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European Union Reference Laboratory for Feed Additives

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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-cysteine monohydrochloride monohydrate
produced by fermentation with
Escherichia coli KCCM 80109 and
Escherichia coli KCCM 80197
(*FAD-2018-0091; CRL/180064*)

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in connection with the Application for Authorisation of a
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Dossier related to: **FAD-2018-0091- CRL/180064**

Name of Product: ***L-cysteine monohydrochloride
monohydrate produced by fermentation
with Escherichia coli KCCM 80109 and
Escherichia coli KCCM 80197***

Active Agent: **L-cysteine**

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
JRC Geel, Belgium**

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Date: **14/05/2019**

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Date: **14/05/2019**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-cysteine monohydrochloride monohydrate produced (L-cysteine HCl H₂O) by fermentation with Escherichia coli KCCM 80109 and Escherichia coli KCCM 80197*, under the category/functional groups 2(b) 'sensory additives/flavouring compounds' according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for all animal species.

According to the Applicant *L-cysteine HCl H₂O* has a minimum purity (mass fraction) of 98.5 %. The *feed additive* is intended to be added directly into *feedingstuffs* or through *premixtures* and *water* for drinking. The Applicant proposed a maximum content of *L-cysteine HCl H₂O* of 25 mg/kg *feedingstuffs*.

For the quantification of *L-cysteine HCl H₂O* in the *feed additive*, the Applicant presented experimental data obtained when analysing the *feed additive* with the VDLUFA method 4.11.6 designed for the analysis of lysine, methionine and threonine in *feed additives* and concentrated *premixtures* and based on ion-exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS/FD). The results presented are considered sufficient to demonstrate the suitability of the procedure for the determination of the amino acid in the *feed additive*. Furthermore, according to the experience of NRLs, the VDLUFA method and other ring trial validated methods designed for the analysis of amino acids and based on a similar analytical procedure are fit-for-purpose. Hence, for official control, the EURL recommends this method (or equivalent) for the determination of *L-cysteine HCl H₂O* in the *feed additive*.

For the quantification of the *L-cysteine HCl H₂O* content in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009) based on IEC-VIS. This method, designed for the analysis of free (synthetic and natural) and of total (peptide-bound and free) amino acids (including *cysteine*) in *premixtures* and *feedingstuffs*, does not distinguish between the salts and the amino acid enantiomers. The Community method was further ring-trial validated by twenty-three laboratories for the determination of *total cyst(e)ine* (sum of *cysteine* and *cystine*, peptide bound and free) in feed and resulted in the equivalent standard method EN ISO 13903:2005. While the Applicant proposed a maximum content of *L-cysteine HCl H₂O* in *feedingstuffs* of 25 mg/kg, a higher limit of quantification of 350 mg/kg *feedingstuffs* was derived for *total cyst(e)ine*. Therefore, the EURL is unable to recommend the Community method for the official control of this product in *feedingstuffs*.

Nevertheless, based on the performance characteristics available, the EURL recommends for official control the ring-trial validated Community method based on IEC-VIS to quantify *L-cysteine HCl H₂O* in *premixtures*.

The Applicant did not submit any method for the official control of *L-cysteine HCl H₂O* in *water*. However, in the frame of the stability studies, the Applicant presented experimental data obtained when analysing the amino acid with the above mentioned ring-trial validated method VDLUFA – Method 4.11.6. The results presented are considered sufficient to demonstrate the suitability of the procedure for the analysis of *L-cysteine HCl H₂O* in *water*. Hence, for official control, the EURL recommends this method (or equivalent) for the determination *L-cysteine HCl H₂O* in *water*.

In the frame of the authorised "Chemical Defined flavouring Group 34 – amino acids" (FAD-2010-0107), for the identification of *L-cysteine HCl H₂O* in the feed additive, the EURL positively evaluated the European Pharmacopeia 2.2.56 (2009) method for amino acids based on IEC coupled with post-column derivatisation and photometric detection (visible – VIS). Furthermore, the EURL recommendation has been included in the corresponding authorising regulation. In order to foster the use of the same method for identical substances, the EURL recommends this European Pharmacopeia method for official control to identify *L-cysteine HCl H₂O* in the *feed additive*.

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-cysteine monohydrochloride monohydrate produced by fermentation with *Escherichia coli* KCCM 80109 and *Escherichia coli* KCCM 80197, sensory additives, flavouring compounds, all animal species and categories

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *L-cysteine monohydrochloride monohydrate* (*L-cysteine HCl H₂O*) produced by fermentation with *Escherichia coli* KCCM 80109 and *Escherichia coli* KCCM 80197, under the category/functional groups 2(b) 'sensory additives/flavouring compounds' according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for all animal species [1-3].

L-cysteine HCl H₂O is already authorised as sensory additive "produced by chemical synthesis or hydrolysis of animal or vegetal protein" for all species (except cats and dogs) with a recommended maximum content of the active substance in complete *feedingstuffs* of 25 mg/kg [4,5].

According to the Applicant, the off-white crystalline powder *L-cysteine HCl H₂O* has a minimum purity (mass fraction) of 98.5 % [1,3,6]. The *feed additive* is produced by fermentation with two non-genetically modified strains of *Escherichia coli* K12. The production strains are deposited in the "Korean Culture Center of Microorganisms" (KCCM) under accession numbers KCCM 80109 and KCCM 80197 [7].

The *feed additive* is intended to be added directly into *feedingstuffs* or through *premixtures* and *water* for drinking [8]. Furthermore, the Applicant proposed a maximum content of *L-cysteine HCl H₂O* of 25 mg/kg *feedingstuffs* [8].

Note: The EURL has previously evaluated the analytical methods for the determination of *L-cysteine HCl H₂O* as sensory feed additive in the frame of the "Chemical Defined flavouring Group 34" [9] and FAD-2018-0042 [10].

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-cysteine monohydrochloride monohydrate* 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 quantification of *L-cysteine HCl H₂O* in the *feed additive* the Applicant submitted an in-house validated analytical method based on reversed phase high performance liquid chromatography (HPLC) and ultraviolet detection (UV) [11,12].

Moreover, in the frame of the stability studies, the Applicant presented experimental data obtained when analysing *L-cysteine HCl H₂O* with the ring-trial validated method by the “Association of German Agricultural Analytical and Research Institutes” (VDLUFA, Germany – Method 4.11.6) which is actually designed for the analysis of free (synthetic and natural but not peptide-bound) lysine, methionine and threonine in *feed additives* and concentrated *premixtures* [13,14]. This method is based on ion-exchange chromatography (IEC) coupled with post-column derivatisation and optical detection (VIS or fluorescence detection - FD). The *feed additive* and/or *premixtures* samples are dissolved or extracted with hydrochloric acid (0.1 mol/l) and diluted with sodium citrate buffer. The internal standard solution (norleucine) is added and the amino acids are separated by IEC and determined after post-column derivatisation with ninhydrin by colourimetric detection or by fluorescence detection after post-column reaction with o-phthalaldehyde (OPA). The results presented are considered sufficient to demonstrate the suitability of the procedure for the analysis of *L-cysteine HCl H₂O* in the *feed additive*. Furthermore, according to the experience of NRLs, the VDLUFA 4.11.6 or other ring trial validated methods designed for the analysis of free amino acids and based on similar analytical procedure are fit for purpose (i.e. ISO 17180:2013) [15]. Hence, for official control, the EURL recommends this method (or equivalent) for the determination of *L-cysteine HCl H₂O* in the *feed additive*.

For the quantification of the *L-cysteine HCl H₂O* content in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method [11,16]. This method applies for the determination of free (synthetic and natural) and of total (peptide-bound and free) amino acids (including *cysteine*), using an amino acid analyser or HPLC equipment provided with an ion-exchange column. The method is intended for *premixtures* and *feedingstuffs*, it does not distinguish between the salts of amino acids and it cannot differentiate 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 IEC and determined by post-column derivatisation with ninhydrin and photometric detection at 570 nm (visible – VIS).

The procedure chosen for the determination of the total amino acids depends on the amino acids under investigation. *Cysteine* 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 disulfite. 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 IEC and determined after post-column derivatisation with ninhydrin by photometric detection at 570 nm. The method does not distinguish between *cysteine* and cystine since both compounds are determined together as cysteic acid in hydrolysates of oxidised sample. Finally, cystine and *cysteine* are both determined as cysteic acid in hydrolysates of oxidised sample, but calculated as cystine (by using a molar weight of 120.15 g/mol).

The Community method was ring-trial validated using three different matrices listed in Table 1. The Community method was further ring-trial validated by twenty-three laboratories for the determination of total *cysteine* (sum of *cysteine* and cystine) in feed and resulted in the equivalent standard method EN ISO 13903:2005 [17]. The reported performance characteristics are listed in Table 1.

A limit of quantification of 350 mg/kg *feedingstuffs* was derived for *total cyst(e)ine* [17]. However, the Applicant proposed a maximum content of *L-cysteine HCl H₂O* in *feedingstuffs* of 25 mg/kg [9]. Therefore, the EURL is unable to recommend the Community method for the official control of this product in *feedingstuffs* when intended as flavouring *feed additive*.

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

Intercomparison study	Matrix	<i>Cyst(e)ine</i> content (g/kg)	RSD _r (%)	RSD _R (%)
Commission Regulation (EC) No 152/2009 [16]	Mixed pig feed	3	3.3	9.9
	Broiler compound	4	2.8	8.8
	Protein concentrate	5	2.6	12.3
ISO 13903:2005 [17]	Poultry meal	8.1	4.6	17.7
	Broiler finisher feed	3.2	3.1	11.3
	Broiler starter feed	3.5	1.7	16
	Corn	1.8	3.9	13.9
	Fishmeal	4.8	4.0	19.0

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

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated Community method based on IEC-VIS to quantify *L-cysteine HCl H₂O* in *premixtures*. In general, the procedure based on quantification of free L-cyst(e)ine content has to be followed in order to avoid an overestimation of L-cyst(e)ine content which may come from *premixtures* containing proteins if the procedure for quantification of total L-cyst(e)ine content is used.

The Applicant did not submit any method for the official control of *L-cysteine HCl H₂O* in *water* [11]. However, in the frame of the stability studies, the Applicant presented experimental data obtained when analysing *L-cysteine HCl H₂O* with the above mentioned ring-trial validated method VDLUFA – Method 4.11.6 [18]. The results presented are considered sufficient to demonstrate the suitability of the procedure for the analysis of *L-cysteine HCl H₂O* in *water*. Hence, for official control, the EURL recommends this method (or equivalent) for the determination of *L-cysteine HCl H₂O* in *water*.

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 present application.

Identification/Characterisation of the feed additive (section 2.6.3 of the dossier - Annex II of Commission Regulation (EC) No 429/2008)

The EURL, in the frame of the authorised "Chemical Defined flavouring Group 34 – amino acids", previously evaluated the analytical method for the identification of *L-cysteine HCl H₂O* in the *feed additive* [4,5,9].

In the above mentioned dossier the Applicant submitted the European Pharmacopeia method for amino acids (analogous to Community method) [19]. The amino acids are separated by IEC and determined by post-column derivatisation with ninhydrin and photometric detection at 570 nm (visible – VIS) using norleucine as internal standard. The method proposed does not distinguish between the salts of amino acids, nor differentiates between D and L forms of amino acids. Each feed additive was identified by comparison with a corresponding amino acid standard. The Applicant provided the typical chromatogram for the *CDG 34* compounds of interest (*L-cysteine HCl H₂O* included).

Based on the satisfactory experimental evidence provided, the EURL recommended for official control the European Pharmacopeia method submitted in the frame of the *CDG 34* application for the qualitative identification of *L-cysteine HCl H₂O* in the *feed*

additive. Furthermore, the EURL recommendation has been included in the corresponding authorising regulation [4,9]. In order to foster the use of the same method for identical substances, the EURL recommends this European Pharmacopoeia method for official control to identify *L-cysteine HCl H₂O* in the *feed additive*.

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.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control (i) the European Pharmacopoeia method based on ion-exchange chromatography coupled to visible detection (IEC-VIS) for the identification of *L-cysteine monohydrochloride monohydrate* in the *feed additive*; (ii) the analytical method described by VDLUFA (4.11.6) (or equivalent i.e. ISO 17180:2013) based on IEC-VIS/FD to quantify *L-cysteine monohydrochloride monohydrate* in the *feed additive* and *water*; and (iii) the ring-trial validated Community method based on ion-exchange chromatography coupled to visible detection (IEC-VIS) for the quantification of *L-cysteine monohydrochloride monohydrate* in *premixtures*.

Recommended text for the register entry (analytical method)

For the identification of *L-cysteine monohydrochloride monohydrate* in the *feed additive*:

- ion-exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS), Ph.Eur. 6.6-2.2.56-Method 1

For the quantification of *L-cysteine monohydrochloride monohydrate* in the *feed additive* and *water*:

- ion-exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS/FD)

For the quantification of *L-cysteine monohydrochloride monohydrate* in *premixtures*:

- ion-exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS), 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-cysteine monohydrochloride monohydrate* produced by fermentation with *Escherichia coli* KCCM 80109 and *Escherichia coli* KCCM 80197 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. 1831/0004-2019
- [3] Annex I – submission number 1544781383072-2334
- [4] Commission Implementing Regulation (EC) No 2018/249 of 15 February 2018 concerning the authorisation of concerning the authorisation of taurine, beta-alanine, L-alanine, L-arginine, L-aspartic acid, L-histidine, D,L-isoleucine, L-leucine, L-phenylalanine, L-proline, D,L-serine, L-tyrosine, L-methionine, L-valine, L-cysteine, glycine, monosodium glutamate and L-glutamic acid as feed additives for all animal species and L-cysteine hydrochloride monohydrate for all species except cats and dogs, O.J. L 53/134, 23.02.2018
- [5] Commission Implementing Regulation (EU) 2018/1567 of 18 October 2018 correcting Implementing Regulation (EU) 2018/249 concerning the authorisation of taurine, betaalanine, L-alanine, L-arginine, L-aspartic acid, L-histidine, D,L-isoleucine, L-leucine, L-phenylalanine, L-proline, D,L-serine, L-tyrosine, L-methionine, L-valine, L-cysteine, glycine, monosodium glutamate and L-glutamic acid as feed additives for all animal species and L-cysteine hydrochloride monohydrate for all species except cats and dogs
- [6] *Technical dossier, Section II: II.2.1.1. Chemical substances
- [7] *Technical dossier, Section II: II.2.1.2. Micro-organisms
- [8] *Technical dossier, Section II: II.2.5.1. Proposed mode of use in animal nutrition
- [9] FAD-2010-0107, Chemically defined flavourings from Chemical Group 34, Ref. Ares(2011)301126 - 18/03/2011 <https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2010-0107.pdf>
- [10] FAD-2018-0042 L-cysteine monohydrochloride monohydrate produced by fermentation with *Escherichia coli* KCCM 80180 and *Escherichia coli* KCCM 80181, Ref. Ares(2019)1054139 – 20/02/2019
- [11] *Technical dossier, Section II: II.6.1. Methods of analysis for the active substance
- [12] *Technical dossier, Section II: Annex_II_6_01 CJ L-Cys HCl Method Validation report.pdf
- [13] VDLUFA MB III 4.11.6 Bestimmung von Lysin, Methionin und Threonin in Aminosäurehandelsprodukten und Vormischungen

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- [14] *Technical dossier, Section II: Annex_II_4_01 CJ L-Cys_Shelf life(18M&Accelerated).pdf
- [15] EN ISO 17180:2013 - Animal feeding stuffs – Determination of lysine, methionine and threonine in commercial amino acid products and premixtures
- [16] 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
- [17] EN ISO 13903:2005- Animal feeding stuffs – Determination of amino acids content
- [18] *Technical dossier, Section II: Annex_II_4_03_CJ L-Cys HCl Stability in water.pdf
- [19] European Pharmacopeia 6.6 – Method 2.2.56 (2009) p.5059

*Refers to Dossier no: FAD-2018-0091

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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