

## Amendment to EURL report

**FAD-2010-0058 & FAD-2010-0081 (L-threonine)**

Ref. JRC.DG.D.6/CvH/SB/ago/ARES(2011)423196 [1]

Geel, 05/07/2012

The EURL identified a new ring-trial validated ISO/DIS 17180 method for the determination of *threonine* in *feed additive* [2]. This method is based on the technique presented in the Community method and recommended for *premixtures* and *feedingstuff*. This multi-analyte technique applies for the determination of *threonine* content in commercial amino acid products, using an amino acid analyzer or HPLC equipment. The method 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 ion exchange chromatography. *Threonine* is determined colorimetrically after post-column derivatisation with ninhydrine and UV detection at 440 and 570 nm or by fluorescence detection (FD) after post column reaction with ortho-phthaldialdehyde (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 standard deviation for *reproducibility* ( $RSD_R$ ) of 2.2 %.

Based on the performance characteristics presented, the EURL recommends for official control the ISO/CD 17180 method, based on ion exchange chromatography coupled with post-column derivatisation and UV or fluorescence detection to determine *threonine* in *feed additives*.

On the basis of the above the "**Recommended text for the register entry (analytical method)**" should read:

For the determination of *threonine* in *feed additive*:

- ion exchange chromatography coupled with post-column derivatisation and photometric detection (HPLC-UV/FD) – ISO/DIS 17180

For the determination of *threonine* in *premixtures* and *feedingstuff*:

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

[1] [http://irmm.jrc.ec.europa.eu/EURLs/EURL\\_feed\\_additives/authorisation/evaluation\\_reports/](http://irmm.jrc.ec.europa.eu/EURLs/EURL_feed_additives/authorisation/evaluation_reports/)

[2] ISO/DIS 17180:2012 *Animal feeding stuffs – Determination of lysine, methionine and threonine in commercial amino acids products and premixtures*



JRC.DG.D.6/CvH/SB/ago/ARES(2011)423196

**EURL 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-2010-0058  
CRL/ 100062

FAD-2010-0081  
CRL/ 100084

Name of Feed Additive: L-Threonine, technically pure

Active Agent (s): L-Threonine

Rapporteur Laboratory: European Union Reference  
Laboratory for Feed Additives  
(EURL-FA)  
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Date: 14/04/2011

## EXECUTIVE SUMMARY

In the current application authorisation is sought for *L-Threonine* under Articles 4(1) and 10(2), category 'nutritional additives' and functional group 3(c) 'amino acids, their salts and analogues' according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *L-Threonine* for all animal species and categories. The *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs* or *water*. The two Applicants (FAD-2010-0058 and FAD-2010-0081) suggested no minimum or maximum *L-Threonine* concentrations in *premixtures*, *feedingstuffs* and *water*.

For the determination of *L-Threonine* in *premixtures* and *feedingstuffs* the Applicants submitted the ring-trial validated Community method for amino acids. The method applies for the determination of *free* (synthetic and natural) and *total* (peptide-bound and free) amino acids, using an amino acid analyzer or High Performance Liquid Chromatography (HPLC) equipment. However, only performance characteristics for the determination of total *L-Threonine* are reported:

- a relative standard deviation for *repeatability* (RSD<sub>r</sub>) ranging from 1.9 to 4.1%;
- a relative standard deviation for *reproducibility* (RSD<sub>R</sub>) ranging from 3.8 to 11.7%.

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 to determine *L-Threonine* in *premixtures* and *feedingstuffs*.

For the determination of the *active substance* in the *feed additive*, both Applicants submitted the abovementioned ring trial validated Community method designed for the analysis of *premixtures* and *feedingstuffs*. One Applicant (FAD-2010-0081) identified an alternative ring-trial validated method developed by the "Association of Official Agricultural Chemists" "Lysine, Methionine and *Threonine* in Feed Grade Amino Acids and Premixes" (AOAC 999:13 - 2004). Additionally the EURL identified a ring-trial validated method developed by the "Association of German Agricultural Analytical and Research Institutes" (VDLUFA, Germany – Method 4.11.6). Both the methods are based on the same principle of the Community method (i.e. extraction of the sample with diluted hydrochloric acid and measuring the target analyte with HPLC coupled with post-column derivatisation system) and are explicitly designed to determine free *L-Threonine* in *feed additive* and *premixtures* at amino acid contents higher than 100 g/kg. The following performance characteristics covering both methods were reported:

- RSD<sub>r</sub> ranging from 0.5 to 2.4%; and
- RSD<sub>R</sub> ranging from 0.9 to 4.7%

Based on the performance characteristics presented, the EURL recommends for official control, the ring trial validated AOAC 999:13 and VDLUFA 4.11.6 methods based on ion exchange chromatography coupled with post-column derivatisation to determine the free *L-Threonine* in the *feed additive*.

The Applicants provided no experimental data for the identification of *L-Threonine* in *water*. Therefore, the EURL cannot evaluate nor recommend a method for the official control to determine *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 for *L-Threonine* under Articles 4(1) (new use in water) and 10(2) (already authorised as feed additive without any restrictions under Commission Directive 88/485/EEC [1]), category of 'nutritional additives' functional group 3(c) 'amino acids, their salts and analogues' according to Annex I of Regulation (EC) No 1831/2003 [2] - [3]. According to the two Applicants (FAD-2010-0058 and FAD 2010-0081) *L-Threonine, technically pure* is produced through fermentation process of substrates of vegetal origin using a selected strain of *Escherichia coli* K-12 [4] - [5]. *L-Threonine*, as obtained at the end of the manufacturing process, is not subject to any further formulation or preparation. The *feed additive* is a white crystalline powder and consists of minimum 98% of the active substance [6] -[9].

Specifically, authorisation is sought for the use of the *L-Threonine* for all animal species and categories. The *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs* [10] or *water* [11]. The Applicants suggested no minimum or maximum *L-Threonine* concentrations in *premixtures, feedingstuffs* and *water* [12] -[13].

## 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 these dossiers, 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 [14].

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

For the determination of *L-Threonine* in *premixtures* and *feedingstuffs* the Applicants submitted the ring-trial validated Community method [15] - [17]. This method applies for the determination of *free* (synthetic and natural) and of *total* (peptide-bound and free) amino acids, 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.

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.

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 ion exchange chromatography 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 ISO 13903:2005 method [18]. The performance characteristics reported for the determination of total *L-Threonine* are listed in Table 1. Furthermore, a limit of quantification was derived for *free L-Threonine* and *total L-Threonine*: 0.03 and 0.2 g/kg *feedingstuffs*, 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, to determine *L-Threonine* in *premixtures* and *feedingstuffs*.

For the determination of *L-Threonine* in the *feed additive*, the two Applicants proposed to apply the abovementioned ring trial validated Community method designed for the analysis of *premixtures* and *feedingstuffs* [15] - [16]. An alternative method was proposed by one Applicant (FAD-2010-0081): the method characterised by the “Association of Official Agricultural Chemists” (AOAC 999:13 - 2004 [19]) "Lysine, Methionine and *Threonine* in Feed Grade Amino Acids and Premixes". Additionally the EURL identified the method characterised by the “Association of German Agricultural Analytical and Research Institutes” (VDLUFA, Germany – Method 4.11.6 [20]). Both the methods are ring-trial validated [20] - [21] and are based on the same principle described in the abovementioned Community method (i.e. extraction of the sample with diluted hydrochloric acid and measuring the target analyte with an amino acid analyser or HPLC coupled with post-column derivatisation system). Both the methods are designed for the determination of free *L-Threonine* in *feed additive* and *premixtures* at amino acid contents higher than 100 g/kg.

The *feed additive* and/or *premixtures* samples are dissolved in hydrochloric acid (0.1 mol/l) and diluted with sodium citrate buffer. The internal standard solution (Norleucine) is added and the amino acids are separated by ion exchange chromatography and determined by post column derivatisation with ninhydrin or by fluorescence detection after post-column reaction with *o*-phthaldialdehyde (OPA). The reported performance characteristics are listed in Table 1.

Based on the performance characteristics presented, the EURL recommends for official control, the two ring trial validated methods - AOAC 999:13 and VDLUFA 4.11.6 - based on amino acid analyser or High Performance Liquid chromatography (HPLC) coupled with post-column derivatisation, to determine *L-Threonine* in the *feed additive*.

**Table 1:** Method performance characteristics obtained in the frame of four different ring-trial validation exercises based on similar methods for the determination of *L-Threonine* in *feed additive, premixtures* and *feedingstuffs*.

The performance characteristics reported refer to:

- *total L-Threonine* determination (peptide bound and free) for the Commission Regulation No 152/2009 & ISO 13903:2005
- *free L-Threonine* determination (synthetic and natural) for AOAC 999:13 & VDLUFA 4:11:6 (highlighted)

Intercomparison study	Matrix	<i>L-Threonine</i> g/kg	RSD <sub>r</sub> (%)	RSD <sub>R</sub> (%)
Commission Regulation (EC) No 152/2009 [17] study carried out in 1990	Mixed pig feed	7	1.9	4.1
	Broiler compound	9	2.1	5.2
	Protein concentrate	22	2.7	3.8
	Premixture	58	2.2	4.3
ISO 13903:2005 [18] study carried out in 1994	Poultry meal	2.3	3.2	9.1
	Broiler finisher feed	0.7	2.7	8.2
	Broiler starter feed	1.12	2.7	9.9
	Corn	0.3	4.1	11.7
	Fishmeal	2.3	3.6	10.7
AOAC 999:13 [19] study carried out in 1996 [21]	9 different Premixtures	80 - 220	0.7 - 1.4	1.9 - 2.3
	L-Threonine	956	1.2	2.2
VDLUFA 4:11:6 [20] study carried out in 1997 and 1998	5 different Premixtures	92 - 350	0.5 - 2.4	0.9 - 4.7

RSD<sub>r</sub>, RSD<sub>R</sub> - relative standard deviation for *repeatability* and *reproducibility*, respectively

The Applicant provided no experimental data for the determination of *L-Threonine* in *water*. Therefore the EURL could not evaluate nor recommend a method for official control to determine *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 ring-trial validated methods, characterised by AOAC (method 999:13) and VDLUFA (method 4.11.6), using High-Performance Liquid Chromatography (HPLC) coupled to post column derivatisation and spectrophotometric or fluorescence detection, to determine *L-Threonine* in the *feed additive*.



- the ring trial validated Community method, using High-Performance Liquid Chromatography (HPLC) coupled to post column derivatisation and photometric detection, to determine *L-Threonine* in *premixtures* and *feedingstuff*.

### ***Recommended text for the register entry (analytical method)***

For the determination of *L-Threonine* in *feed additive*:

- ion exchange chromatography method with post-column derivatisation and UV or fluorescence detection (AOAC method 999:13 or VDLUFA method 4.11.6)

For the determination of *L-Threonine* in *premixtures* and *feedingstuff*:

- ion exchange chromatography method with post-column derivatisation and UV or fluorescence detection: 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 dossiers have 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] \*Application/Ref:SANCO/D/2:Forw.Appl.1831/0041-2010
- [3] +Application/Ref:SANCO/D/2:Forw.Appl.1831/058-2010
- [4] \*Technical dossier, Section II: 3.1 Active substance(s)/agent(s)
- [5] +Technical dossier, Section II: 3.1 Active substance(s)/agent(s)
- [6] \*Application, (Annex A), FAD-2010-0058\_Description\_ *L-Threonine*
- [7] +Application, (Annex A), FAD-2010-0081\_Description\_ *L-Threonine*
- [8] \*Technical dossier, Section II: 3.2 Additive
- [9] +Technical dossier, Section II: 3.2 Additive
- [10] +Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
- [11] \*Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
- [12] \*Application, (Annex A), FAD-2010-0058\_Conditions of use\_ *L-Threonine*
- [13] +Application, (Annex A), FAD-2010-0081\_Conditions of use\_ *L-Threonine*
- [14] 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
- [15] \*Technical dossier, Section II: 2.6.1 Method of analysis for the active substances



- [16] +Technical dossier, Section II: 2.6.1 Method of analysis for the active substances
- [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 (EN ISO 13903:2005)
- [19] AOAC Official Method 999:13 – Lysine, Methionine and Threonine in Feed Grade Amino Acids and Premixes
- [20] Bestimmung von Lysin, Methionin und Threonin in Aminosäurehandelsprodukten und Vormischungen – 4.11.6, Methodenbuch III, 5. Erg. 2004, VDLUFA – Verlag, Darmstadt
- [21] Fontaine and Eudaimon, J. of AOAC Int. (2000), Vol. 83, No. 4, 771-783
- \*Refers to Dossier no: FAD-2010-0058  
+Refers to Dossier no: FAD-2010-0081

## **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**

The following National Reference Laboratories contributed to this report:

- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer, Speyer (DE)
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)
- Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 — Landwirtschaftliches Untersuchungswesen, Leipzig (DE)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
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