EUROPEAN COMMISSION
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European Union Reference Laboratory for Feed Additives

JRC F.5/CvH/SB/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-histidine monohydrochloride monohydrate
using strain NITE BP-02526
(FAD-2018-0070; CRL/180061)

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:	**FAD-2018-0070 - CRL/180061**
Name of Product:	***L-histidine monohydrochloride monohydrate using strain NITE BP-02526***
Active Agent:	**L-histidine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Zigmas Ezerskis** **22/03/2019**
Report approved by: Date:	**Christoph von Holst** **22/03/2019**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-histidine monohydrochloride monohydrate using* the bacteria *strain NITE BP-02526*, under the category/functional groups 3(c) 'nutritional additives'/'amino acids, their salts and analogues' and 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-histidine monohydrochloride monohydrate* has a minimum purity (mass fraction) of 98 %. As nutritional feed additive, the amino acid is intended to be added directly into *feedingstuffs* or through *premixtures* and *water* for drinking. As sensory feed additive, *L-histidine monohydrochloride monohydrate* is intended to be added into *feedingstuffs* and *water* for drinking through flavouring *premixtures*. However, the Applicant did not propose any minimum or maximum content of *L-histidine monohydrochloride monohydrate* in *feedingstuffs*.

For the quantification of *L-histidine monohydrochloride monohydrate* in the *feed additive* and *premixtures* the Applicant submitted the ring-trial validated method EN ISO 17180:2013 specifically designed for lysine, methionine and threonine in products containing more than 10 % of amino acid. This standard method is based on ion exchange chromatography coupled to visible or fluorescence detection (IEC-VIS/FLD). It does not distinguish between the salts of amino acids and cannot differentiate between enantiomers. The Applicant presented results from validation and verification studies demonstrating the extension of the scope of the above mentioned ISO method for the determination of *L-histidine monohydrochloride monohydrate* in the *feed additive* and *premixtures* (containing more than 10 % *histidine*). The following performance characteristics are reported: a relative standard deviation for repeatability (RSD_r) ranging from 0.6 to 4.3 %, a relative standard deviation for intermediate precision (RSD_{ip}) ranging from 1.1 to 4.8 % and a recovery rate from 91 to 103 %.

For the quantification of *L-histidine monohydrochloride monohydrate* in *feedingstuffs* the Applicant submitted the ring-trial validated Community method (Commission Regulation (EC) No 152/2009) based on IEC coupled with photometric detection (VIS). The method, designed only for the analysis of amino acids in *premixtures* and *feedingstuffs*, does not distinguish between the salts and the amino acid enantiomers. This method was further ring-trial validated by twenty-three laboratories, resulting in the EN ISO 13903:2005 method. The following performance characteristics were reported for the quantification of total *histidine*: RSD_r ranging from 2.4 to 7.0 % and RSD_R ranging from 13 to 23 %. In the frame of the stability studies the Applicant presented experimental data obtained analysing the *feed additive* in *water* according to EN ISO 13903:2005 thus demonstrating its applicability for the determination of *L-histidine monohydrochloride monohydrate* in *water*.

In the frame of this authorisation the EURL recommends for official control (i) the ring-trial validated method EN ISO 17180:2013 based on ion exchange chromatography coupled to visible or fluorescence detection (IEC-VIS/FLD) to quantify free *L-histidine monohydrochloride monohydrate* in the *feed additive* and *premixtures* (containing more than 10 % *histidine*); (ii) the ring-trial validated Community method based on IEC-VIS for the quantification of *L-histidine monohydrochloride monohydrate* in *premixtures* and *feedingstuffs*; and (iii) the ring-trial validated EN ISO 13903:2005 method based on IEC-VIS for the quantification of *L-histidine monohydrochloride monohydrate* 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), as last amended by Regulation (EU) 2015/1761) is not considered necessary.

KEYWORDS

L-histidine monohydrochloride monohydrate produced using strain NITE BP-02526, nutritional additives, amino acids, all animal species and categories, sensory additives, flavouring compounds.

1. BACKGROUND

In the current application authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *L-histidine monohydrochloride monohydrate using* the bacteria *strain NITE BP-02526*, under the category/functional groups 3(c) 'nutritional additives'/'amino acids, their salts and analogues' and 2(b) 'sensory additives/flavouring compounds' according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for all animal species [1,2]. *L-histidine monohydrochloride monohydrate (L-histidine HCl H_2O)* produced by Escherichia coli (ATCC 9637) is already authorised as nutritional *feed additive* [3]. As sensory *feed additive*, the product is authorised only in the form of *L-histidine* produced by "chemical synthesis or protein hydrolysis" [4].

According to the Applicant, the white crystalline powder *L-histidine HCl H_2O* has a minimum purity (mass fraction) of 98 % [1,5]. The *feed additive* is produced by fermentation using a genetically modified strain of Escherichia coli [6,7]. The production strain is deposited in the "National Institute of Technology and Evaluation" (NITE, Japan) with reference BP-02526. As nutritional feed additive, *L-histidine HCl H_2O* is intended to be added directly into *feedingstuffs* or through *premixtures* and *water* for drinking [8]. As sensory feed additive, *L-histidine HCl H_2O* is intended to be added into *feedingstuffs* and *water* for drinking through flavouring *premixtures* [8]. Furthermore, when intended for ruminants, the *feed additive* is placed on the market as preparation [5]. The Applicant did not propose any minimum or

maximum content of *L-histidine* in *feedingstuffs* [1,8]. However, when authorised as sensory additive, it is recommended to use the amino acid at a level of 25 mg/kg complete feed [8].

Note: The EURL has previously evaluated the analytical methods for the determination of *L-histidine HCl* H_2O as nutritional *feed additive* in the frame of FAD-2006-0022&FAD-2018-0013 and as sensory *feed additive* in the frame of the Chemical Defined flavouring Group 34 [9-11].

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-histidine 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 determination of *L-histidine HCl* H_2O in the *feed additive* and *premixtures* the Applicant submitted the ring-trial validated method EN ISO 17180:2013 - "Animal feeding stuffs – Determination of lysine, methionine and threonine in commercial amino acid products and premixtures" [12,13]. This standard method is based on the experimental protocol described in the Community method for the determination of amino acids in feed (including *histidine*) [14]. The method does not distinguish between the salts of amino acids and it cannot differentiate between enantiomers. It applies for products and *premixtures* containing more than 10 % of amino acid.

The Applicant presented validation and verification studies with the aim to demonstrate the extension of scope of the above mentioned ISO method for the determination of *L-histidine HCl* H_2O in the *feed additive* and *premixtures* [15,16]. Minor modifications from the original standard operating procedure are described in the corresponding documents [15,16].

Free *histidine* is extracted with diluted hydrochloric acid and further diluted with sodium citrate buffer. After addition of norleucine as internal standard, the amino acids are separated

by High Performance Liquid Chromatography (HPLC) with an Ion Exchange Column (IEC). Free *histidine* is quantified either after post-column derivatisation with ninhydrine and Visible (VIS) detection at 440 nm and 570 nm or by fluorescence detection (FLD) after post-column reaction with ortho-phthaldialdehyde with a detector excitation wavelength at 330 nm and emission at 460 nm. The following performance characteristics are reported: a relative standard deviation for repeatability (RSD_r) ranging from 0.6 to 4.3 %, a relative standard deviation for intermediate precision (RSD_{ip}) ranging from 1.1 to 4.8 % and a recovery rate from 91 to 103 % [15,16].

For the quantification of *L-histidine HCl* H_2O in *feedingstuffs* the Applicant submitted the ring-trial validated Community method mentioned above [12,14]. This method can only be applied in *premixtures* and *feedingstuffs* for the quantification of *free* (synthetic and natural) and *total* (peptide-bound and free) amino acids, using an amino acid analyser or IEC coupled with post-column derivatisation and VIS detection. It does not distinguish between the salts of amino acids and cannot differentiate between 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. The procedure chosen for the determination of the *total* amino acids depends on the amino acids under investigation. *L-histidine* can be determined in either oxidised or unoxidised samples. The oxidation is performed at 0 °C with a performic acid/phenol mixture. An excess of oxidation reagent is decomposed with sodium disulfite. The oxidised or unoxidised sample 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 by post-column derivatisation with ninhydrin and photometric detection at 570 nm.

The Community method was ring-trial validated using four different matrices for threonine, cyst(e)ine, methionine and lysine only. This method was further ring-trial validated by twenty-three laboratories, resulting in the EN ISO 13903:2005 method (*histidine* included) [17]. The performance characteristics reported for the quantification of total *L-histidine* are listed in Table 1.

For the quantification of *L-histidine HCl* H_2O in *water* the Applicant submitted the ring-trial validated EN ISO 13903:2005 method mentioned above [12,17]. The method, equivalent to the Community method, is intended for *premixtures* and *feedingstuffs* only. For the analysis in *water* minor adaptations of the protocol have been presented [18]. The Applicant did not perform any validation/verification study to demonstrate the suitability of the slightly modified ISO method for the determination of *histidine* in *water*.

Table 1: Method performance characteristics obtained in the frame of EN ISO 13903:2005 [17] for the determination of total *L-histidine* in *feed*.

Ring-Trial	Matrix	*histidine* content g/kg	RSD_r %	RSD_R %
[17]	Poultry meal	13.1	2.8	18.5
	Broiler finisher feed	5.0	4.0	19.8
	Broiler starter feed	6.5	2.8	15.4
	Corn	2.7	7.0	23.3
	Fishmeal	13.7	2.4	12.9

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

However, in the frame of the stability studies, the Applicant performed the analysis successfully applying the proposed method for the determination of *histidine* in *water* [18,19]. Hence the EURL recommends for official control the ring-trial validated EN ISO 13903:2005 method based on IEC-VIS to quantify *histidine* in *water*.

Based on the performance characteristics available, the EURL recommends for official control: i) the ring-trial validated method EN ISO 17180:2013 based on IEC-VIS/FLD for the quantification of free *L-histidine monohydrochloride monohydrate* in the *feed additive* and *premixtures* (containing more than 10 % *histidine*); ii) the ring-trial validated Community method, based on IEC-VIS to quantify *L-histidine monohydrochloride monohydrate* in *premixtures* and *feedingstuffs*; and iii) the ring-trial validated EN ISO 13903:2005 method, based on IEC-VIS to quantify *L-histidine monohydrochloride monohydrate* 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)

An evaluation of corresponding methods of analysis is not considered necessary by the EURL.

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 ring-trial validated method EN ISO 17180:2013 based on ion exchange chromatography coupled to visible or fluorescence detection (IEC-VIS/FLD) to quantify free *L-histidine monohydrochloride monohydrate* in the *feed additive* and *premixtures* (containing more than 10 % *histidine*); (ii) the ring-trial validated Community method based on IEC-VIS for the quantification of *L-histidine monohydrochloride monohydrate* in *premixtures* and *feedingstuffs*; and (iii) the ring-trial validated EN ISO 13903:2005 method based on IEC-VIS for the quantification of *L-histidine monohydrochloride monohydrate* in *water*.

Recommended text for the register entry (analytical method)

For the quantification of *L-histidine monohydrochloride monohydrate* in the *feed additive* and *premixtures* (containing more than 10 % *histidine*):

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

For the quantification of *L-histidine monohydrochloride monohydrate* in *premixtures* and *feedingstuffs*:

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

For the quantification of *L-histidine monohydrochloride monohydrate* in *water*:

- ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *L-histidine monohydrochloride monohydrate using strain NITE BP-02526* 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. 1831-0073-2018 & Annex I – submission number 1538421406123-2296

[3] Commission Regulation (EC) No 244/2007 of 7 March 2007 concerning the authorisation of L-histidine monohydrochloride monohydrate as a feed additive, O.J. L 73/6, 13.3.2007

[4] Commission Implementing Regulation (EU) 2018/249 of 15 February 2018 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, 23.2.2018

[5] *Technical dossier, Section II: 2.1.2 Proposal for classification

[6] *Technical dossier, Section II: 2.1.3 Qualitative and quantitative composition

[7] *Technical dossier, Section II: 2.3 Manufacturing process, including any processing procedures

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

[9] FAD-2006-0022, L-histidine, Ref. D08/FSQ/CVH/GS/(2006) D/13915 https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2006-0022.pdf

[10] FAD-2018-0013, L-histidine monohydrochloride monohydrate produced by Corynebacterium glutamicum KCCM80172, Ref. Ares(2018)5739711 - 09/11/2018 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2018-0013_histidinehcl.pdf

[11] 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

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

[13] EN ISO 17180:2013 - Animal feeding stuffs – Determination of lysine, methionine and threonine in commercial amino acid products and premixtures

[14] 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

[15] *Technical dossier, Section II: An_II_54_Raterink_2018_Triskelion_V21163_Validation_Meth_in_CP

[16] *Technical dossier, Section II: An_II_56_Raterink_2018_Triskelion_V21164_Validation _Meth_in_premix

[17] EN ISO 13903:2005- Animal feeding stuffs – Determination of amino acids content

[18] *Technical dossier, Section II: An_II_61_Jurgens_et_al_ 2017_R11616_Stab_in_water

[19] *Technical dossier, Section II: 2.4.1.3 Stability of L-histidine HCl H_2O used in water

*Refers to Dossier no: FAD-2018-0070

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:

- Państwowy Instytut Weterynaryjny, Pulawy (PL)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- RIKILT Wageningen UR, Wageningen (NL)
- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
- 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)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft. Geschäftsbereich 6 — Labore Landwirtschaft, Nossen (DE)
- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA), Speyer (DE)
- Laboratoire de Rennes (SCL L35), Service Commun des Laboratoires DGCCRF et DGDDI, Rennes (FR)
- Elintarviketurvallisuusvirasto/Livsmedelssäkerhetsverket (Evira), Helsinki/Helsingfors (FI)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)