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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 fermentation with *Corynebacterium glutamicum* KCCM 80367
(FEED-2022-10610; CRL/220063)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FEED-2022-10610 - CRL/220063**
Name of Product:	***L-threonine produced by fermentation with Corynebacterium glutamicum KCCM 80367***
Active Agent (s):	**L-threonine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**María José González de la Huebra**
Report checked by: Date:	**Zigmas Ezerskis** **27/11/2023**
Report approved by: Date:	**Christoph von Holst** **27/11/2023**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *L-threonine produced by fermentation with Corynebacterium glutamicum* KCCM 80367, 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. The authorisation is sought for all animal species.

According to the Applicant, the *feed additive* contains *L-threonine* as an active substance at a minimum of 75 % (w/w).

L-threonine is intended to be mixed either in *premixtures* or added directly into *compound feed*. However, the Applicant did not propose a minimum or maximum *L-threonine* content in *compound feed*.

For the determination of *threonine* in the *feed additive* and *premixtures* the Applicant submitted the ring-trial validated method EN ISO 17180:2013 based on ion-exchange chromatography coupled to post-column derivatisation and optical (visible or fluorescence) detection (IEC-VIS/FLD). The method is dedicated for the determination of free lysine, methionine and *threonine* in commercial amino acid products and *premixtures* containing more than about 10 % of the amino acid. The following performance characteristics were reported for the determination of *threonine*: 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 %.

Based on the performance characteristics available, the EURL recommends for official control the EN ISO 17180:2013 method based on IEC-VIS/FLD for the determination of *threonine* in the *feed additive* and *premixtures.*

For the determination of *threonine* in *compound feed* the Applicant submitted the ring-trial validated European Union (EU) method (Commission Regulation (EC) No 152/2009). The method is based on ion-exchange chromatography coupled to optical detection (IEC-VIS) and is dedicated for the analysis of free and total amino acids in *premixtures* containing less than 10 % of the amino acid and *compound feed*. The EU method was initially ring-trial validated using four different matrices and further validated in the second ring-trial, resulting in the EN ISO 13903:2005 method. The following combined performance characteristics were reported for the determination of total *threonine* in animal feedingstuffs: RSD_r ranging from 1.9 to 4.1 % and RSD_R ranging from 3.8 to 11.7 %. Furthermore, limits of quantification (LOQ) of 0.03 and 0.2 g/kg *compound feed* were reported for free and total *threonine*, respectively.

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated European Union (EU) method, based on IEC-VIS to determine *threonine* in *premixtures* and *compound feed*.

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.

KEYWORDS

L-threonine produced by fermentation with Corynebacterium glutamicum KCCM 80367, nutritional additives, amino acids, all animal species.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (new feed additive) for *L-threonine produced by fermentation with Corynebacterium glutamicum* KCCM 80367, 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]. The authorisation is sought for all animal species [1,2]. *L-threonine* produced by other *Corynebacterium glutamicum* and *Escherichia coli* strains is already authorised as nutritional *feed additive* for all animal species under several Commission Implementing Regulations [3-7].

According to the Applicant, the *feed additive* contains as an active substance a minimum of 75 % (w/w) of *L-threonine* [8], which is produced by fermentation with the *Corynebacterium glutamicum* KCCM 80367 strain [9].

L-threonine is intended to be mixed either in *premixtures* or added directly into *compound feed* [10]. However, the Applicant did not propose a minimum or maximum *L-threonine* content in *compound feed* [10].

Note: The EURL has previously evaluated the analytical methods in the frame of several *L-threonine* related dossiers [11].

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 fermentation with Corynebacterium glutamicum* KCCM 80367 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 determination of *threonine* in the *feed additive* and *premixtures* the Applicant proposed [12] the ring-trial validated method EN ISO 17180:2013 dedicated for the determination of free lysine, methionine and *threonine* in commercial amino acid products and *premixtures* [13]. This method does not distinguish between the salts of amino acids and cannot differentiate between enantiomers. It is applicable for products containing more than about 10 % of the 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 ion-exchange chromatography (IEC). The *threonine* is determined either after post-column derivatisation with ninhydrine and optical detection: (VIS) detection at 440 nm 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 wavelength at 460 nm [13].

The performance characteristics reported for the determination of free *threonine* in commercial amino acid products and *premixtures* when using the EN ISO 17180:2013 method 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 determination of free *threonine* in the *feed additive* and *premixtures.*

For the determination of *threonine* in *compound feed* the Applicant proposed [12] and submitted the ring-trial validated European Union (EU) method [14].

The EU method is designed for the determination of *free* (synthetic and natural) and of *total* (peptide-bound and free) amino acids in *premixtures* and *compound feed*, using an amino acid analyser or IEC coupled to post-column derivatisation and optical (VIS) detection. It does not distinguish between the salts of amino acids and cannot differentiate between enantiomers. It is applicable for *compound feed* and *premixtures* containing less than 10 % of the amino acid.

Table 1: Method performance characteristics obtained in the frame of ring-trial validation studies (EN ISO 17180:2013 [13], EU method [14] and EN ISO 13903:2005 [15]) for the determination of *threonine* in animal *feedingstuffs*.

Ring-Trial	Matrix	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, 15]	Mixed pig feed	6.9	1.9	4.1
	Broiler compound	9.3	2.1	5.2
	Protein concentrate	22	2.7	3.8
	Premix	58	2.2	4.3
[15]	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 *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 optical 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 disulfite. 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 optical detection at 570 nm [14].

The EU method was ring-trial validated using four different matrices. This method was further ring-trial validated by 23 laboratories, resulting in the EN ISO 13903:2005 method [15].

The performance characteristics reported for the determination of total *threonine* in different animal feedingstuffs when using the EU method (or equivalent EN ISO 13903:2005 method) are listed in Table 1. Furthermore, the following limits of quantification (LOQ) were reported for free and total *threonine*: 0.03 and 0.2 g / kg *compound feed*, respectively [15].

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated European Union (EU) method, based on IEC-VIS to determine *threonine* in *premixtures* and *compound feed*.

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)

An evaluation of corresponding methods of analysis is not relevant for the present application.

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 ring-trial validated method EN ISO 17180:2013 based on ion-exchange chromatography coupled to optical detection (IEC-VIS/FLD) to determine *threonine* in the *feed additive* and *premixtures* and (ii) the European Union (EU) method based on ion-exchange chromatography coupled to optical detection (IEC-VIS) for the determination of *threonine* in *premixtures* and *compound feed*.

Recommended text for the register entry (analytical method)

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

- Ion-exchange chromatography coupled to post-column derivatisation and optical detection (IEC-VIS/FLD) – EN ISO 17180

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

- Ion-exchange chromatography coupled to post-column derivatisation and optical detection (IEC-VIS/FLD) – EN ISO 17180 and/or
- Ion-exchange chromatography coupled to post-column derivatisation and optical detection (IEC-VIS), Commission Regulation (EC) No 152/2009 (Annex III, F)

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

- Ion-exchange chromatography coupled to post-column derivatisation and optical detection (IEC-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-threonine produced by fermentation with Corynebacterium glutamicum* KCCM 80367 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] *Forwarding of applications for authorisation of feed additives in accordance with Regulation (EC) No 1831/2003 – E-Submission Food Chain platform – https://webgate.ec.europa.eu/esfc/#/applications/12310 https://open.efsa.europa.eu/questions/EFSA-Q-2022-00873

[2] *Application – Annex 1

[3] Commission Implementing Regulation (EU) 2021/969 of 16 June 2021 concerning the authorisation of L-threonine produced by Escherichia coli CGMCC 13325 as a feed additive for all animal species, *OJ L 214, 17.6.2021*

[4] Commission Implementing Regulation (EU) 2020/1091 of 24 July 2020 concerning the authorisation of L-threonine as a feed additive for all animal species, *OJ L 241, 27.7.2020*

[5] Commission Implementing Regulation (EU) 2020/238 of 20 February 2020 concerning the authorisation of L-threonine as a feed additive for all animal species, OJ L 48, 21.2.2020

[6] Commission Implementing Regulation (EU) 2019/894 of 28 May 2019 concerning the authorisation of L-threonine produced by *Escherichia coli* CGMCC 7.232 as a feed additive for all animal species, OJ L 142, 29.5.2019

[7] 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, OJ L 201, 27.7.2016

[8] *Technical dossier, Section II : II.1.3. Qualitative and quantitative composition

[9] *Technical dossier, Section II: 2.1.2. Micro-organisms

[10] *Technical dossier, Section II: II.5.1. Proposed mode of use in animal nutrition

[11] EURL reports:
https://joint-research-centre.ec.europa.eu/publications/fad-2020-0017_en
https://joint-research-centre.ec.europa.eu/publications/fad-2018-0035_en
https://joint-research-centre.ec.europa.eu/publications/fad-2018-0003_en
https://joint-research-centre.ec.europa.eu/publications/fad-2017-0037_en
https://joint-research-centre.ec.europa.eu/publications/fad-2016-0003_en
https://joint-research-centre.ec.europa.eu/publications/fad-2013-0028_en

[12] *Technical dossier, Section II: II.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] EN ISO 13903:2005- Animal feeding stuffs – Determination of amino acids content

*Refers to Dossier no: FEED-2022-10610

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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- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
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- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Wageningen Food Safety Research (WFSR)[1] (NL)

[1] Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: RIKILT Wageningen UR, Wageningen.