



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers & Reference Materials (Geel/Ispra)
European Union Reference Laboratory for Feed Additives

JRC.F.5/CvH/ZE/AS/Ares

Subject: Addendum to the EURL evaluation reports

References:

FAD-2017-0071 – Copper chelates of lysine and glutamic acid
(JRC F.5/CvH/ZE/AS/Ares (2018)3218372)

FAD-2017-0072 – Zinc chelates of lysine and glutamic acid
(JRC F.5/CvH/ZE/AS/Ares (2018)2861090)

FAD-2018-0009 – Manganese chelates of lysine and glutamic acid
(JRC F.5/CvH/MGH/AS/Ares(2018)3918699)

FAD-2018-0010 – Iron chelates of lysine and glutamic acid
(JRC F.5/CvH/ZE/AS/Ares(2018)3918376)

Upon the request from DG SANTE [1], the EURL evaluated the supplementary information provided [2-7] in the frame of the following feed additive dossiers: 2017-0071, 2017-0072, 2018-0009 and 2018-0010, in order to confirm the chelated structures of the corresponding *feed additives*.

For proving the chelated structures of the *feed additives* the Applicant has proposed the measurements of the chelated products by mid-infrared (IR) spectrometry and visual interpretation of the acquired spectra of the chelated products and the reference spectra of *lysine* and *glutamic acid* [2].

The Applicant provided the mid-infrared (IR) spectra of the *feed additives* [8-11], of the individual chelated products of the trace elements and of lysine and glutamic acid [3-6].

The comparison of the mid-IR spectra, namely i) of the *feed additives*; ii) of the individual chelated products of the trace elements; and iii) of lysine and glutamic acid indicates the different shape of the peaks/bands, shifts of the specific wavelengths and appearance or disappearance of certain signals: a) in the region of the amino and carboxylic hydroxyl group stretching vibrations (3500 to 2800 cm^{-1}); b) in the region of stretching vibrations of the carboxylic group and bending vibrations of the amino group (1730 to 1400 cm^{-1}); and c) in the 'fingerprint region' (1400 to 800 cm^{-1}).

Based on the available data, the EURL recommends for official control the measurements by mid-IR spectrometry together with the determination of the content of the *trace element* (*copper, zinc, manganese and iron*) and *lysine* and *glutamic acid* in the products for proving the chelated structure of the *feed additives*.

Recommended text for the registry entry (analytical method)

For proving the chelated structure of the *feed additive*:

- mid-infrared (IR) spectrometry together with the determination of the content of the *trace element* and *lysine* and *glutamic acid* in the *feed additive*

References

- [1] ^{1,2,3,4} Supplementary Information – DG SANTE request cf. Copper, Zinc, Manganese and Iron chelates of lysine and glutamic acid from Zinpro Animal Nutrition (Europe), Ref. Ares(2019)7043415
- [2] ^{1,2,3,4} Supplementary Information – Zinpro IR method for determination of chelation_September 2019
- [3] ¹ Supplementary Information – EFSA ProPath Cu Spontaneous Supplementary Information Response_20181122
- [4] ² Supplementary Information – EFSA ProPath Zn Spontaneous Supplementary Information Response_20181122
- [5] ³ Supplementary Information – EFSA ProPath Mn Spontaneous Supplementary Information Response_20181122
- [6] ⁴ Supplementary Information – EFSA ProPath Fe Spontaneous Supplementary Information Response_20181122
- [7] ^{1,2,3,4} Supplementary Information – F. H. A. Al-Jeboori and T. A. M. Al-Shimiesawi, *J. of Chem. and Pharm. Research*, 2013, 5(11) 318
- [8] ¹ Technical dossier, Section II: 2.2. Characterisation of the active substance(s)/agent(s)
- [9] ² Technical dossier, Section II: 2.2. Characterisation of the active substance(s)/agent(s)
- [10] ³ Technical dossier, Section II: 2.2. Characterisation of the active substance(s)/agent(s)
- [11] ⁴ Technical dossier, Section II: 2.2. Characterisation of the active substance(s)/agent(s)

¹ Refers to Dossier no: FAD-2017-0071

² Refers to Dossier no: FAD-2017-0072

³ Refers to Dossier no: FAD-2018-0009

⁴ Refers to Dossier no: FAD-2018-0010



EUROPEAN COMMISSION
DIRECTORATE GENERAL
JOINT RESEARCH CENTRE
Directorate F – Health, Consumers and Reference Materials
European Union Reference Laboratory for Feed Additives

 Ref. Ares(2018)3218372 - 18/06/2018

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**

Copper chelates of lysine and glutamic acid
(FAD-2017-0071; CRL/170061)

**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-2017-0071 - CRL/170061**

Name of Product: ***Copper chelates of lysine and glutamic acid***

Active Agent (s): **Copper**

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

Report prepared by: **Zigmas Ezerskis**

Report checked by: **María José González de la Huebra**
Date: **18/06/2018**

Report approved by: **Christoph von Holst**
Date: **18/06/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *copper chelates of lysine and glutamic acid* under the category/ functional group (3b) "nutritional additives"/"compounds of trace elements", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of the *feed additive* for all categories and species.

Copper chelates of lysine and glutamic acid is a solid preparation with a minimum content of 17 % (w/w) of *copper*, 19 % (w/w) of *lysine* and 19 % (w/w) of *glutamic acid*.

The *feed additive* is intended to be incorporated into *premixtures* and *feedingstuffs*. The Applicant proposed maximum levels of *total copper* in *feedingstuffs* ranging from 15 to 170 mg/kg – depending of the animal species/category – and thus complying with the limits set in the Regulations (EC) No 1334/2003 and (EC) No 479/2006.

For the quantification of *total copper* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted two internationally recognised ring-trial validated CEN methods based on inductively coupled plasma atomic emission spectrometry (ICP-AES): EN 15510 and EN 15621. These two methods together with the Community method based on atomic absorption spectrometry, which was further ring-trial validated by the UK Food Standards Agency (FSA), were previously evaluated and recommended by the EURL in the frame of the Copper group dossier.

In addition, during the review process the EURL identified two ring-trial validated methods, namely: ISO 6869 based on atomic absorption spectrometry and EN 17053 based on inductively coupled plasma mass spectrometry (ICP-MS).

Based on the acceptable method performance characteristics available, the EURL recommends for official control the five ring-trial validated methods: i) EN 15621 and ISO 6869 for the quantification of *total copper* in the *feed additive*, *premixtures* and *feedingstuffs*; ii) EN 15510 and EN 17053 for the quantification of *total copper* in *premixtures* and *feedingstuffs*; and iii) the Community method (Commission Regulation (EC) No 152/2009 – Annex IV-C) for the quantification of *total copper* in *feedingstuffs*.

For the quantification of *lysine* and *glutamic acid* in the *feed additive* the Applicant submitted the ring-trial validated EN ISO 13903 method based on ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS). This standard method is equivalent to the experimental protocol described in the Community method designed for the determination of *free* (synthetic and natural) and *total* (peptide-bound and free) amino acids including *lysine* and *glutamic acid*, using an amino acid analyser or a High

Performance Liquid Chromatography (HPLC) equipment. This method does not distinguish between the salts and the amino acid enantiomers.

The Applicant applied the above mentioned IEC-VIS method for analysis of five batches of the *feed additive* with an average content of 20 % (w/w) for *lysine* and *glutamic acid* and obtained a relative standard deviation for *repeatability* (RSD_r) ranging from 1.8 % to 2.0 %. This is in agreement with the precision values reported in the frame of the two ring-trial validation studies.

Based on the performance characteristics available, the EURL recommends for official control the method based on IEC-VIS to quantify *lysine* and *glutamic acid* 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.

KEYWORDS

Copper, copper chelates of lysine and glutamic acid, nutritional feed additives, compounds of trace elements, all animal species

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (new *feed additive*) for *copper chelates of lysine and glutamic acid* under the category/ functional group (3b) "nutritional additives"/"compounds of trace elements", according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of the *feed additive* for all categories and species [1,2].

Copper chelates of lysine and glutamic acid is a solid preparation with a minimum content of 17 % (w/w) of *copper*, 19 % (w/w) of *lysine* and 19 % (w/w) of *glutamic acid* [3,4].

The *feed additive* is intended to be incorporated into *premixtures* and *feedingstuffs* [4]. The Applicant proposed maximum levels of *total copper* in *feedingstuffs* complying to the limits set in Regulations (EC) No 1334/2003 and 479/2006: 170 mg/kg for piglets; 25 mg/kg for other pigs and fish; 15 mg/kg for bovines before the start of rumination and ovine; 35 mg/kg for other bovines; and 25 mg/kg for crustaceans and other species [3,4].

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 *copper chelates of lysine and glutamic acid* 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 *total copper* in the *feed additive, premixtures* and *feedingstuffs* the Applicant submitted two internationally recognised ring-trial validated CEN methods: EN 15510 based on inductively coupled plasma atomic emission spectrometry (ICP-AES) after ash or wet digestion with hydrochloric acid [5] and EN 15621 based on ICP-AES after pressure digestion [6].

These two methods together with the Community method based on atomic absorption spectrometry [7], which was further ring-trial validated by the UK Food Standards Agency (FSA) [8], were previously evaluated and recommended by the EURL in the frame of the Copper group dossier (including FAD-2010-0031; FAD-2010-0070; FAD-2010-0331) [9].

In addition, during the review process the EURL identified two ring-trial validated methods, namely: ISO 6869 based on atomic absorption spectrometry [10] and EN 17053 based on inductively coupled plasma mass spectrometry (ICP-MS) [11].

The performance characteristics reported for the five methods mentioned above are summarised in Table 1.

Based on the acceptable method performance characteristics available, the EURL recommends for official control the five ring-trial validated methods: i) EN 15621 and ISO 6869 for the quantification of *total copper* in the *feed additive, premixtures* and *feedingstuffs*; ii) EN 15510 and EN 17053 for the quantification of *total copper* in *premixtures* and *feedingstuffs*; and iii) the Community method (Commission Regulation (EC) No 152/2009 – Annex IV-C) for the quantification of *total copper* in *feedingstuffs*.

Table 1: Performance characteristics for the quantification of *total copper* in *premixtures* and *feedingstuffs*

	EN 15510	EN 15621	UK FSA	ISO 6869	EN 17053
Method	ICP-AES	ICP-AES	AAS	AAS	ICP-MS
Mass fraction (mg/kg)	6.8 – 775	11.6 – 264900	17 – 39	6 – 39100	2.5 – 1389
RSD _r (%)	2.9 – 12 ^(*)	1.9 – 16.1	2.4 – 9.2	1.2 – 14.5	2.9 – 6.7
RSD _R (%)	8.0 – 22 ^(*)	7.1 – 18.9	5.4 – 9.2	2.9 – 23.8 ^(**)	5.7 – 9.8
LOQ (mg/kg)	3	1	-	5	-
Reference	[5]	[6]	[8]	[10]	[11]

RSD_r and RSD_R: relative standard deviation for *repeatability* and *reproducibility*; LOQ: limit of quantification; (*) the larger precision values were obtained for mineral premixes; (**) the larger precision values were obtained for mixed feed.

Even though the methods EN 15510 and EN 17053 were ring-trial validated at narrower range for *total copper* content than the methods EN 15621 and ISO 6869, the first two ones still might be considered for the quantification of *total copper* in the *feed additive* after appropriate dilution with the condition that the methods are proven as fit-for-purpose.

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)

For the quantification of *lysine* and *glutamic acid* in the *feed additive* the Applicant submitted the ring-trial validated EN ISO 13903 method based on ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS) [12].

This standard method is equivalent to the experimental protocol described in the Community method designed for the determination of *free* (synthetic and natural) and *total* (peptide-bound and free) amino acids including *lysine* and *glutamic acid*, using an amino acid analyser or a High Performance Liquid Chromatography (HPLC) equipment [13]. This method does not distinguish between the salts and the amino acid 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 ion exchange chromatography and determined after post-column derivatisation with ninhydrin by

photometric detection at 570 nm. The procedure chosen for the determination of the *total* amino acids depends on the amino acids under investigation. Total *lysine* and total *glutamic acid* can be determined from unoxidised or oxidised samples. The oxidation is performed at 0 °C with the mixture of performic acid and phenol. An excess of the oxidation reagent is decomposed with sodium disulfite. The unoxidised or oxidised samples are hydrolysed with hydrochloric acid (6 mol/l) for 23 hours. The hydrolysate is adjusted to pH 2.2. The amino acids are separated by ion exchange chromatography and determined by post-column derivatisation with ninhydrin and photometric detection at 570 nm [13].

The Applicant applied the above mentioned IEC-VIS method for the analysis of five batches of the *feed additive* with an average content of 20 % (w/w) for *lysine* and *glutamic acid* and obtained a relative standard deviation for *repeatability* (RSD_r) ranging from 1.8 % to 2.0 %. This is in agreement with the precision values reported in the frame of the two ring-trial validation studies [12,13].

Based on the performance characteristics available, the EURL recommends for official control the method based on IEC-VIS to quantify *lysine* and *glutamic acid* 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:

- the EN 15621 and ISO 6869 for the quantification of *total copper* in the *feed additive*, *premixtures* and *feedingstuffs*;
- the EN 15510 and EN 17053 for the quantification of *total copper* in *premixtures* and *feedingstuffs*; and
- the Community method based on atomic absorption spectrometry (AAS) for the quantification of *total copper* in *feedingstuffs* (only);
- the method based on ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS) for the quantification of *lysine* and *glutamic acid* in the *feed additive*

Recommended text for the register entry (analytical method)

For the quantification of *total copper* in the *feed additive, premixtures* and *feedingstuffs*:

- Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) – EN 15621; or
- Atomic Absorption Spectrometry (AAS) – ISO 6869; or
- Inductively Coupled Plasma Atomic Emission Spectrometry after pressure digestion, (ICP-AES) – EN 15510 (for *premixtures* and *feedingstuffs* only); or
- Inductively Coupled Plasma Mass Spectrometry (ICP-MS) – EN 17053 (for *premixtures* and *feedingstuffs* only); or
- Atomic Absorption Spectrometry (AAS) – Commission Regulation (EC) No 152/2009 (for *feedingstuffs* only)

For the quantification of *lysine* and *glutamic acid* in the *feed additive*:

- ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *copper chelates of lysine and glutamic acid* 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. 1831-0002-2018
- [2] *Application, Annex I – submission number 1513701728385-2164
- [3] *Application, Proposal for Register Entry – Annex A
- [4] *Technical dossier, Section II: Identity, characterisation and conditions of use of the feed additive; methods of analysis
- [5] EN 15510:2007 – *Animal feeding stuffs – Determination of calcium, sodium, phosphorus, magnesium, potassium, iron, zinc, copper, manganese, cobalt, molybdenum, arsenic, lead and cadmium by ICP-AES*
- [6] EN 15621:2012 – *Animal feeding stuffs – Determination of calcium, sodium, phosphorus, magnesium, potassium, sulphur, iron, zinc, copper, manganese and cobalt after pressure digestion by ICP-AES*
- [7] Commission Regulation (EC) No 152/2009 laying down the methods of sampling and analysis for official control of feed – Annex IV-C

- [8] Food Standards Agency – Information Bulletin on Methods of Analysis and Sampling for Foodstuffs, No 102; March 2010
- [9] #Copper Group – JRC.D.5/CvH/ZE/mds/Ares(2017)2353146
- [10] ISO 6869:2000 *Animal feeding stuffs – Determination of the contents of calcium, copper, iron, magnesium, manganese, potassium, sodium and zinc – Method using atomic absorption spectrometry*
- [11] EN 17053:2018 *Animal feeding stuffs: Methods of sampling and analysis – Determination of trace elements, heavy metals and other elements in feed by ICP-MS (multi-method)*
- [12] EN ISO 13903:2005 – *Animal feeding stuffs – Determination of amino acids content*
- [13] Commission Regulation (EC) No 152/2009 laying down the methods of sampling and analysis for official control of feed – Annex III-F

*Refers to Dossier no: FAD-2017-0071

<https://ec.europa.eu/jrc/en/eurl/feed-additives/evaluation-reports>

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:

- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Fødevarestyrelsens Laboratorie Aarhus (kemisk) (DK)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- RIKILT Wageningen UR, Wageningen (NL)
- Elintarviketurvallisuusvirasto/Livsmedelssäkerhetsverket (Evira), Helsinki/Helsingfors (FI)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 – Labore Landwirtschaft, Nossen (DE)