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JRC F.5/CvH/ZE/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-valine produced by fermentation with *Escherichia coli*
CCTCC M2020321
(FAD-2021-0032; CRL/210030)**



**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-2021-0032 - CRL/210030**

Name of Product: **L-valine produced by fermentation with
Escherichia coli CCTCC M2020321**

Active Agent (s): **L-valine**

Rapporteur Laboratory: **European Union Reference Laboratory for
Feed Additives (EURL-FA)
JRC Geel, Belgium**

Report prepared by: **Zigmas Ezerskis**

Report checked by: **María José González de la Huebra**
Date: **13/07/2021**

Report approved by: **Christoph von Holst**
Date: **14/07/2021**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *L-valine produced by fermentation with Escherichia coli* CCTCC M2020321, under the category/functional groups 3(c) 'nutritional additives/'amino acids, their salts and analogues', according to Annex I of Regulation (EC) No 1831/2003. Specifically, the authorisation is sought for all animal species.

According to the Applicant, *L-valine* has a minimum purity (mass fraction) of 98 %. The *feed additive* is intended to be mixed either in *premixtures* or added directly to *feedingstuffs* or *water* for drinking. However, the Applicant did not propose any minimum or maximum content of *L-valine* in *feedingstuffs*.

For the characterisation of the *feed additive*, the EURL found the "L-valine monograph" of Food Chemical Codex (FCC), where different tests (including the test based on an optical rotation) are used for the identification of *L-valine*.

For the quantification of *valine* in *feedingstuffs* the Applicant submitted the ring-trial validated European Union (EU) method based on ion-exchange chromatography (IEC) coupled with post-column derivatisation and optical (visible – VIS) detection. This method applies for the determination of free (synthetic and natural) and total (peptide-bound and free) amino acids. The EU method is intended for *premixtures* and *feedingstuffs*, it 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 following performance characteristics were reported for the quantification of total *valine* in *feedingstuffs*: a relative standard deviation for *repeatability* (RSD_r) ranging from 1.7 to 3.8 % and a relative standard deviation for *reproducibility* (RSD_R) ranging from 8.8 to 16.1 %.

In addition, the EURL recently evaluated and recommended in the frame of two dossiers (FAD-2019-0072 and FAD-2020-0033) the EU method for the determination of *valine* in very similar *feed additives* and *water*. Furthermore, in the frame of another *L-valine* dossier (FAD-2017-0032), the EURL previously evaluated and recommended VDLUFA official method (4.11.6) or equivalent method based on ion-exchange chromatography coupled with post-column derivatisation and optical (visible or fluorescence) detection (IEC-VIS/FLD) for the quantification of *valine* in *water*. The EURL considers that these recommendations are also valid in the frame of the current application.

In the frame of this authorisation the EURL recommends for official control (i) the "L-valine monograph" of the Food Chemical Codex (FCC) for the identification of *L-valine* in the *feed additive*; (ii) the ring-trial validated European Union method based on IEC-VIS for the quantification of *valine* in the *feed additive*, *premixtures*, *feedingstuffs* and *water*; and (iii) the

analytical method described by VDLUFA (4.11.6) or equivalent method based on IEC-VIS/FLD to quantify *valine* 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-valine produced by fermentation with Escherichia coli CCTCC M2020321, nutritional additives, amino acids, 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-valine produced by fermentation with Escherichia coli* CCTCC M2020321, under the category/functional groups 3(c) 'nutritional additives'/amino acids, their salts and analogues', according to Annex I of Regulation (EC) No 1831/2003 [1,2]. Specifically, the authorisation is sought for all animal species [2].

L-valine, produced by different microorganisms, is already authorised as nutritional *feed additive* under Commission Implementing Regulations, namely (EU) 2015/1114 [3], (EU) 2019/1289 [4], (EU) 2020/1797 [5] and (EU) 2021/719 [6].

According to the Applicant, *L-valine* has a minimum purity (mass fraction) of 98 % [2,7]. The *feed additive* is produced by fermentation using a genetically modified strain of *Escherichia coli* CCTCC M2020321 [8].

The *feed additive* is intended to be mixed either in *premixtures* or added directly to *feedingstuffs* or *water* for drinking [9]. However, the Applicant did not propose any minimum or maximum content of *L-valine* in *feedingstuffs* [2,9].

Note: The EURL has previously evaluated the analytical methods for the determination of *L-valine* in the frame of several dossiers [10-19].

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-valine produced by fermentation with Escherichia coli* CCTCC M2020321 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 *valine* in the *feed additive* and *premixtures* the Applicant proposed [20] the EN ISO 17180 method without submitting corresponding experimental proofs of the extension of scope, while for the quantification of *valine* in *feedingstuffs* the Applicant submitted the ring-trial validated European Union (EU) method [21].

The EU method applies for the determination of free (synthetic and natural) and total (peptide-bound and free) amino acids, using an amino acid analyser or High Performance Liquid Chromatography equipment provided with an ion-exchange column (IEC). The method is intended for *premixtures* and *feedingstuffs*, it 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 [21].

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 ion-exchange chromatography (IEC) and determined by post-column derivatisation with ninhydrin and optical (VIS) detection at 570 nm. The procedure chosen for the determination of the *total* amino acids depends on the amino acids under investigation. *Valine* 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 h. 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 [21].

The EU method was ring-trial validated using four different matrices for threonine, cyst(e)ine, methionine and lysine only [21]. This method was further ring-trial validated by 23 laboratories, resulting in the EN ISO 13903 method (*valine* included) [22]. The performance characteristics reported for the quantification of total *valine* in feed are listed in Table 1.

Table 1: Method performance characteristics obtained in the frame of the ring-trial validation of the EN ISO 13903 method for the quantification of total *valine* in *feedingstuffs*

Ring-Trial	Matrix	<i>valine</i> content g/kg	RSD _r %	RSD _R %
[22]	Poultry meal	28.2	3.2	12.8
	Broiler finisher feed	9.2	3.8	12.7
	Broiler starter feed	11.1	1.7	8.8
	Corn	3.8	2.4	16.1
	Fishmeal	27.8	2.3	11.2

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

In addition, the EURL recently evaluated and recommended in the frame of two dossiers [18,19] the EU method for the determination of *valine* in very similar *feed additives* and *water*. Furthermore, in the frame of another *L-valine* dossier [16] the EURL previously evaluated and recommended VDLUFA official method (4.11.6) [23] or equivalent method based on ion-exchange chromatography coupled with post-column derivatisation and optical (visible or fluorescence) detection (IEC-VIS/FLD) for the quantification of *valine* in *water*. The EURL considers that these recommendations are also valid in the frame of the current application.

Based on the available data, the EURL recommends for official control (i) the ring-trial validated European Union method based on IEC-VIS to quantify *valine* in the *feed additive*, *premixtures*, *feedingstuffs* and *water*; and (ii) the analytical method described by VDLUFA (4.11.6) or equivalent method based on IEC-VIS/FLD to quantify *valine* 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)

The EURL found the "L-valine monograph" of Food Chemical Codex (FCC), where different tests (including the test based on an optical rotation) are used for the identification of *L-valine* in the *feed additive* [24]. The EURL recommends for official control the approach of the Food Chemical Codex for the identification of *L-valine* 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-valine monograph" of the Food Chemical Codex (FCC) for the identification of *L-valine* in the *feed additive*; (ii) the European Union method based on IEC-VIS for the quantification of *valine* in the *feed additive, premixtures, feedingstuffs* and *water*; and (iii) the analytical method described by VDLUFA (4.11.6) or equivalent method based on IEC-VIS/FLD to quantify *valine* in *water*.

Recommended text for the register entry (analytical method)

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

- Food Chemical Codex "L-valine monograph"

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

- Ion-exchange chromatography coupled with post-column derivatisation and optical detection (IEC-VIS)

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

- 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 *valine* in *water*:

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

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *L-valine produced by fermentation with Escherichia coli* CCTCC M2020321 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

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*Refers to Dossier no: FAD-2021-0032

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

² Name and address according to according COMMISSION IMPLEMENTING REGULATION (EU) 2015/1761: Elintarviketurvallisuusvirasto/Livsmedelssäkerhetsverket (Evira), Helsinki/Helsingfors.