Ref. Ares(2023)2779465 - 19/04/2023

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health and Food
Food and Feed Compliance

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

Iron (II) betaine complex

(FEED-2022-9491; CRL/220043)

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

Dossier related to:	**FEED-2022-9491 - CRL/220043**
Name of Product:	***Iron (II) betaine complex***
Active Agent (s):	***Iron (II) betaine / Iron***
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Stefano Bellorini** **18/04/2023**
Report approved by: Date:	**Christoph von Holst** **19/04/2023**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4 for *iron (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 all animal species and categories.

According to the Applicant, the *feed additive* is a solid preparation of *iron (II) betaine complex* containing minimum of 14 % (w/w) of *iron* and minimum of 36 % (w/w) of *betaine* as main components.

The *feed additive* is intended to be incorporated into *premixtures* and *compound feed* with the proposed levels of total *iron* are ranging from 450 to 750 mg/kg in *compound feed*.

For the quantification of total *iron* in the *feed additive*, *premixtures* and *compound feed* 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 ISO 6869 method based on AAS have been previously evaluated and recommended by the EURL for the quantification of total *iron* in *feed additives*, *premixtures* and *compound feed* in the frame of several iron dossiers.

Furthermore, the European Union (EU) method based on atomic absorption spectrometry (AAS), which was further ring-trial validated by the UK Food Standards Agency (FSA) has been previously recommended by the EURL for the quantification of total *iron* in *compound feed*.

The EURL is also aware of the ring-trial validated EN 17053 method based on inductively coupled plasma-mass spectrometry (ICP-MS), which has been recommended for the quantification of total *iron* in *premixtures* and *compound feed* in the frame of some former iron dossiers.

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 *iron* in the *feed additive*; (ii) EN 15621, EN 15510, ISO 6869 and EN 17053 for the quantification of total *iron* in *premixtures*; and iii) EN 15621, EN 15510, ISO 6869, EN 17053 and EU method for the quantification of total *iron* in *compound feed*.

For the quantification of *betaine* in the *feed additive* the Applicant proposed 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.

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*.

In addition, for proving the formation of *iron (II) betaine* complex, the Applicant proposed the qualitative X-ray powder diffraction (XRD) method, where the diffraction patterns are compared to those of the starting materials. The EURL considers the X-ray powder diffraction (XRD) method suitable for proving the complex formation between *iron* 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

Iron (II) betaine complex, *iron*, nutritional feed additives, compounds of trace elements, all animal species and categories.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new *feed additive*) for *iron (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 all animal species and categories [1,2].

According to the Applicant, the *feed additive* is a solid preparation of *iron (II) betaine complex* containing minimum of 14 % (w/w) of *iron* and minimum of 36 % (w/w) of *betaine* as main components [3].

The *feed additive* is intended to be incorporated into *premixtures* and *compound feed* with the following proposed maximum levels of total *iron* in *compound feed*: 250 mg/day for piglets; 450 mg/kg for bovines and poultry; 500 mg/kg for ovines; 600 mg/kg for pets and 750 mg/kg for other species [4]. The proposed levels are in agreement with the current established limits as indicated in Commission Implementing Regulation (EU) 2017/2330 [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 *iron (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 *iron* in the *feed additive*, *premixtures* and *compound feed* 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 ISO 6869 based on AAS [9] have been previously evaluated and recommended by the EURL for the quantification of total *iron* in the *feed additives*, *premixtures* and *compound feed* in the frame of several *iron* dossiers [10-13].

Furthermore, the European Union (EU) method based on atomic absorption spectrometry (AAS) [14], which was further ring-trial validated by the UK Food Standards Agency (FSA) [15] has been previously recommended by the EURL for the quantification of total *iron* in *compound feed* in the frame of the above-mentioned dossiers [10-13].

The EURL is also aware of the ring-trial validated EN 17053 method based on inductively coupled plasma-mass spectrometry (ICP-MS) [16], which has been recommended for the quantification of total *iron* in *premixtures* and *compound feed* in the frame of some of the above-mentioned *iron* dossiers [11-13].

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 *iron* is detected by ICP-AES at selected specific wavelengths (259.94 or/and 238.20 nm; however 238.20 nm is interfering with the signal of cobalt) 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) [9,14]. 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 [14]. The analyte is detected by an air-acetylene flame AAS at selected specific wavelength (248.30 nm). The quantification is performed using an external standard calibration curve [9,14].

According to the EN 17053 method, the sample is digested with concentrated nitric acid under pressure. The elemental *iron* is detected by ICP-MS at mass-to-charge (m/z) of 56 (low resolution) or 55.9344 (high resolution). The quantification of the analyte is performed using an external standard calibration or standard additions [16].

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 *iron* in the *feed additive*; (ii) EN 15621, EN 15510, ISO 6869 and EN 17053 for the quantification of total *iron* in *premixtures*; and iii) EN 15621, EN 15510, ISO 6869, EN 17053 and EU method for the quantification of total *iron* in *compound feed*.

Note: When applying the recommended methods for the quantification of *iron* 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.

Table 1: Performance characteristics for the quantification of *total iron* in animal feedingstuffs.

	EN 15621	EN 15510	ISO 6869	UK FSA	EN 17053
Method	ICP-AES	ICP-AES	AAS	AAS	ICP-MS
Mass fraction (mg/kg)	277 – 15940	293 – 8182	79 – 31000	197 – 340	36.1 – 3114
RSD_r (%)	2.9 – 6.3	2.4 – 4.8	0.9 – 16(*)	2.3 – 9.5	3.0 – 4.3
RSD_R (%)	9.6 – 12.4	5.1 – 10	6.0 – 24(*)	5.3 – 9.5	5.7 – 13.7
LOQ (mg/kg)	1	3	5	20	5
Reference	[7]	[8]	[9]	[15]	[16]

RSD_r and RSD_R: relative standard deviation for *repeatability* and *reproducibility*; LOQ: limit of quantification;
(*) based on dry weight.

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 proposed [6] 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 [17]. This method was previously evaluated and recommend by the EURL in the frame of the dossiers for zinc (II) betaine and copper (II) betaine complexes [18,19].

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 2.4 % was derived for an average betaine content of 37.8 % (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*.

In addition, for proving the formation of *iron (II) betaine* complex, the Applicant proposed the qualitative X-ray powder diffraction (XRD) method, where the diffraction patterns are compared to those of the starting materials. The appearance of new lines in the diffractograms of the additive indicates the formation of new phases. If intense lines of the starting materials are no longer present, conversion into the additive are considered to be complete [6].

The EURL considers the qualitative X-ray powder diffraction (XRD) method suitable for proving the complex formation between *iron* 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.

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 *iron* in the *feed additive*;

- the EN 15621, EN 15510, ISO 6869 and the EN 17053 method based on inductively coupled plasma-mass spectrometry (ICP-MS) for the quantification of total *iron* in *premixtures*;
- the EN 15621, EN 15510, ISO 6869, EN 17053 and the European Union (EU) method based on AAS for the quantification of total *iron* in *compound feed*;
- 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*.

In addition, the EURL considers the qualitative X-ray powder diffraction (XRD) method suitable for proving the complex formation between *iron* and *betaine*.

Recommended text for the register entry (analytical method)

For the quantification of total *iron* 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 *iron* 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 *iron* in *compound feed*:

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

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Iron (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] *Forwarding of applications for authorisation of feed additives in accordance with Regulation (EC) No 1831/2003 – E-Submission Food Chain platform – https://open.efsa.europa.eu/questions/EFSA-Q-2022-00624 https://webgate.ec.europa.eu/esfc/#/applications/11111

[2] *Application, Annex 1

[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) 2017/2330 of 14 December 2017 concerning the authorisation of Iron(II) carbonate, Iron(III) chloride hexahydrate, Iron(II) sulphate monohydrate, Iron(II) sulphate heptahydrate, Iron(II) fumarate, Iron(II) chelate of amino acids hydrate, Iron(II) chelate of protein hydrolysates and Iron(II) chelate of glycine hydrate as feed additives for all animal species and of Iron dextran as feed additive for piglets and amending Regulations (EC) No 1334/2003 and (EC) No 479/2006, OJ L 333, 15.12.2017

[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] 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*

[10] #Iron Group – JRC.D.5/CvH/ZE/mds/Ares(2017) 2353385

[11] #FAD-2018-0010 – JRC F.5/CvH/ZE/AS/Ares(2019)7167892

[12] #FAD-2018-0086 – JRC F.5/CvH/MGH/AS/Ares(2019)3011269

[13] #FAD-2019-0094 – JRC F.5/CvH/ZE/AS/Ares(2020)2629421

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

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

[16] 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)*

[17] 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

[18] #FAD-2021-0072 – JRC F.5/CvH/ZE/AS/Ares(2021)5798124

[19] #FAD-2021-0073 – JRC F.5/CvH/ZE/AS/Ares(2021)6231218

*Refers to Dossier no: FEED-2022-9491

#https://joint-research-centre.ec.europa.eu/eurl-fa-eurl-feed-additives/eurl-fa-authorisation/eurl-fa-evaluation-reports_en

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)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- [1]Wageningen Food Safety Research (WFSR) (NL)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)

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