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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 11674
(FAD-2016-0032; CRL/160004)

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-2016-0032 - CRL/160004**
Feed Additive:	***L-tryptophan*** *produced by Escherichia coli CGMCC 11674*
Active Agent (s):	**L-tryptophan**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Piotr Robouch (EURL-FA)** **03/01/2017**
Report approved by: Date:	**Christoph von Holst** **03/01/2017**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-tryptophan produced by Escherichia coli CGMCC 11674*, 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 of 26 July 1988 amending the Annex to Council Directive 82/471/EEC concerning certain products used in animal nutrition.

For the quantification of *L-tryptophan* in *premixtures*, *feedingstuffs* and *water* the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009). This High Performance Liquid Chromatography equipment coupled with fluorescence detection (HPLC-FD) method applies for the determination of *free* (synthetic and natural) and of *total* (peptide-bound and free) amino acid in *feedingstuffs* only. The following performance characteristics were reported for the quantification of *tryptophan*: a relative standard deviation for *repeatability* (RSD_r) ranging from 0.8 to 1.9 % and a relative standard deviation for *reproducibility* (RSD_R) ranging from 1.5 to 6.3 %.

For the quantification of *L-tryptophan* in the *feed additive* the Applicant submitted the ring-trail validated EN ISO 13904:2005 based on HPLC-FD and specifically designed for *feedingstuffs*. The standard was further ring-trial validated to assess the performance characteristics of the analytical method for the determination of the amino acid in *feed additives* and *premixtures* thus resulting in the recent standard EN ISO 13904:2016. The following performance characteristics were reported for the quantification of *free tryptophan*: RSD_r ranging from 0.5 to 5.3 % and RSD_R ranging from 1.0 to 9.5 %.

Based on the performance characteristics presented, the EURL recommends for official control (i) the ring-trial validated EN ISO 13904:2016 based on reversed phase HPLC-FD, to determine *tryptophan* in *feed additives* and *premixtures* and (ii) the Community method, based on reversed phase HPLC-FD, to determine *tryptophan* in *feedingstuffs*. In addition, the EURL identified the "L-tryptophan monograph" of the Food Chemical Codex (FCC) for the characterisation of the *feed additive*.

Since the Applicant provided no experimental data to determine *L-tryptophan* in *water* the EURL is neither able to evaluate nor to recommend a method for official control.

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-tryptophan produced by Escherichia coli CGMCC 11674, 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 11674*, 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 light brown powder with a minimum purity of 98 % [1,4-5]. The *feed additive* is produced by fermentation with a genetically modified strain of Escherichia coli. The production strain is deposited in the "Chinese General Microbiological Culture Collection Centre" (CGMCCC) with reference Escherichia coli CGMCCC 11674 [6].

L-tryptophan is intended to be mixed either in *premixtures* or added directly to *feedingstuffs* or *water* for drinking [7], however the Applicant did not propose a minimum or maximum *L-tryptophan* content in *feedingstuffs* [1,7].

Note: The EURL has previously evaluated the analytical methods in the frame of two *L-tryptophan* related dossiers [8,9].

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

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 [10].

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

For the quantification of *L-tryptophan* in *premixtures, feedingstuffs* and *water* the Applicant submitted the ring-trial validated Community method [11,12]. This method applies for the determination of *free* (synthetic and natural) and of *total* (peptide-bound and free) amino acid, using High Performance Liquid Chromatography (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 could 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.

The solutions obtained are diluted with methanol (with a volume ranging between 10 to 30% of the total) and water, in order to have 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 1.

For the quantification of *L-tryptophan* in *feed additive* the Applicant submitted the ring-trail validated "EN ISO 13904:2005 Animal feeding stuffs - Determination of tryptophan content" [11,13]. The scope of the method, originally intended for *feedingstuffs* only, has been extended to the determination of *free tryptophan* in commercial products and *premixtures* (containing more than 2 % of *tryptophan*) and resulted in the recently published EN ISO 13904:2016 (supersedes EN ISO 13904-2005) [14]. The analytical procedure for the determination of the amino acid is identical to the one described for the Community method

apart from the inclusion of a specific sample preparation for the extraction of *tryptophan* in the more concentrated matrices.

A fourth inter-laboratory comparison study carried out specifically to assess the performance characteristics of the method for the determination of free tryptophan in pure products and *premixtures* has been included in the updated standard [14].

The performance characteristics are listed in Table 1.

Based on the performance characteristics presented, the EURL recommends for official control (i) the ring-trial validated EN ISO 13904:2016 based on reversed phase HPLC/FD, to determine *tryptophan* in *feed additives* and *premixtures* and (ii) the Community method, based on reversed phase HPLC-FD, to determine *tryptophan* in *feedingstuffs*.

Table 1: Method performance characteristics obtained in the frame of four ring-trial validation exercises (Community method [12] and EN ISO 13904:2016 [14]) 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 (%)
[12,14]	**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
[12,14]	**FS**	Wheat & soya mixture	*free*	0.39	1.3	4.7
		Wheat & soya mixture with *L-tryptophan*		0.93	1.3	5.1
[12,14]	**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
[14]	**FA**	Pure product 1	*free*	900	0.7	1.3
		Pure product 2		940	0.8	1.2
		Pure product 3		960	0.9	1.3
		Pure product 4		980	0.5	1.0
	PM	Premix 1	*free*	25	5.3	9.5
		Premix 2		10	1.4	3.0
		Premix 3		15	2.2	3.3
		Premix 4		50	0.9	2.1

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

Furthermore, 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 quantification on titration with perchloric acid (0.1N) [15].

The Applicant provided no experimental data to demonstrate the applicability of the Community method for the determination of *L-tryptophan* in *water* [11]. Therefore the EURL is neither able to evaluate nor to recommend a method for official control to determine *L-tryptophan* 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 (i) the "L-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* and (ii) the ring-trial validated EN ISO 13904:2016, based on High-Performance Liquid Chromatography (HPLC) coupled to fluorescence detection (FD), to quantify *tryptophan* in *feed additives* and *premixtures*. Furthermore, the EURL recommends for the quantification of *tryptophan* in the *feedingstuffs* the Community method based on HPLC-FD. Since the Applicant provided no experimental data nor experimental method for the quantification of *L-tryptophan* in *water*, the EURL cannot evaluate nor recommend a method for the official control to determine *L-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 *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 fermentation with Escherichia coli CGMCCC 11674"* 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 SANTE/E5: Forw. Appl. 1831/0030-2016
[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, O.J. L 239 , 30/08/1988 P. 0036 – 0039
[4] *Application: Annex 1
[5] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition
[6] *Technical dossier, Section II: 2.2.1.2 Micro-organism
[7] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
[8] 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
[9] 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
[10] 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
[11] *Technical dossier, Section II: II.6.1 Methods of analysis for the active substance
[12] 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)
[13] EN ISO 13904:2005 - Animal feeding stuffs – Determination of tryptophan content
[14] EN ISO 13904:2016 - Animal feeding stuffs – Determination of tryptophan content
[15] Food Chemical Codex monograph "*L-Tryptophan*", FCC 7 (2010), p. 1060
*Refers to Dossier no: FAD-2016-0032

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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- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Instytut Zootechniki - Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
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