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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-histidine monohydrochloride monohydrate
produced by fermentation with ***Escherichia coli*** **NITE SD 00329**
(FAD-2020-0087; CRL/200072)

European Union Reference Laboratory
Feed Additives

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Dossier related to:	**FAD-2020-0087 - CRL/200072**
Name of Product:	***L-histidine monohydrochloride monohydrate produced by fermentation with Escherichia coli* NITE SD 00329**
Active Agent (s):	**L-histidine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**María José González de la Huebra**
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Report approved by: Date:	**Christoph von Holst** **23/06/2021**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *L-histidine monohydrochloride monohydrate* produced by fermentation with *Escherichia coli* NITE SD 00329, under the categories/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. The authorisation is sought for all animal species.

According to the Applicant, *L-histidine monohydrochloride monohydrate* has a minimum purity (mass fraction) of 98 %. The *feed additive* is intended to be added directly into *feedingstuffs* or through *premixtures* and complementary feed. The Applicant did not propose any minimum or maximum content of *L-histidine monohydrochloride monohydrate* in *feedingstuffs* when used as nutritional additive, however a typical inclusion level of 5 mg/kg *feedingstuffs* and a maximum recommended level of 25 mg/kg *feedingstuffs* were proposed by the Applicant when used as a sensory additive.

For the quantification of *L-histidine monohydrochloride monohydrate* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant proposed the ring-trial validated European Union (EU) method for the determination of amino acids in feed (including *histidine*). This method is dedicated for the quantification of *free* (synthetic and natural) and *total* (peptide-bound and free) amino acids in *premixtures* and *feedingstuffs*, using ion-exchange chromatography (IEC) coupled with post-column derivatisation and optical (visible - VIS) detection. The method does not distinguish between the amino acids and their salts, or between different salts of the same amino acids, and it cannot differentiate between enantiomers. This method was further ring-trial validated by 23 laboratories, resulting in the EN ISO 13903 method. The following performance characteristics were reported for the quantification of total *histidine*: a relative standard deviation for *repeatability* (RSD_r) ranging from 2.4 to 7.0 % and a relative standard deviation for *reproducibility* (RSD_R) ranging from 13 to 23 %.

The extension of scope of the European Union (EU) method to the *feed additive* has been demonstrated by the Applicant in the frame of the stability studies of another similar product of *L-histidine monohydrochloride monohydrate* produced by fermentation with a different *Escherichia coli* strain.

In addition, the EURL is aware of the ring-trial validated method EN ISO 17180 dedicated for the determination of lysine, methionine and threonine in commercial amino acid products and *premixtures*. This standard method is based on the experimental protocol described in the ring-trial validated EU method for the determination of the mentioned amino acids in feed. The EN ISO 17180 method does not distinguish between the amino acids and their salts, or

between different salts of the same amino acids, and it cannot differentiate between enantiomers. The method is applicable for products and *premixtures* containing more than 10 % in mass fraction of the amino acid. The extension of the scope of the EN ISO 17180 method for the determination of *histidine* in products has also been demonstrated in the frame of recent dossiers.

Furthermore, in the frame of previous *L-histidine monohydrochloride monohydrate* dossiers the EURL has evaluated and recommended a single-laboratory validated and further verified high performance liquid chromatography coupled with spectrophotometric detection (HPLC-UV) for the determination of *histidine* in another almost identical *feed additive*.

In a recent *L-histidine monohydrochloride monohydrate* authorisation a maximum limit has been set for histamine, an impurity of *L-histidine monohydrochloride monohydrate*. Consequently, the EURL has evaluated and recommended for official control a method based on high performance liquid chromatography coupled with a spectrophotometric detection (HPLC-UV) for the quantification of histamine in the *feed additive.*

Based on the performance characteristics available, the EURL recommends for official control: (i) the methods based on ion-exchange chromatography coupled with optical (visible or fluorescence) detection (IEC-VIS/FLD or IEC-VIS) or the single-laboratory validated and further verified method based on HPLC-UV for the quantification of *histidine* in the *feed additive*; (ii) the European Union (EU) method based on IEC-VIS for the quantification of *histidine* in *premixtures* and *feedingstuffs* (for *feedingstuffs* only when used as nutritional additive); and (iii) the method based on high performance liquid chromatography coupled with spectrophotometric detection (HPLC-UV) for the quantification of *histamine* in the *feed additive*.

The EURL is unable to recommend the EU method for the official control of this product in *feedingstuffs* when intended to be used as flavouring compound, due to the facts that its use as flavouring compound typically implies very low inclusion levels in *feedingstuffs* (e.g. 25 mg/kg) and a limit of quantification of this method was not established for *histidine.*

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 by fermentation with *Escherichia coli* NITE SD 00329, *histidine*, nutritional additives, sensory additives, amino acids, all animal species.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (an authorisation of a new feed additive) for *L-histidine monohydrochloride monohydrate* produced by fermentation with *Escherichia coli* NITE SD 00329, under the categories/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 [1]. The authorisation is sought for all animal species [1,2]. *L-histidine monohydrochloride monohydrate feed additives* produced by fermentation with *Escherichia Coli* strains are already authorised as by Commission Regulations (EC) No 2020/2116 and (EC) No 2020/1090 [3,4].

According to the Applicant, *L-histidine monohydrochloride monohydrate* has a minimum purity (mass fraction) of 98 % [5].

The *feed additive* is produced by fermentation with a genetically modified strain of *Escherichia coli* NITE SD 00329 [6]. The production strain is deposited in the "Biological Resource Centre of the Japanese National Institute of Technology and Evaluation" (NBRC) under accession number NITE SD 00329 [7].

The *feed additive* is intended to be added directly into *feedingstuffs* or through *premixtures* and complementary feed [8]. The Applicant did not propose any minimum or maximum content of *L-histidine monohydrochloride monohydrate* in *feedingstuffs* when used as nutritional additive, however a typical inclusion level of 5 mg/kg *feedingstuffs* and a maximum recommended level of 25 mg/kg *feedingstuffs* were proposed by the Applicant when used as sensory additive [8].

Note: The EURL has previously evaluated analytical methods for the determination of *L-histidine monohydrochloride monohydrate* in the frame of several dossiers [9].

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 produced by fermentation with strain Escherichia coli* NITE SD 00329 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 *histidine* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant proposed the ring-trial validated European Union (EU) method for the determination of amino acids in feed (including *histidine*) [10]. This method is dedicated for the quantification of *free* (synthetic and natural) and *total* (peptide-bound and free) amino acids in *premixtures* and *feedingstuffs*, using ion-exchange chromatography (IEC) coupled with post-column derivatisation and optical (VIS) detection. The method does not distinguish between the amino acids and their salts, or between different salts of the same amino acids, and it cannot differentiate between enantiomers. The EU method was ring-trial validated using four different matrices for threonine, cyst(e)ine, methionine and lysine [10].

Following the EU method, *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 after post-column derivatisation with ninhydrin by optical detection at 570 nm. The procedure chosen for the determination of the *total* amino acids depends on the amino acids under investigation. *Histidine* can be determined in either oxidised or non-oxidised 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 non-oxidised 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 after post-column derivatisation with ninhydrin by optical (VIS) detection at 570 nm [10].

This method was further ring-trial validated by 23 laboratories, resulting in the EN ISO 13903:2005 method (*histidine* included) [11]. The performance characteristics reported for the quantification of total *histidine* are listed in Table 1.

The Applicant applied, in the frame of the stability studies [12], an equivalent method described in the Japanese Pharmacopoeia and harmonised with the European and the US Pharmacopoeias to *feed additive* and *premixture* samples of *L-histidine monohydrochloride monohydrate* produced by fermentation with a different *Escherichia coli* strain [12-13], thus demonstrating the extension of the scope of the above mentioned EU method for the determination of *histidine* in the *feed additive* and *premixtures*.

Table 1: Method performance characteristics obtained in the frame of EN ISO 13903:2005 [11] for the determination of total *histidine* in *feedingstuffs*.

Ring-Trial	Matrix	*histidine* content g/kg	RSD_r %	RSD_R %
[15]	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.

In addition, the EURL is aware of the ring-trial validated method EN ISO 17180 dedicated for the determination of lysine, methionine and threonine in commercial amino acid products and *premixtures* [14]. This standard method is based on the experimental protocol described in the ring-trial validated EU method for the determination of the mentioned amino acids in feed [10]. The EN ISO 17180 method does not distinguish between the amino acids and their salts, or between different salts of the same amino acids, and it cannot differentiate between enantiomers. The method is applicable for products and *premixtures* containing more than 10 % in mass fraction of the amino acid. The extension of the scope of the EN ISO 17180 method for the determination of *histidine* in products has been demonstrated in the frame of recent dossiers [9].

Following the EN ISO 17180 method, *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 IEC. *Histidine* is quantified (i) after post-column derivatisation with ninhydrine by visible (VIS) detection at 440 nm and 570 nm or (ii) after post-column reaction with ortho-phthaldialdehyde by fluorescence detection (FLD) at an excitation wavelength of 330 nm and an emission wavelength of 460 nm [14].

Furthermore, in the frame of previous *L-histidine monohydrochloride monohydrate* dossiers [9] the EURL has evaluated the suitability of a single-laboratory validated and further verified method based on high performance liquid chromatography coupled with spectrophotometric detection (HPLC-UV) for the determination of *histidine* in another almost identical *feed additive*. This HPLC-UV method has been included in the corresponding Commission Implementing Regulations [3-4].

The EURL is unable to recommend the European Union method for the official control of this product in *feedingstuffs* when intended to be used as sensory additive/flavouring compound, due to the facts that its use as flavouring compound typically implies very low inclusion levels in *feedingstuffs* (e.g. 25 mg/kg) and a limit of quantification of this method was not established for *histidine.*

Based on the performance characteristics available, the EURL recommends for official control: (i) the methods based on ion-exchange chromatography coupled with optical (visible or fluorescence) detection (IEC-VIS/FLD or IEC-VIS) or the single-laboratory validated and further verified method based on high performance liquid chromatography method coupled with spectrophotometric detection (HPLC-UV) for the quantification of *histidine* in the *feed additive*; and (ii) European Union (EU) method based on IEC-VIS for the quantification of *histidine* in *premixtures* and *feedingstuffs* (for *feedingstuffs* only when used as nutritional additive).

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)

In the frame of a recent *L-histidine monohydrochloride monohydrate* authorisation [3] a maximum limit has been set for histamine, an impurity of *L-histidine monohydrochloride monohydrate.* Consequently, the EURL, upon request of DG SANTE, has evaluated and recommended for official control a method based on high performance liquid chromatography coupled with a spectrophotometric detection (HPLC-UV) for the quantification of *histamine* in the *feed additive* [15]. The EURL considers that this recommendation is valid also in frame of the current application.

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 methods based on ion-exchange chromatography coupled with optical (visible or fluorescence) detection (IEC-VIS/FLD or IEC-VIS) or the single-validated and further verified method based on high performance liquid chromatography coupled with spectrophotometric detection (HPLC-UV) for the quantification of *histidine* in the *feed additive*; (ii) the European Union (EU) method based on IEC-VIS for the quantification of *histidine* in *premixtures* and *feedingstuffs* (for *feedingstuffs* only when used as nutritional additive); and (iii) the method based on high performance liquid chromatography coupled with spectrophotometric detection (HPLC-UV) for the quantification of *histamine* in the *feed additive*.

Recommended text for the register entry (analytical method)

For the quantification of *histidine* in the *feed additive:*

- Ion-exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS/FLD) or
- Ion-exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS) or
- High performance liquid chromatography coupled with spectrophotometric detection (HPLC-UV)

For the quantification of *histidine* in *premixtures* and *feedingstuffs* (for *feedingstuffs* only when used as nutritional additive):

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

For the quantification of histamine in the *feed additive*:

- High performance liquid chromatography coupled with spectrophotometric detection (HPLC-UV)

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* produced by *Escherichia coli* NITE SD 00329 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, Reference SANTE/E5: Forw. Appl. 1831/0080-2020

[2] *Application, Annex I – Submission number 1604482326296-2722

[3] Commision Implementing Regulation (EU) 2020/2116 of 16 December 2020 concerning the renewal of the authorisation of L-histidine monohydrochloride monohydrate produced by Escherichia coli ATCC 9637 as a feed additive for salmonids and its extension of use to other finfish, and repealing Regulation (EC) No 244/2007

[4] Commision Implementing Regulation (EU) 2020/1090 of 24 July 2020 concerning the authorisation of L-histidine monohydrochloride monohydrate as a feed additive for all animal species

[5] *Technical dossier, Section II – II.1 Identity of the additive

[6] *Technical dossier, Section II – II.2 Characterisation of the active substance(s)/agent(s)

[7] *Technical dossier, Section II – Annex II.1

[8] *Technical dossier, Section II – II.5 Conditions of use of the additive

[9] EURL evaluation reports :
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2020-0016-histidine.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2018-0070_l-histidine.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2018-0040-histidine.pdf
https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2018-0013_histidinehcl.pdf

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

[12] *Technical dossier, Section II – Annex II.47

[13] *Technical dossier, Section II – Annex II.51

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

[15] EURL evaluation report:
https://ec.europa.eu/jrc/sites/default/files/ammended-updated-fad-2006-0022-histidine.pdf

*Refers to Dossier no: FAD-2020-0087

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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- Państwowy Instytut Weterynaryjny, Pulawy (PL)
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- 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)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural**.** Generalitat de Catalunya, Cabrils (ES)
- Wageningen Food Safety Research[1] (WFSR) (NL))

[1] Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: RIKILT Wageningen UR, Wageningen.