EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F – Health and Food
Food and Feed Compliance

European Union Reference Laboratory
Feed Additives

JRC F.5/CvH/ZE/AS/Ares

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 Corynebacterium glutamicum KCCM80346
(FEED-2022-6311; CRL/220062)

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:	**FEED-2022-6311 - CRL/220062**
Name of Product:	***L-tryptophan produced by fermentation with Corynebacterium glutamicum KCCM80346***
Active Agent (s):	***L-tryptophan***
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**Zigmas Ezerskis**
Report checked by: Date:	**María José González de la Huebra** **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-tryptophan produced by fermentation with Corynebacterium glutamicum* KCCM80346, 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-tryptophan* with a minimum purity of 60 % (w/w). The *feed additive* is intended to be added directly to *compound feed* or through *premixtures*. The Applicant did not propose a minimum or maximum *L-tryptophan* content in *compound feed*.

For the determination of *tryptophan* in the *feed additive*, *premixtures* and *compound feed* the Applicant proposed a ring-trial validated European Union (EU) method applicable for the determination of the free (synthetic and natural) and total (peptide-bound and free) *tryptophan* in feed using an HPLC method with fluorescence detection (FLD). The method does not distinguish between the amino acid enantiomers. Furthermore, the EURL is aware of another ring-trial validated EN ISO 13904 method based on high performance liquid chromatography with fluorescence detection (HPLC-FLD) which is dedicated for the determination of free *tryptophan* in commercial products and *premixtures* (containing more than 2 % (w/w) of *tryptophan*) and for the determination of free and total *tryptophan* in *compound feed*.
Based on the performance characteristics available, the EURL recommends for official control the above mentioned two ring-trial validated methods based on HPLC-FLD to determine *tryptophan* in the *feed additive*, *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-tryptophan produced by fermentation with Corynebacterium glutamicum KCCM80346, nutritional additives, amino acids, their salts and analogues, all animal species.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *L-tryptophan produced by fermentation with Corynebacterium glutamicum* KCCM80346, 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 [1,2]. *L-tryptophan* produced by *Corynebacterium glutamicum* is already authorised as *feed additive* under Commission Implementing Regulation (EU) 2020/229 [3].

According to the Applicant, the *feed additive* contains *L-tryptophan* with a minimum purity of 60 % (w/w) [4]. The *feed additive* is intended to be added directly to *compound feed* or through *premixtures*. However, the Applicant did not propose a minimum or maximum *tryptophan* content in *compound feed* [5].

Note: The EURL has previously evaluated the analytical methods in the frame of several *L-tryptophan* related dossiers [6].

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 fermentation with Corynebacterium glutamicum* KCCM80346 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 *tryptophan* in the *feed additive*, *premixtures* and *compound feed* the Applicant proposed [7] a ring-trial validated European Union (EU) method [8].

The EU method is applicable for the determination of free (synthetic and natural) and total (peptide-bound and free) *tryptophan* in feed using an HPLC with fluorescence detection (FLD). The method does not distinguish between the amino acid enantiomers.

For determination of free *tryptophan* in *compound feed*, the sample (1 to 5 g) is mixed with 0.1 M hydrochloric acid and an internal standard (alpha-methyltryptophan). The mixture is shaken or stirred for 60 min. After letting particles to settle down, an aliquot is taken and mixed with ortho-phosphoric acid. The resulting solution is adjusted with sodium hydroxide to pH 3. For the determination of total *tryptophan* in *compound feed*, the sample (0.1 to 1.0 g) is hydrolysed under alkaline conditions in an autoclave using a saturated barium hydroxide

solution at 110 °C for 20 h. After the hydrolysis, the internal standard (alpha-methyltryptophan) and phosphoric acid are added, and the solution is adjusted to pH 3 with a hydrochloric acid. The mixtures obtained by applying the two above mentioned procedures are diluted with methanol (with a volume ranging between 10 to 30 % of the total volume) and water, to reach approximately the same concentration of *tryptophan* as in the calibration standard solution. After a filtration step, a chromatographic analysis by HPLC with fluorescence detection (excitation and emission wavelengths at 280 nm and 356 nm, respectively) is performed. The quantification of *tryptophan* is performed by an internal standard calibration [8].

Furthermore, the EURL is aware of another ring-trial validated EN ISO 13904 method based on high performance liquid chromatography with fluorescence detection (HPLC-FLD) which is dedicated for the determination of free *tryptophan* in commercial products and *premixtures* (containing more than 2 % (w/w) of *tryptophan*) and for the determination of free and total *tryptophan* in *compound feed* [9].

When applying the EN ISO 13904 method, the procedure to determine free and total *tryptophan* in *compound feed* is identical to the one described in the above mentioned EU method. For the determination of free *tryptophan* in commercial products and *premixtures*, the sample (0.5 to 5.0 g) is mixed with 0.1 M hydrochloric acid and stirred for 30 min. After letting particles to settle down, an aliquot is taken, mixed with an internal standard (alpha-methyltryptophan) and diluted with 0.1 M hydrochloric acid. The mixture is then filtered for further chromatographic analysis by HPLC with fluorescence detection (excitation and emission wavelengths at 280 nm and 356 nm, respectively). The quantification of *tryptophan* is performed by an internal standard calibration [9].

The EU and EN ISO 13904 methods were ring-trial validated for the determination of free and total *tryptophan* in various matrices in the frame of several inter-laboratory comparisons. The performance characteristics reported 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 HPLC-FLD to determine *tryptophan* in the *feed additive*, *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 current 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.

Table 1: Method performance characteristics obtained in the frame of four ring-trial validation studies (EU method [8] and EN ISO 13904 [9]) for the determination of free and total *tryptophan* in concentrated products containing *tryptophan* (FA), *premixtures* (PM) and *compound feed* (FS).

References	Matrix		*Tryptophan* content (g/kg)		RSD_r (%)	RSD_R (%)
[8,9]	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
[8,9]	FS	Wheat & soya mixture	free	0.39	1.3	4.7
		Wheat & soya mixture with *L-tryptophan*		0.93	1.3	5.1
[8,9]	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
[9]	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 and RSD_R: relative standard deviations for *repeatability* and *reproducibility,* respectively.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control the two ring-trial validated methods (EU method and EN ISO 13904) based on high performance liquid chromatography and fluorescence detection (HPLC-FLD) to quantify *tryptophan* in the *feed additive*, *premixtures* and *compound feed*.

Note: Only the version of 2016 (and onwards) of the EN ISO 13904 standard method is applicable as older versions do not include the relevant modifications in the standard operating procedure.

Recommended text for the register entry (analytical method)

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

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

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

- High performance liquid chromatography with fluorescence detection (HPLC-FLD) - 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 Corynebacterium glutamicum* KCCM80346 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/7971 https://open.efsa.europa.eu/questions/EFSA-Q-2022-00882

[2] *Application, Annex 1

[3] Commission Implementing Regulation (EU) 2020/229 of 19 February 2020 concerning the authorisation of L-tryptophan as a feed additive for all animal species, OJ L 47, 20.2.2020

[4] *Technical dossier, Section II: II.1.3. Qualitative and quantitative composition (active substance, other components, impurities, batch to batch variation)

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

[6] EURL reports:
https://joint-research-centre.ec.europa.eu/publications/fad-2020-0038_en
https://joint-research-centre.ec.europa.eu/publications/fad-2018-0038_en
https://joint-research-centre.ec.europa.eu/publications/fad-2018-0033_en
https://joint-research-centre.ec.europa.eu/publications/fad-2017-0033_en
https://joint-research-centre.ec.europa.eu/publications/fad-2017-0052_en
https://joint-research-centre.ec.europa.eu/publications/fad-2017-0038_en
https://joint-research-centre.ec.europa.eu/publications/fad-2017-0019_en
https://joint-research-centre.ec.europa.eu/publications/fad-2016-0032_en
https://joint-research-centre.ec.europa.eu/publications/fad-2013-0025_en
https://joint-research-centre.ec.europa.eu/publications/fad-2010-0056_en

[7] *Technical dossier, Section II: II.6.1. Methods of analysis for the active substance

[8] 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)

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

*Refers to Dossier no: FEED-2022-6311

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

The following National Reference Laboratories contributed to this report:

- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- Instytut Zootechniki — Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- [1]Wageningen Food Safety Research (WFSR) (NL)

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