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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 (technically pure) produced using strain AG8012X derived from E-coli K-12

(FAD-2013-0025; CRL/130008)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2013-0025 - CRL/130008**
Additive name:	***L-tryptophan (technically pure) produced using strain AG8012X derived from E-coli K-12***
Active Agent (s):	***L-tryptophan***
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **Geel, Belgium**
Report prepared by:	**Stefano Bellorini**
Report checked by: Date:	**Piotr Robouch (EURL-FA)** **03/12/2013**
Report approved by: Date:	**Christoph von Holst** **03/12/2013**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Articles 4(1) for *L-tryptophan (technically pure)* produced using strain AG8012X derived from *Escherichia coli K-12*, 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. The *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs* or *water*. The Applicant suggested no minimum or maximum *L-tryptophan* concentrations in *premixtures* and *feedingstuffs*.

For the determination of *L-tryptophan* in *feedingstuffs,* the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009) based on High Performance Liquid Chromatography coupled with fluorescence detection (HPLC/FD). The method applies for the determination of *free* (synthetic and natural) and *total* (peptide-bound and free) amino acid in *feedingstuffs*. The following performance characteristics are reported for free *L-tryptophan:* a relative standard deviation for *repeatability* (RSD_r) of 1.3%, and a relative standard deviation for *reproducibility* (RSD_R) ranging from 4.7 to 5.1%; while for total *L-tryptophan:* a RSD_r ranging from 0.8 to 1.9%, and a RSD_R ranging from 1.5 to 6.3%. Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated Community method, based on HPLC/FD, to determine *L-tryptophan* in *feedingstuffs*.

For the determination of *L-tryptophan* in the *feed additive* and *premixtures* the Applicant submitted a ring-trial validated method based on the Community method mentioned above. The following performance characteristics are reported for *feed additive*: RSD_r ranging from 0.5 to 0.9%, and RSD_R ranging from 1.0 to 1.3%; while for *premixtures*: RSD_r ranging from 0.9 to 5.3%, and RSD_R ranging from 2.1 to 9.5%. The experimental data presented is currently being evaluated by the ISO committee in order to extend the scope of the EN ISO method 13904:2005 ('Animal feeding stuffs - Determination of tryptophan content') to *feed additives* and *premixtures*. In addition, the EURL identified the "*L-tryptophan"* monograph of the Food Chemical Codex (FCC), describing the identification of *L-tryptophan* based on infrared absorption and optical rotation. Relying on the experimental evidence provided, the EURL recommends for official control to combine the identification methods described in the FCC "L-tryptophan" monograph to the quantification ring-trial validated Community method, based on reversed phase HPLC/FD, for the determination of *L-tryptophan* in the *feed additive* and *premixtures.*

The Applicant provided neither experimental data nor experimental method for the determination of *L-tryptophan* in *water*. Therefore, the EURL cannot evaluate nor recommend a method for the 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.

KEYWORDS

L-tryptophan (technically pure) produced by E-coli K-12 (AG8012X), nutritional additives, amino acids, all animal species and categories

1. BACKGROUND

In the current application authorisation is sought under Articles 4(1) (authorisation of a new feed additive) for *L-tryptophan (technically pure)* produced using genetically modified strain AG8012X derived from *Escherichia coli K-12*, 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 [1,2]. *L-tryptophan (technically pure)* has been already authorised as feed additive without any restrictions under Commission Directive 88/485/EEC [3,4]. The strain is deposited in the National Institute of Advanced Industrial Science and Technology (NITE) in Japan (registered as FERM BP-11354) [5]. Authorisation is sought for all animal species [1-3].

According to the Applicant *L-tryptophan* is a white to yellow crystalline powder with a minimum purity of 98% [1]. As described in the "*L-tryptophan monograph"* of the Food Chemical Codex, the feed additive has a specific optical rotation ranging from -29.7º and -33.0º [6].

The *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs* or *water* [7]. The Applicants suggested no minimum or maximum *L-tryptophan* concentrations in *premixtures* and *feedingstuffs* [1,7].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, 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 (technically pure) produced using strain AG8012X derived from Escherichia coli K-12* 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 [8]

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

For the determination of *L-tryptophan* in *feedingstuffs* the Applicant submitted the ring-trial validated Community method [9,10]. 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*. The method does not distinguish between the amino acid enantiomers.

Depending on the nature of the *L-tryptophan*, two different sample preparations could be applied:

- to analyse *free L-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 L-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). This method is identical to the EN ISO method 13904:2005 [11].

The Community method was ring trial validated for *free* and *total L-tryptophan* determination in various matrices in the frame of three interlaboratory comparisons. The performance characteristics, as reported in the Official Journal, are listed in Table 1.

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated Community method, based on reversed phase HPLC/FD, to determine *L-tryptophan* in *feedingstuffs*.

Table 1: Method performance characteristics obtained in the frame of three different ring-trial validation exercises based on the Community method for the determination of *free* and *total L-tryptophan* in *feedingstuffs* [10].

Commission Regulation (EC) No 152/2009	Matrix	*L-tryptophan* g/kg	RSD_r (%)	RSD_R (%)
Ring trial 1 - *total*	Pig feed	2.4	1.9	6.3
	Pig feed supplemented with *L-tryptophan*	3.4	1.6	6
	Feed concentrate for pigs	4.2	1.9	2.2
Ring trial 3 - *total*	Mixed pig feed	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
Ring trial 2 - *free*	Wheat & soya mixture	0.39	1.3	4.7
	Wheat & soya mixture with *L-tryptophan*	0.93	1.3	5.1

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

For the determination of *L-tryptophan* in the *feed additive* and *premixtures* the Applicant submitted a ring-trial validated method based on the Community method mentioned above [9]. The satisfactory performance characteristics reported by the Applicant are summarised in Table 2. The experimental data presented is currently being evaluated by a ISO committee in order to extend the scope of the EN ISO method 13904:2005 ('Animal feeding stuffs - Determination of tryptophan content') to *feed additives* and *premixtures* [11-14]. In addition, the EURL identified the "*L-tryptophan"* monograph of the Food Chemical Codex (FCC), describing the identification based on infrared absorption and optical rotation [6].

Based on the experimental evidence provided the EURL recommends for official control to combine the identification methods described in the FCC "L-tryptophan" monograph together with the quantification ring-trial validated Community method, based on reversed phase HPLC/FD, to determine *L-tryptophan* in *the feed additive* and *premixtures.*

Table 2: Method performance characteristics obtained in the frame of a ring-trial validation exercise based on the Community method for the determination of *free tryptophan* in *feed additives* and *premixtures* [12-13].

Bipea Interlaboratory comparison	Feed additive				Premix			
	1	2	3	4	1	2	3	4
tryptophan g/kg	900	940	960	980	25	10	15	50
RSD_r (%)	0.7	0.8	0.9	0.5	5.3	1.4	2.2	0.9
RSD_R (%)	1.3	1.2	1.3	1.0	9.5	3.0	3.3	2.1

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

The Applicant provided neither experimental data nor experimental method for the determination of *L-tryptophan* in *water* [9]. Therefore, the EURL cannot evaluate nor recommend a method for the 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 the ring-trial validated Community method, using High-Performance Liquid Chromatography (HPLC) coupled to fluorescence detection (FD), to quantify *L-tryptophan* in the *feed additive*, *premixtures* and *feedingstuffs.* Furthermore, the EURL recommends for identification the "*L-tryptophan"* monograph of the Food Chemical Codex (FCC) based on infrared absorption and optical rotation.

The Applicant provided neither experimental data nor experimental method for the determination of *L-tryptophan* in *water* [9]. Therefore, 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 *feed additive:*

- Food Chemical Codex "L- *tryptophan* monograph"

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

- High Performance Liquid Chromatography (HPLC) coupled to fluorescence detection, 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* 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 SANCO/G1: Forw. Appl. 1831/0020-2013

[3] *Application, Annex_1

[4] 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

[5] *Application, An_Supp_II_1_1_Deposition certificate_AG8012X.pdf

[6] Food Chemical Codex monograph "L-Tryptophan", FCC 7 (2010), p.1060-61

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

[8] 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

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

[10] 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

[11] Animal feeding stuffs – Determination of tryptophan content (EN ISO 13904:2005)

[12] *Technical dossier, An_II_30_prNF EN ISO_13904_DRAFT_Dec_2012.pdf

[13] *Supplementary Information, Rapport Bipea TRP final_ 980 2012 04 n334 (2).pdf

[14] Analytical method for free tryptophan on pure products and mineral premixtures containing minimum 5% tryptophan (ISO/WD 13904)

*Refers to Dossier no: FAD-2013-0025

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was European Union Reference Laboratory for Feed Additives, IRMM, 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 (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

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- Fødevarestyrelsen, Ringsted[1] (DK)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, Labore Landwirtschaft, Nossen[2] (DE)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
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- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)

[1] Official name and address according to Regulation (EC) No 885/2009: Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby

[2] Official name and address according to Regulation (EC) No 885/2009: Sächsische Landesanstalt für Landwirtschaft. Fachbereich 8 – Landwirtschaftliches Untersuchungswesen, Leipzig