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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

Zinc (II) betaine complex

(FAD-2021-0072; CRL/210008)

European Union Reference Laboratory
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Dossier related to:	**FAD-2021-0072 - CRL/210008**
Name of Product:	***Zinc (II) betaine complex***
Active Agent (s):	**Zinc (II) betaine / Zinc**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Stefano Bellorini** **21/09/2021**
Report approved by: Date:	**Christoph von Holst** **22/09/2021**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *zinc (II) betaine complex* 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, the authorisation is sought for the use of the *feed additive* for dogs, cats, milk replacers for calves, suckling and weaned piglets, sows, rabbits, salmonids, all fish other than salmonids and other species and categories.

According to the Applicant, the *feed additive* is a solid preparation of *Zinc (II) betaine complex* containing minimum of 20 % (w/w) of *zinc* and minimum of 41 % (w/w) of *betaine* and 9 to 12 % (w/w) of *sulphur* corresponding to 27 to 36 % (w/w) of *sulfate*.

The *feed additive* is intended to be incorporated into *premixtures* and *feedingstuffs* with the following proposed maximum levels of total *zinc* in *feedingstuffs*: 200 mg/kg for dogs and cats; 180 mg/kg for salmonids and milk replacers for calves; 150 mg/kg for piglets, sows, rabbits and all fish other than salmonids; and 120 mg/kg for other species and categories. The proposed levels are in agreement with the Commission Implementing Regulation (EU) 2016/1095.

For the quantification of total *zinc* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant proposed the internationally recognised ring-trial validated EN 15621 method based on inductively coupled plasma-atomic emission spectrometry (ICP-AES). In addition, the EN 15510 method based on ICP-AES and the European Union (EU) method based on atomic absorption spectrometry (AAS), which was further ring-trial validated by the UK Food Standards Agency (FSA), have been previously evaluated and recommended by the EURL in the frame of the Zinc group dossiers (including FAD-2010-0059; FAD-2010-0063; FAD-2010-0072; FAD-2010-0142; FAD-2010-0228). The EURL is also aware of two ring-trial validated methods, namely: ISO 6869 based on AAS and EN 17053 based on inductively coupled plasma-mass spectrometry (ICP-MS).

Based on the available method performance characteristics, the EURL recommends for official control the above mentioned five ring-trial validated methods: (i) EN 15621, EN 15510 and ISO 6869 for the quantification of total *zinc* in the *feed additive*, *premixtures* and *feedingstuffs*; (ii) the EN 17053 for the quantification of total *zinc* in *premixtures* and *feedingstuffs*; and (iii) the EU method for the quantification of total *zinc* in *feedingstuffs*.

For the quantification of *betaine* in the *feed additive* the Applicant submitted the official method of International Commission for Uniform Methods of Sugar Analysis (ICUMSA) based on high performance liquid chromatography with refraction index detection (HPLC-RI) which is dedicated for the determination of *betaine* in beet molasses. The Applicant

performed batch-to-batch analysis of 5 samples of the *feed additive* using the above mentioned HPLC-RI method and a relative standard deviation for *repeatability* (RSD_r) of 0.9 % was derived for an average betaine content of 42 % (w/w).

Based on the available data, the EURL recommends for official control the above mentioned method based on HPLC-RI for the quantification of *betaine* content in the *feed additive*.

For the quantification of *sulphur* and *sulfate* content in the *feed additive* the Applicant proposed no methods for official control. However, several batches of the *feed additive* have been analysed for *sulphur* and *sulfate* content by using the above mentioned ring-trial validated EN 15621 method. The standard deviation for *repeatability* (RSD_r) of 3.1 % was obtained for the average *sulphur* content of 10.8 % (w/w), which corresponds to *sulfate* content of 32.5 % (w/w).

Based on the performance profile, the EURL recommends for official control the EN 15621 method for the quantification of *sulphur* and *sulfate* content in the *feed additive*.

For proving the formation of *zinc (II) betaine* complex, the Applicant applied a qualitative method based on powder X-ray diffraction (XRD). Based on the available data, the EURL considers that this method is suitable for proving the complex formation between *zinc* and *betaine*.

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

Zinc (II) betaine complex, *zinc*, nutritional feed additives, compounds of trace elements, dogs, cats, milk replacers for calves, suckling and weaned piglets, sows, rabbits, salmonids, all fish other than salmonids and other species and categories.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new *feed additive*) for *zinc (II) betaine complex* 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 [1,2]. Specifically, the authorisation is sought for the use of the *feed additive* for dogs, cats, milk replacers for calves, suckling and weaned piglets, sows, rabbits, salmonids, all fish other than salmonids and other species and categories [2].

According to the Applicant, the *feed additive* is a solid preparation of *zinc (II) betaine complex* containing minimum of 20 % (w/w) of *zinc* and minimum of 41 % (w/w) of *betaine*.

The additive contains also 9 to 12 % (w/w) of sulphur corresponding to 27 to 36 % (w/w) of *sulfate* and maximum of 5 % (w/w) of water [3].

The *feed additive* is intended to be incorporated into *premixtures* and *feedingstuffs* with the following proposed maximum levels of total *zinc* in *feedingstuffs*: 200 mg/kg for dogs and cats; 180 mg/kg for salmonids and milk replacers for calves; 150 mg/kg for piglets, sows, rabbits and all fish other than salmonids; and 120 mg/kg for other species and categories [2, 4]. The proposed levels are in agreement with the Commission Implementing Regulation (EU) 2016/1095 [5].

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 *zinc (II) betaine complex* 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 *zinc* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant proposed [6] the internationally recognised ring-trial validated EN 15621 method based on inductively coupled plasma-atomic emission spectrometry (ICP-AES) [7]. In addition, the EN 15510 method based on ICP-AES [8] and the European Union (EU) method based on atomic absorption spectrometry (AAS) [9], which was further ring-trial validated by the UK Food Standards Agency (FSA) [10], have been previously evaluated and recommended by the EURL in the frame of the Zinc group dossiers (including FAD-2010-0059; FAD-2010-0063; FAD-2010-0072; FAD-2010-0142; FAD-2010-0228) [11]. The EURL is also aware of two ring-trial validated methods, namely: ISO 6869 based on AAS [12] and EN 17053 based on inductively coupled plasma-mass spectrometry (ICP-MS) [13].

According to the EN 15621 method, the sample is digested under pressure with the mixture of nitric acid and hydrogen peroxide (or only using nitric acid), while in case of EN 15510

method, 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) [7,8]. The elemental *zinc* is detected by ICP-AES at selected specific wavelengths (206.200 and/or 213.856 nm) and the quantification is performed using an external standard calibration or standard additions [7,8].

According to the EU and EN ISO 6869 methods, 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) [9,12]. When applying the EU method, other methods of digestion such as a microwave pressure may be used provided they have been demonstrated giving similar results [9]. The analyte is detected by an air-acetylene flame AAS at selected specific wavelength (213.8 nm). The quantification is performed using an external standard calibration curve [9,12].

According to the EN 17053 method, the sample is digested with concentrated nitric acid under pressure. The elemental *zinc* is detected by ICP-MS at mass-to-charge (m/z) of 66 and/or 68. The quantification of the analyte is performed using an external standard calibration or standard additions [13].

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

Based on the acceptable method performance characteristics, the EURL recommends for official control the five ring-trial validated methods: (i) EN 15621, EN 15510 and ISO 6869 for the quantification of total *zinc* in the *feed additive*, *premixtures* and *feedingstuffs*; (ii) the EN 17053 for the quantification of total *zinc* in *premixtures* and *feedingstuffs*; and (iii) the EU method for the quantification of total *zinc* in *feedingstuffs*.

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

	EN 15621	EN 15510	UK FSA	ISO 6869	EN 17053
Method	ICP-AES	ICP-AES	AAS	AAS	ICP-MS
Mass fraction (mg/kg)	127 – 10310	27.4 – 3826	93 – 199	29 – 14600	55 – 6286(**)
RSD_r (%)	2.1 – 3.5	1.7 – 8.8(*)	1.0 – 6.1	1.7 – 7.6	2.3 – 5.5
RSD_R (%)	5.5 – 13.2	5.0 – 19(*)	4.1 – 9.5	3.3 – 15.0	5.2 – 11.3
LOQ (mg/kg)	1	3	20	5	5
Reference	[7]	[8]	[10]	[12]	[13]

RSD_r and RSD_R: relative standard deviation for *repeatability* and *reproducibility*; LOQ: limit of quantification; (*) the largest precision values were obtained for mineral premixes; (**) based on dry weight.

Note: When applying the recommended methods for the quantification of *zinc* in the *feed additive*, the EURL suggests to perform an appropriate additional dilution of the samples after the digestion in a way that the mass fraction of the analyte would fit the ring-trial validation ranges of the methods.

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 quantification of *betaine* in the *feed additive* the Applicant submitted the official method of International Commission for Uniform Methods of Sugar Analysis (ICUMSA) based on high performance liquid chromatography with refraction index detection (HPLC-RI) and which is dedicated for the determination of *betaine* in beet molasses [14].

Following the method, the sample of 5 g is dissolved in 100 ml of water. The obtained solution as such or after additional dilution is filtered through membrane filter before the chromatographic analysis. The analysis is performed by using cation exchange column kept at 70 to 90 ^{o}C and alkaline (pH 9 to 9.5) mobile phase. The analyte is detected using a differential refractometry and the quantification is performed by using *betaine* as a standard substance for the calibration [14].

The Applicant performed batch-to-batch analysis of 5 samples of the *feed additive* using the above mentioned HPLC-RI method and a relative standard deviation for *repeatability* (RSD_r) of 0.9 % was derived for an average betaine content of 42 % (w/w) [3].

Based on the available data, the EURL recommends for official control the above mentioned method based on HPLC-RI for the quantification of *betaine* content in the *feed additive*.

For the quantification of *sulphur* and *sulfate* content in the *feed additive* the Applicant proposed no methods for official control.

However, several batches of the *feed additive* have been analysed for *sulphur* and *sulfate* content by using the above mentioned ring-trial validated EN 15621 method [7].

When applying the method to several batches of the *feed additive* the Applicant reported the results of *sulphur* content, which were experimentally determined; in addition, the Applicant indicated the results of the *sulfate* content, which were calculated from the *sulphur* content using a conversion factor of 3 [3]. The standard deviation for *repeatability* (RSD_r) of 3.1 % was obtained for the average *sulphur* content ranging of 10.8 % (w/w), which corresponds to *sulfate* content of 32.5 % (w/w) [3].

Based on the performance profile, the EURL recommends for official control the EN 15621 method for the quantification of *sulphur* and *sulfate* content in the *feed additive*.

Note: Given the stoichiometry of *sulfate* group and atomic, and molecular masses of *sulphur* and of *sulfate*, respectively, for the quantification of *sulfate* content in the *feed additive* laboratories have to multiply the experimentally determined content of *sulphur* by factor of 3.

In addition, the Applicant applied a qualitative method based on powder X-ray diffraction (XRD) for proving the formation of *zinc (II) betaine* complex. The samples of the *feed additive* are measured at room temperature in transmission mode using Cu-Kα1 radiation [6]. The Applicant presented the diffractogram of the *feed additive* sample where the XRD powder pattern is compared to those of the starting materials (zinc sulfate and betaine) [15]. The comparison demonstrated new lines occurring in the diffractogram of the additive indicating the formation of new phases [15]. As the intense lines of the starting materials are no longer present in the difractogram of the *feed additive*, the conversion of the materials into the additive can be considered as complete [6,15].

The EURL considers that the above mentioned XRD powder diffraction method is suitable for proving the complex formation between *zinc* and *betaine*.

Furthermore, the Applicant proposed an indirect method for proving the binding between *zinc* and *betaine*, which is based on the extraction of potentially free *betaine* from the *feed additive* with dichloromethane in a soxhlet apparatus, followed by a gravimetric and XRD analysis of extraction products [6,16,17]. The Applicant presented data of two samples containing 3.0 and 4.5 % (w/w) of added (free) *betaine* to the *feed additive*, which were treated with dichloromethane resulting in added *betaine* recoveries of 89 % and 94 %, respectively [16].

For the identification and the estimation of the quantity of the extracted free *betaine*, the EURL would suggest instead analysing the concentrated dichloromethane extracts of the *feed additive* by using the above mentioned HPLC-RI method. However, given that there are not enough experimental data presented, the EURL is not able to conclude on suitability of the proposed method for the determination of free *betaine* 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 EN 15510 methods based on inductively coupled plasma-atomic emission spectrometry (ICP-AES), and ISO 6869 method based on atomic

absorption spectrometry (AAS) for the quantification of total *zinc* in the *feed additive*, *premixtures* and *feedingstuffs*;

- the EN 17053 method based on inductively coupled plasma-mass spectrometry (ICP-MS) for the quantification of total *zinc* in *premixtures* and *feedingstuffs*;
- the European Union (EU) method based on AAS for the quantification of total *zinc* in *feedingstuffs*;
- the official method of International Commission for Uniform Methods of Sugar Analysis (ICUMSA) based on high performance liquid chromatography with refraction index detection (HPLC-RI) for the quantification of *betaine* content in the *feed additive*; and
- the EN 15621 method based on inductively coupled plasma-atomic emission spectrometry (ICP-AES) for the quantification of *sulphur* and *sulfate* content in the *feed additive*.

In addition, the EURL considers that the qualitative XRD powder diffraction method proposed by the Applicant is suitable for proving the complex formation between *zinc* and *betaine*.

Recommended text for the register entry (analytical method)

For the quantification of total *zinc* in the *feed additive*:

- Inductively coupled plasma-atomic emission spectrometry, ICP-AES (EN 15621 or EN 15510) or
- Atomic absorption spectrometry, AAS (ISO 6869)

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

- Inductively coupled plasma-atomic emission spectrometry, ICP-AES (EN 15621 or EN 15510) or
- Atomic absorption spectrometry, AAS (ISO 6869) or
- Inductively coupled plasma-mass spectrometry, ICP-MS (EN 17053)

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

- Inductively coupled plasma-atomic emission spectrometry, ICP-AES (EN 15621 or EN 15510) or
- Atomic absorption spectrometry, AAS (Commission Regulation (EC) No 152/2009 (Annex IV-C) or ISO 6869) or

– Inductively coupled plasma-mass spectrometry, ICP-MS (EN 17053)

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

– High performance liquid chromatography with refraction index detection (HPLC-RI)

For the quantification of *sulphur* and *sulfate* in the *feed additive*:

– Inductively coupled plasma-atomic emission spectrometry, ICP-AES (EN 15621)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *zinc (II) betaine complex* 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. 1831-0021-2021

[2] *Application, Annex 1 – Submission Number 1615282926030-2862

[3] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition (active substance/agent, other components, impurities, batch to batch variation)

[4] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition

[5] Commission Implementing Regulation (EU) 2016/1095 of 6 July 2016 concerning the authorisation of Zinc acetate dihydrate, Zinc chloride anhydrous, Zinc oxide, Zinc sulphate heptahydrate, Zinc sulphate monohydrate, Zinc chelate of amino acids hydrate, Zinc chelate of protein hydrolysates, Zinc chelate of glycine hydrate (solid) and Zinc chelate of glycine hydrate (liquid) as feed additives for all animal species and amending Regulations (EC) No 1334/2003, (EC) No 479/2006, (EU) No 335/2010 and Implementing Regulations (EU) No 991/2012 and (EU) No 636/2013, *OJ L 182, 7.7.2016*

[6] *Technical dossier, Section II: 2.6.1 Method of analysis of the active substance

[7] EN 15621:2017 – *Animal feeding stuffs: Methods of sampling and analysis – Determination of calcium, sodium, phosphorus, magnesium, potassium, sulphur, iron, zinc, copper, manganese and cobalt after pressure digestion by ICP-AES*

[8] EN 15510:2017 – *Animal feeding stuffs: Methods of sampling and analysis – Determination of calcium, sodium, phosphorus, magnesium, potassium, iron, zinc, copper, manganese, cobalt, molybdenum and lead by ICP-AES*

[9] Commission Regulation (EC) No 152/2009 laying down the methods of sampling and analysis for official control of feed – Annex IV-C

[10] Food Standards Agency – Information Bulletin on Methods of Analysis and Sampling for Foodstuffs, No 102; March 2010

[11] #Zinc Group – JRC.D.5/CvH/ZE/mds/Ares(2016)99245

[12] 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*

[13] 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)*

[14] International Commission for Uniform Methods of Sugar Analysis (ICUMSA) – Official Method GS 4/8-21: The Determination of Betaine in Beet Molasses by a High Performance Liquid Chromatography (HPLC), Proc. 22nd Session ICUMSA, 1998, 305

[15] *Technical dossier, Section II – Annex_II_10

[16] *Technical dossier, Section II – Annex_II_11

[17] *Technical dossier, Section II – Annex_II_12

*Refers to Dossier no: FAD-2021-0072

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)
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- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
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- [2]Ruokavirasto Helsinki (FI)
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[2] Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: Elintarviketurvallisuusvirasto/Livsmedelssäkerhetsverket (Evira), Helsinki/Helsingfors.