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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
(FAD-2016-0003; CRL/150038)



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in connection with the Application for Authorisation of a
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Dossier related to: **FAD-2016-0003 - CRL/150038**

Name of Product / Feed Additive: ***L-threonine***

Active Agent (s): **L-threonine**

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
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Date: **07/07/2016**

Report approved by: **Christoph von Holst**
Date: **08/07/2016**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Articles 4(1) for *L-threonine*, 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. The *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs* or *water*. The Applicant suggested no minimum or maximum *L-threonine* concentrations in *premixtures* and *feedingstuffs*.

For the quantification of *threonine* in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method for amino acids using an amino acid analyzer or High Performance Liquid Chromatography (HPLC) equipped with ion exchange column (IEC). The method applies for the determination of *free* (synthetic and natural) and *total* (peptide-bound and free) amino acids. Only performance characteristics for the determination of total *threonine* are reported: - a relative standard deviation for *repeatability* (RSD_r) ranging from 1.9 to 4.1%; and - a relative standard deviation for *reproducibility* (RSD_R) ranging from 3.8 to 11.7%. Based on the performance characteristics available, the EURL recommends for official control this Community method to quantify *threonine* in *premixtures* and *feedingstuffs*.

For the quantification of *L-threonine* in the *feed additive* and *water*, the Applicant suggested to apply the above-mentioned Community method specifically designed for the analysis of *premixtures* and *feedingstuffs*. No experimental data were provided to demonstrate the applicability of this method to the quantification of *L-threonine* in these two matrices. However, the EURL recommends for official control, based on previous "*L-threonine*" reports (e.g. FAD-2013-0028), (i) the "*L-threonine*" monograph of the Food Chemical Codex (FCC) for the identification of *L-threonine* in the *feed additive*, using infrared absorption together with optical rotation and (ii) the ring-trial validated method (EN ISO 17180:2013) based on IEC coupled with post-column derivatisation and Ultraviolet (UV) or fluorescence detection (FD) for the determination of *L-threonine* in the *feed additive* and concentrated *premixtures* (more than 10%). Precisions of the order of 1 to 2 % were reported for a commercial product with *threonine* content of 96 %.

The EURL could not evaluate nor recommend any method for the official control to quantify *L-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 Articles 4(1) (authorisation of a new feed additive) for *L-threonine* produced using genetically modified strain derived from *Escherichia coli K-12*, 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 [1,2]. The strain is deposited in the Chinese General Microbiological Culture Collection Centre (registered as CGMCC 11473) [4]. Authorisation is sought for all animal species [1-2]. *L-threonine* has been already authorised as feed additive without any restrictions under Commission Directive 88/485/EEC [3].

According to the Applicant *L-threonine* is a light brown powder with a minimum purity of 98.5% [1,5]. The *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs* or *water* [6]. The Applicants suggested no minimum or maximum *L-threonine* concentrations in *premixtures* and *feedingstuffs* [1].

Note: The EURL previously evaluated the analytical methods for the determination of *L-threonine* in the frame of several dossiers: FAD-2013-0028, FAD-2010-0058&0081.

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* 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 [7].

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 *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method [8,9]. 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 High Performance Liquid Chromatography (HPLC) equipped with ion exchange (IE) column. 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 (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. *L-threonine* can be determined in either oxidised or un-oxidised samples. Oxidation is performed at 0° C with a performic acid/phenol mixture. Excess oxidation reagent is decomposed with sodium disulphite. 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 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 listed in Table 1. This method was further ring-trial validated by twenty-three laboratories, resulting in the EN ISO 13903:2005 method [10]. The performance characteristics reported for the determination of total *L-threonine* are listed in Table 1. Furthermore, limits of quantification (LOQ) of 0.03 and 0.2 g/kg *feedingstuffs* were determined for *free L-threonine* and *total L-threonine*, respectively [10].

Table 1: Method performance characteristics obtained in the frame of two ring-trial validation exercises for the determination of *L-threonine* in *premixtures* and *feedingstuffs* [9,10]. Data refer to total *L-threonine*.

Ring-Trial	Matrix	L-threonine g/kg	RSD _r %	RSD _R %
Commission Regulation (EC) No 152/2009 [9] study carried out in 1990	Mixed pig feed	6.9	1.9	4.1
	Broiler compound	9.3	2.1	5.2
	Protein concentrate	22	2.7	3.8
EN ISO 13903:2005 [10] study carried out in 1994	Premixture	58	2.2	4.3
	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

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated Community method, based on IEC coupled with post-column derivatisation, to quantify *L-threonine* in *premixtures* and *feedingstuffs*.

For the quantification of *L-threonine* in the *feed additive* and *water*, the Applicant proposed to apply the above mentioned Community method specifically designed for the analysis of *premixtures* and *feedingstuffs* [8,9]. However, no experimental data were provided to demonstrate the applicability of this method to the quantification of *L-threonine* in these two matrices.

However, based on previous "*L-threonine*" reports (e.g. FAD-2013-0028), the EURL recommends for official control:

- 1) the "*L-threonine*" monograph of the Food Chemical Codex (FCC) [11] for the identification *L-threonine* in the *feed additive*, using infrared absorption together with optical rotation; and
- 2) 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]) based on IEC coupled with post-column derivatisation and Ultraviolet (UV) or fluorescence detection (FD) for the determination of *L-threonine* in the *feed additive* and concentrated *premixtures* (more than 10%).

The EN ISO standard is based on the experimental protocol described in the Community method for *threonine*. It does not distinguish between the salts and the amino acid enantiomers and it applies for products containing more than 10% of amino acid.

Threonine 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 IEC. *Threonine* is determined colorimetrically after post-column derivatisation with ninhydrine and UltraViolet (UV) detection at 440 and 570 nm or by fluorescence detection (FD) after post column reaction with ortho-phthalaldehyde (OPA).

The following performance characteristics are reported for a commercial product with *threonine* content of 96 %: - a relative standard deviation for *repeatability* (RSD_r) of 1.2 %; and - a relative standard deviation for *reproducibility* (RSD_R) of 2.2 %.

The EURL could not evaluate nor recommend any method for the official control to quantify L-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.

4. CONCLUSIONS AND RECOMMENDATIONS

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

- the FCC "*L-threonine*" monograph based on infrared absorption and optical rotation to identify *L-threonine* in the *feed additive*;
- the ring-trial validated EN ISO 17180 method, based on ion exchange chromatography (IEC) coupled with post-column derivatisation and photometric detection (UV/FD), to quantify *threonine* in the *feed additive* and *premixtures*; and
- the ring-trial validated Community method, using ion exchange chromatography (IEC) coupled with photometric detection (UV), to quantify *threonine* in *premixtures* and *feedingstuff*.

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*:

- ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-UV/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-UV), 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-threonine* 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. 1831/0001-2016
- [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: 2.2.1 Description
- [5] *Application: Annex 1
- [6] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
- [7] 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
- [8] *Technical dossier, Section II: 2.6.1 Methods of analysis for the active substance
- [9] 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
- [10] EN ISO 13903:2005- Animal feeding stuffs – Determination of amino acids content;
- [11] Food Chemical Codex monograph "*L*-Threonine", FCC 7 (2010), p.1031-32
- [12] EN ISO 17180:2013 - Animal feeding stuffs – Determination of lysine, methionine and threonine in commercial amino acid products and premixtures;

*Refers to Dossier no: FAD-2016-0003

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 (EU) 2015/1761.

8. ACKNOWLEDGEMENTS

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