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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-leucine
produced using strain NITE BP-02351
(FAD-2018-0041; CRL/180022)

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-2018-0041 - CRL/180022**
Name of Product:	***L-leucine*** ***produced using strain NITE BP-02351***
Active Agent:	**L-leucine**
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
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Report checked by: Date:	**Zigmas Ezerskis** **20/11/2018**
Report approved by: Date:	**Christoph von Holst** **20/11/2018**

EXECUTIVE SUMMARY

In the current application authorisation is sought under Article 4(1) for *L-leucine produced using* the bacteria *strain NITE BP-02351*, 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-leucine* has a minimum purity (mass fraction) of 98 %. As nutritional feed additive, *L-leucine* is intended to be added directly into *feedingstuffs* or through *premixtures* and *water* for drinking. As sensory feed additive, *L-leucine* 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-leucine* in *feedingstuffs*.

For the quantification of *leucine* 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 *leucine* in the *feed additive* and *premixtures*. The following performance characteristics were reported: a relative standard deviation for repeatability (RSD_r) ranging from 0.7 to 2.7 %, a relative standard deviation for intermediate precision (RSD_{ip}) ranging from 0.6 to 3.2 % and a recovery rate from 98 to 105 %. In addition, the EURL identified the "L-leucine monograph" of the Food Chemical Codex (FCC) for the identification of *L-leucine* in the *feed additive*.

For the quantification of *L-leucine* in *premixtures* and *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 *leucine*: RSD_r ranging from 1.7 to 2.7 % and RSD_R ranging from 6.3 to 7.6 %. In the frame of the stability studies the Applicant presented experimental data obtained analysing *the feed additive* in *water* according to ISO 13903:2005 thus demonstrating its applicability for the determination of *leucine* in *water*.

In the frame of this authorisation the EURL recommends for official control (i) the "L-leucine monograph" of the Food Chemical Codex (FCC) based on infrared absorption for the identification of *L-leucine* in the *feed additive*; (ii) 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 *leucine* in the *feed additive* and *premixtures* (containing more than 10 % *leucine*); (iii) the ring-trial validated Community method based on IEC-VIS for the quantification of *leucine* in *premixtures* and *feedingstuffs*; and (iv) the ring-trial validated EN ISO 13903:2005 method based on IEC-VIS for the quantification of *leucine* 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) is not considered necessary.

KEYWORDS

L-leucine produced using strain NITE BP-02351, 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-leucine produced using* the bacteria *strain NITE BP-02351*, 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-leucine* as feed flavouring produced by "chemical synthesis or protein hydrolysis" is already authorised as *feed additive* [3].

According to the Applicant, the white crystalline powder *L-leucine* has a minimum purity (mass fraction) of 98 % [1,4,5]. The *feed additive* is produced by fermentation using a genetically modified strain of *Escherichia coli K-12* [6]. The production strain is deposited in the "National Institute of Technology and Evaluation" (NITE) with reference BP-02351. As nutritional feed additive, *leucine* is intended to be added directly into *feedingstuffs* or through *premixtures* and *water* for drinking [7]. As sensory feed additive, *leucine* is intended to be added into *feedingstuffs* and *water* for drinking through flavouring *premixtures* [7]. However the Applicant did not propose any minimum or maximum content of *L-leucine* in *feedingstuffs* [1,7].

Note: The EURL has previously evaluated the analytical methods for the determination of *leucine* as sensory *feed additive* in the frame of the Chemical Defined flavouring Group 34 [8].

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-leucine* 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 *leucine* 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" [9,10]. This standard method is based on the experimental protocol described in the Community method for the determination of amino acids (incl. *leucine*) [11]. It 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 *leucine* [12,13].

Free *leucine* 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 *leucine* 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 0.7 to 2.7 %, a relative standard deviation for intermediate precision (RSD_{ip}) ranging from 0.6 to 3.2 % and a recovery rate from 98 to 105 % [12,13].

For the quantification of *L-leucine* in *premixtures* and *feedingstuffs* the Applicant submitted the ring-trial validated Community method mentioned above [9,11]. 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-leucine* 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 (*leucine* included) [14]. The performance characteristics reported for the quantification of total *L-leucine* are listed in Table 1.

For the quantification of *L-leucine* in *water* the Applicant submitted the ring-trial validated EN ISO 13903:2005 method mentioned above [9,14]. The method, equivalent to the Community method, is intended for *premixtures* and *feedingstuffs* only [14]. For the analysis in *water* minor adaptations of the protocol have been presented [15]. The Applicant did not perform any validation/verification studies to demonstrate the suitability of the slightly modified ISO method for the determination of *leucine* in *water*.

However, in the frame of the stability studies, the Applicant performed the analysis successfully applying the proposed method for the determination of *leucine* in *water* [15,16]. Hence the EURL recommends for official control the ring-trial validated EN ISO 13903:2005 method based on IEC-VIS to quantify *leucine* 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 *leucine* in the *feed additive* and *premixtures* (containing more than 10 % *leucine*); ii) the ring-trial validated Community method, based on IEC-VIS to quantify *leucine* in *premixtures* and *feedingstuffs*; and iii) the ring-trial validated EN ISO 13903:2005 method, based on IEC-VIS to quantify *leucine* in *water*.

Table 1: Method performance characteristics obtained in the frame of EN ISO 13903:2005 [14] for the determination of total *L-leucine* in *feed*.

Ring-Trial	Matrix	*leucine* content g/kg	RSD_r %	RSD_R %
[14]	Poultry meal	4.1	2.5	7.6
	Broiler finisher feed	1.7	2.7	6.3
	Broiler starter feed	2.0	1.7	6.3
	Corn	1.0	1.9	7.0
	Fishmeal	4.1	1.9	6.8

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

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)

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-leucine monograph" of the Food Chemical Codex (FCC) where identification is based on infrared absorption [17].

The EURL recommends for official control the Food Chemical Codex for the identification of *L-leucine* 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-leucine monograph" of the Food Chemical Codex (FCC) based on infrared absorption for the identification of *L-leucine* in the *feed additive*; (ii) 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 *leucine* in the *feed additive* and *premixtures* (containing more than 10 % *leucine*); (iii) the ring-trial validated Community method based on IEC-VIS for the quantification of *leucine* in *premixtures* and *feedingstuffs*; and (iv) the ring-trial validated EN ISO 13903:2005 method based on IEC-VIS for the quantification of *leucine* in *water*.

Recommended text for the register entry (analytical method)

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

- Food Chemical Codex "L-leucine monohydrochloride monograph"

For the quantification of *leucine* in the *feed additive* and *premixtures* (containing more than 10 % *leucine*):

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

For the quantification of *leucine* 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 *leucine* 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-leucine produced using strain NITE BP-02351* 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. 1831-0049-2018 & Annex I – submission number 1529072978554-2240

[3] 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

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

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

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

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

[8] 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

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

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

[11] 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

[12] *Technical dossier, Section II: An_II_53_Raterink_2018_Triskelion_V21163_Validation_Analytical_Meth_6AA_in_CP

[13] *Technical dossier, Section II: An_II_55_Raterink_2018_Triskelion_V21164_Validation _Analytical_Meth_6AA_in_premix

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

[15] *Technical dossier, Section II: An_II_59_'Jurgens et al., 2017_R11616

[16] *Technical dossier, Section II: 2.4.1.3 Stability of L-leucine in water

[17] Food Chemical Codex monograph "L-leucine", FCC 7 (2010), p.577

*Refers to Dossier no: FAD-2018-0041

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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