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Feed Additives

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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

L-methionine (min. 98.5 %) and **L-methionine (min. 90 %)**
produced by fermentation with
***Corynebacterium glutamicum* KCCM 80245** *and*
***Escherichia coli* KCCM 80246**
(FAD-2021-0018; CRL/210002)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2021-0018 - CRL/210002**
Name of Product:	***L-methionine (min. 98.5 %) and L-methionine (min. 90 %) produced by fermentation with Corynebacterium glutamicum KCCM 80245 and Escherichia coli KCCM 80246***
Active Agent (s):	**L-methionine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA) JRC Geel, Belgium**
Report prepared by:	**Stefano Bellorini**
Report checked by: Date:	**Zigmas Ezerskis 08/10/2021**
Report approved by: Date:	**Christoph von Holst 08/10/2021**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-methionine (min. 98.5 %) and L-methionine (min. 90 %) produced by fermentation with Corynebacterium glutamicum KCCM 80245 and Escherichia coli KCCM 80246*, under the category/functional group 3(c) 'nutritional additives'/'amino acids, their salts and analogues', according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for all animal species.

According to the Applicant the *feed additive* has a minimum purity (mass fraction on dry matter basis) of 98.5 % and 90 % with respect to the *L-methionine* content, respectively. The *feed additive* is intended to be added directly into *feedingstuffs* or through complementary feed, *premixtures* and *water* for drinking. However, the Applicant did not propose any minimum or maximum content of *L-methionine* in *feedingstuffs*.

For the quantification of *L-methionine* in the *feed additive* the Applicant submitted (i) the method based on titration described in the corresponding European Pharmacopoeia monograph and (ii) a single-laboratory validated method based on high performance liquid chromatography (HPLC) coupled to spectrophotometric detection at 210 nm (UV).

For the quantification of *methionine* in the *feed additive* the EURL identified instead, the ring-trial validated method EN ISO 17180:2013 - "Animal feeding stuffs – Determination of lysine, *methionine* and threonine in commercial amino acid products and premixtures" based on ion-exchange chromatography coupled to visible or fluorescence detection (IEC-VIS/FLD). This standard method does not distinguish between the salts of amino acids and cannot differentiate between enantiomers. It applies for products containing more than 10 % of amino acid. The following performance characteristics were reported: a relative standard deviation for repeatability (RSD_r) ranging from 0.5 to 1.6 % and a relative standard deviation for reproducibility (RSD_R) ranging from 1.5 to 2.6 %. In addition, the EURL identified the "L-methionine monograph" of the Food Chemical Codex (FCC) for the identification of *L-methionine* in the *feed additive*.

For the quantification of *L-methionine* in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated European Union method (Commission Regulation (EC) No 152/2009) based on IEC coupled to photometric detection (IEC-VIS). This method, designed only for the analysis of amino acids in *premixtures* and *feedingstuffs*, does not distinguish between the salts and the amino acid enantiomers. The European Union method was ring-trial validated using four different matrices. This method was further ring-trial validated by 23 laboratories, resulting in the EN ISO 13903:2005 method. The following performance characteristics were reported for the quantification of total *methionine* in *premixtures* and *feedingstuffs*: RSD_r ranging from 1.1 to 5.6 % and RSD_R ranging from 6.9 to 13 %.

The Applicant did not provide experimental data to determine L-methionine in water. Nevertheless, for the quantification of methionine in water, as concluded in the previous EURL reports and further specified in the corresponding legislation, the EURL recommended for official control the European Union method.

In the frame of this authorisation the EURL recommends for official control (i) the "L-methionine monograph" of the Food Chemical Codex (FCC) based on infrared absorption for the identification of *L-methionine* in the *feed additive*; (ii) the ring-trial validated method EN ISO 17180:2013 based on ion-exchange chromatography coupled to visible or fluorescence detection (IEC-VIS/FLD) to quantify free *methionine* in the *feed additive* and *premixtures* (containing more than 10 % *methionine*); and (iii) the European Union method based on IEC-VIS for the quantification of *methionine* in *premixtures*, *feedingstuffs* and *water*.

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

L-methionine (min. 98.5 %) and L-methionine (min. 90 %) produced by fermentation with *Corynebacterium glutamicum KCCM 80245 and Escherichia coli KCCM 80246*, nutritional additives, amino acids, all animal species

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *L-methionine (min. 98.5 %)* and *L-methionine (min. 90 %)* produced by fermentation with *Corynebacterium glutamicum KCCM 80245 and Escherichia coli KCCM 80246*, under the category/functional group 3(c) 'nutritional additives'/'amino acids, their salts and analogues', according to Annex I of Regulation (EC) No 1831/2003. The authorisation is sought for all animal species [1,2].

According to the Applicant, the white crystalline powdered products contain as active substance *L-methionine* with a minimum purity (mass fraction) respectively of 98.5 % and 90 % [3]. The *feed additive* is produced by fermentation with genetically modified strains of *Corynebacterium glutamicum* and *Escherichia coli* [4]. The production strains are deposited in the "Korean Culture Centre of Microorganisms" (KCCM) with the accession number *80245* for *Corynebacterium glutamicum* and 80246 for *Escherichia coli*.

The two forms of the *feed additive* are intended to be added directly into *feedingstuffs* or through complementary feed, *premixtures* and *water* for drinking. The Applicant did not propose any minimum or maximum content of *L- methionine* in *feedingstuffs* [5].

Note: The EURL has previously evaluated the analytical methods for the determination of *methionine* in the frame of several dossiers [6-8].

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 *L-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 *L-methionine* in the *feed additive* the Applicant submitted the method based on titration described in the corresponding European Pharmacopoeia monograph [9,10]. Furthermore, for the quantification of *L-methionine* in the *feed additive* the Applicant submitted a single-laboratory validated method based on high performance liquid chromatography (HPLC) coupled to spectrophotometric detection at 210 nm (UV) [9,11]. In the frame of the validation study, the Applicant reported satisfactory performance characteristics [11]. However, the Applicant did not present a verification study or any additional test performed by a second independent laboratory applying the above mentioned method. Therefore, the EURL cannot conclude on the fitness-for-purpose of the method for official control purposes.

For the quantification of *methionine* in the *feed additive* the EURL identified instead, the ring-trial validated method EN ISO 17180:2013 - "Animal feeding stuffs – Determination of lysine, *methionine* and threonine in commercial amino acid products and premixtures" [12]. This standard method is based on the experimental protocol described in the European Union method for the analysis of free amino acids (including *methionine*) [13]. It does not

distinguish between the salts of amino acids and cannot differentiate between enantiomers. It applies for products containing more than 10 % of amino acid.

Free *methionine* is extracted with diluted hydrochloric acid and further diluted with sodium citrate buffer. After addition of norleucine as internal standard, the amino acids are separated by high performance liquid chromatography (HPLC) with an ion-exchange column. Free methionine 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. The performance characteristics reported for the quantification of free methionine are listed in Table 1.

Based on the performance characteristics available, the EURL recommends for official control the EN ISO 17180:2013 method for the quantification of free *methionine* in the feed additive and premixtures (containing more than 10 % *methionine*).

For the quantification of *L-methionine* in *premixtures* and *feedingstuffs* the Applicant submitted the mentioned above ring-trial validated European Union method [9,13]. This method was designed for the quantification of free (synthetic and natural) and total (peptide-bound and free) amino acids in *premixtures* and *feedingstuffs*, using an amino acid analyser or ion exchange chromatography (IEC) coupled with post-column derivatisation and VIS detection. It does not distinguish between the salts of amino acids and cannot differentiate between enantiomers.

According to the method, the free amino acids (including *methionine*) are extracted with diluted hydrochloric acid. Co-extracted nitrogenous macromolecules are precipitated with sulfosalicylic acid and removed by filtration. The solution is filtered and adjusted to pH 2.2. The amino acids are separated by IEC and determined by post-column derivatisation with ninhydrin and photometric detection at 570 nm. The procedure chosen for the determination of the total amino acids depends on the amino acid under investigation. *Methionine* must be oxidised to *methionine* sulfone prior to hydrolysis. Oxidation is performed at 0 °C with a performic acid/phenol mixture. The excess of oxidation reagent is decomposed with sodium disulfite. The oxidised 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 IEC and determined by post-column derivatisation with ninhydrin and photometric detection at 570 nm. *Methionine* is determined as *methionine* sulfone in hydrolysates of oxidised sample and calculated as *methionine* by introducing the molar mass of the amino acid in the equation used for the calculation of results.

The European Union method was ring-trial validated using four different matrices. This method was further ring-trial validated by 23 laboratories, resulting in the EN ISO

13903:2005 method [14]. The performance characteristics reported for the quantification of total *methionine* are listed in Table 1. Furthermore, the following limits of quantification were reported for free *methionine* and total *methionine*: 0.04 and 0.25 g/kg *feedingstuffs*, respectively [14].

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated European Union method based on IEC-VIS to quantify *methionine* in *premixtures* and *feedingstuffs*.

The Applicant did not provide experimental data to determine *L-methionine* in *water* [9]. Nevertheless, even if the determination of *methionine* in *water* is not explicitly stated in the scope, as concluded in the previous EURL reports and further specified in the corresponding legislation, the EURL recommends the European Union method for official control for the quantification of *methionine* in *water* [6,7,13,15-17].

Table 1: Method performance characteristics obtained in the frame of ring-trial validation studies (EN ISO 17180:2013 [12], European Union method [13] and EN ISO 13903:2005 [14]) for the determination of total *methionine* in the *feed additive*, *premixtures* and *feedingstuffs*.

Ring-Trial	Matrix	*methionine* content g/kg	RSD_r %	RSD_R %
[12]	Premix 2	270	1.6	1.9
	Premix 3	110	1.2	1.8
	Premix 4	140	0.9	1.9
	Premix 5	320	0.5	2.6
	Premix 6	307	1.1	2.5
	Premix 7	90	0.9	1.5
	Premix 8	89	1.2	1.9
	Premix 9	190	1.0	1.7
	Premix 10	184	0.7	1.5
	DL-methionine	936	0.9	1.5
[13]	Mixed pig feed	3.3	3.4	7
	Broiler compound	5.1	3.1	10.9
	Protein concentrate	12	2.2	13
	Premix	90.2	2.4	6.9
[14]	Poultry meal	11.7	2.1	12
	Broiler finisher feed	5.3	1.1	7.6
	Broiler starter feed	6.2	2.1	10.2
	Corn	1.8	5.6	11.7
	Fishmeal	16.1	1.9	9.7

RSD_r, RSD_R - relative standard deviation for *repeatability* and *reproducibility,* respectively

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)

The 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)

The EURL found the "L-methionine monograph" of the Food Chemical Codex (FCC) where identification is based on infrared absorption [18]. The EURL recommends the Food Chemical Codex for the identification of *L-methonine* 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 (i) the "L-methionine monograph" of the Food Chemical Codex (FCC) based on infrared absorption for the identification of *L-methionine* in the *feed additive*; (ii) the ring-trial validated method EN ISO 17180:2013 based on ion-exchange chromatography coupled to visible or fluorescence detection (IEC-VIS/FLD) to quantify free *methionine* in the *feed additive* and *premixtures* (containing more than 10 % *methionine*); and (iii) the European Union method based on IEC-VIS for the quantification of *methionine* in *premixtures*, *feedingstuffs* and *water*.

Recommended text for the register entry (analytical method)

For the identification of *L-methionine* in the *feed additive*:

- Food Chemical Codex " *L-methionine* monograph"

For the quantification of *methionine* in the *feed additive and premixtures* (containing more than 10% *methionine*)*:*

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

For the quantification of *methionine* in *premixtures* and *feedingstuffs:*

- ion-exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS), Commission Regulation (EC) No 152/2009 (Annex III, F)

For the quantification of *methionine* in *water*:

- ion-exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *L-methionine (min. 98.5 %) and L-methionine (min. 90 %)* 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: FORW. APPL. 1831/0015-2021

[2] *Technical dossier, Section II: II.1.2 Proposal for classification

[3] *Technical dossier, Section II: II.2.1.1 Chemical substances

[4] *Technical dossier, Section II: II.2.2.2 Microorganisms (as source of the additive)

[5] *Technical dossier, Section II: II.5.1 Proposed mode of use in animal nutrition

[6] FAD-2018-0085, L-methionine produced by fermentation with Corynebacterium glutamicum KCCM80184 and Escherichia coli K12 KCCM80096, Ref. Ares(2019)3043147 - 07/05/2019, https://ec.europa.eu/jrc/sites/default/files/finrep_fad-2018-0085-methionine.pdf

[7] FAD-2012-0016, L-methionine, Ref. JRC.D.5/SFB/CvH/SB/ag/ARES(2012)1459970, https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2012-0016-L-Methionine.pdf

[8] FAD-2010-0023, DL-Methionine; DL-Methionine sodium salt; DL-Methionine protected with ethylcellulose; DL-Methionine protected with copolymer vinylpiridine/styrene; Hydroxy analogue of Methionine; Calcium salt of hydroxy analogue of Methionine; Isopropyl ester of the hydroxylated analogue of Methionine, Ref.JRC.D.5/FSQ/CvH/SB/ag/ARES(2012)240861, https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2010-0023.pdf

[9] *Technical dossier, Section II: II.6.1 Methods of analysis for the active substance

[10] European Pharmacopoeia 7.2, 01/2008:1027, p. 2379

[11] *Technical dossier, Section II: Annex_II_6_01 Method validation.pdf

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

[13] Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed, O.J. L 54, 26.02.2009

[14] EN ISO 13903:2005- Animal feeding stuffs – Determination of amino acids content

[15] Commission Implementing Regulation (EU) No 469/2013 of 22 May 2013 concerning the authorisation of DL-methionine, DL-methionine sodium salt, hydroxy analogue of methionine, calcium salt of hydroxy analogue of methionine, isopropyl ester of hydroxy analogue of methionine, DL-methionine protected with copolymer vinylpyridine/styrene and DL-methionine protected with ethylcellulose as feed additives, O.J. L 136/1, 23.5.2013 Food Chemical Codex monograph "L-lysine monohydrochloride", FCC 7 (2010), p.598

[16] Commission Implementing Regulation (EU) No 852/2014 of 5 August 2014 concerning the authorisation of L-methionine as a feed additive for all animal species, O.J. L 233/22, 6.8.2014
[17] Commission Implementing Regulation (EU)) 2020/1497 of 15 October 2020 concerning the authorisation of L-methionine produced by Corynebacterium glutamicum KCCM 80 184 and Escherichia coli KCCM 80 096 as a feed additive for all animal species, O.J. L 342/1, 16.10.2020
[18] Food Chemical Codex monograph "L-methionine", FCC 7 (2010), p. 642-643

*Refers to Dossier no: FAD-2021-0018

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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- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
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- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Ruokavirasto Helsinki (FI)[2]

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

[2] Name and address according to COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: Elintarviketurvallisuusvirasto/Livsmedelssäkerhetsverket (Evira), Helsinki/Helsingfors.