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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-threonine
produced by Escherichia coli CGMCC 7.232
(FAD-2017-0037; CRL/170034)

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-2017-0037 - CRL/170034**
Name of Product:	***L-threonine*** *produced by Escherichia coli CGMCC 7.232*
Active Agent:	**L-threonine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
Report prepared by:	**Stefano Bellorini**
Report checked by: Date:	**Piotr Robouch (EURL-FA)** **02/02/2018**
Report approved by: Date:	**Christoph von Holst** **02/02/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Articles 4(1) for *L-threonine* produced by Escherichia coli CGMCC 7.232, 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. *L-threonine* is already authorised as feed additive under Commission Directive 88/485/EEC.

For the quantification of *L-threonine* in the *feed additive* the Applicant submitted the ring-trial validated method EN ISO 17180:2013 based on Ion Exchange Chromatography coupled with post-column derivatisation and Visible or Fluorescence Detection (IEC-VIS/FD). The following performance characteristics are reported: a relative standard deviation for repeatability (RSD_r) ranging from 0.7 to 1.4 %; and a relative standard deviation for reproducibility (RSD_R) ranging from 1.9 to 2.3 %. In addition, the EURL identified the "L-threonine monograph" of the Food Chemical Codex (FCC) for the identification of *L-threonine* in the *feed additive*.

For the quantification of *L-threonine* in *premixtures, feedingstuffs* and *water* the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009) based on IEC coupled with photometric detection (IEC-VIS). This method, designed only for the analysis of *premixtures* and *feedingstuffs*, does not distinguish between the salts and the amino acid enantiomers. The following performance characteristics were reported for the quantification of total *threonine*: RSD_r ranging from 1.9 to 2.7 %, and RSD_R ranging from 3.8 to 5.2 %. In the frame of the stability studies, the Applicant presented experimental data obtained analysing *threonine* in *water* with the VDLUFA official method based on IEC-VIS/FD. The results presented are considered sufficient to demonstrate the suitability of the procedure for the analysis of the amino acid in *water*. Hence the EURL recommends for official control this method to quantify *threonine* in *water*.

In the frame of this authorisation the EURL recommends for official control (i) the "*L-threonine* monograph" of the FCC based on infrared absorption for the identification of *L-threonine* in the *feed additive*; (ii) the ring-trial validated method EN ISO 17180:2013 based on IEC-VIS/FD to quantify free *threonine* in *feed additive* and *premixtures* (containing more than 10 % threonine); (iii) the Community method based on IEC-VIS for the quantification of *threonine* in *premixtures* and *feedingstuffs*; and (iv) the analytical method described by VDLUFA (4.11.6) based on IEC-VIS/FD to quantify *threonine* 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-threonine, nutritional additives, amino acids, all animal species and categories

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *L-threonine* produced by Escherichia coli CGMCC 7.232, 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 [1-2]. *L-threonine* is already authorised as *feed additive* under 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 [3].

According to the Applicant, the product is a white granulated powder with a minimum purity of 98.5 % [4,5]. The *feed additive* is produced by fermentation with a genetically modified strain of Escherichia coli. The production strain is deposited in the "China General Microbiological Culture Collection Centre" (CGMCC) with reference Escherichia coli CGMCC 7.232 [6].

L-threonine is intended to be mixed either in *premixtures* or added directly to *feedingstuffs* or in addition to *water* [7]. However, the Applicant did not propose a minimum or maximum *L-threonine* content in *feedingstuffs* [1].

Note: The EURL has previously evaluated the analytical methods in the frame of four *L-threonine* related dossiers [8-10].

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-threonine* produced by Escherichia coli CGMCC 7.232 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 substance in feed additive, premixtures, feedingstuffs and water

For the quantification of *L-threonine* in the *feed additive* the Applicant submitted 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" [12,13]. This standard method is based on the experimental protocol described in the Community method for *threonine* [14]. It does not distinguish between the salts of amino acids and it cannot differentiate between enantiomers. It applies for products containing more than 10 % of amino acid.

Free *threonine* 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 with an Ion Exchange Column (IEC). Free *threonine* is quantified either after post-column derivatisation with ninhydrine and Visible (VIS) detection at 440 and 570 nm, or by fluorescence detection (FD) 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 *threonine* are listed in Table 1.

In addition, the EURL found the "L-threonine monograph" of the Food Chemical Codex (FCC) for the characterisation of *L-threonine* in the *feed additive*, where identification is based on infrared absorption and quantification via titration with perchloric acid [15].

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

For the quantification of *L-threonine* in *premixtures*, *feedingstuffs* and *water* the Applicant submitted the ring-trial validated Community method mentioned above [12,14].

Table 1: Method performance characteristics obtained in the frame of ring-trial validation studies (EN ISO 17180:2013 [13], Community method [14] and EN ISO 13903:2005 [16]) for the determination of total *L-threonine* in *feed additive*, *premixtures* and *feedingstuffs*.

Ring-Trial	Matrix	L-threonine g/kg	RSD_r %	RSD_R %
[13]	Feed Additive	955	1.2	2.2
	Premix 2	149	1.3	2.3
	Premix 3	82	0.7	1.9
	Premix 4	112	0.8	2.2
	Premix 5	221	0.8	1.9
	Premix 6	213	1.2	1.9
	Premix 7	140	0.7	2.1
	Premix 8	138	1.4	2.3
	Premix 9	151	1.0	2.0
	Premix 10	147	1.0	2.2
[14,16]	Mixed pig feed	6.9	1.9	4.1
	Broiler compound	9.3	2.1	5.2
	Protein concentrate	22	2.7	3.8
	Premixture	58	2.2	4.3
[16]	Corn	2.9	4.1	11.7
	Broiler finisher feed	7.3	2.7	8.2
	Broiler starter feed	11	2.7	9.9
	Poultry meal	23	3.2	9.1
	Fishmeal	23	3.6	10.7

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

This method was designed for the quantification of *free* (synthetic and natural) and of *total* (peptide-bound and free) amino acids in *premixtures* and *feedingstuffs*, using an amino acid analyser or IEC coupled with post-column derivatisation and VIS detection. It does not distinguish between the salts of amino acids and it cannot differentiate between 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 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 acids under investigation. *Threonine* can be determined in either oxidised or non-oxidised

samples. Oxidation is performed at 0 °C with a performic acid/phenol mixture. The excess of oxidation reagent is decomposed with sodium disulphite. The oxidised or non-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.

The Community method was ring-trial validated using four different matrices. This method was further ring-trial validated by twenty-three laboratories, resulting in the EN ISO 13903:2005 method [16]. The performance characteristics reported for the quantification of total *threonine* are listed in Table 1. Furthermore, the following limits of quantification were reported for free and total *threonine*: 0.03 and 0.2 g/kg *feedingstuffs*, respectively [16].

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

The Applicant provided no experimental data to demonstrate the applicability of the Community method for the determination of *L-threonine* in *water* [12,14]. However, in the frame of the stability studies in water, the Applicant presented experimental data obtained analysing *threonine* with the VDLUFA official method which is actually designed for the analysis in feed [17-19]. This method can be applied for the determination of *threonine* using IEC coupled with VIS or FD. The results presented are considered sufficient to demonstrate the suitability of the procedure for the analysis of the amino acid in *water*. Hence the EURL recommends this method for official control.

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 (i) the "*L-threonine* monograph" of the Food Chemical Codex (FCC) based on infrared absorption for the identification of *L-threonine* 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/FD) to quantify free *threonine* in *feed additive* and *premixtures* (containing more than 10 % threonine); (iii) the Community method based on IEC-VIS for the quantification of *threonine* in *premixtures* and *feedingstuffs*; and (iv) the analytical method described by VDLUFA (4.11.6) based on IEC-VIS/FD to quantify *threonine* in *water*.

Recommended text for the register entry (analytical method)

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

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

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

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

For the quantification of *threonine* 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 threonine in water:

- ion exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS/FD)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *L-threonine* produced by Escherichia coli CGMCC 7.232 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, Proposal of Registry Entry – Annex A

[2] *Application, Reference SANCO/G1: Forw. Appl. 1831/0030-2017

[3] 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

[4] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition

[5] *Technical dossier: Annex MSDS OF L-THREONINE98.5%-BIOTECH.doc

[6] *Technical dossier, Section II: 2.2.1.2 Micro-organisms

[7] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition

[8] FAD-2010-0058 and FAD-2010-0081, L-Threonine, technically pure, Ref. Ares(2012)822925 - 06/07/2012 https://ec.europa.eu/jrc/sites/jrcsh/files/amend-FinRep-FAD-2010-0058%2B0081.pdf

[9] FAD-2010-0028, L-threonine (technically pure) produced using strain AG7056X derived from E-coli K-12, Ref. Ares(2013)3628717 - 03/12/2013 https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2013-0028-L-Threonine.doc_.pdf

[10] FAD-2016-0003, L-threonine, Ref. Ares(2016)3271131 - 08/07/2016 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad2016_0003_l_threonine.pdf

[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: 2.6.1 Methods of analysis for the active substance

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

[14] 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

[15] Food Chemical Codex monograph "L-Threonine", FCC 7 (2010), p.1031-32

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

[17] *Technical dossier, Section II: 2.4.1 Stability

[18] *Technical dossier: Annex 2.1.4.a

[19] VDLUFA MB III 4.11.6 Bestimmung von Lysin, Methionin und Threonin in Aminosäurenhandelsprodukten und Vormischungen

*Refers to Dossier no: FAD-2017-0037

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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- Instytut Zootechniki — Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, PESCA, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
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