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European Union Reference Laboratory

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
(FAD-2012-0016 ; CRL/120010)



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Dossier related to: FAD-2012-0016
CRL/120010

Name of Feed Additive: L-methionine

Active Substance(s): L-methionine

Rapporteur Laboratory: European Union Reference
Laboratory for Feed Additives
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EXECUTIVE SUMMARY

In the current application authorisation is sought under Articles 4(1) (authorisation of a feed additive) for *L-methionine*, 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. Authorisation is sought for *all animal species*.

According to the Applicant, the *feed additive* is produced by fermentation process with *Escherichia Coli* K-12. The strain is deposited in the 'Korean Culture Centre of Microorganisms'. The *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs* or *water*. The Applicant proposed no minimum or maximum *L-methionine* concentrations in *feedingstuffs*.

For the determination of *L-methionine* in the *feed additive*, the Applicant proposed the titrimetric method as described in the relevant European Pharmacopoeia monograph. For the identification, the EURL proposed instead to apply the tests described in the Food Chemical Codex (FCC) "*L-Methionine* monograph": infrared absorption in combination with the analysis of the optical rotation. Furthermore, for the quantification of *methionine* in the *feed additive*, the EURL identified the ring-trial validated ISO/DIS 17180 method. This multi-analyte technique applies for the determination of *methionine* content in commercial amino acid products and *premixtures* containing more than 10% active substance, using an amino acid analyzer or High Performance Liquid Chromatography (HPLC) equipment coupled with post-column derivatisation and Visible or fluorescence detection (VIS/FD). The following performance characteristics are reported for a *methionine* content ranging from 30.6 to 93.3%:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 0.5 to 1.1%; and
- a standard deviation for *reproducibility* (RSD_R) ranging from 1.5 to 2.6%.

Based on the performance characteristics presented, the EURL recommends for official control the FCC monograph methods based on infrared absorption and optical rotation to identify the *L-methionine* in the *feed additive* and the ISO/DIS 17180 method, based on ion exchange chromatography coupled with post-column derivatisation VIS/FD detection, to quantify *methionine* in the *feed additive*.

For the determination of *methionine* in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method - Commission Regulation (EC) No 152/2009 (further ring-trial validated - CEN EN ISO 13903:2005). The following performance characteristics were reported for the determination of *total methionine*:

- RSD_r ranging from 1.1 to 5.6 %;
- RSD_R ranging from 6.9 to 13%; and
- a limit of quantification (LOQ) of 0.25 g/kg.

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated Community method, based on ion exchange chromatography coupled with post-column derivatisation and VIS or FD detection to determine *methionine* in *feedingstuffs* and *premixtures* containing less than 10% of the active substance. For *premixtures* containing more than 10% of *methionine*, the EURL recommends the ring-trial validated ISO/DIS 17180 method, based on ion exchange chromatography coupled with post-column derivatisation and VIS or fluorescence detection.

The Applicant did not provide any experimental method or data for the determination of *L-methionine* in *water*. However, in *FAD-2010-0023*, the concerned Applicant has successfully demonstrated the extension of scope of the Community method for the determination of *methionine* in *water*. Therefore the EURL recommends this method for official control to determine *methionine* in *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) is not considered necessary.

KEYWORDS

L-methionine, nutritional additives, amino acids, their salts and analogues, all animal species and categories.

1. BACKGROUND

In various forms and analogues, synthetically produced *methionine* is already authorised as *feed additive*, without any restrictions [1-5]. In the current application authorisation is sought under Articles 4(1) (authorisation of a feed additive) for the "L-isomer" form, *L-methionine*, 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 [6,7]. Authorisation is sought for *all animal species* [7].

According to the Applicant, the *feed additive* is produced by fermentation process with *Escherichia Coli* K-12. The strain is deposited in the 'Korean Culture Centre of Microorganisms'. *L-methionine* is a white crystalline powder with a minimum purity of 98.5% and a specific optical rotation between +21.1° and +25.1° [8,9]

The *feed additives* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs* or *water*. The Applicant proposed no minimum or maximum *L-methionine* concentrations in *feedingstuffs* [6, 10].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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 (EFSA) for each application or group of applications. For this 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

Identification /Characterisation of the feed additive

Qualitative and quantitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (e.g. arsenic, cadmium, lead, mercury, aflatoxin B1 and dioxins) are available from the respective European Union Reference Laboratories [11].

Description of the analytical methods for the determination of the active substances in feed additive, premixtures, feedingstuffs and water.

For the determination of *L-methionine* in the *feed additive*, the Applicant proposed the titrimetric method as described in the relevant European Pharmacopoeia monograph [12-14]. However, the analytical technique proposed is not considered suitable for the distinction between the *L* and the *D* forms of *methionine*.

For the identification, the EURL proposed instead, infrared absorption test as described in the Food Chemical Codex (FCC) *L-Methionine* monograph: "the spectrum of the sample exhibits maxima at the same wavelength as the spectrum of the Reference standard" [15]. Moreover, the EURL suggests combining the test with the analysis of the optical rotation as described in the same FCC monograph. Furthermore, for the quantification of *methionine* in the *feed additive*, the EURL identified the ring-trial validated ISO/DIS 17180 method [16]. This multi-analyte technique applies for the determination of *methionine* content in commercial amino acid products and *premixtures* containing more than 10% *methionine*, using an amino acid analyzer or High Performance Liquid Chromatography (HPLC) equipment. The method does not distinguish between the salts and the amino acid enantiomers. *Methionine* is extracted with diluted hydrochloric acid and diluted with sodium citrate buffer. After addition of norleucine as internal standard, the amino acids are separated by ion exchange chromatography. *Methionine* is determined by VIS detection at 440 and 570 nm after post-

column derivatisation with ninhydrine or by fluorescence detection (FD) after post column reaction with ortho-phthalaldehyde (OPA).

The following performance characteristics are reported for a *methionine* content ranging from 30.6 to 93.3 %:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 0.5 to 1.1 %; and
- a standard deviation for *reproducibility* (RSD_R) ranging from 1.5 to 2.6 %.

Based on the performance characteristics presented, the EURL recommends for official control the FCC monograph methods based on infrared absorption and optical rotation to identify the *L-methionine* in the *feed additive* and the ISO/DIS 17180 method, based on ion exchange chromatography coupled with post-column derivatisation and VIS or fluorescence detection, to quantify *methionine* in the *feed additive*.

For the determination of *L-methionine* in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method [12,17]. This method applies for the determination of *free* and of *total* (peptide-bound and free) amino acids, using an amino acid analyzer or HPLC equipment. The method does not distinguish between the salts and the amino acid enantiomers. The *free* amino acids 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 ion exchange chromatography and determined by post column derivatisation with ninhydrin and photometric detection at 570 nm. For the determination of the *total methionine*, the sample must be oxidised to methionine sulphone prior to hydrolysis. Oxidation is performed at 0° C with a formic acid/phenol mixture. Excess oxidation reagent is decomposed with sodium disulphite. Methionine sulphone is hydrolysed with hydrochloric acid (6 mol/L) containing 1g phenol/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 by spectrophotometric detection at 570 nm. The analytical result is expressed as mass fraction of *methionine* calculated from the measured methionine sulphone and using a conversion factor (149.21 g/mol). The Community method was ring trial validated using four different matrices listed in Table 1. This method was further ring-trial validated by twenty-three laboratories, resulting in the CEN EN ISO 13903:2005 method [18]. Furthermore, a limit of quantification of 0.25 g/kg *feedingstuffs* was reported in the CEN/ISO standard. The corresponding performance characteristics are listed in Table 1.

Table 1: Method performance characteristics obtained in the frame of two different ring-trial validation exercises for the determination of *total methionine* in *premixtures* and *feedingstuffs*.

Intercomparison study	Matrix	Methionine g/kg	RSD _r (%)	RSD _R (%)
Commission Regulation (EC) No 152/2009 [17] <i>study carried out in 1990</i>	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
ISO 13903:2005 [18] <i>study carried out in 1994</i>	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 and RSD_R - relative standard deviation for *repeatability* and *reproducibility*, respectively

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated Community method, based on ion exchange chromatography coupled with post-column derivatisation and photometric detection to determine *methionine* in *feedingstuffs* and *premixtures* containing less than 10% *methionine*. For *premixtures* containing more than 10% of the active substance, the EURL recommends the ring-trial validated ISO/DIS 17180 method, based on ion exchange chromatography coupled with post-column derivatisation and VIS or fluorescence detection.

The Applicant did not provide any experimental method or data for the determination of *L-methionine* in *water*. However, in *FAD-2010-0023*, the concerned Applicant has successfully demonstrated the extension of scope of the Community method for the determination of *methionine* in *water* [19]. Therefore the EURL recommends this method for official control to determine *methionine* in *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) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation, the EURL recommends for official control:

- the FCC monograph methods based on infrared absorption and optical rotation to identify the *L-methionine* in the *feed additive* and the ISO/DIS 17180 method, based on ion exchange chromatography coupled with post-column derivatisation and photometric or fluorescence detection, to quantify *methionine* in the *feed additive* and *premixtures* containing more than 10% of *methionine*;
- the ring trial validated Community method, using High Performance Liquid Chromatography (HPLC) coupled to post column derivatisation and photometric detection to determine *methionine* in *feedingstuffs*, *water* and *premixtures* containing less than 10% of *methionine*.

Recommended text for the register entry (analytical method)

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

- infrared absorption and optical rotation - FCC monographs methods

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

- ion exchange chromatography coupled with post-column derivatisation and photometric or fluorescence detection (HPLC-VIS/FD) - ISO/DIS 17180.

For the determination of *methionine* in *feedingstuffs*, *water* and *premixtures* containing less than 10% of *methionine*:

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

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *L-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] Commission Directive 88/485/EEC of 26 July 1988 amending the Annex to Council Directive 82/471/EEC concerning certain products used in animal nutrition.
 - [2] Commission Directive 89/520/EEC of 6 September 1989 amending the Annex to Council Directive 82/471/EEC concerning certain products used in animal nutrition.
 - [3] Commission Directive 93/26/EEC of 4 June 1993 amending Council Directive 82/471/EEC concerning certain products used in animal nutrition.
 - [4] Commission Directive 93/26/EEC of 4 June 1993 amending Council Directive 82/471/EEC concerning certain products used in animal nutrition.
 - [5] Commission Directive 2003/104/EC of 12 November 2003 authorising isopropyl ester of the hydroxylated analogue of methionine.
 - [6] *Application, Proposal of Registry Entry – Annex A
 - [7] *Application/Ref: SANCO/G1: Forw.Appl.1831/0035-2012
 - [8] *Technical dossier, Section II: II.2. Characterisation of the active substance/agent
 - [9] *Technical dossier, Section II: II.2.2 Relevant properties
 - [10] *Technical dossier, Section II: II.5.1 Proposed mode of use in animal nutrition
 - [11] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories.
 - [12] *Technical dossier, Section II: II.6.1 Methods of analysis for the active substance
 - [13] *Technical dossier, Section II: Ref_II_1_14_PhEur_2008.pdf
 - [14] European Pharmacopoeia 7.2, tests 01/2008:1027
 - [15] Food Chemical Codex monograph "*L-Methionine*", FCC 7 (2010), p. 642-643
 - [16] Animal feeding stuffs – Determination of lysine, methionine and threonine in commercial amino acids products and premixtures; ISO/DIS 17180:2011
 - [17] 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
 - [18] Animal feeding stuffs – Determination of amino acids content; CEN EN ISO 13903:2005
 - [19] FAD-2010-0023 / CRL/100003 – Methionine – Ref. Ares(2012)240861 – 01/03/2012
- *Refers to Dossier No FAD-2012-0016

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was European Union Reference Laboratory for Feed Additives, IRMM, 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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

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- Fødevarestyrelsen, Ringsted (DK)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
- Laboratorio Arbitral Agroalimentario, Ministerio de Agricultura, Pesca y Alimentación, Madrid (ES)
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