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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

Ferric citrate chelate

(FAD-2018-0065; CRL/180046)

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-2018-0065 - CRL/180046**
Name of Product:	***Ferric citrate chelate***
Active Agent (s):	**Ferric Citrate**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report approved by: Date:	**Christoph von Holst** **12/08/2019**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *ferric citrate chelate* under the category/ functional group (4 b, d) "zootechnical additives"/ "gut flora stabilisers", "other zootechnical additives", 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 suckling/weaned piglets and minor porcine species.

The *feed additive (ferric citrate chelate)* is an orange-brown powder containing of 16.5 to 20 % (w/w) of *iron (III)* and a maximum of 3 % (w/w) of *iron (II)*. In addition and on request of the EURL, the Applicant provided the content of total *iron* ranging from 16.5 to 23 % (w/w) and the content of *citrate* ranging from 50.2 % to 58.6 % (w/w) in the *feed additive*. Additionally, the Applicant has set a criterion for *citrate* content in the *feed additive*, expressed as the ratio of the percentage of *citrate* and of total *iron*, ranging from 2.3 to 3.2. According to the Applicant the active substance of the *feed additive* is *ferric citrate*. The *feed additive* is intended to be incorporated into *premixtures* and *feedingstuffs* with a proposed minimum *ferric citrate chelate* content of 500 mg/kg *feedingstuffs*.

For the determination of the *ferric citrate* content added to *premixtures* and *feedingstuffs* the Applicant proposed an indirect single-laboratory validated and verified method, based on the enumeration of colour coated graphite particles (a non-nutrient marker) which are included in the *feed additive*. For the quantification of the *ferric citrate* content added to *premixtures*, the Applicant suggested applying this enumeration method after diluting the premixture samples with blank feed. The following performance characteristics were reported in the frame of the homogeneity study: a relative standard deviation for *repeatability* of 12.5 % and an average *recovery* of 86 %, which were considered acceptable by the EURL.

Based on the available performance characteristics, the EURL recommends for official control this indirect method (using a non-nutrient marker as proposed by the Applicant) for the quantification of the added content of *ferric citrate* in *premixtures* and *feedingstuffs* provided that the following criteria are fulfilled: (1) the marker is well characterised; (2) the marker is added into the *feed additive* before the mixing of the product with the compound feed; and (3) the inclusion content of the marker, expressed as number of graphite particles per mass of the *feed additive*, is specified and kept constant (e.g. 72 particles /mg micro-tracered *feed additive* in the case of 10 % addition rate of the marker into the *feed additive*). Moreover, it is recommended that these conditions are included in the Regulation authorising the *feed additive*. In addition, the official control for quantification of the added content of *ferric citrate* in *premixtures* and *feedingstuffs* is not possible when the specific marker is used also for another feed additive(s), in case both (all) are added to the same feed.

For the identification/characterisation of the *feed additive*, the Applicant proposed to quantify total *iron* and *citrate* in the *feed additive*. For the quantification of total *iron* the Applicant submitted three internationally recognised ring-trial validated CEN methods: the EN 15510 method based on inductively coupled plasma-atomic emission spectrometry (ICP-AES) after ashing or wet digestion with hydrochloric acid, the EN 15621 method based on ICP-AES after pressure digestion and the EN ISO 6869 method based on atomic absorption spectrometry. These methods were previously evaluated and recommended by the EURL in the frame of the *iron* group dossier for the quantification of total *iron* in different *feed additives*.

Based on the acceptable performance characteristics available, the EURL recommends for official control of the content of total *iron* in the *feed additive* the three ring-trial validated methods described in EN 15510, EN 15621 and ISO 6869.

For the quantification of *citrate* in the *feed additive* the Applicant submitted a single-laboratory validated and further verified method based on ion-exchange high performance liquid chromatography (HPLC) coupled to ultraviolet (UV) detection to quantify *citrate* in the *feed additive*. The following performance characteristics were reported in the frame of validation and verification studies: a relative standard deviation for a *repeatability* (RSD_r) ranging from 0.3 to 0.4 %; a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 0.3 to 1.6 %; and a recovery rate of 99 %.

Based on the available performance characteristics the EURL recommends for official control of the content of *citrate* in the *feed additive* the single-laboratory validated and further verified method based on ion-exchange high performance liquid chromatography (HPLC) coupled to ultraviolet (UV) detection.

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

Ferric citrate chelate, *ferric citrate*, zootechnical additives, gut flora stabilisers, other zootechnical additives, suckling/weaned piglets and minor porcine species

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (new *feed additive*) for *ferric citrate chelate* under the category/ functional group (4 b, d) "zootechnical additives"/ "gut flora stabilisers", "other zootechnical additives", 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 suckling/weaned piglets and minor porcine species [1-3].

The *feed additive (ferric citrate chelate)* is an orange-brown powder containing of 16.5 to 20 % (w/w) of *iron (III)* and a maximum of 3 % (w/w) of *iron (II)* [4]. In addition, on request of the EURL the Applicant provided the content of total *iron* ranging from 16.5 to 23 % (w/w) and the content of *citrate* ranging from 50.2 % to 58.6 % (w/w) in the *feed additive* [5]. Additionally, the Applicant has set a criterion for *citrate* content in the *feed additive*, expressed as the ratio of the percentage of *citrate* and of total *iron*, ranging from 2.3 to 3.2 [5]. According to the Applicant the active substance of the *feed additive* is *ferric citrate* [4].

The *feed additive* is intended to be incorporated into *premixtures* and *feedingstuffs* with a proposed minimum *ferric citrate chelate* content of 500 mg/kg *feedingstuffs* [2,3].

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 *ferric citrate chelate* 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 premixtures, feedingstuffs and when appropriate water (section 2.6.1 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

For the determination of the *ferric citrate* content added to *premixtures* and *feedingstuffs* the Applicant proposed an indirect single-laboratory validated and verified method, based on the enumeration of colour coated graphite particles (a non-nutrient marker) which are included in the *feed additive* [6]. The marker used is Microtracers^{TM} G-Green, consisting of graphite particles, coated with a green food colour and containing *ca.* 72 particles per milligram of the marker [4]. The inclusion of this marker in the *feed additive* was originally intended to

monitor the homogeneity of the *feed additive* in *premixtures* and *feedingstuffs* after mixing [4].

According to the method, the sample (25 to 200 g) of *feedingstuffs* is transferred into a beaker and sufficient tetrachloroethylene (or another solvent with high density) is added to wet thoroughly the sample and to force the graphite particles to sediment at the bottom of the beaker. After addition of the solvent, the mixture is stirred thoroughly and left to stay for 30 s. The floating feed is removed and the solvent is decanted. The sediment is dried on a hot plate for 5 to 10 min and the dry residue is transferred on filter paper. After a developing solution is applied on the dry sediment, the coloured particles are dried and counted by visual inspection [6]. For the quantification of the *ferric citrate chelate* content added to *premixtures*, the Applicant suggested applying this method after diluting the premixture samples with blank feed [4].

In the frame of the homogeneity studies [4], feed samples were prepared by taking 500 g of *ferric citrate* mixed with 50 g of the marker and added to 1000 kg of feed. An aliquot of 25 g of homogenised feed was taken for the estimation of the homogenous distribution of the *feed additive*. Given the fact that *ca.* 72000 graphite particles are present per gram of the marker this results in an expected number of graphite particles of *ca.* 90 per 25 g of feed. The following performance characteristics were reported in the frame of the homogeneity study: a relative standard deviation for *repeatability* of 12.5 % and an average *recovery* of 86 %. Similar performance characteristics were presented in the frame of the validation and verification studies [6,7] of the method for the determination of ferric tyrosine chelate in feed, which has been previously evaluated and recommended by the EURL in the frame of the corresponding dossier [8].

Based on the available performance characteristics, the EURL recommends for official control this indirect method (using a non-nutrient marker as proposed by the Applicant) for the quantification of the added content of *ferric citrate* in *premixtures* and *feedingstuffs* provided that the following criteria are fulfilled: (1) the marker is well characterised; (2) the marker is added into the *feed additive* before the mixing of the product with the compound feed; and (3) the inclusion content of the marker, expressed as number of graphite particles per mass of the *feed additive*, is specified and kept constant (e.g. 72 particles /mg micro-tracered *feed additive* in the case of 10 % addition rate of the marker into the *feed additive*). Moreover, it is recommended that these conditions are included in the Regulation authorising the *feed additive*. In addition, the official control for quantification of the added content of ferric citrate in *premixtures* and *feedingstuffs* is not possible when the specific marker is used also for another feed additive(s), in case both (all) are added to the same 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)

In the frame of the additional identification/characterisation of the *feed additive*, the Applicant proposed to quantify total *iron* and *citrate* in the *feed additive* [4]. For the quantification of total *iron* in the *feed additive* the Applicant submitted three internationally recognised ring-trial validated CEN methods: the EN 15510 method based on inductively coupled plasma-atomic emission spectrometry (ICP-AES) after ashing or wet digestion with hydrochloric acid [9], the EN 15621 method based on ICP-AES after pressure digestion [10] and the EN ISO 6869 method based on atomic absorption spectrometry [11]. These methods were previously evaluated and recommended by the EURL in the frame of the *iron* group dossier for the quantification of total *iron* in different *feed additives* [12].

The following performance characteristics were reported for the three above mentioned CEN methods in the frame of the ring-trial validation studies for quantification of total *iron* content ranging from 79 to 31000 mg/kg in matrices of the scope: a relative standard deviation for *repeatability* (RSD_r) ranging from 1 % to 16 %; and a relative standard deviation for *reproducibility* (RSD_R) ranging from 5 % to 24 % [9-11].

Based on the acceptable performance characteristics available, the EURL recommends for official control of total *iron* in the *feed additive* the three ring-trial validated methods described in EN 15510, EN 15621 and ISO 6869.

For the quantification of *citrate* in the *feed additive*, the Applicant initially proposed the European Pharmacopeia Monographs 0400 [13] and 0412 [14], where the determination of citrates is based on acid/base titration with 0.1 M perchloric acid and naphtholbenzein as an indicator. On request of the EURL, the method was tested by the Applicant and it was found that the original protocol is not applicable for the quantification of *citrate* in *ferric citrate chelate* [15]. As a consequence, the Applicant submitted another single-laboratory validated and further verified method based on ion-exchange high performance liquid chromatography (HPLC) coupled to ultraviolet (UV) detection to quantify *citrate* in the *feed additive* [16].

The sample (0.5 g) is treated with concentrated hydrochloric acid at 80 ^{o}C, sonicated until complete dissolution, diluted with water and filtered for further chromatographic analysis. The analyte is detected by UV at 220 nm. The quantification is performed by using an external calibration with citric acid as the standard substance [16].

The following performance characteristics were reported in the frame of validation [17] and verification [18] studies: a relative standard deviation for a *repeatability* (RSD_r) ranging from

0.3 to 0.4 %; a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 0.3 to 1.6 %; and a recovery rate of 99 %.

Based on the available performance characteristics the EURL recommends for official control of *citrate* in the *feed additive* the single-laboratory validated and further verified method based on ion-exchange high performance liquid chromatography (HPLC) coupled to ultraviolet (UV) detection.

In addition, qualitative spectroscopic studies were used to demonstrate chelation [19,20]. According to the Applicant, the analysis by Mössbauer spectroscopy of 5 batches of the *feed additive* showed a characteristic features indicating that *iron* and *citrate* is present in a chelated form [5,19]. The analysis of the *feed additive* and citric acid by infrared spectroscopy and the comparison of their spectra indicated also the chelation of *iron* with *citrate* [5,20].

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 ring-trial validated methods based on inductively coupled plasma-atomic emission spectrometry (ICP-AES) with or without pressure digestion (EN 15510 and EN 15621), and the EN ISO 6869 method based on atomic absorption spectrometry (AAS) for the quantification of total *iron* in the *feed additive*
- the single-laboratory validated and further verified method based on ion-exchange high performance liquid chromatography (HPLC) coupled to ultraviolet (UV) detection for the quantification of *citrate* in the *feed additive*
- the single-laboratory validated and verified method, based on the enumeration of colour coated graphite particles added to the *feed additive* for the determination of the added content of *ferric citrate chelate* in *premixtures* and *feedingstuffs*

However, the official control for the determination of the added content of *ferric citrate chelate* in *premixtures* and *feedingstuffs* requires that the amount of the specific marker added to the *feed additive* is fixed and preferably included in the Regulation. Moreover it has to be underlined that this method is not applicable when the same marker is used also for another feed additive(s), in case both (all) are added to the same feed.

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 15510); or
- inductively coupled plasma-atomic emission spectrometry, ICP-AES with pressure digestion (EN 15621); or
- atomic absorption spectrometry, AAS (EN ISO 6869)

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

- ion-exchange high performance liquid chromatography (HPLC) coupled to ultraviolet (UV) detection

For the determination of the added content of *ferric citrate chelate* in *premixtures* and *feedingstuffs*:

- enumeration of colour coated particles of the marker present at fixed mass ratio in the *feed additive*

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *ferric citrate chelate* 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. 1831-0068-2018

[2] *Application, Annex I – submission number 1533653445127-2280

[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] *Supplementary information – 0_Reply_to_EURL_28_6_2019

[6] *Technical dossier, Section II: Annex_II_6_1_1

[7] *Technical dossier, Section II: Annex_II_6_1_2

[8] #FAD-2017-0027 – JRC F.5/CvH/ZE/acs/Ares(2017)4362608

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

[10] 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*

[11] EN 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*

[12] #Iron Group – JRC.D.5/CvH/ZE/mds/Ares(2017)2353146

[13] European Pharmacopoeia Monograph 6.0, 01/2008:0400

[14] European Pharmacopoeia Monograph 6.0, 01/2008:0412

[15] *Supplementary information – Annex_II_1_3_3

[16] *Supplementary information – Annex_II_6_1_3

[17] *Supplementary information – Annex_II_6_1_4

[18] *Supplementary information – Annex_II_6_1_5

[19] *Supplementary information – Annex_II_1_3_4

[20] *Supplementary information – Annex_II_1_3_5

*Refers to Dossier no: FAD-2018-0065

#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:

- Państwowy Instytut Weterynaryjny, Pulawy (PL)
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- [1]Wageningen Food Safety Research (WFSR) (NL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Laboratorio Arbitral Agroalimentario. Ministerio de Agricultura, Alimentación y Medio Ambiente, Madrid (ES)

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