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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 fermentation with **Escherichia coli KCCM 10534**
(FAD-2018-0038; CRL/180030)

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-2018-0038 - CRL/180030**
Name of Product:	***L-tryptophan produced by fermentation with Escherichia coli KCCM 10534***
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:	**Zigmas Ezerskis (EURL-FA)** **17/12/2018**
Report approved by: Date:	**Christoph von Holst** **17/12/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-tryptophan produced by fermentation with Escherichia coli KCCM 10534*, 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. According to the Applicant, the product has a minimum purity of 98 % and it is intended to be mixed either in *premixtures* or added directly to *feedingstuffs* or *water* for drinking. However, the Applicant did not propose a minimum or maximum *L-tryptophan* content in *feedingstuffs*.

For the quantification of *L-tryptophan* in the *feed additive* the Applicant submitted a single-laboratory validated analytical method based on High Performance Liquid Chromatography (HPLC) and photometric detection. Furthermore, for the quantification of *L-tryptophan* in *premixtures* and *feedingstuffs* the Applicant submitted the VDLUFA 4.11.2 method, based on HPLC coupled with fluorescence detection (FLD).

However, the EURL previously evaluated and recommended (i) the ring-trial validated EN ISO 13904:2016 method based on HPLC-FLD for the quantification of *L-tryptophan* in *feed additive* and *premixtures* (containing more than 2 % of *tryptophan*); and (ii) the ring-trial validated Community method based on HPLC-FLD for the quantification of *L-tryptophan* in *feedingstuffs*. 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*.

Furthermore, in the frame of the stability and homogeneity studies, the Applicant presented experimental data obtained analysing *tryptophan* in *water* with a slightly modified version of the VDLUFA method 4.11.2 based on HPLC-FLD and dedicated for the determination of *tryptophan* in *feed*. The results presented are considered sufficient to demonstrate the suitability of the method for the analysis of the amino acid in *water*. Hence, the EURL recommends for official control this method to quantify *tryptophan* in *water*.

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 fermentation with Escherichia coli KCCM 10534, 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 fermentation with Escherichia coli KCCM 10534*, 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* (produced by *Escherichia coli*) is already authorised as *feed additive* under Commission Implementing Regulation (EU) 2017/873 [3].

According to the Applicant, the product is a pale brownish powder with a minimum purity of 98 % [4,5]. The *feed additive* is produced through fermentation with a genetically modified strain of *Escherichia coli* [6]. The production strain is deposited in the "Korean Culture Centre of Microorganisms" (KCCM) with accession number KCCM 10534 [6,7].

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

Note: The EURL has previously evaluated the analytical methods in the frame of several *L-tryptophan* related dossiers [9-16].

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* 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-tryptophan* in the *feed additive* the Applicant submitted an in-house validated analytical method based on reversed phase high performance liquid chromatography (HPLC) and ultraviolet detection (UV) [17,18].

The *feed additive* is diluted in distilled water and an aliquot is directly injected into the HPLC system equipped with a C18 column. The analyte is eluted via a potassium phosphate buffer.

L-tryptophan is detected with a photodiode array detector (PDA) at 254 nm and quantified using an external calibration curve.

In the frame of the validation study, the Applicant reported the following performance characteristics: relative standard deviation for repeatability (RSD_r) and intermediate precision (RSD_{ip}) ranging from 0.1 to 2.0 % and a recovery rate (R_{rec}) ranging from 97 to 104 % [18].

Furthermore, for the quantification of the *L-tryptophan* content in *premixtures* and *feedingstuffs* the Applicant submitted the official method of the "Association of German Agricultural Analytical and Research Institutes" (VDLUFA, Germany – Method 4.11.2), based on HPLC coupled with fluorescence detection (FLD) [19,20].

However, for the determination of *L-tryptophan* in *feedingstuffs* a ring-trial validated Community method exists [21]. This method, similar to the VDLUFA method 4.11.2, is applicable for the determination of *free* (synthetic and natural) and *total* (peptide-bound and free) amino acid using HPLC with fluorescence detection (FLD). 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 an 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 solution is adjusted to pH 3.

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

The Community method was ring-trial validated for the determination of free and total *tryptophan* in various matrices in the frame of three inter-laboratory comparisons. The performance characteristics reported are shown in Table 1.

Moreover, for the quantification of free *tryptophan* in commercial products and *premixtures* (containing more than 2 % of *tryptophan*), the EURL recommended in previous reports the ring-trial validated method EN ISO 13904:2016 specifically designed for these matrices [22]. The analytical procedure for the determination of the amino acid is identical to the one described for the Community method but includes a specific sample preparation step for the extraction of *tryptophan* from these more complex matrices.

Table 1: Method performance characteristics obtained in the frame of ring-trial validation studies (Community method [21] and EN ISO 13904:2016 [22]) 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 (%)
[21,22]	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
[21,22]	FS	Wheat & soya mixture	*free*	0.39	1.3	4.7
		Wheat & soya mixture with *L-tryptophan*		0.93	1.3	5.1
[21,22]	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
[22]	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

A fourth inter-laboratory comparison study was organised to assess the performance characteristics when analysing pure products and *premixtures* [22]. The performance characteristics are shown in Table 1.

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

The Applicant did not submit a suitable method for the determination of *L-tryptophan* in *water* [17]. However, in the frame of the stability and homogeneity studies the Applicant presented experimental data in *water* obtained analysing *tryptophan* with a slightly modified version of the above mentioned VDLUFA method 4.11.2 designed for the determination of *L-tryptophan* in *feedingstuffs* [20,23-26]. 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, as in former reports, 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)

An 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)

For the identification of the *feed additive* the EURL recommends the "L-tryptophan monograph" of the Food Chemical Codex (FCC), where a test based on infrared absorption is described [27].

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 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 and fluorescence detection (HPLC-FLD) to quantify *tryptophan* in the *feed additive*, *premixtures* and/or *feedingstuffs*; and (iii) the analytical method described by VDLUFA (4.11.2) based on high performance liquid chromatography with fluorescence detection (HPLC-FLD) to quantify *tryptophan* in *water*.

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 the *feed additive* and *premixtures*:

- High performance liquid chromatography with fluorescence detection (HPLC-FLD) - EN ISO 13904

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

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

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

- High performance liquid chromatography with fluorescence detection (HPLC-FLD)

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 fermentation with Escherichia coli KCCM 10534* 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. 1831-0043-2018

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

[3] Commission Implementing Regulation (EU) 2017/873 of 22 May 2017 concerning the authorisation of L-tryptophan produced by Escherichia coli as a feed additive for all animal species, OJ L 134, 22.05.2017, p. 14

[4] *Application: Annex I

[5] *Technical dossier, Section II: II.2.1.1. Chemical substances

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

[7] *Technical dossier, Section II: II.1.1. Name of the additive

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

[9] 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

[10] 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

[11] 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

[12] FAD-2017-0019, L-tryptophan produced by Escherichia coli CGMCC 7248, Ref. Ares(2017)5629822 - 17/11/2017 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2017-0019_tryptophan.pdf

[13] FAD-2017-0038, L-tryptophan produced by Escherichia coli CGMCC 7.267, Ref. Ares(2018)650989 - 02/02/2018 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2017-0038-tryptophan.pdf

[14] FAD-2017-0052, L-tryptophan produced by Escherichia coli K12 KCCM 80152, Ref. Ares(2018)2609807 - 18/05/2018 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2017-0052-tryptophan.pdf

[15] FAD-2017-0033, L-tryptophan produced by fermentation with Escherichia coli K12 KCCM80135, Ref. Ares(2018)3172506 - 15/06/2018 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2017-0033-tryptophan.pdf

[16] FAD-2018-0033, produced by fermentation with Corynebacterium glutamicum KCCM80176, Ref. Ares(2018)5743025 - 09/11/2018 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2018-0033_tryptophan.pdf

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

[18] *Technical dossier, Section II: Annex_II_6_01

[19] *Technical dossier, Section II: II.6.3 Methods of the analysis relating to the identity and characterisation of the additive

[20] Bestimmung von Tryptophan HPLC-Verfahren – 4.11.2, Methodenbuch III, 2. Erg. 1988, VDLUFA – Verlag, Darmstadt
[21] 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)
[22] EN ISO 13904:2016 - Animal feeding stuffs – Determination of tryptophan content
[23] *Technical dossier, Section II: II.4.1.3 Stability of the additive used in water
[24] *Technical dossier, Section II: Annex_II_4_05
[25] *Technical dossier, Section II: II.4.2 Homogeneity
[26] *Technical dossier, Section II: Annex_II_4_08
[27] Food Chemical Codex monograph "*L-Tryptophan*", FCC 7 (2010), p. 1060
*Refers to Dossier no: FAD-2018-0038

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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- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 — Labore Landwirtschaft, Nossen (DE)
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