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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-isoleucine
produced by fermentation with Corynebacterium glutamicum KCCM 80189
(FAD-2019-0022; CRL/180083)

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-2019-0022 - CRL/180083**
Name of Product:	***L-isoleucine produced by fermentation with Corynebacterium glutamicum KCCM 80189***
Active Agent:	**L-isoleucine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Zigmas Ezerskis** **04/10/2019**
Report approved by: Date:	**Christoph von Holst** **04/10/2019**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-isoleucine produced by fermentation with Corynebacterium glutamicum KCCM 80189*, under the category/functional group 3(c) 'nutritional additives'/'amino acids, their salts and analogues', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for all animal species.

According to the Applicant *L-isoleucine* has a minimum purity (mass fraction) of 90 %. The *feed additive* is intended to be added directly into *feedingstuffs* (or through *premixtures*) and *water* for drinking. However, the Applicant did not propose any minimum or maximum content of *L-isoleucine* in *feedingstuffs*.

For the quantification of *L-isoleucine* in the *feed additive*, *premixtures* and *fedingstuffs* the Applicant submitted an in-house validated analytical method based on high performance liquid chromatography coupled to fluorescence detection (HPLC-FLD). The Applicant did not present a verification study and therefore, the EURL cannot recommend the method for official control purposes.

For the quantification of *isoleucine* in feed a ring-trial validated European Union (EU) method (Commission Regulation (EC) No 152/2009) based on ion exchange chromatography (IEC) coupled to photometric detection exists (IEC-VIS). This method, designed 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 resulting in the EN ISO 13903:2005 method. The following performance characteristics were reported for the quantification of total *isoleucine*: relative standard deviation for repeatability (RSD_r) ranging from 2.0 to 5.4 % and relative standard deviation for reproducibility (RSD_R) ranging from 6.8 to 14.3 %.

The Applicant did not provide experimental data to determine *isoleucine* in *water*. Nevertheless, as concluded in a previous EURL report, the EURL recommends the EU method for official control for the quantification of *isoleucine* in the *feed additive* and *water*.

In addition, the EURL found the "L-isoleucine monograph" of the Food Chemical Codex (FCC) for the identification of *L-isoleucine* in the *feed additive*.

In the frame of this authorisation the EURL recommends for official control (i) the "L-isoleucine monograph" of the Food Chemical Codex (FCC) based on infrared absorption for the identification of *L-isoleucine* in the *feed additive*; and (ii) the European Union (EU) method based on IEC-VIS for the quantification of *isoleucine* in the *feed additive*, *premixtures*, *feedingstuffs* and *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-isoleucine produced by fermentation with Corynebacterium glutamicum KCCM 80189, nutritional additives, amino acids, 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-isoleucine produced by fermentation with Corynebacterium glutamicum KCCM 80189*, under the category/functional group 3(c) 'nutritional additives'/'amino acids, their salts and analogues', according to Annex I of Regulation (EC) No 1831/2003. Authorisation is sought for all animal species [1-2]. *L-isoleucine* produced by fermentation with Escherichia Coli is already authorised as nutritional feed additive under Regulation (EU) 348/2010 [3].

According to the Applicant, the white to yellowish crystalline powdered *L-isoleucine* has a minimum purity (mass fraction) of 90 % [1,4].

The *feed additive* is produced by fermentation with a non-genetically modified strain of *Corynebacterium glutamicum* [5]. The production strain is deposited in the "Korean Centre of Microorganisms" (KCCM) under the accession number KCCM80189 [6].

The *feed additive* is intended to be added directly into *feedingstuffs* (or through *premixtures*) and *water* for drinking [5]. However the Applicant did not propose any minimum or maximum content of *L-isoleucine* in *feedingstuffs* [1,5].

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-isoleucine produced by fermentation with Corynebacterium glutamicum KCCM 80189* 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-isoleucine* in the *feed additive, premixtures* and *feedingsuffs* the Applicant submitted an in-house validated method based on High Performance Liquid Chromatography (HPLC) coupled to fluorescence detection (FLD) [7,8]. The amino acid is quantified after post-column reaction with ortho-phthaldialdehyde with a detector excitation wavelength at 338 nm and emission at 425 nm [9].

In the frame of the validation study, the Applicant reported satisfactory performance characteristics [9]. However, the Applicant did not present a verification study or any additional test performed by a second independent laboratory applying the above mentioned method. Therefore, the EURL cannot conclude on the fitness-for-purpose of the method for official control purposes.

For the quantification of *isoleucine* in feed a ring-trial validated EU method exists [10]. This method was designed for the quantification of free (synthetic and natural) and of total (peptide-bound and free) amino acids in *premixtures* and *feedingstuffs*, using an amino acid analyser or Ion Exchange Chromatography (IEC) coupled to post-column derivatisation and visible detection (VIS). 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. *Isoleucine* can be determined in either oxidised or non-oxidised samples. Oxidation is performed at 0 °C with a performic acid/phenol mixture. The 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 by post-column derivatisation with ninhydrin and photometric detection at 570 nm.

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

Table 1: Method performance characteristics reported in EN ISO 13903:2005 for the determination of total *isoleucine* [11]

Ring-Trial	Matrix	*Isoleucine* content g/kg	RSD_r %	RSD_R %
[11]	Poultry meal	22.4	2.7	11.7
	Broiler finisher feed	7.6	3.2	6.8
	Broiler starter feed	1.0	2.0	10.3
	Corn	2.8	5.4	14.6
	Fishmeal	23.2	2.1	10.3

RSD_r, 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 EU method, based on IEC-VIS to quantify *isoleucine* in *premixtures* and *feedingstuffs*.

The Applicant did not provide experimental data to determine *isoleucine* in *water* [7,8]. Nevertheless, as concluded in a previous EURL report (even if the determination of *isoleucine* in the *feed additive* and *water* is not explicitly stated in the scope of the EU method), the EURL recommends the EU method for official control for the quantification of *isoleucine* in the *feed additive* and *water* [3,10,12].

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)

The 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 found the "L-isoleucine monograph" of the Food Chemical Codex (FCC) where identification is based on infrared absorption [13].

The EURL recommends the Food Chemical Codex for the identification of *L-isoleucine* 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 "L-isoleucine monograph" of the Food Chemical Codex (FCC) based on infrared absorption for the identification of *L-isoleucine* in the *feed additive*; and (ii) the European Union (EU) method based on IEC-VIS for the quantification of *isoleucine* in the *feed additive*, *premixtures*, *feedingstuffs* and *water*.

Recommended text for the register entry (analytical method)

For the identification of *L-isoleucine* in the *feed additive*:

- Food Chemical Codex "L-isoleucine monograph"

For the quantification of *isoleucine* in the *feed additive* and *water*:

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

For the quantification of *isoleucine* in *premixtures* and *feedingstuffs*:

- ion exchange chromatography coupled to 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-isoleucine produced by fermentation with Corynebacterium glutamicum KCCM 80189* 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. 1831/0030-2019 & Annex I – submission number 1553608810597-2388

[3] Commission Regulation (EU) No 348/2010 of 23 April 2010 concerning the authorisation of L-isoleucine as a feed additive for all animal species, O.J. L104/29, 24.4.2010

[4] *Technical dossier, Section II: II.2.1.1. Chemical substances

[5] *Technical dossier, Section II: II.2.2.2. Micro-organisms

[6] *Technical dossier, Section II: II.5.1. Proposed mode of use in animal nutrition

[7] *Technical dossier, Section II: II.6.1. Methods of analysis for the active substance

[8] *Technical dossier, Section II: II.6.3. Methods of the analysis relating to the identity and characterisation of the additive

[9] *Technical dossier, Section II – Annex_II_06_01

[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] FAD-2009-0001, L-isoleucine, Ref. Ares(2009)175417 - 16/07/2009 https://ec.europa.eu/jrc/sites/jrcsh/files/FinRep-FAD-2009-0001.pdf

[13] Food Chemical Codex monograph "L-Isoleucine", FCC 7 (2010), p.544

*Refers to Dossier no: FAD-2019-0022

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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