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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

Ferrous lysinate sulfate

(FAD-2019-0094; CRL/190061)

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:	**FAD-2019-0094 - CRL/190061**
Name of Product / Feed Additive:	***Ferrous lysinate sulfate***
Active Agent (s):	**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** **18/05/2020**
Report approved by: Date:	**Christoph von Holst** **19/05/2020**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *ferrous lysinate sulfate* 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.

The *feed additive* is a preparation with a minimum content of 15 % (w/w) of *iron* and 40 % (w/w) of *lysine*.

The *feed additive* is intended to be incorporated into *feedingstuffs* through *premixtures*. The Applicant proposed maximum levels of *total iron* in *feedingstuffs* ranging from 450 to 750 mg/kg or 250 mg/day depending on the animal species.

For the quantification of *total iron* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant proposed several internationally recognised ring-trial validated methods, namely ISO 6869 based on atomic absorption spectrometry (AAS), EN 15621 and EN 15510 based on inductively coupled plasma-atomic emission spectrometry (ICP-AES). In addition, for the quantification of *total iron* in *premixtures* and *feedingstuffs* the Applicant proposed the internationally recognised ring-trial validated EN 17053 method based on inductively coupled plasma-mass spectrometry (ICP-MS) and the European Union method based on atomic absorption spectrometry (AAS). The above mentioned methods were previously evaluated and recommended by the EURL in the frame of several iron based feed additive dossiers.

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

For the quantification of *lysine* in the *feed additive* the Applicant proposed the ring-trial validated method EN ISO 17180 based on ion-exchange chromatography (IEC) with post-column derivatisation coupled to optical (visible (VIS) or fluorescence (FLD)) detection.

Based on the performance characteristics available, the EURL recommends for official control the above mentioned EN ISO 17180 method based on IEC-VIS/FLD to quantify *lysine* in the *feed additive*.

In addition, the EURL recommends for official control for the identification of the sulfate in the *feed additive* the European Pharmacopoeia monograph 20301.

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

Ferrous lysinate sulfate, *iron*, nutritional additives, compounds of trace elements, all animal species

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new *feed additive*) for *ferrous lysinate sulfate* 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 [1].

The *feed additive* is an olive green-to-black granulate, free flowing solid preparation with a minimum content of 15 % (w/w) of *iron* and 40 % (w/w) of *lysine* [2,3].

The *feed additive* is intended to be incorporated into *feedingstuffs* through *premixtures* [4]. The Applicant, complying with the limits set in the Corrigendum to Commission Implementing Regulation (EU) 2017/2330, proposed maximum levels of *total iron* in *feedingstuffs*: 450 mg/kg for bovines and poultry; 500 mg/kg for ovines; 600 mg/kg for pet animals; 750 mg/kg for other species; and 250 mg/day for piglets up to one week before weaning [1,4,5].

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 *ferrous lysinate sulfate* 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 *feedingstuffs* the Applicant proposed [6] several internationally recognised ring-trial validated methods, namely ISO 6869 based on atomic absorption spectrometry (AAS) [7], EN 15621 [8] and EN 15510 [9] based on inductively coupled plasma-atomic emission spectrometry (ICP-AES).

According to the ISO 6869 method, the sample (1 to 5 g) is dissolved in 6 M hydrochloric acid. If necessary, the sample is ashed at 550±15 °C before its treatment with the hydrochloric acid. The resulting solution after the precipitation and filtration is diluted for its further analysis by acetylene-air flame atomic absorption spectrometry (AAS). Iron is detected at an absorption wavelength of 248.30 nm and the quantification is performed via an external calibration using the standard solutions of the analyte of interest [7].

According to the EN 15621 method, the sample (0.5 g) is mixed with nitric acid or a mixture of nitric acid and hydrogen peroxide and digested under pressure by a microwave equipment until complete dissolution of the sample. The resulting solution is further analysed after an appropriate dilution by ICP-AES. Iron is detected at emission wavelengths of 259.94 nm or/and 238.20 nm; however 238.20 nm is interfering with the signal of cobalt. The quantification is performed via an external calibration using the standard solutions of the analyte of interest or by standard additions [8].

According to the EN 15510 method, the sample (1 to 5 g) 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). The resulting solution is further analysed after appropriate dilution by ICP-AES. The quantification is performed at emission wavelengths of 259.94 nm or/and 238.20 nm by using an external calibration or standard additions [9].

Moreover, the internationally recognised ring-trial validated method EN 17053 based on inductively coupled plasma-mass spectrometry (ICP-MS) [10] and the European Union method based on AAS (Commission Regulation (EC) No 152/2009 – Annex IV-C) [11], which has been further ring-trial validated by the UK Food Standards Agency (FSA) [12], have been proposed by the Applicant [6] for the quantification of *total iron* in *premixtures* and *feedingstuffs*, respectively. The above mentioned methods were previously evaluated and recommended by the EURL in the frame of several iron based feed additive dossiers [13-18].

According to the EN 17053 method, the sample (0.5 g) is mixed with nitric acid or a mixture of nitric acid and hydrogen peroxide and digested under pressure by a microwave equipment

until complete dissolution of the sample. The resulting solution is further analysed after appropriate dilution by ICP coupled to low or high resolution mass spectrometry (MS). Iron is detected at mass-to-charge (m/z) of 56.00 (low resolution) or 55.9344 (high resolution). The quantification is performed via an external calibration or standard additions. In addition, an internal standard (rhodium solution) or the isotope dilution technique should be used, if there is the need to cope with matrix interferences [10].

According to the European Union method, the sample (5 to 20 g) is ashed at 475 ^{o}C for 16 h. Then, the residue is cooled down, treated with a concentrated hydrochloric acid and the resulting solution is filtered after a dilution with water for further analysis by acetylene-air flame atomic absorption spectrometry (AAS). Iron is detected at an absorption wavelength of 248.30 nm and the quantification is performed via an external calibration using the standard solutions of the analyte of interest [11].

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

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

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

	ISO 6869	EN 15621	EN 15510	EN 17053	UK FSA
Method	**AAS**	**ICP-AES**	**ICP-AES**	**ICP-MS**	**AAS**
Mass fraction (mg/kg)	79 – 31000	277 – 15940	293 – 8182	36.1 – 3114	197 – 340
RSD_r (%)	0.9 – 16$^{(*)}$	2.9 – 6.3	2.4 – 4.8	3.0 – 4.3	2.3 – 9.5
RSD_R (%)	6.0 – 24$^{(*)}$	9.6 – 12.4	5.1 – 10	5.7 – 13.7	5.3 – 9.5
LOQ (mg/kg)	5	1	3	5	20
Reference	[7]	[8]	[9]	[10]	[12]

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

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 *lysine* in the *feed additive* the Applicant proposed the ring-trial validated method EN ISO 17180 based on ion-exchange chromatography (IEC) coupled to post-column derivatisation and optical (visible (VIS) or fluorescence (FLD)) detection [19].

The method is dedicated for the determination of *lysine*, methionine and threonine in commercial amino acid products and premixtures containing more than 10 % of amino acid. It does not distinguish between the salts of amino acids and cannot differentiate between enantiomers. According to the method, free *lysine* is extracted with diluted hydrochloric acid. After addition of norleucine as internal standard, the amino acids are separated by ion-exchange chromatography (IEC). Free *lysine* is quantified either after post-column derivatisation with ninhydrine and visible (VIS) detection at 440 nm and 570 nm or by fluorescence detection (FLD) after post-column reaction with ortho-phthaldialdehyde with a detector excitation wavelength at 330 nm and emission at 460 nm [19].

The following performance characteristics were reported in the frame of the ring-trial validation study for the quantification of *lysine* in products and *premixtures* with the content of *lysine* ranging from 10.2 to 76 % (w/w): a RSD_r and RSD_R ranging from 0.7 to 1.7 % and from 1.5 to 2.5 %, respectively [19].

Based on the performance characteristics available, the EURL recommends for official control the above mentioned EN ISO 17180 method based on IEC-VIS/FLD to quantify *lysine* in the *feed additive*.

For the identification of the sulfate in the *feed additive* the Applicant proposed the generic European Pharmacopoeia monograph (Ph. Eur. 01/2008:20301) on identification of ions and functional groups [20]. The EURL recommends for official control for the identification of sulfate in the *feed additive* the European Pharmacopoeia monograph mentioned above.

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 ISO 6869, EN 15621 and EN 15510 methods for the quantification of *total iron* in the *feed additive*, *premixtures* and *feedingstuffs*;
- the EN 17053 method for the quantification of *total iron* in *premixtures* and *feedingstuffs*;
- the European Union method (Commission Regulation (EC) No 152/2009 – Annex IV-C) for the quantification of *total iron* in *feedingstuffs* (only);
- the European Pharmacopoeia monograph 20301 for the identification of the sulfate in the *feed additive*; and
- the EN ISO 17180 method based on ion-exchange chromatography coupled to post-column derivatisation and optical detection (IEC-VIS/FLD) for the quantification of *lysine* in the *feed additive*

Recommended text for the register entry (analytical method)

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

- Atomic Absorption Spectrometry (AAS) – ISO 6869; or
- Inductively Coupled Plasma-Atomic Emission Spectrometry after pressure digestion (ICP-AES) – EN 15621; or
- Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) – EN 15510; 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 identification of sulfate in the *feed additive*:

- European Pharmacopoeia monograph 20301

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

- ion-exchange chromatography coupled to post-column derivatisation and optical detection (IEC-VIS/FLD) – EN ISO 17180

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *ferrous lysinate sulfate* 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. 1831-0005-2020 & Annex I – submission number 1576668415553-2506

[2] *Technical dossier, Section II – 2.1.3. Qualitative and Quantitative composition of the additive

[3] *Technical dossier, Section II – Annex_II_1

[4] *Technical dossier, Section II – 2.5. Conditions of use of the additive

[5] Corrigendum to 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, O.J. L 333, 15.12.2017

[6] *Technical dossier, Section II – 2.6. Methods of analysis and reference samples

[7] 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*

[8] 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*

[9] 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*

[10] 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)*

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

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

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

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

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

[16] #FAD-2013-0020 – JRC.D.5/CvH/RFO/mds/Ares(2015)4392419

[17] #FAD-2012-0035 – JRC.D.5/SFB/CvH/ZE/mds/AreAres(2014)3101870

[18] #Iron oxides – JRC.DG.D.6/CvH/RM/ag/ARES(2011)1139661

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

[20] European Pharmacopoeia monograph 01/2008:20301 – *Identification reactions of ions and functional groups*

*Refers to Dossier no: FAD-2019-0094

#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

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- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Wageningen Food Safety Research[1] (WFSR) (NL)
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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)

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