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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-2010-0261 CRL/ 100260
Name of Feed Additive:	L-Cystine
Active Agent (s):	L-Cystine
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-Cystine* under Articles 4(1), 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-Cystine* for all animal species and categories. The *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs*. The Applicant suggested no minimum or maximum *L-Cystine* concentrations in *premixtures* and *feedingstuffs*.

For the determination of the *active substance* in the *feed additive* the Applicant submitted the internationally recognised European Pharmacopoeia titrimetric method. No performance characteristics of this method are provided. However, the EURL considers this method suitable to determine *L-Cysteine* in the *feed additive* within the frame of official control.

For the determination of *Cystine* 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 acids, using an amino acid analyzer or High Performance Liquid Chromatography (HPLC) equipment. However, only performance characteristics for the determination of *total Cystine* are reported:

- a relative standard deviation for *repeatability* (RSD_r) ranging from 1.7 to 4.6%;
- a relative standard deviation for *reproducibility* (RSD_R) ranging from 8.8 to 19%.

Based on the performance characteristics presented, the EURL recommends for official control, the ring-trial validated Community method based on ion exchange chromatography coupled with post-column derivatisation and photometric detection to determine *Cystine* in *premixtures* and *feedingstuffs*.

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

1. BACKGROUND

L-Cystine is already authorised as *feed additive* as "sensory additive", functional group "flavouring compounds", under the category "natural or corresponding synthetic chemically defined flavouring" [1]. In the current application authorisation is sought for *L-Cystine* under Articles 4(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-Cystine* is produced by extraction from a natural source (i.e. keratin present in poultry feathers) [3]. The *feed additive* is a white solid crystalline powder with a minimum purity of 98.5% of the active substance [4, 5].

Specifically, authorisation is sought for the use of *L-Cystine* for all animal species and categories. The *feed additive* is intended to be mixed either in *premixtures* or added directly to complete *feedingstuffs* [6]. The Applicant suggested no minimum or maximum *L-Cystine* concentrations in *premixtures* and *feedingstuffs* [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-Cystine*, 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 and feedingstuffs.

For the determination of *L-Cystine* in the *feed additive* the Applicant proposed to apply the internationally recognised European Pharmacopoeia methods [9, 10]:

- for the identification, the optical rotation and infrared absorption spectrophotometry methods (test A & B respectively), and

- for the determination, the titrimetric method.

No performance characteristics of these methods are provided. However, the EURL considers these methods suitable to determine *L-Cystine* in the frame of official control.

For the determination of *Cystine* in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method [11]. This method applies for the determination of *free* (synthetic and natural) and of *total* (peptide-bound and free) amino acids, using an amino acid analyzer or High Performance Liquid Chromatography (HPLC) equipment. The method does not distinguish between the salts and the amino acid enantiomers.

The *free* amino acids are extracted with diluted hydrochloric acid. Co-extracted nitrogenous macromolecules are precipitated with sulfosalicylic acid and removed by filtration. The solution is filtered and adjusted to pH 2.2. The amino acids are separated by ion exchange chromatography and determined by post column derivatisation with ninhydrin and photometric detection at 570 nm. The procedure chosen for the determination of the *total* amino acids depends on the amino acids under investigation. *Cystine* must be oxidised to cysteic acid prior to hydrolysis. Oxidation is performed at 0° C with a performic acid/phenol mixture. Excess oxidation reagent is decomposed with sodium disulphite. Cysteic acid is hydrolysed with hydrochloric acid (6 mol/l) for 23 hours. The hydrolysate is adjusted to pH 2.2. The amino acids are separated by ion exchange chromatography and determined after post column derivatisation with ninhydrin by photometric detection at 570 nm. The method doesn't distinguish between *Cystine* and *Cysteine* since both compounds are determined together as cysteic acid in hydrolysates of oxidised sample. Finally, the analytical result it is expressed as mass fraction of *Cystine* calculated from the measured cysteic acid and using a conversion factor (120.15 g/mol).

The Community method was ring trial validated using three different matrices listed in Table 1. This method was further ring-trial validated by twenty-three laboratories, resulting in the CEN EN ISO 13903:2005 method [12]. Furthermore, a limit of quantification of 0.35 g/kg *feedingstuffs* was derived for *total Cystine*. The performance characteristics reported for the determination of total *Cystine* are listed in Table 1.

Table 1: Method performance characteristics obtained in the frame of two different ring-trial validation exercises for the determination of *Cystine* in *premixtures* and *feedingstuffs*. The performance characteristics reported refer to *total Cystine* determination (peptide bound and free) and are a sum of *Cystine* and Cysteine (indicated as *Cyst(e)ine*).

Intercomparison study	Matrix	*Cyst(e)ine* g/kg	RSD_r (%)	RSD_R (%)
Commission Regulation (EC) No 152/2009 [11]	Mixed pig feed	3	3.3	9.9
	Broiler compound	4	2.8	8.8
study carried out in 1990	Protein concentrate	5	2.6	12.3
ISO 13903:2005 [12]	Poultry meal	0.8	4.6	17.7
	Broiler finisher feed	0.3	3.1	11.3
	Broiler starter feed	0.4	1.7	16
	Corn	0.2	3.9	13.9
study carried out in 1994	Fishmeal	0.5	4.0	19.0

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 ion exchange chromatography coupled with post-column derivatisation and photometric detection to determine *Cystine* in *premixtures* and *feedingstuffs*.

Furthermore, the EURL would like to underline that 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 *Cystine* in the *feed additive* in the frame of 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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control:

- the European Pharmacopoeia 6.0, method 01/2008-0998, using titration to determine *L-Cystine* in *feed additive*;

– the ring trial validated Community method, using ion exchange chromatography coupled to post column derivatisation and photometric detection, to determine *Cystine* in *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

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

– Titrimetry, European Pharmacopoeia (Ph. Eur. 6.0, method 01/2008-0998)

For the determination of *Cystine* in *premixtures* and *feedingstuffs*:

– ion exchange chromatography method with post-column derivatisation and photometric detection: Commission Regulation (EC) No 152/2009 (Annex III, F)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *L-Cystine* 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] Council Directive 70/524/EEC concerning additives in feedingstuffs – List of authorised additives in feedingstuffs (2004/C50/01)
[2] *Application/Ref:SANCO/D/2:Forw.Appl.1831/0153-2010
[3] *Technical dossier, Section II: 2.3.1 Active substance(s)/agent(s)
[4] *Technical dossier, Section II: 2.1.5 Physical state of each form of the product
[5] *Technical dossier, Section I: 1.1.2.b Identification of the additive
[6] *Technical dossier, Section II: 2.5.1 Proposed mode of use in animal nutrition
[7] *Application, (Annex A), FAD-2010-0261_Conditions of use_ *L-Cystine*
[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] European Pharmacopoeia 6.0, method 01/2008-0998
[11] 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
[12] Animal feeding stuffs – Determination of amino acids content (CEN EN ISO 13903:2005)
*Refers to Dossier no: FAD-2010-0261

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

The following National Reference Laboratories contributed to this report:

- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Schwerpunktlabor Futtermittel des Bayerischen Landesamtes für Gesundheit und Lebensmittelsicherheit (LGL), Oberschleißheim (DE)
- Sächsische Landesanstalt für Landwirtschaft, Fachbereich 8 — Landwirtschaftliches Untersuchungswesen, Leipzig (DE)
- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA) Speyer, Speyer (DE)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen. Jena (DE)
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)
- Kmetijski inštitut Slovenije, Ljubljana (SI)