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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**

Chromium chelate of DL-methionine
(FAD-2018-0021; CRL/180026)

**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-2018-0021 - CRL/180026**

Name of Product: ***Chromium chelate of DL-methionine***

Active Agent (s): ***Chromium DL-methionine***

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
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Date: **03/10/2019**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *chromium chelate of DL-methionine* under the category/ functional group (4d) "zootechnical additives"/"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 lactating cows.

The *feed additive* is to be marketed as a grey-tan powder preparation (*Availa-Cr*) with a content of *chromium chelate of DL-methionine* of 3.4 % (w/w), including calcium carbonate and vegetable oil as carriers. The content of *chromium* in *Availa-Cr* is ranging from 1004 to 1474 mg/kg and the content of *methionine* in the preparation is in the range from 13800 to 17500 mg/kg. According to the Applicant the active substance of the *feed additive* is *chromium DL-methionine*.

The *feed additive* is intended to be incorporated into *premixtures* and *feedingstuffs*. The Applicant proposed minimum and maximum levels of *chromium* ranging from 0.3 to 0.5 mg/kg *feedingstuffs*.

For the quantification of the *chromium DL-methionine* content in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant did not submit any method. Instead, the Applicant proposed the separate determination of the *chromium* and *methionine* contents in the above mentioned matrices and submitted the corresponding methods.

For the quantification of the *chromium* content in the *feed additive (Availa-Cr)* the Applicant submitted a single-laboratory validated and further verified method based on inductively coupled plasma-mass spectrometry (ICP-MS). The following performance characteristics were reported in the frame of the validation and verification studies: a relative standard deviation for *repeatability* (RSD_r) ranging from 1.0 to 4.6 %; a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 1.3 to 5.9 %; and a *recovery* rate (R_{rec}) ranging from 90 to 116 %.

Based on the acceptable performance characteristics available, the EURL recommends for official control the single-laboratory validated and further verified method based on ICP-MS for the quantification of the *chromium* content in the *feed additive (Availa-Cr)*.

For the quantification of *chromium* in *feedingstuffs* the Applicant proposed the AOAC 2006.03 method and an in-house method based on ICP-MS. However no experimental proof of the applicability of both methods to quantify *chromium* content in feed, at the proposed added levels of 0.3 to 0.5 mg/kg *feedingstuffs*, was submitted.

Therefore, the EURL cannot evaluate nor recommend any method for official control to quantify the proposed added *chromium* content in *feedingstuffs*.

For the characterisation of the *feed additive* the Applicant proposed to quantify the *methionine* content by the ring-trial validated AOAC 999.13 method based on ion-exchange chromatography coupled to post-column derivatisation and colorimetric or fluorescence detection. The EURL instead identified the ring-trial validated EU and EN ISO 13903 methods based on ion-exchange high performance liquid chromatography coupled to post-column derivatisation and photometric detection (IEC-VIS), already evaluated and recommended by the EURL in the frame of a previous *methionine chelate* dossier.

Based on the performance characteristics available, the EURL recommends for official control the above mentioned EU and EN ISO 13903 methods based on IEC-VIS to quantify *methionine* in the *feed additive*.

Furthermore, for proving the chelated structure of the *feed additive* the Applicant has proposed an additional experiment, namely the measurement of the product (*Availa-Cr*) by mid-infrared (IR) spectrometry.

Based on the available data, the EURL recommends for official control the measurement by mid-IR spectrometry together with the determination of the content of *chromium* and *methionine* in the product, for proving the chelated structure of 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.

KEYWORDS

Chromium chelate of DL-methionine, chromium, DL-methionine, zootechnical additive, lactating cows

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (new *feed additive*) for *chromium chelate of DL-methionine* under the category/ functional group (4d) "zootechnical additives"/"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 lactating cows [1,2].

The *feed additive* is to be marketed as a grey-tan powder preparation (*Availa-Cr*) with content of *chromium chelate of DL-methionine* of 3.4 % (w/w), including calcium carbonate and vegetable oil as carriers [3]. The content of *chromium* in *Availa-Cr* is ranging from 1004 to

1474 mg/kg and the content of *methionine* in the preparation is in the range from 13800 to 17500 mg/kg [3]. According to the Applicant the active substance of the *feed additive* is *chromium DL-methionine* [3,4].

The *feed additive* is intended to be incorporated into *premixtures* and *feedingstuffs* [3]. The Applicant proposed minimum and maximum levels of *chromium* ranging from 0.3 to 0.5 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 *chromium chelate of DL-methionine* 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 the *chromium DL-methionine* content in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant did not submit any method. Instead, the Applicant proposed the separate determination of the *chromium* and *methionine* contents in the above mentioned matrices and submitted the corresponding methods [3].

For the quantification of the *chromium* content in the *feed additive* the Applicant proposed an in-house method based on inductively coupled plasma-mass spectrometry (ICP-MS) [3]. On the request from the EURL, the Applicant submitted the protocol of this method [5] and performed a single-laboratory validation [5] and verification [6] studies for the determination of the *chromium* content in the *feed additive* (*Availa-Cr*).

The sample (0.5 to 1.1 g) is mixed with nitric acid and is heated at 110 °C for 1.5 h. After cooling down, hydrogen peroxide is added to the digestion mixture, which is kept at 110 °C for another 0.5 h. Afterwards, the mixture is cooled down, diluted and fortified with an internal standard (scandium) before the analysis by ICP-MS. The quantification of the

chromium content is performed using chromium standard solutions as calibrants, containing also the internal standard [5].

The performance characteristics reported for the quantification of the *chromium* content in the *feed additive (Availa-Cr)* in the frame of the validation [5] and verification [6] studies are summarised in Table 1.

Table 1: Performance characteristics for the quantification of *chromium* in the *feed additive (Availa-Cr)* in the frame of the validation and verification studies

	Validation	Verification
Method	ICP-MS	
Mass fraction (mg/kg)	1 – 2500	1000
RSD _r (%)	1 – 4.6	3.5
RSD _{ip} (%)	1.3 – 5.9	3.7
R _{rec} (%)	90 – 99	116
Reference	[5]	[6]

RSD_r and RSD_{ip}: a relative standard deviation for *repeatability* and for *intermediate precision*; R_{rec}: a recovery rate.

Based on the acceptable performance characteristics available, the EURL recommends for official control the single-laboratory validated and further verified method based on ICP-MS for the quantification of the *chromium* content in the *feed additive (Availa-Cr)*.

For the quantification of the *chromium* content in *premixtures* the Applicant proposed the AOAC Official method 2006.03 [3] based on inductively coupled plasma-atomic emission spectrometry (ICP-AES) suitable for the analysis of certain elements (*chromium* included) in fertilisers [7] and applied it in the frame of homogeneity studies [3,8]. A relative standard deviation for *repeatability* (RSD_r) of 8.8 % and a *recovery rate* (R_{rec}) of 109 % were derived for an average total *chromium* content of 192 mg/kg *premixtures* [3,8].

For the quantification of *chromium* in *feedingstuffs* the Applicant proposed [3] the above mentioned AOAC method and an in-house method based on ICP-MS. However he did not provide experimental proofs of the applicability of both methods to quantify *chromium* content in feed at the proposed levels of *chromium* of 0.3 to 0.5 mg/kg *feedingstuffs*. Much higher naturally occurring levels of *chromium* in animal feed were stated by the Applicant [9] and reported in the two peer-reviewed publications [10,11]. Therefore, the EURL cannot evaluate nor recommend any method for official control to quantify the proposed added *chromium* content in *feedingstuffs*.

In the frame of the homogeneity studies in feed, containing much higher than the proposed levels of *chromium*, the Applicant applied a method based on ICP-MS and a RSD_r of 6.1 % was obtained for an average total *chromium* content of 28 mg/kg *feedingstuffs* [12].

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 the *methionine* content in the *feed additive (Availa-Cr)* the Applicant proposed [3] the ring-trial validated AOAC 999.13 method, based on ion-exchange chromatography coupled to post-column derivatisation and colorimetric or fluorescence detection [13] and used the method in the frame of stability studies of the *feed additive* [3, 14,15]. However, the above mentioned AOAC method applies for products/premixtures containing more than 10 % of amino acid content while the content of *methionine* in *Availa-Cr* is lower than 10 % (w/w).

The EURL identified instead the ring-trial validated EU method [16] for the quantification of total *methionine* in the *feed additive*. This method applies for the determination of free (synthetic and natural) and of total (peptide-bound and free) amino acids, using an amino acid analyser or ion-exchange high performance liquid chromatography coupled to post-column derivatisation and photometric detection (IEC-VIS). The method does not distinguish between the salts and the amino acid enantiomers. Furthermore, the EURL already evaluated and recommended this method in the frame of the zinc methionine dossier FAD-2015-0025 for official control for the quantification of the *methionine* content in the formulation of the *feed additive* with a *methionine* content lower than 10 % (w/w) [17].

Total methionine can be determined in either oxidised or unoxidised samples. Oxidation is performed at 0 °C with a performic acid/phenol mixture. Excess oxidation reagent is decomposed with sodium disulfite. 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 after post-column derivatisation with ninhydrin and photometric detection at 570 nm or analysed by an amino acid analyser [16]. This method was further ring-trial validated, resulting in the EN ISO 13903 method [18], in which the following performance characteristics were reported for the determination of the total *methionine* content in protein concentrates, *premixtures* and *feed ranging* from 0.18 to 90.2 g/kg: 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* is specified [18].

Based on the performance characteristics available, the EURL recommends for official control the above mentioned EU and EN ISO 13903 methods based on IEC-VIS to quantify *methionine* in the *feed additive*.

Furthermore, for proving the chelated structure of the *feed additive* the Applicant has proposed the measurement of the product (*Availa-Cr*) (after its pelleting with KBr) by mid-infrared (IR) spectrometry [19,20], as the chelation causes a wavenumber shift of specific methionine bonds. The comparison of the two mid-IR spectra, namely of the product and of *methionine* demonstrates i) the shift of the amino group (-NH₂) stretching vibration from 3466 cm⁻¹ in the case of *methionine* to 3561 cm⁻¹ for the product; ii) the stretching vibration of the carbonyl group (-C=O) is also shifted from 1583 cm⁻¹ (*methionine*) to 1662 cm⁻¹ (*Availa-Cr*). Therefore, the corresponding mid-IR spectra of the chelated product (*Availa-Cr*) and of *methionine* [19] differ from each other. In contrast, the vibration of the methyl group (-CH₃) at 1511 cm⁻¹ does not shift as this group is not involved in the formation of the chelated structure [19]. The distinction of both spectra is done by visual examination using reference spectra of the chelated product (*Availa-Cr*) and of *methionine*, which are provided by the Applicant [19].

Based on the available data, the EURL recommends for official control the measurement by mid-IR spectrometry together with the determination of the content of *chromium* and *methionine* in the product for proving the chelated structure of 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 single-laboratory validated and further verified method based on inductively coupled plasma-mass spectrometry (ICP-MS) for the quantification of the *chromium* content in the *feed additive (Availa-Cr)*
- the ring-trial validated EU or EN ISO 13903 methods based on ion-exchange chromatography coupled to post-column derivatisation and photometric detection (IEC-VIS) for the quantification of *methionine* in the *feed additive*
- mid-infrared (IR) spectrometry together with the determination of the content of *chromium* and *methionine* in the *feed additive (Availa-Cr)* for proving the chelated structure of the *feed additive*

For the quantification of the *chromium* content in *feedingstuffs* the EURL cannot evaluate nor recommend any method for official control to quantify the proposed added *chromium* content in *feedingstuffs* (0.3 to 0.5 mg/kg) due to much higher naturally occurring levels of *chromium* in animal feed.

Recommended text for the register entry (analytical method)

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

- inductively coupled plasma-mass spectrometry (ICP-MS)

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

- ion-exchange chromatography coupled to post-column derivatisation and photometric detection (IEC-VIS) (EU or EN ISO 13903 methods)

For proving the chelated structure of the *feed additive*:

- mid-infrared (IR) spectrometry together with the determination of the content of *chromium* and *methionine* 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 *Chromium chelate of DL-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 SANTE_E5_FWD. APPL. 1831-0027-2018 & Application, Annex I – submission number 1524152505120-2210
- [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] *Technical dossier, Section II: Characterisation of the active substance(s) / agent(s)
- [5] *Supplementary information – 1586-007-final-audited_Cr validation
- [6] *Supplementary information – EURL-FA-Veri-form-Availa-Cr-1000_June 2019
- [7] AOAC Official Method 2006.03 – Arsenic, Cadmium, Cobalt, Chromium, Lead, Molybdenum, Nickel and Selenium in fertilisers
- [8] *Technical dossier, Section II – II-27 Homogeneity AvCr final report ws_20161028
- [9] *Supplementary information – 16-07-2019 FW Request for clarification methods for Cr in Feed FAD-2018-0021 (Availa-Cr)
- [10] Y. Li *et al*, *J. Dairy Sci.* **88**, 2911 – 2922
- [11] J. W. Spears *et al*, *J. Dairy Sci.*, **100**, 3584 – 3590

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- [12] *Technical dossier, Section II – II-28 Homogeneity of Availa-Cr in pelleted feed_final report
- [13] AOAC Official Method 999.13 – Lysine, methionine and Threonine in Feed Grade amino Acids and Premixes
- [14] *Technical dossier, Section II – II-25 Stability 5C CoA_s_Availa Cr_HPA15005_0 to 36 months
- [15] *Technical dossier, Section II – II-26 Stability EC CoA_s_Availa Cr_HPA15005_0 to 36 months
- [16] Commission Regulation (EC) No 152/2009 laying down the methods of sampling and analysis for official control of feed – Annex III-F
- [17] #FAD-2015-0025, Zinc Chelate of Methionine JRC.D.5/CvH/ZE/mds/Ares (2016) 1124662
- [18] EN ISO 13903:2005 – *Animal feeding stuffs – Determination of amino acids content*
- [19] *SIN – EFSA_Availa-Cr_Supplementary_Information_Request_20181024
- [20] *SIN – Zinpro IR method for determination of chelation_September 2019
- *Refers to Dossier no: FAD-2018-0021

<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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- 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)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- ¹Wageningen Food Safety Research (WFSR) (NL)
- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 — Labore Landwirtschaft, Nossen (DE)

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