EUROPEAN COMMISSION
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**European Union Reference Laboratory for Feed Additives**

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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**
***(FAD-2013-0027; CRL/130010)***

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

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| Dossier related to: | **FAD-2013-0027 - CRL/130010** |
| Name of Product: | **L-lysine as**<br>**- L-lysine monohydrochloride technically pure**<br>**- concentrated liquid L-lysine monohydrochloride**<br>**- concentrated liquid L-lysine (base)** |
| Active Agent (s): | **L-lysine** |
| Rapporteur Laboratory: | **European Union Reference Laboratory for Feed Additives (EURL-FA)**<br>**Geel, Belgium** |
| Report prepared by: | **Stefano Bellorini** |
| Report checked by:<br>Date: | **Piotr Robouch (EURL-FA)**<br>**24/03/2014** |
| Report approved by:<br>Date: | **Christoph von Holst**<br>**04/04/2014** |

## EXECUTIVE SUMMARY

In the current application authorisation is sought under Articles 4(1) (authorisation of a new feed additive) for three different forms of *L-lysine* 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 following three forms of *L-lysine* are submitted for authorisation: *L-lysine monohydrochloride technically pure* (*L-lysine*: minimum 78%)*; concentrated liquid L-lysine monohydrochloride* (*L-lysine*: minimum 22.4%)*;* and *concentrated liquid L-lysine (base)* (*L-lysine*: minimum 50%). Specifically, authorisation is sought for the use of *L-lysine* for all animal species and categories. The solid form of the *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs* while the liquid forms are intended to be added only to complete *feedingstuffs*.

For the quantification of *L-lysine* in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method based on Ion Exchange Chromatography (IEC) coupled with post-column derivatisation using amino acid analyser or high performance liquid chromatography equipped with ion exchange column. The method does not distinguish between the salts and the amino acid enantiomers and it is designed for the analysis of *premixtures* and *feedingstuffs*. The following performance characteristics were reported for the determination of total *lysine*: a relative standard deviation for *repeatability* ($RSD_r$) ranging from 2.1 to 3.5% and relative standard deviation for *reproducibility* ($RSD_R$) ranging from 3.0 to 13.1%. Based on the performance characteristics presented, the EURL recommends for official control, the ring-trial validated Community method to quantify *lysine* in *premixtures* and *feedingstuffs*.

For the quantification of *L-lysine* in the *feed additive*, the Applicant suggested the Community method designed for the analysis of *premixtures* and *feedingstuffs*, together with the method of the "Association of Official Agricultural Chemists" (AOAC 999:13 – 2004) "*Lysine*, Methionine and Threonine in Feed Grade Amino Acids and Premixes". The EURL identified instead the ring-trial validated ISO method (EN ISO 17180:2013 "Animal feeding stuffs – Determination of *lysine*, methionine and threonine in commercial amino acid products and premixtures" containing more than 10% *lysine*) based on IEC coupled with post-column derivatisation and Ultraviolet (UV) or fluorescence detection (FD). The following performance characteristics are reported: $RSD_r$ ranging from 0.7 to 1.7%; $RSD_R$ ranging from 1.5 to 2.5% and a recovery rate ($R_{Rec}$) ranging from 97.8 to 100%. Based on the performance characteristics available, the EURL recommends for official control the EN ISO 17180:2013 method, based on IEC-UV/FD for the quantification of *lysine* in *feed additive* and *premixtures* containing more than 10% *lysine*.

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, L-lysine monohydrochloride technically pure, concentrated liquid L-lysine monohydrochloride, concentrated liquid L-lysine (base),* nutritional additives, amino acids, all animal species and categories

## 1. BACKGROUND

In the current application authorisation is sought under Articles 4(1) (authorisation of a new feed additive) for *L-lysine*, 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 following three forms of *L-lysine* are submitted for authorisation:

- *L-lysine monohydrochloride (HCl) technically pure* (*L-lysine*: minimum 78%)*;*
- *concentrated liquid L-lysine monohydrochloride (HCl)* (*L-lysine*: minimum 22.4%)*;* and
- *concentrated liquid L-lysine (base)* (*L-lysine*: minimum 50%) [1].

According to the Applicant all these forms are produced through a fermentation process using a specific genetically modified strain derived from *Escherichia coli K-12* and grown on substrates of mainly vegetal origin [3-5]. The *L-lysine HCl technically pure* is a solid white to pale yellow crystalline powder, while the *concentrated liquid L-lysine HCl* and the *concentrated liquid L-lysine (base)* are dark-brown liquids [3,6].

All the *L-lysine* forms of concern have already been authorised as feed additives without any restrictions under Commission Directive 88/485/EEC [7]. Furthermore, the EURL has already evaluated the analytical methods in the frame of dossier FAD-2010-0067 (four forms of *lysine* produced from *Corynebacterium glutamicum*) [8].

Specifically, authorisation is sought for the use of *L-lysine* for all animal species and categories. The solid form of the *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs*, while the liquid forms are intended to be sprayed directly on *feedingstuffs* [9]. The Applicant suggested no minimum or maximum *L-lysine* concentrations in *premixtures* and *feedingstuffs* [1].

While use in water was originally foreseen [9], the Applicant informed EURL and EFSA, in the supplementary information [10], about the intention to withdrawn this specific condition of use.

## 2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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* and their suitability to be used for official controls in the frame of the authorisation were evaluated.

## 3. EVALUATION

***Identification /Characterisation of the feed additive***

*Qualitative and quantitative composition of impurities in the additive*

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. arsenic, cadmium, lead, mercury, aflatoxin B1 and dioxins) are available from the respective European Union Reference Laboratories [11]

***Description of the analytical methods for the determination of the active substance in feed additive, premixtures and feedingstuffs***

For the quantification of *L-lysine* in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method [12,13]. This method applies for the determination of *free* (synthetic and natural) and of *total* (peptide-bound and free) amino acids, using an amino acid analyser or high performance liquid chromatography equipped with ion exchange column. The method does not distinguish between the salts and the amino acid enantiomers and is designed for the analysis of *premixtures* and *feedingstuffs*.

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 Ion Exchange Chromatography (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. *L-lysine* can be determined in either oxidised or unoxidised samples. Oxidation is performed at 0° C with a performic acid/phenol mixture. Excess oxidation reagent is decomposed with sodium disulphite. The oxidised or unoxidised 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 Community 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 [14]. The performance characteristics reported for the determination of total *L-lysine* are listed in Table 1. Furthermore, the following limits of quantification were derived for *free lysine* and *total lysine*: 0.04 and 0.3 g/kg *feedingstuffs*, respectively [14].

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated Community method, based on IEC-UV to determine *L-lysine* in *premixtures* and *feedingstuffs*.

For the quantification of *L-lysine* in the *feed additive*, the Applicant suggested the above mentioned ring-trial validated Community method designed for the analysis of *premixtures* and *feedingstuffs* [12,13], together with the method of the "Association of Official Agricultural Chemists" (AOAC 999:13 – 2004; "*Lysine*, Methionine and Threonine in Feed Grade Amino Acids and Premixes") [12,15].

**Table 1:** Method performance characteristics obtained in the frame of ring-trial validation exercises for the determination of (total) *lysine* in *feed additives*, *premixtures* and *feedingstuffs*.

| Ring-Trial | Matrix | *lysine* g/kg | $RSD_r$ % | $RSD_R$ % |
|---|---|---|---|---|
| Commission Regulation (EC) No 152/2009 [13] | 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 |
| EN ISO 13903:2005 [14] | 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 |
| | | | | |
| EN ISO 17180:2013 [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 |

$RSD_r$, $RSD_R$ - relative standard deviation for *repeatability* and *reproducibility,* respectively

The EURL identified instead the recently published ring-trial validated ISO method (EN ISO 17180:2013 - "Animal feeding stuffs – Determination of *lysine*, methionine and threonine in commercial amino acid products and premixtures") [16]. This standard method is based on the experimental protocol described in the Community method for *lysine*. It does not distinguish between the salts and the amino acid enantiomers and 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 IEC. Free *lysine* is determined photometrically after post-column derivatisation with ninhydrine and UltraViolet (UV) detection at 440 and 570 nm or by fluorescence detection (FD) after post column reaction with ortho-phthaldialdehyde with detector set at excitation 330nm and emission 460 nm. The performance characteristics reported for the determination of free *lysine* are listed in Table 1. Furthermore, recovery rates ranging from 97.5 to 100% were obtained.

Based on the performance characteristics available, the EURL recommends for official control the EN ISO 17180:2013 method, based on IEC-UV/FD for the quantification of free *lysine* in *feed additive* and *premixtures* containing more than 10% *lysine*.

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.

## 4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control:

− the ring-trial validated EN ISO 17180, based on ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-UV/FD), to quantify free *lysine* in the *feed additive* and *premixtures* containing more than 10% of lysine;

− the ring-trial validated Community method, using ion exchange chromatography coupled with photometric detection (IEC-UV), to quantify *lysine* in *premixtures* and *feedingstuff*.

***Recommended text for the register entry (analytical method)***

For the quantification of *lysine* in *feed additive*:

− ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-UV/FD) – EN ISO 17180

For the quantification of *lysine* in *premixtures*:

- ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-UV/FD) – EN ISO 17180; or
- ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-UV), Commission Regulation (EC) No 152/2009

For the quantification of *lysine* in *feedingstuffs*:

- ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-UV), Commission Regulation (EC) No 152/2009

## 5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *L-lysine* 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 SANCO/G1: Forw. Appl. 1831/0021-2013

[3] *Application, Annex_1

[4] *Technical dossier[ABC]: 2.1.3 Qualitative and quantitative composition

[5] *Technical dossier[ABC]: 2.3 Manufacturing process

[6] *Technical dossier[ABC]: Table 2.1.5.1 Appearance, density and solubility

[7] 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

[8] #FAD-2010-0067, Lysine, Ref. JRC.DG.D.6/CvH/SB/mds/ARES(2011)301126 – 18/03/2011

[9] *Technical dossier[ABC]: 2.5.1 Proposed mode of use in animal nutrition

[10] *Supplementary information, Letter to EFSA 11 March 2014_FAD-20132-0027_Lys_Sin

[11] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories

[12] *Technical dossier[ABC]: 2.6 Methods of analysis and reference samples

[13] 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

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

[15] AOAC Official Method 999:13 – Lysine, Methionine and Threonine in Feed Grade Amino Acids and Premixes

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

*Refers to Dossier no: FAD-2013-0027

[A]Refers to Sect_II_Identity_CONC_LIQ_LYS_BASE.pdf

[B]Refers to Sect_II_Identity_CONC_LIQ_LYS-HCl.pdf

[C]Refers to Sect_II_Identity_LYS-HCl_TECH_PURE.pdf

[#] http://irmm.jrc.ec.europa.eu/SiteCollectionDocuments/FinRep-FAD-2010-0067.pdf

## 7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was European Union Reference Laboratory for Feed Additives, IRMM, 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 (EC) No 885/2009.

## 8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Sächsische Landesanstalt für Landwirtschaft. Fachbereich 8 – Landwirtschaftliches Untersuchungswesen, Leipzig (DE)[1]
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia i Pesca, Generalitat de Catalunya,Cabrils (ES)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen, Jena (DE)
- Sachgebiet Futtermittel des Bayrischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)[2]
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)

[1] Name and address according to Regulation (EC) No 885/2009: Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, Labore Landwirtschaft, Nossen (DE)

[2] Name and address according to Regulation (EC) No 885/2009: Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)