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EURL 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-2010-0056 CRL/ 100047
Name of Feed Additive:	L-Tryptophan
Active Agent (s):	L-Tryptophan
Rapporteur Laboratory:	European Union Reference Laboratory for Feed Additives (EURL-FA) Geel, Belgium
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EXECUTIVE SUMMARY

In the current application authorisation is sought for *L-Tryptophan* under Articles 4(1) and 10(2), category 'nutritional additives' and functional group 3(c) 'amino acids, their salts and analogues' according to Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of *L-Tryptophan* for all animal species and categories. 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*, *feedingstuffs* and *water*.

For the determination of *L-Tryptophan* in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009). The method applies for the determination of *free* (synthetic and natural) and *total* (peptide-bound and free) amino acid, using High Performance Liquid Chromatography (HPLC) equipment. The following performance characteristics are reported:

* For free *L-Tryptophan:*

- a relative standard deviation for *repeatability* (RSD_r) of 1.3%;
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 4.7 to 5.1%.

* For total *L-Tryptophan:*

- a relative standard deviation for *repeatability* (RSD_r) ranging from 0.8 to 1.9%;
- a relative standard deviation for *reproducibility* (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 reversed phase HPLC with fluorescence detection, to determine *L-Tryptophan* in *premixtures* and *feedingstuffs*.

For the determination of the *active substance* in the *feed additive* the Applicant submitted the above-mentioned ring trial validated Community method designed for the analysis of *premixtures* and *feedingstuffs*. Nevertheless the EURL identified, for the determination of the amino acid in the *feed additive*, a titrimetric method among the internationally recognised European Pharmacopoeia methods. No performance characteristics of this method are provided. However, the EURL considers this method suitable to be used within the frame of official control.

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, nutritional additives, amino acids, all animal species and categories.

1. BACKGROUND

In the current application authorisation is sought for *L-Tryptophan* under Articles 4(1) (new use in water) and 10(2) (already authorised as feed additive without any restrictions under Commission Directive 88/485/EEC [1]), category of 'nutritional additives' functional group 3(c) 'amino acids, their salts and analogues' according to Annex I of Regulation (EC) No 1831/2003 [2]. According to the Applicant *L-Tryptophan* is produced through fermentation process using a strain of Escherichia coli K-12 or alternatively with a strain of Corynebacterium Glutamicum [3]. The *feed additive* is white/yellow/light grey crystalline powder with a minimum purity of 98% of the active substance [4, 5].

Specifically, authorisation is sought for the use of *L-Tryptophan* for all animal species and categories. The *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs* or *water* [6]. The Applicants suggested no minimum or maximum *L-Tryptophan* concentrations in *premixtures*, *feedingstuffs* and *water* [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 (EFSA) for each application or group of applications. For this 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 [8].

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

For the determination of *L-Tryptophan* in *premixtures* and *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. 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:

- in order 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;

- in order to extract 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 approximatly the same concentration of the calibration standard solution. Following a filtration step, the solutions are finally injected and determined by reversed phase HPLC with fluorescence detection (excitation and emission at 280 nm and 356 nm respectively). This method is identical to the ISO-CEN 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.

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 *premixtures* and *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

Based on the performance characteristics presented, the EURL recommends for official control the ring-trial validated Community method, based on reversed phase HPLC with fluorescence detection, to determine *L-Tryptophan* in *premixtures* and *feedingstuffs*.

For the determination of *L-Tryptophan* in the *feed additive* the Applicant proposed the above-mentioned ring trial validated Community method, specifically designed for the analysis of *premixtures* and *feedingstuffs*, without providing experimental evidence for the possible extension of scope to the analysis of *L-Tryptophan* in the *feed additive per se*. On the other hand, an additional interlaboratory comparison of the above-mentioned ISO-CEN method is scheduled for 2011 to prove its applicability to high content products (e.g. *feed additive*). When the corresponding validation results will be published, the EURL will consider this method suitable for the quantification of *L-Tryptophan* in the *feed additive* in the frame of official control.

In the mean time, the EURL identified for the determination of the amino acid in the *feed additive*, the internationally recognised European Pharmacopoeia methods [12]. Specifically, the EURL advises to use as first identification the optical rotation and infrared absorption spectrophotometry methods (test A & B respectively) and for the assay, the titrimetric method described. No performance characteristics of these methods are provided. However, the EURL considers these methods suitable to be used within the frame of official control.

The Applicant provided neither experimental data nor analytical method for the determination of *L-Tryptophan* in *water.* Therefore the EURL could not evaluate nor 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:

- the European Pharmacopoeia 6.0, method 01/2008-1272, using titration to determine *L-Tryptophan* in *feed additive*;
- the ring trial validated Community method, using High-Performance Liquid Chromatography (HPLC) coupled to fluorescence detection, to determine *L-Tryptophan* in *premixtures* and *feedingstuff*.

Recommended text for the register entry (analytical method)

For the determination of *L-Tryptophan* in *feed additive*:

- Titrimetry, European Pharmacopoeia (Ph. Eur. 6.0, method 01/2008-1272)

For the determination of *L-Tryptophan* in *premixtures* and *feedingstuff*:

- 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 dossiers have been made available to the EURL by EFSA.

6. REFERENCES

[1] 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
[2] *Application/Ref:SANCO/D/2:Forw.Appl.1831/0042-2010
[3] *Technical dossier, Section II: 2.3.1 Active substance(s)/agent(s)
[4] *Application, (Annex A), FAD-2010-0056_Description_ *L-Tryptophan*
[5] *Technical dossier, Section II: 2.1.1 Chemical substances
[6] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
[7] *Application, (Annex A), FAD-2010-0056_Conditions of use_ *L-Tryptophan*
[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 Method of analysis for the active substances
[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] European Pharmacopoeia 6.0, method 01/2008-1272
*Refers to Dossier no: FAD-2010-0056

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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