



EUROPEAN COMMISSION

DIRECTORATE GENERAL

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Directorate D: Institute for Reference Materials and Measurements

European Union Reference Laboratory for Feed Additives

 Ref. Ares(2015)3811738 - 15/09/2015

JRC.D.5/SFB/CvH/ZE/mds/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**

Zinc Chelate of Methionine
(FAD-2015-0002; CRL/150001)

**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-2015-0002 - CRL/150001**

Name of Product: **Zinc Chelate of Methionine**

Active Agent (s): **Zinc Chelate of Methionine**

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
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Date: **11/09/2015**

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Date: **14/09/2015**

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for *Zinc Chelate of Methionine* 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.

Zinc Chelate of Methionine is a dark brown powder with minimum contents of 18 % total zinc and 36 % *methionine*. The *feed additive* is intended to be incorporated directly into *feedingstuffs* or through *premixtures*. The Applicant suggested maximum levels of total zinc in complete *feedingstuffs* complying with the limits set in Regulation (EC) No 1334/2003, ranging from 150 to 250 mg/kg – depending of the animal species/category. In addition, the Applicant intends using the *feed additive* in *water* through complementary feed at maximum levels of total zinc twice lower than the doses used in complete *feedingstuffs*.

For the *determination* of total zinc in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted the internationally recognised ring-trial validated method EN 15510 based on inductively coupled plasma atomic emission spectroscopy (ICP-AES). Two additional methods were previously evaluated and recommended by the EURL in the frame of the Zinc group dossier: the ring-trial validated EN 15621 method based on ICP-AES after pressure digestion and the Community method based on atomic absorption spectroscopy. The Community method was further ring-trial validated by the UK Food Standards Agency (FSA). Based on the acceptable method performance characteristics available, the EURL recommends for official control the two CEN methods (EN 15510 or EN 15621) for the quantification of total zinc in the *feed additive*, *premixtures* and *feedingstuffs*, together with the Community method (Com Reg (EC) No 152/2009 – Annex IV-C) for the quantification of total zinc in *feedingstuffs*.

For the quantification of total zinc in *water* the Applicant suggested to use EN 15510 method. However, the EURL recommended in the frame of Zinc group dossier the ring trial validated EN ISO 11885 method, based on inductively coupled plasma optical emission spectroscopy (ICP-OES). Based on the acceptable performance characteristics the EURL recommends for official control the ICP-OES CEN method (EN ISO 11885) to quantify total zinc content in the *water*.

For the quantification of *methionine* content in the *feed additive* and *premixtures* the Applicant suggested using an Official AOAC 999.13 method. However, the EURL previously evaluated – in the frame of FAD-2010-0254 dossier - the ring-trial validated EN ISO 17180 method based on ion-exchange chromatography coupled with post-column derivatisation and

colourimetric or fluorescence detection. The method does not distinguish between the salts and the amino acid enantiomers and applies for the products/premixtures containing more than 10 % of amino acid content. In addition, for the quantification of *methionine* content in the *feed additive* and *premixtures* the EURL identified the ring-trial validated method by the “Association of German Agricultural Analytical and Research Institutes” (VDLUFA, Germany – Method 4.11.6), based on ion-exchange chromatography coupled with post-column derivatisation and colourimetric or fluorescence detection. This method was designed for the determination of free methionine (not protein bound) in commercial products and premixtures with amino acid contents higher than 100 g/kg. Based on acceptable performance characteristics available, the EURL recommends for official control the two ring-trial validated methods (EN ISO 17180 and VDLUFA 4.11.6) to quantify *methionine* content in the *feed additive* and *premixtures*.

For the quantification of total methionine in *feedingstuffs* the EURL already evaluated and recommended for official control - in the frame of the Methionine group dossier (FAD-2010-0023) - the ring-trial validated Community method. This method applies for the determination of free (synthetic and natural) and of total (peptide-bound and free) amino acids, using an amino acid analyzer or High Performance Liquid Chromatography (HPLC) equipment. The method does not distinguish between the salts and the amino acid enantiomers. This method was further ring-trial validated, resulting in the EN ISO 13903 method.

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

Zinc Chelate of Methionine, nutritional additives, compounds of trace elements, all animal species and categories

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new *feed additive*) for *Zinc Chelate of Methionine* 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].

Zinc Chelate of Methionine is a dark brown powder with minimum contents of 18 % total zinc and 36 % *methionine* [2,3].

The *feed additive* is intended to be incorporated directly in *feedingstuffs* or through *premixtures* [3]. The Applicant suggested maximum levels of total zinc in complete *feedingstuffs* [2,3] complying to the limits set in Regulation (EC) No 1334/2003: 250 mg/kg for pet animals; 200 mg/kg for fish and milk replacers; and 150 mg/kg for other species. In addition, the Applicant intends using the *feed additive* in *water* through complementary feed at maximum levels of total zinc twice lower than the doses used in complete *feedingstuffs* [2,3].

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 *Zinc Chelate of Methionine* 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 [4].

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

For the characterisation of the *feed additive*, the Applicant used Fourier transformed infrared spectroscopy (FTIR) in absorption mode at the wave range from 4600 to 400 cm^{-1} and monitored the following seven bands: 3487, 3124, 2920, 1639, 1477, 1411 and 1398 cm^{-1} [3].

For the *determination of total zinc* in the *feed additive, premixtures and feedingstuffs* the Applicant submitted the internationally recognised ring-trial validated method EN 15510 based on inductively coupled plasma atomic emission spectroscopy (ICP-AES) [5]. For the determination of *total zinc*, a test portion of the sample is ashed and dissolved in hydrochloric acid (in the case of organic *feedingstuffs*) or wet digested with hydrochloric acid (in the case of mineral compounds).

Two additional methods were previously evaluated and recommended by the EURL in the frame of the Zinc group dossier (including FAD-2010-0059; FAD-2010-0063; FAD-2010-0072; FAD-2010-0142; FAD-2010-0228) [6]: the ring-trial validated EN 15621 method based on ICP-AES after pressure digestion [7] and the Community method based on atomic absorption spectroscopy [8]. The Community method was further ring-trial validated by the UK Food Standards Agency (FSA) [9], using samples such as dog biscuits, layer pellets, beef nuts, sow rolls or rabbit pellets.

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

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

	EN 15510 [5]	EN 15621 [7]	UK FSA [9]
Method	ICP-AES	ICP-AES	AAS
Content (mg/kg)	27.4 – 3826	26.6 – 3618	93 – 199
RSD _r (%)	1.7 – 8.8 ^(*)	1.5 – 5.4	1.0 – 6.1
RSD _R (%)	5.0 – 19 ^(*)	2.7 – 22	4.1 – 9.5
LOQ (mg/kg)	3	1	20

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

For the quantification of *total zinc* in *water* the Applicant suggested to use EN 15510 method. However, the EURL recommended in the frame of Zinc group dossier [6] the ring trial validated EN ISO 11885 method [10], based on inductively coupled plasma optical emission spectroscopy (ICP-OES). The following performance characteristics were reported for drinking water, surface water (filtered) and waste water, where the *total zinc* content ranged from 124 to 1251 µg/L: - RSD_F ranging from 1.5 to 2.4 %; - RSD_R ranging from 4.9 to 5.9 %; and - LOQ = 1 µg/L.

Based on the acceptable performance characteristics the EURL recommends for official control the ICP-OES CEN method (EN ISO 11885) to quantify *total zinc* content in the *water*.

For the quantification of *methionine* content in the *feed additive* and *premixtures* the Applicant suggested using an Official AOAC 999.13 method [11]. However, the EURL previously evaluated in the frame of FAD-2010-0254 dossier [12] - an equivalent ring-trial validated EN ISO 17180 method [13] based on ion-exchange chromatography coupled with post-column derivatisation and colourimetric or fluorescence detection. The method does not distinguish between the salts and the amino acid enantiomers and applies for the products/premixtures containing more than 10 % of amino acid content.

Methionine is extracted with diluted hydrochloric acid and mixed with sodium citrate buffer. After addition of norleucine as internal standard, the amino acids are separated by an amino acid analyser or High Performance Liquid Chromatography (HPLC) equipment using a cation exchange column and sodium citrate buffer as an eluent. *Methionine* is determined colourimetrically at 440 and 570 nm, after post-column derivatisation with ninhydrine or by fluorescence detection after post column reaction with orthophthaldialdehyde [13].

The following performance characteristics were reported in the frame of the ring-trial for *methionine* content in the products/premixtures ranging from 90 to 930 g/kg: - a relative standard deviation for *repeatability* (RSD_F) ranging from 0.5 to 1.7 %; and - a relative standard deviation for *reproducibility* (RSD_R) ranging from 1.5 to 2.6 %.

In addition, for the quantification of *methionine* content in the *feed additive* and *premixtures* the EURL identified the ring-trial validated method by the "Association of German Agricultural Analytical and Research Institutes" (VDLUFA, Germany – Method 4.11.6), based on ion-exchange chromatography coupled with post-column derivatisation and colourimetric or fluorescence detection [14]. This method was designed for the determination of *free methionine* (not protein bound) in commercial products and premixtures with amino acid contents higher than 100 g/kg.

The *feed additive* and/or *premixtures* samples are dissolved or extracted with hydrochloric acid (0.1 mol/L) and diluted with sodium citrate buffer. The internal standard solution

(Norleucine) is added and the amino acids are separated by ion exchange chromatography and determined after post column derivatisation with ninhydrin by colourimetric detection; or by fluorescence detection after post-column reaction with o-phthalaldehyde (OPA) or analysed by amino acid analyser [14].

The following performance characteristics were reported in the frame of the ring-trial for *methionine* content in the products/premixtures ranging from 120 to 300 g/kg: - a relative standard deviation for *repeatability* (RSD_r) ranging from 0.6 to 1.5 %; and - a relative standard deviation for *reproducibility* (RSD_R) ranging from 2.4 to 4.6 %.

Based on acceptable performance characteristics available, the EURL recommends for official control the two ring-trial validated methods (EN ISO 17180 and VDLUFA 4.11.6) to quantify *methionine* content in the *feed additive* and *premixtures*.

For the quantification of *total methionine* in *feedingstuffs* the EURL already evaluated and recommended for official control - in the frame of the Methionine group dossier FAD-2010-0023 [15] - the ring-trial validated Community method [16]. This method applies for the determination of free (synthetic and natural) and of total (peptide-bound and free) amino acids, using an amino acid analyzer or High Performance Liquid Chromatography (HPLC) equipment. The method does not distinguish between the salts and the amino acid enantiomers.

Total methionine can be determined in either oxidised or un-oxidised 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 ion exchange chromatography and determined by post column derivatisation with ninhydrin and photometric detection at 570 nm or analysed by amino acid analyser [16].

This method was further ring-trial validated, resulting in the EN ISO 13903 method [17], in which the following performance characteristics were reported for the determination of *total methionine* in *feedingstuffs*: RSD_r ranging from 1.1 to 5.6 %; and RSD_R ranging from 7.0 to 13 %. Furthermore, a limit of quantification of 250 mg *total methionine* /kg *feedingstuffs* was derived.

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 EN 15510 method based on inductively coupled plasma atomic emission spectroscopy (ICP-AES) and the EN 15621 method based on ICP-AES after pressure digestion for the quantification of *total zinc* in the *feed additive, premixtures* and *feedingstuffs*; and
- The Community method based on atomic absorption spectroscopy (AAS) for the quantification of *total zinc* in *feedingstuffs* (only)
- The EN ISO 11885 method based on inductively coupled plasma optical emission spectroscopy (ICP-OES) for the quantification of *total zinc* content in *water*
- The two ring trial validated methods (EN ISO 17180 and VDLUFA 4.11.6), based on ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-UV/FD), for the quantification of *methionine* in the *feed additive* and *premixtures*

Recommended text for the register entry (analytical method)

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

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

For the quantification of *total zinc* in the *water*:

- Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) – EN ISO 11885

For the quantification of *methionine* content in the *feed additive* and *premixtures*:

- ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-UV/FD) – EN ISO 17180 or VDLUFA 4.11.6

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Zinc Chelate of Methionine* 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 SANCO/G1: Forw. Appl.1831/0006-2015
- [2] *Application, Proposal for Register Entry – Annex A
- [3] *Technical dossier, Section II: Identity, characterisation and conditions of use of the feed additive; methods of analysis
- [4] 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
- [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] #Zinc Group – JRC.DG.D.6/CvH/PR/mds/ARES(2011)1156545
- [7] EN 15621:2012 – *Animal feeding stuffs – Determination of cadmium, sodium, phosphorus, magnesium, potassium, sulphur, iron, zinc, copper, manganese, cobalt and molybdenum after pressure digestion by ICP-AES*
- [8] Commission Regulation (EC) No 152/2009 laying down the methods of sampling and analysis for official control of feed – Annex IV-C
- [9] Food Standards Agency – Information Bulletin on Methods of Analysis and Sampling for Foodstuffs, No 102; March 2010
- [10] EN ISO 11885:2009 – *Water quality – Determination of selected elements by inductively coupled plasma optical emission spectrometry (ICP-OES – ICP-AES)*
- [11] AOAC Official Method 999.13 – Lysine, Methionine and Threonine in feed grade amino acids and premixes
- [12] #FAD-2010-0254, Methionine-Zinc – JRC.DG.D.6/CvH/GB/ag/ARES(2011)1156545
- [13] EN ISO 17180:2013 – *Animal feeding stuffs - Determination of lysine, methionine and threonine in commercial amino acid products and premixtures*
- [14] Bestimmung von Lysin, Methionin und Threonin in Aminosäurehandelsprodukten und Vormischungen – 4.11.6, Methodenbuch III, 5. Erg. 2004, VDLUFA – Verlag, Darmstadt)
- [15] #FAD-2010-0023, Methionine Group – JRC.D.5/FSQ/CvH/SB/ag/Ares(2012)240861
- [16] Commission Regulation (EC) No 152/2009 laying down the methods of sampling and analysis for official control of feed – Annex III-F
- [17] EN ISO 13903:2005 – *Animal feeding stuffs – Determination of amino acids content*

*Refers to Dossier no: FAD-2015-0002

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

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)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 - Labore Landwirtschaft, Nossen (DE)¹
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Elintarviketurvallisuusvirasto/Livsmedelssäkerhetsverket (Evira), Helsinki/Helsingfors (FI)
- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Ministerio de Agricultura, Alimentación y Medio Ambiente, Madrid² (ES)
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