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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-isoleucine produced by fermentation with *Corynebacterium glutamicum* CGMCC 20437

(FEED-2023-13997; CRL/220072)

European Union Reference Laboratory
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Dossier related to:	**FEED-2023-13997 - CRL/220072**
Name of Product / Feed Additive:	***L-isoleucine produced by fermentation with Corynebacterium glutamicum CGMCC 20437***
Active Agent (s):	**L-isoleucine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Stefano Bellorini** **04/12/2023**
Report approved by: Date:	**Christoph von Holst** **05/12/2023**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4 for *L-isoleucine produced by fermentation with Corynebacterium glutamicum CGMCC 20437*, 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. The authorisation is sought for all animal species.

According to the Applicant, the *feed additive* contains *L-isoleucine* as an active substance with a minimum purity of 93.5 % (w/w, based on dry matter). The *feed additive* is intended to be added directly to *compound feed* or through *premixtures* and into *water*. However, the Applicant did not propose a minimum or maximum *L-isoleucine* content in *compound feed*.

For the determination of *isoleucine* in the *feed additive* and *premixtures* the Applicant proposed the ring-trial validated method EN ISO 17180 dedicated for the determination of free lysine, methionine and threonine in commercial amino acid products and *premixtures*. The method is based on ion-exchange chromatography (IEC) with optical (visible - VIS or fluorescence - FLD) detection. It does not distinguish between the salts of amino acids and cannot differentiate between enantiomers. This method is applicable for products containing more than 10 % of the amino acid.

The above mentioned method has been already evaluated and recommended by the EURL in the frame of the previous dossier (FAD-2019-0035) for the determination of free *isoleucine* in very similar *feed additive* and *premixtures* containing this additive. The EURL considers that this method is also applicable for the determination of *isoleucine* in the *feed additive* and *premixtures* of the current dossier.

Based on the available information, the EURL recommends for official control the ring-trial validated method EN ISO 17180 based on IEC-VIS/FLD for the determination of free *isoleucine* in the *feed additive* and *premixtures*.

For the determination of *isoleucine* in *compound feed* the Applicant proposed the ring-trial validated European Union (EU) method (Commission Regulation (EC) No 152/2009) based on ion-exchange chromatography with optical detection (IEC-VIS). This method is designed for the quantification of free (synthetic and natural) and of total (peptide-bound and free) amino acids in *premixtures* containing less than 10 % of the amino acid and *compound feed*, it does not distinguish between the salts of amino acids and cannot differentiate between enantiomers.

In addition, in the frame of the stability studies of *L-isoleucine* in the *feed additive* in *water* the Applicant provided experimental data demonstrating the applicability of the above mentioned EU method for the determination of *isoleucine* in the *feed additive* and *water*.

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated European Union (EU) method, based on IEC-VIS to determine *isoleucine* in the *feed additive*, *premixtures*, *compound feed* and *water*.

In addition, the EURL recommends "L-isoleucine monograph" of the Food Chemical Codex monograph 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.

KEYWORDS

L-isoleucine produced by fermentation with Corynebacterium glutamicum CGMCC 20437, nutritional additives, amino acids, their salts and analogues, all animal species.

1. BACKGROUND

In the current application an authorisation is sought under Article 4(1) (authorisation of a new feed additive) for *L-isoleucine produced by fermentation with Corynebacterium glutamicum CGMCC 20437*, 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. The authorisation is sought for all animal species [1,2]. *L-isoleucine* produced by fermentation with *Corynebacterium glutamicum* and *Escherichia coli* is already authorised as nutritional feed additive under Commission Implementing Regulation (EU) 2020/1397 [3].

According to the Applicant, the *feed additive* contains *L-isoleucine* as an active substance with a minimum purity of 93.5 % (w/w, based on dry matter) [4]. The *feed additive* is intended to be added directly to *compound feed* or through *premixtures* and into *water*. However, the Applicant did not propose a minimum or maximum *L-isoleucine* content in *compound feed* [5].

Note: The EURL has previously evaluated the analytical methods in the frame of several *L-isoleucine* related dossiers [6].

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 CGMCC 20437* 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 *isoleucine* in the *feed additive* and *premixtures* the Applicant proposed [7] the ring-trial validated method EN ISO 17180:2013 dedicated for the determination of free lysine, methionine and threonine in commercial amino acid products and *premixtures* [8]. The method is based on ion-exchange chromatography (IEC) with optical (visible - VIS or fluorescence - FLD) detection. It does not distinguish between the salts of amino acids and cannot differentiate between enantiomers. It is applicable for products containing more than 10 % of the amino acid [8].

The above mentioned method has been already evaluated and recommended by the EURL in the frame of the previous dossier (FAD-2019-0035) for the determination of free *isoleucine* in very similar *feed additive* and *premixtures* containing this additive [6]. The EURL considers that this method is also applicable for the determination of *isoleucine* in the *feed additive* and *premixtures* of the current dossier.

Based on the available information, the EURL recommends for official control the ring-trial validated method EN ISO 17180:2013 based on IEC-VIS/FLD for the determination of free *isoleucine* in the *feed additive* and *premixtures*.

For the determination of *isoleucine* in *compound feed* the Applicant proposed [10] the ring-trial validated European Union (EU) method [9].

The EU method is designed for the determination of *free* (synthetic and natural) and of *total* (peptide-bound and free) amino acids in *premixtures* containing less than 10 % of the amino acid and *compound feed*, using an amino acid analyser or IEC coupled to post-column derivatisation and optical (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 optical 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 optical detection at 570 nm [9].

The EU method was ring-trial validated using four different matrices. This method was further ring-trial validated by 23 laboratories, resulting in the equivalent EN ISO 13903:2005 method [10]. The performance characteristics reported for the quantification of total *isoleucine* in different animal feedingstuffs when using the EN ISO 13903:2005 method are listed in Table 1.

In addition, limits of quantification (LOQ) ranging between 30 and 350 mg/kg *compound feed* are specified for various amino acids [10].

In addition, in the frame of the stability studies of *L-isoleucine* in the *feed additive* in *water* [11], the Applicant provided experimental data demonstrating the applicability of the above mentioned EU method for the determination of *isoleucine* in the *feed additive* and *water*.

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated European Union (EU) method, based on IEC-VIS to determine *isoleucine* in the *feed additive*, *premixtures*, *compound feed* and *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.

Table 1: Performance characteristics of the ring-trial validated EN ISO 13903:2005 method for the determination of total *isoleucine* in animal feedingstuffs [10].

Matrix	*Isoleucine* content (g/kg)	RSD_r (%)	RSD_R (%)	Reference
Poultry meal	22.4	2.7	11.7	[10]
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.

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

For the identification of *L-isoleucine* in the *feed additive*, the EURL found "*L-isoleucine* monograph" of the Food Chemical Codex (FCC) where different tests (including one based on an optical specific rotation) are used for the identification of *L-isoleucine* [12].

Hence, the EURL recommends the Food Chemical Codex monograph 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 Food Chemical Codex (FCC) "*L-isoleucine* monograph" for the identification of *L-isoleucine* in the *feed additive*; (ii) the ring-trial validated method EN ISO 17180:2013 based on ion-exchange chromatography coupled to optical detection (IEC-VIS/FLD) to determine *isoleucine* in the *feed additive* and *premixtures*; and (iii) the European Union (EU) method based on ion-exchange chromatography coupled to optical detection (IEC-VIS) for the determination of *isoleucine* in the *feed additive*, *premixtures*, *compound feed* 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 determination of *isoleucine* in the *feed additive*:

- Ion-exchange chromatography coupled to post-column derivatisation and optical detection (IEC-VIS/FLD or IEC-VIS)

For the determination of *isoleucine* in *premixtures*:

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

For the determination of *isoleucine* in *compound feed*:

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

For the determination of *isoleucine* in *water*:

– Ion-exchange chromatography coupled to post-column derivatisation and optical 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-isoleucine produced by fermentation with Corynebacterium glutamicum CGMCC 20437* 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] *Forwarding of applications for authorisation of feed additives in accordance with Regulation (EC) No 1831/2003 – E-Submission Food Chain platform: https://webgate.ec.europa.eu/esfc/#/applications/42277 https://open.efsa.europa.eu/questions/EFSA-Q-2023-00207

[2] *Application, Annex 1

[3] Commission Implementing Regulation (EU) 2020/1397 of 5 October 2020 concerning the renewal of the authorisation of L-isoleucine produced by *Escherichia coli* FERM ABP-10641 as a nutritional additive, its extension of use and the authorisation of L-isoleucine produced by *Corynebacterium glutamicum* KCCM 80189 as a feed additive for all animal species, and repealing Regulation (EU) No 348/2010, OJ L 324, 6.10.2020

[4] *Technical dossier, Section II: II.1.3. Qualitative and quantitative composition

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

[6] EURL reports: https://joint-research-centre.ec.europa.eu/publications/fad-2019-0079_en https://joint-research-centre.ec.europa.eu/publications/fad-2019-0035_en https://joint-research-centre.ec.europa.eu/publications/fad-2019-0022_en

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

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

[9] 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

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

[11] *Technical dossier, Section II: II.4.1. Stability

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

*Refers to Dossier no: FEED-2023-13997

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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- Univerza v Ljubljani. Veterinarska fakulteta. Nacionalni veterinarski inštitut. Enota za patologijo prehrane in higieno okolja, Ljubljana (SI)
- Laboratori Agroalimentari, Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. Generalitat de Catalunya, Cabrils (ES)
- [1]Wageningen Food Safety Research (WFSR) (NL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)

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