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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-tryptophan
produced by Escherichia coli CGMCC 7248
(FAD-2017-0019; CRL/170003)

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-2017-0019 - CRL/170003**
Name of Product:	***L-tryptophan*** *produced by Escherichia coli CGMCC 7248*
Active Agent (s):	**L-tryptophan**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA) JRC Geel, Belgium**
Report prepared by:	**Stefano Bellorini**
Report checked by: Date:	**Piotr Robouch (EURL-FA)** **17/11/2017**
Report approved by: Date:	**Christoph von Holst** **17/11/2017**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-tryptophan produced by Escherichia coli CGMCC 7248*, 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-tryptophan* is already authorised as *feed additive* under Commission Directive 88/485/EEC.

For the quantification of *L-tryptophan* in the *feed additive*, the Applicant validated and further verified the method based on titration described in the European Pharmacopoeia monograph 01/2017:1272. For the quantification of *L-tryptophan* in *premixtures* and *feedingstuffs*, the Applicant submitted two single-laboratory validated and further verified analytical methods based on High Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD).

However, the EURL previously evaluated (i) the ring-trial validated Community method based on HPLC coupled with fluorescence detection (FD) for the quantification of *L-tryptophan* in *feedingstuffs;* and (ii) the ring-trial validated EN ISO 13904:2016 method "Animal feeding stuffs - Determination of tryptophan content" for the quantification of *L-tryptophan* in *feed additive* and *premixtures* (containing more than 2 % of *tryptophan*). Based on the performance characteristics available, the EURL recommends for official control these two ring-trial validated methods to quantify *tryptophan* in the *feed additive, premixtures* and/or *feedingstuffs*. In addition, the EURL identified the "L-tryptophan monograph" of the Food Chemical Codex (FCC) for the identification of 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.

KEYWORDS

L-tryptophan produced by Escherichia coli CGMCC 7248, nutritional additives, amino acids, their salts and analogues, all animal species

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *L-tryptophan produced by Escherichia coli CGMCC 7248*, 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-tryptophan* 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 or slightly yellow crystalline powder with a minimum purity of 98 % [4-6]. 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 7248 [6,7].

L-tryptophan is intended to be mixed either in *premixtures* or added directly to *feedingstuffs* [8]. The *feed additive* is not intended for use in *water* for drinking [9]. However, the Applicant did not propose a minimum or maximum *L-tryptophan* content in *feedingstuffs* [2,8].

Note: The EURL has previously evaluated the analytical methods in the frame of three L-tryptophan related dossiers [10-12].

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-tryptophan produced by Escherichia coli CGMCC 7248* 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 [13].

Description of the analytical methods for the determination of the active substance in the feed additive, premixtures and feedingstuffs

For the quantification of *L-tryptophan* in the *feed additive* the Applicant submitted the method described in the European Pharmacopoeia monograph 01/2017:1272 [14,15]. The method, based on titration with perchloric acid (0.1 M), was successfully validated and further verified by an independent external laboratory [16]. The corresponding performance characteristics are presented in Table 1.

For the quantification of free (synthetic and natural) *L-tryptophan* in *premixtures* and *feedingstuffs* the Applicant submitted a single-laboratory validated and further verified analytical method based on High Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD) [14,17-19].

The amino acid is extracted with diluted hydrochloric acid in the presence of internal standard. Depending of the matrix analysed, a different dilution factor is applied. The sediment is allowed to settle. Part of the solution is centrifuged, filtered and injected in the HPLC-DAD system and measured at 280 nm (reference 390 nm). The corresponding performance characteristics are reported in Table 1.

For the quantification of total (peptide-bound and free) *L-tryptophan* in *feedingstuffs* the Applicant submitted another single-laboratory validated and further verified analytical method based HPLC-DAD [14,20,21].

The sample is dissolved in a water solution containing barium hydroxide and kept for several hours in an oven (120°C). Once removed from the oven, it is further diluted with water and the internal standard is added. The solution is manually homogenised and transferred to a glass beaker and the pH adjusted to 3. The resulting solution is diluted with ultrapure water, filtered and injected in the HPLC-DAD system and measured at 280 nm (reference 390 nm). The corresponding performance characteristics are reported in Table 1.

Table 1: Method performance characteristics obtained in the frame of validation and verification studies, for the quantification of *free* and *total tryptophan* in the *feed additive* (FA), *premixtures* (PM) and *feedingstuffs* (FS).

Ref.	Method	Matrix	L-tryptophan (g/kg)		RSD_r (%)	RSD_{ip} (%)	Rec (%)
[15,16]	Titration	FA	*total*	983	0.46-0.62	0.39-0.84	100.2-101
[17-19]	HPLC-DAD	PM	*free*	2.5-7.7	0.56-1.87	1.68	99.1-101.9
		FS	*free*	0.6	0.77-0.85	1.19-1.3	95.7-99.4
[20,21]		FS	*total*	3.2	2.05-2.99	2.9-3.6	101.9-104

RSD_r, RSD_{ip} - relative standard deviation for *repeatability,* for *intermediate precision,* respectively; *Rec - recovery*

However, the EURL previously evaluated - in the frame of other *tryptophan* dossiers [10-12] - two ring-trial validated methods for the quantification of *tryptophan* in the *feed additive*, *premixtures* and *feedingstuffs*, presented hereafter.

The ring-trial validated Community method [22] designed for the quantification of free (synthetic and natural) and of total (peptide-bound and free) amino acid, using HPLC equipment coupled with fluorescence detection (FD) in *feedingstuffs* only. The method does not distinguish between the amino acid enantiomers.

Depending on the nature of the *L-tryptophan*, two different procedures can be applied:

- to analyse free *tryptophan*, the amino acid is extracted with diluted hydrochloric acid in the presence of internal standard; the sediment is allowed to settle and the supernatant is transferred into a beaker, where the solution is adjusted to pH 3 with sodium hydroxide;
- to determine the total *tryptophan*, the sample is hydrolysed under alkaline conditions using a saturated barium hydroxide solution and autoclaved at 110 °C for 20 hours. After hydrolysis the internal standard is added and the pH adjusted to 3.

These solutions are then diluted with methanol (with a volume ranging between 10 to 30% of the total) and water, to reach approximately the same concentration of the calibration standard solution. Following a filtration step, the solutions are finally injected and determined by reversed phase HPLC-FD (excitation and emission at 280 nm and 356 nm respectively).

The Community method was ring trial validated for free and total *tryptophan* determination in various matrices in the frame of three interlaboratory comparisons. The performance characteristics reported in the Official Journal are listed in Table 2.

The ring-trial validated CEN method EN ISO 13904:2016 [23] designed for the determination of free *tryptophan* in commercial products and *premixtures* (containing more than 2 % of *tryptophan*). The analytical procedure for the determination of the amino acid is identical to the one described for the Community method but including a specific sample preparation step for the extraction of *tryptophan* from more concentrated matrices. A fourth inter-laboratory comparison study was organised to assess the performance characteristics when analysing pure products and *premixtures* [23]. The performance characteristics are listed in Table 2.

Based on the performance characteristics available, the EURL recommends for official control the two ring-trial validated methods mentioned above - based on reversed phase HPLC-FD - to quantify *tryptophan* in the *feed additive, premixtures* and/or *feedingstuffs*.

Table 2: Method performance characteristics obtained in the frame of four ring-trial validation exercises (Community method [22] and EN ISO 13904:2016 [23]) for the determination of *free* and *total tryptophan* in *feed additives* (FA), *premixtures* (PM) and *feedingstuffs* (FS).

References	Matrix		*L-tryptophan* content g/kg		RSD_r (%)	RSD_R (%)
[22,23]	FS	Pig feed	*total*	2.4	1.9	6.3
		Pig feed supplemented with *L-tryptophan*		3.4	1.6	6.0
		Feed concentrate for pigs		4.2	1.9	2.2
[22,23]	FS	Wheat & soya mixture	*free*	0.39	1.3	4.7
		Wheat & soya mixture with *L-tryptophan*		0.93	1.3	5.1
[22,23]	FS	Mixed pig feed	*total*	2.1	1.0	1.5
		Low fat fish meal		8.8	1.2	4.7
		Soybean meal		6.9	1.3	4.1
		Skimmed milk powder		5.2	0.8	4.2
[23]	FA	Pure product 1	*free*	903	0.7	1.3
		Pure product 2		938	0.8	1.2
		Pure product 3		958	0.9	1.3
		Pure product 4		998	0.5	1.0
	PM	Premix 1	*free*	13	5.3	9.5
		Premix 2		99	1.4	3.0
		Premix 3		193	2.2	3.3
		Premix 4		500	0.9	2.1

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

In addition to the above mentioned European Pharmacopoeia monograph (Eur.Ph 01/2017:1272), the EURL identified the "L-tryptophan monograph" of the Food Chemical Codex (FCC) for the characterisation of *L-tryptophan* in the *feed additive*, where identification is based on infrared absorption and optical rotation and quantification on titration with perchloric acid (0.1N) [24].

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 "*tryptophan*" monograph of the Food Chemical Codex (FCC) based on infrared absorption and optical rotation for the identification of L-tryptophan in the feed additive; (ii) two ring-trial validated methods (EN ISO 13904:2016 and Community method) based on High-Performance Liquid Chromatography coupled to fluorescence detection (HPLC-FD) to quantify *tryptophan* in the *feed additive*, *premixtures* and/or *feedingstuffs*.

Recommended text for the register entry (analytical method)

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

- Food Chemical Codex "L-tryptophan monograph"

For the quantification of *tryptophan* in *feed additive* and *premixtures*:

- High performance liquid chromatography coupled to fluorescence detection (HPLC-FD) - EN ISO 13904-2016

For the quantification of *tryptophan* in *feedingstuffs*:

- High performance liquid chromatography coupled to fluorescence detection (HPLC-FD) - Commission Regulation (EC) No 152/2009 (Annex III, G)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *L-tryptophan produced by Escherichia coli CGMCC 7248* 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, Reference SANTE/E5: FWD. APPL. 1831-0013-2017

[2] *Application, Proposal of Registry Entry – Annex A

[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, O.J. L 239 , 30/08/1988 P. 0036 – 0039

[4] *Technical dossier, Section II: 2.1.5 Physical state of each form of the product

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

[6] *Technical dossier, Section II: Introduction

[7] *Technical dossier, Section II: 2.2.1.2 Microorganisms

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

[9] *Technical dossier, Section II: 2.4.1.3 Stability of the feed additive used in water or aqueous media

[10] FAD-2010-0056, L-tryptophan, Ref. JRC.DG.D.6/CvH/SB/ag/ARES(2011)480045 https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2010-0056.pdf

[11] FAD-2013-0025, L-tryptophan, Ref. Ares(2013)3628454 - 03/12/2013 https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep%20FAD-2013-0025-L-Tryptophan.doc_.pdf

[12] FAD-2016-0032, L-tryptophan produced by Escherichia coli CGMCC 11674, Ref. Ares(2017)34659 – 04/01/2017 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2016-0032_tryptophan.pdf

[13] 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

[14] *Technical dossier, Section II: 2.6.1 Methods of analysis for the active substance

[15] European Pharmacopoeia monograph "Tryptophan" 01/2017:1272

[16] *Technical dossier, Section II: Annex II_6_1d

[17] *Technical dossier, Section II: Annex II_6_1a

[18] *Technical dossier, Section II: Annex II_6_1b

[19] *Technical dossier, Section II: Annex II_6_1e

[20] *Technical dossier, Section II: Annex II_6_1c

[21] *Technical dossier, Section II: Annex II_6_1f

[22] 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 (Annex III, G)

[23] EN ISO 13904:2016 - Animal feeding stuffs – Determination of tryptophan content

[24] Food Chemical Codex monograph "*L-Tryptophan*", FCC 7 (2010), p. 1060

*Refers to Dossier no: FAD-2017-0019

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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