs* the Applicants proposed the European Union (EU) method dedicated for the determination of amino acids in feed. This method is 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 ion-exchange chromatography (IEC) coupled with post-column derivatisation and optical (VIS) detection. The method does not distinguish between the amino acids and their salts, or between different salts of the same amino acids, and it cannot differentiate between enantiomers. The following performance characteristics were reported in the frame of the ring-trial validation studies 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 %.

Based on the performance characteristics available, the EURL recommends for official control (i) the European Pharmacopoeia monograph (Ph. Eur. 01/2008:20301) for the identification of the sulphate in *L-lysine sulphate*; (ii) the ring-trial validated method EN ISO 17180:2013 based on ion-exchange chromatography coupled with optical (visible or fluorescence) detection (IEC-VIS/FLD) to quantify free *lysine* in the *feed additive* and *premixtures* (containing more than 10 % *lysine*); and (iii) the ring-trial validated European Union (EU) method based on IEC-VIS for the quantification of *lysine* in *premixtures* and *feedingstuffs*.

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 sulphate produced by fermentation with *Corynebacterium glutamicum* KCCM 80227, *L-lysine sulphate* produced by fermentation with *Escherichia coli* CGMCC 7.398, nutritional additives, amino acids, all animal species.

1. BACKGROUND

In the current applications authorisations are sought under Article 4(1) (authorisation of a new feed additive) for *L-lysine sulphate* produced by fermentation with *Corynebacterium glutamicum* KCCM 80227 and *L-lysine sulphate* produced by fermentation with *Escherichia coli* CGMCC 7.398, 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 [1,2]. The authorisations are sought for all animal species [1,2]. *L-lysine sulphate* produced by fermentation with different strains of microorganisms are currently authorised as *feed additives* under several Commission Implementing Regulations, namely (EU) 2017/439 [3], (EU) 2019/1964 [4], (EU) 2020/997 [5] and (EU) 2020/1798 [6].

According to the Applicants, the *feed additives* contain a minimum of 52 % (w/w) of *L-lysine* in *L-lysine sulphate* produced by fermentation with *Corynebacterium glutamicum* KCCM 80227 [7] or a minimum of 55 % (w/w) of *L-lysine* in *L-lysine sulphate* produced by fermentation with *Escherichia coli* CGMCC 7.398 [8].

The *feed additives* are produced by fermentation with a non-genetically modified strain of *Corynebacterium glutamicum* or with a genetically modified strain of *Escherichia coli*, which are deposited in the "Korean Culture Center of Microorganisms" (KCCM) and the "Chinese General Microbiological Culture Collection Center" (CGMCC), respectively, with the references *Corynebacterium glutamicum* KCCM 80227 [9] and *Escherichia coli* CGMCC 7.398 [10], respectively.

The *feed additives* are intended to be added directly into *feedingstuffs* or through *premixtures* [11,12]. However, the Applicants did not propose any minimum or maximum content of *L-lysine sulphate* in *feedingstuffs* [1,2,11,12].

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

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* KCCM 80227 and *L-lysine sulphate* produced by fermentation with *Escherichia coli* CGMCC 7.398 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 additives* and *premixtures* the Applicants proposed [14,15] the ring-trial validated method EN ISO 17180:2013 based on ion-exchange chromatography coupled with optical (visible or fluorescence) detection (IEC-VIS/FLD) which is dedicated for the determination of lysine, methionine and threonine in commercial amino acid products and *premixtures* containing more than 10 % of amino acid [16]. The method does not distinguish between the amino acids and their salts, or between different salts of the same amino acids, and it cannot differentiate between enantiomers.

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 ion-exchange chromatography (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 [16]. 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*).

Table 1: Method performance characteristics obtained in the frame of ring-trial validation studies (EN ISO 17180:2013 [16], European Union method [17] and EN ISO 13903:2005 [18]) for the determination of *lysine* in the *feed additive*, *premixtures* and *feedingstuffs*.

Reference	Matrix	*lysine* content g/kg	RSD_r %	RSD_R %
[16]	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
[17]	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
[18]	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 and RSD_R - relative standard deviations for *repeatability* and *reproducibility,* respectively

For the quantification of *lysine* in *feedingstuffs* the Applicants proposed [14,15] the European Union (EU) method dedicated for the determination of amino acids in feed [17]. This method is 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 optical (VIS) detection. The method does not distinguish between the amino acids and their salts, or between different salts of the same 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 [17].

The EU method was ring-trial validated using four different matrices listed in Table 1. This method was further ring-trial validated by 23 laboratories, resulting in the EN ISO 13903:2005 method [18]. The corresponding 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 [18].

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*.

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 sulphate in *L-lysine sulphate* the EURL found the generic European Pharmacopoeia monograph on sulphates (Ph. Eur. 01/2008:20301) [19]. The EURL recommends for official control the European Pharmacopoeia monograph for the identification of the sulphate 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 in *L-lysine sulphate*; (ii) the ring-trial validated method EN ISO 17180:2013 based on ion-exchange chromatography coupled with optical (visible or fluorescence) detection (IEC-VIS/FLD) to quantify free *lysine* in the *feed additive* and *premixtures* (containing more than 10 % *lysine*); and (iii) the ring-trial validated European Union (EU) method based on ion-exchange chromatography coupled with optical (visible) detection (IEC-VIS) for the quantification of *lysine* in *premixtures* and *feedingstuffs*.

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 additives* 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 optical detection (IEC-VIS) – 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* KCCM 80227 and *L-lysine sulphate* produced by fermentation with *Escherichia coli* CGMCC 7.398 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_FWD. APPL. 1831-0070-2020 & Annex 1 – submission number 1603112176764-2706

[2] +Application, Reference SANTE_E5_FWD. APPL. 1831-0074-2020 & Annex 1 – submission number 1604479671513-2720

[3] 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

[4] 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, O.J. L 307, 28.11.2019

[5] 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, 10.7.2020

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

[8] +Technical dossier, Section II: II.1.3. Qualitative and quantitative composition

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

[10] +Technical dossier, Section II: II.2.1.2. Micro-organisms

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

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

[13] EURL Evaluation Reports:
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-2013-0045_l-lysine%20sulphate.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0052_lysine.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2017-0024_lysine.pdf
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-2018-0019_l-lysinehcl_sulphate.pdf
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-2018-0037-lysine.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2019-0015_l-lysine_sulphate.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2019-0014-lysine.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2019-00160028-lysinehcl.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2020-0008-l-lysine.pdf

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

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

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

[17] 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

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

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

*Refers to Dossier no: FAD-2020-0082
+Refers to Dossier no: FAD-2020-0085

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)
- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Instytut Zootechniki — Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- [1]Wageningen Food Safety Research (WFSR) (NL)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 — Labore Landwirtschaft, Nossen (DE)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- [2]Ruokavirasto Helsinki (FI)

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

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