



EUROPEAN COMMISSION
DIRECTORATE GENERAL
JOINT RESEARCH CENTRE
Directorate F – Health, Consumers and Reference Materials
European Union Reference Laboratory for Feed Additives

 Ref. Ares(2018)3868557 - 20/07/2018

JRC F.5/CvH/SB/ZE/Ares

**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 fermentation with *Corynebacterium glutamicum* KCCM80118
(*FAD-2018-0003; CRL/170056*)



**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-2018-0003 - CRL/170056**

Name of Product: ***L-threonine*** produced by fermentation with
Corynebacterium glutamicum KCCM80118

Active Agent: **L-threonine**

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
JRC Geel, Belgium**

Report prepared by: **Stefano Bellorini**

Report checked by: **Zigmas Ezerskis**
Date: **19/07/2018**

Report approved by: **Christoph von Holst**
Date: **19/07/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-threonine* produced by *Corynebacterium glutamicum KCCM80118*, 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 Implementing Regulation (EU) 2016/1220.

For the quantification of *L-threonine* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009) based on Ion Exchange Chromatography 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_T ranging from 1.9 to 2.7 %, and RSD_R ranging from 3.8 to 5.2 %.

For the quantification of *L-threonine* in the *feed additive* the EURL identified the ring-trial validated method EN ISO 17180:2013 based on IEC coupled with post-column derivatisation and Visible or Fluorescence Detection (IEC-VIS/FLD). The following performance characteristics are reported: a relative standard deviation for repeatability (RSD_T) 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*.

Within the dossier, the Applicant presented experimental data obtained analysing *threonine* in *water* with the AOAC official method 999.13 based on IEC-VIS/FLD. 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/FLD to quantify free *threonine* in the *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 AOAC (999.13) based on IEC-VIS/FLD 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 *Corynebacterium glutamicum* KCCM80118, 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* - produced by *Escherichia coli* - is authorised as feed additive for all animal species under Commission Implementing Regulation (EU) 2016/1220 of 26 July 2016 [3].

According to the Applicant, the product is an off-white powder with a minimum purity of 98.5 % [4]. The *feed additive* is produced by fermentation with a genetically modified strain of *Corynebacterium glutamicum* [5]. The production strain is deposited in the "Korean Centre of Microorganisms" (KCCM) with reference KCCM80118.

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 several *L-threonine* related dossiers [7-10].

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 Corynebacterium glutamicum* KCCM80118 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-threonine* in the *feed additive, premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method [11,12]. 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 High Performance Liquid Chromatography (HPLC) equipped with an Ion Exchange Column (IEC) and 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 [13]. 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 [13].

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*.

For the quantification of *L-threonine* in the *feed additive* a specific ring-trial validated standard method exists (EN ISO 17180:2013 - "Animal feeding stuffs – Determination of lysine, methionine and *threonine* in commercial amino acid products and premixtures") [14]. This standard method is based on the experimental protocol described in the Community method for *threonine* [12]. 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 HPLC with IEC. Free *threonine* is quantified either after post-column derivatisation with ninhydrine and VIS detection at 440 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 *threonine* 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 *threonine* in the *feed additive* and *premixtures* (containing more than 10 % *threonine*).

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

Ring-Trial	Matrix	L-threonine g/kg	RSD _r %	RSD _R %
[14]	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
[12,13]	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
[13]	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

The Applicant didn't submit any method for the official control of *L-threonine* in *water* [11]. However, within the dossier, the Applicant presented experimental data obtained analysing *threonine* in *water*. The tests were carried out using the ring trial validated AOAC Official Method 999.13 designed for the analysis of threonine in feed additives and premixtures [15,16].

This method, equivalent to EN ISO 17180:2013, is based on IEC coupled with VIS or FLD. 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.

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)

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-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 [17].

The EURL recommends the Food Chemical Codex for the identification of *L-threonine* 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-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/FLD) 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 AOAC (999.13) 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/FLD) – 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/FLD)

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 Corynebacterium glutamicum KCCM80118* 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 SANTE/E5: Forw. Appl. 1831/0006-2018
- [3] Commission Implementing Regulation (EU) 2016/1220 of 26 July 2016 concerning the authorisation of L-threonine produced by *Escherichia coli* as a feed additive for all animal species, O.J. L 201/11, 27.07.2016
- [4] *Technical dossier, Section II: II.2.1.1. Chemical substances
- [5] *Technical dossier, Section II: II.2.2.2. Microorganisms
- [6] *Technical dossier, Section II: II.5.1. Proposed mode of use in animal nutrition
- [7] 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>
- [8] 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
- [9] FAD-2016-0003, L-threonine, Ref. Ares(2016)3271131 - 08/07/2016 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad2016_0003_1_threonine.pdf

- [10] FAD-2017-0037, L-threonine produced by Escherichia coli CGMCC 7.232, Ref. Ares(2018)799054 - 12/02/2018 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2017-0037_threonine.pdf
- [11] *Technical dossier, Section II: II.6.1. Methods of analysis for the active substance
- [12] 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
- [13] EN ISO 13903:2005- Animal feeding stuffs – Determination of amino acids content
- [14] EN ISO 17180:2013 - Animal feeding stuffs – Determination of lysine, methionine and threonine in commercial amino acid products and premixtures
- [15] *Technical dossier, Section II: II.4.2. Homogeneity
- [16] AOAC Official Method 999:13 – Lysine, Methionine and Threonine in Feed Grade Amino Acids and Premixes
- [17] Food Chemical Codex monograph "L-Threonine", FCC 7 (2010), p.1031-32
- *Refers to Dossier no: FAD-2018-0003

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:

- Fødevarestyrelsens Laboratorie Aarhus (kemisk) (DK)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Instytut Zootechniki – Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Elintarviketurvallisuusvirasto/Livsmedelssäkerhetsverket (Evira), Helsinki/Helsingfors (FI)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 – Labore Landwirtschaft, Nossen (DE)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)