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Safety and efficacy of L-valine produced by fermentation using *Corynebacterium glutamicum* CGMCC 7.358 as a feed additive for all animal species

EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), Vasileios Bampidis, Giovanna Azimonti, Maria de Lourdes Bastos, Henrik Christensen, Birgit Dusemund, Mojca Fašmon Durjava, Maryline Kouba, Marta López-Alonso, Secundino López Puente, Francesca Marcon, Baltasar Mayo, Alena Pechová, Mariana Petkova, Fernando Ramos, Yolanda Sanz, Roberto Edoardo Villa, Ruud Woutersen, Pier Sandro Cocconcelli, Boet Glandorf, Lieve Herman, Miguel Prieto Maradona, Maria Saarela, Montserrat Anguita, Orsolya Holczknecht, Jaume Galobart, Paola Manini, Elisa Pettenati, Fabiola Pizzo and Jordi Tarrés-Call

Abstract

Following a request from the European Commission, the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) was asked to deliver a scientific opinion on L-valine produced by fermentation using a non-genetically modified strain of *Corynebacterium glutamicum* (CGMCC 7.358). The additive is intended to be used in feed and water for drinking for all animal species and categories. The production strain meets the qualifications for the qualified presumption of safety (QPS) approach to safety assessment and is considered safe. L-Valine produced using *C. glutamicum* CGMCC 7.358 is safe for the target species when supplemented in appropriate amounts to the diet according to the nutritional needs of the target species, for the consumer and for the environment. No conclusion could be drawn on the potential of L-valine produced using *C. glutamicum* CGMCC 7.358 to be toxic by inhalation, irritant to the skin or eyes, or a dermal sensitiser due to the lack of data. The product L-valine produced by fermentation using *C. glutamicum* CGMCC 7.358 is regarded as an efficacious source of the essential amino acid L-valine for non-ruminant nutrition. For the supplemental L-valine to be as efficacious in ruminants as in non-ruminant species, it requires protection against degradation in the rumen.

Keywords: nutritional additive, amino acids, L-valine, *C. glutamicum* CGMCC 7.358, safety, efficacy

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Table of contents

1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

Regulation (EC) No 1831/2003[1] establishes the rules governing the Community authorisation of additives for use in animal nutrition. In particular, Article 4(1) of that Regulation lays down that any person seeking authorisation for a feed additive or for a new use of a feed additive shall submit an application in accordance with Article 7.

The European Commission received a request from Welding GmbH[2] for authorisation of the product L-valine, when used as a feed additive for all animal species (category: nutritional additives; functional group: amino acids, their salts and analogues).

According to Article 7(1) of Regulation (EC) No 1831/2003, the Commission forwarded the application to the European Food Safety Authority (EFSA) as an application under Article 4(1) (authorisation of a feed additive or new use of a feed additive). The particulars and documents in support of the application were considered valid by EFSA as of 29 January 2020.

According to Article 8 of Regulation (EC) No 1831/2003, EFSA, after verifying the particulars and documents submitted by the applicant, shall undertake an assessment in order to determine whether the feed additive complies with the conditions laid down in Article 5. EFSA shall deliver an opinion on the safety for the target animals, consumer, user and the environment and on the efficacy of the product L-valine (≥ 98.0%), produced by fermentation using *Corynebacterium glutamicum* CGMCC 7.358 for all animal species, when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

L-Valine (minimum 98.0%) produced by fermentation using *C. glutamicum* CGMCC 7.358 has not been previously assessed as a feed additive in the European Union. L-Valine produced by different microbial strains is authorised as a feed additive for all animal species.[3]

The FEEDAP Panel has issued several scientific opinions on the safety and efficacy of L-valine produced by fermentation using different strains of *C. glutamicum* or *Echerichia coli* (EFSA 2008a,b; EFSA FEEDAP Panel, 2013, 2014a, 2015a,b, 2019a,b, 2020) or when used as a feed flavouring compound (EFSA FEEDAP Panel, 2014b).

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier[4] in support of the authorisation request for the use of L-valine (minimum 98.0%) produced by fermentation using *C. glutamicum* CGMCC 7.358, as additive in feed and water for drinking for all animal species.

The FEEDAP Panel used the data provided by the applicant together with data from other sources, such as previous risk assessments by EFSA or other expert bodies to deliver the present output.

EFSA has verified the European Union Reference Laboratory (EURL) report as it relates to the methods used for the control of the L-valine produced by fermentation with *C. glutamicum* CGMCC 7.358 in animal feed. The Executive Summary of the EURL report can be found in Annex A.[5]

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of the additive under assessment is in line with the principles laid down in Regulation (EC) No 429/2008 and the

[1] Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.
[2] Welding GmbH, Esplanade 39, 20354, Hamburg (Germany).
[3] Regulation (EC) No 403/2009 of 14 May 2009 /Amended by Commission Implementing Regulation (EU) No 848/2014 of 4 August 2014/Amended by Commission Implementing Regulation (EU) No 2015/1114 of 9 July 2015; Commission Implementing Regulation (EU) No 848/2014 of 4 August 20144/Amended by Commission Implementing Regulation (EU) No 2015/1114 of 9 July 2015; Commission Implementing Regulation (EU) No 1236/2014 of 18 November 2014/Amended by Commission Implementing Regulation (EU) No 2015/1114 of 9 July 2015; Commission Implementing Regulation (EU) No 2015/1114 of 9 July 2015; and Commission Implementing Regulation (EU) 2019/1289 of 31 July 2019.
[4] FEED dossier reference: FAD-2019-0072.
[5] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2019-0072-valine.pdf

relevant guidance documents: Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017a), Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a), Guidance on the assessment of the safety of feed additives for the target species (EFSA FEEDAP Panel, 2017b), Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c), Guidance on studies concerning the safety of use of feed additives for user/workers (EFSA FEEDAP Panel, 2012), Guidance on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018b) and Guidance on the assessment of the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019c).

3. Assessment

L-Valine (minimum 98.0%) produced by *C. glutamicum* CGMCC 7.358 is proposed as a nutritional feed additive (functional group: amino acids, their salts and analogues) in feed for all animal species.

3.1. Characterisation

3.1.1. Characterisation of the production organism

L-Valine is produced by a non-genetically modified strain of *C. glutamicum* which is deposited in the China General Microbiological Culture Collection Center with accession number CGMCC 7.358.[6]

The identity of the production strain was confirmed [redacted]

The production strain is derived from [redacted]

The WGS of the production strain *C. glutamicum* CGMCC 7.358 was aligned with the genome of [redacted]

The production strain was tested for its susceptibility to all the antimicrobials listed for '*Corynebacterium* and other Gram-positive' bacteria in the Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a).[10] All minimum inhibitory concentration (MIC) values were below the corresponding cut off values. Thus, the strain is considered phenotypically susceptible to those antibiotics.

The WGS of the production strain was interrogated for the presence of antimicrobial resistance (AMR) genes [redacted] No hits were identified.

3.1.2. Manufacturing process

L-Valine is produced by fermentation using *C. glutamicum* CGMCC 7.358. [redacted]

3.1.3. Characterisation of the active substance/additive

L-Valine (International Union of Pure and Applied Chemistry (IUPAC)) name: (2*S*)-2-amino-3-methylbutanoic acid; synonyms: α-amino isovaleric acid, 2-amino-3-methylbutyric acid), a compound

[6] Technical dossier/Section II/Annex 2.2.1.2a and supplementary information July 2020/FAD-2019-0072 SIN 110320 Answers 2000707, answer to Q1.

[redacted]

[10] Technical dossier/Supplementary information July 2020/Annex 2.

[redacted]

identified by Chemical Abstracts Service (CAS) No 72-18-4 and European Inventory of Existing Commercial Chemical Substances (EINECS) No 200-773-6, has a molecular weight of 117.15 g/mol. The molecular formula is $C_5H_{11}NO_2$ and its molecular structure is given in Figure 1.

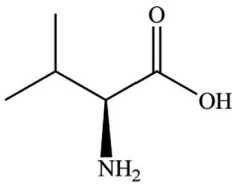

Figure 1: Molecular structure of L-valine

The additive is specified to contain ≥ 98% L-valine on dry matter basis and ≤ 0.5% moisture.[14]

The analysis of five batches showed an average of 99.0% valine on a dry matter basis (range 98.8–99.2%) and a loss on drying of 0.3% (range 0.2–0.3%).[15] The amount of unidentified material was on average 1%.

The specific optical rotation measured in three batches ranged from +27.7 to +28.1° which fall within the range set in the European Pharmacopoeia (+25.6 to +29.0°) and confirm the L-enantiomer of valine.[16]

3.1.3.1. Impurities

Five batches of the additive were analysed for heavy metals (cadmium, lead and mercury) and arsenic. All values were below the respective limit of detection (LOD).[17] Polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), dioxin-like polychlorinated biphenyls (DL-PCBs) and non-dioxin-like PCBs were found below the LOD in the three batches analysed. The sum of PCDD/Fs calculated according to Regulation EU 2017/644 was 0.121 ng TEQ/kg (upper bound 137 ng/kg for wet weight), the sum of DL-PCBs 0.117 ng TEQ/kg (upper bound 132 ng/kg for wet weight), and the sum of dioxin and DL-PCBs 0.237 ng TEQ/kg (upper bound 269 ng/kg for wet weight).[18] The concentration of mycotoxins, aflatoxins (not specified), ochratoxin A, zearalenone, fumonisins (B1, B2 and B3), deoxynivalenol (DON) and citrinin in the additive were analysed in three batches of the additive and found below the LOD.[19] The detected amounts of these undesirable substances do not raise safety concerns.

The microbial quality of the additive was analysed in three batches. *Salmonella* spp. was absent in 25-g samples. In the same amount of product, *Escherichia coli*, yeast and moulds and enterobacteria were not detected.[20]

The antimicrobial activity of the additive was tested against *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* ATCC 25922. No antimicrobial activity was detected.[21] The results of the test provided supporting evidence of the absence of antimicrobial activity of *C. glutamicum* CGMCC 7.358.

The presence of viable cells of the production strain in the final additive was investigated in three batches of L-valine, [redacted] No microbial growth was observed.

[14] Technical dossier/Supplementary information July 2020/FAD-2019-0072 SIn 110320 Answers 2000707.
[15] Technical dossier/Section II/Annexes 2.1.3a to e and supplementary information July 2020/FAD-2019-0072 SIn 110320 Answers 2000707 and Annex 4. Valine was analysed following the EU official method (Regulation (EU) 152/2009).
[16] Technical dossier/Section II/Annex 2.1.4e.
[17] Technical dossier/Section II/Annex 2.1.4a. LOD in mg/kg were 0.01 for arsenic and lead, and 0.002 for cadmium and mercury. The procedure is not accredited.
[18] Technical dossier/Section II/Annex 2.1.4b to d.
[19] Technical dossier/Section II/Annex 2.1.4a. LOD in μg/kg were 0.05 for aflatoxins, 5 for ochratoxin A, 17 for zearalenone, 25 for the sum of the three fumonisins, 134 for DON and 15 for citrinin.
[20] Technical dossier/Section II/Annex 2.1.4a and supplementary information July 2020/FAD-2019-0072 SIn 110320 Answers. Microbiological methods according to VDLUFA. LOD of 1 colony forming unit/g.
[21] Technical dossier/Section II/Annex 2.2.1.2e.
[redacted]

3.1.3.2. Physical-chemical properties

The additive is a white crystalline powder with a solubility of 85 g/L in water (at 25°C), and a bulk density of 575 kg/m^3.[23]

The dusting potential was analysed (Stauber–Heubach method) in three batches of the final product. The values ranged from 0.6 to 1.0 g/m^3.[24]

The particle size distribution of three batches of the final product was measured by laser diffraction. In all cases 100% of the particles had a diameter < 100 μm. The fractions of particles with diameters < 50 and < 10 μm ranged 58–60% and 3–5%, respectively.[25]

3.1.3.3. Stability and homogeneity

The shelf life of the additive (three batches) was tested at 25°C and 40°C when stored in typical packaging paper bags protected from light for two years.[26] No losses were observed.

The stability of the additive (three batches) in a vitamin–mineral premixture containing choline chloride (25,000 mg/kg) was studied when supplemented at an inclusion rate of 0.12%.[27] The samples were stored in paper bags at 25°C and 60% relative humidity (RH) for 6 months. Only one batch showed a loss of 7.7%.

The stability of the additive (nine batches) was studied in a complete feed for pigs for fattening, another for sows and a third one for laying hens (three different batches of additive were used for each species) when supplemented at 0.16, 0.34 and 0.06%, respectively.[28] The basal diet of the complete feed for pigs for fattening consisted on maize and soybean meal; that for sows on maize, soybean meal and peanut meal; and that for laying hens on maize, soybean meal and meat and bone meal.[29] Mash and pelleted feeds were tested after storage at 20–25°C and 40–60% RH in paper bags for 3 months. Pelleting temperature was about 80°C. Pelleting of the mash feeds led to loses up to 6% in both the feed for pigs for fattening and sows, and up to 2% in the feed for laying hens. After 3 months storage of the mash and pelleted feeds, no losses were observed in the feed for pigs for fattening and losses up to 2% were observed in the other two feeds.

The stability of the additive (three batches) in water for drinking was studied at a concentration of 500 mg/L (0.05%) when stored at 20°C in opened plastic containers for 48 h.[30] Only a loss of 2% was detected in one of the batches.

One of the pelleted feeds for pig for fattening described above was used to study the capacity of the additive to distribute homogeneously in feed.[31] Free valine was analysed in 10 subsamples. The coefficient of variation was 3%.

3.1.4. Conditions of use

L-valine is intended to be used in feeds for all animal species to achieve an adequate amino acid profile and to meet the L-valine requirements. It can be added directly to complete feed or complementary feedingstuffs, or via a premixture. No inclusion levels have been proposed, as the requirements, in quantitative terms, depend on the species, the physiological state of the animal, the performance level, the environmental conditions and the amino acid composition of the unsupplemented diet. The applicant proposes also the use of the additive in water for drinking but specifies that care should be taken to avoid amino acids imbalances in target species by supplementation through feed and water for drinking at the same time.

[23] Technical dossier/Section II/Annexes 2.1.5d to f.
[24] Technical dossier/Section II/Subsection 2.1.5.
[25] Technical dossier/Section II/Annexes 2.1.5a1 to c2.
[26] Technical dossier/Section II/Annexes 2.4.1a to f.
[27] Technical dossier/Section II Annex 2.4.1h and premixture composition in Annex 2.4.1g.
[28] Technical dossier/Section II/Annexes 2.4.2j to l and supplementary information July 2020/FAD-2019-0072 SIn 110320 Answers.
[29] Technical dossier/Section II/Annex 2.4.1i.
[30] Technical dossier/Section II/Annexes 2.4.1m and n.
[31] Technical dossier/Section II/Annexes 2.4.3o and 2.4.1i.

3.2. Safety

3.2.1. Safety for the target species, the consumer and the environment

L-Valine requirements of different species (non-ruminant and ruminant) and animal categories, absorption and metabolic fate of L-valine, and tolerance to L-valine excess in the diet were described in previous opinions (EFSA FEEDAP Panel, 2013, 2014a).

The additive contains > 98% L-valine in a dry matter basis and it is highly purified. Safety concerns from the additive may derive from the residues of the fermentation process/production strain remaining in the final product. The production strain belongs to a species, *C. glutamicum,* that qualifies for the qualified presumption of safety (QPS) approach to safety assessment (EFSA, 2007) when used for production purposes (EFSA BIOHAZ Panel, 2020). The strain was unambiguously identified as *C. glutamicum* and was shown not to harbour acquired antimicrobial resistance determinants for antibiotics of human and veterinary importance. No viable cells of the production strain were found in the final product. Consequently, no safety concerns for the target animals, consumers and the environment would rise from the fermentation residues that may be present in the final additive.

The FEEDAP Panel, in its previous statement (EFSA FEEDAP Panel, 2010), identified hygienic concerns when amino acids are administered in water for drinking.

The amino acid L-valine, supplemented to feed, will be incorporated into proteins of tissues and/or products of animal origin and any of their potential excess will be metabolised and excreted as urea/ uric acid and carbon dioxide. Therefore, the composition of tissues and products of animal origin will not be affected by the use of L-valine in animal nutrition.

The amino acid L-valine is a physiological and natural component of the proteins of living organisms. When consumed, it will be absorbed, and the non-absorbed fraction will be incorporated into the intestinal microbial mass and excreted as such.

Conclusions on the safety for the target species, consumer and the environment

The FEEDAP Panel concludes that L-valine produced using *C. glutamicum* CGMCC 7.358 is safe for the target species when supplemented in appropriate amounts to the diet according to the nutritional needs of the target species, for the consumer and for the environment. The FEEDAP Panel identified hygienic concerns regarding the administration of amino acids via water for drinking.

3.2.2. Safety for the user

The additive has a dusting potential up to 1 g/m^3 and the particle size distribution shows that all particles are < 100 μm diameter. Consequently, the user may be exposed by inhalation.

No studies were provided to support the safety of the additive for the user or workers.[32] In absence of data, the FEEDAP Panel cannot conclude on the potential of L-valine under assessment to be toxic by inhalation, irritant to skin or eyes or to be a dermal sensitiser.

3.3. Efficacy

Efficacy studies are not required for amino acids that occur naturally in plant and animal proteins. The nutritional role of the amino acid L-valine is well established in the scientific literature. The product L-valine produced by fermentation using *C. glutamicum* CGMCC 7.358 is regarded as an efficacious source of the essential amino acid L-valine for non-ruminant nutrition. The Panel indicated in a previous opinion (EFSA FEEDAP Panel, 2013) that ruminant metabolism would reduce the delivery of the amino acid to the abomasum, and therefore, measures to ensure a more efficient delivery should be considered.

3.4. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[33] and Good Manufacturing Practice.

[32] Technical dossier/Supplementary information July 2020/ FAD-2019-0072 SIn 110320 Answers.

[33] Regulation (EC) No 183/2005 of the European Parliament and of the Council of 12 January 2005 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

4. Conclusions

L-Valine produced using *C. glutamicum* CGMCC 7.358 is safe for the target species when supplemented in appropriate amounts to the diet according to the nutritional needs of the target species, for the consumer and for the environment. The FEEDAP Panel identified hygienic concerns regarding the administration of amino acids via water for drinking.

The FEEDAP Panel cannot conclude on the potential of L-valine produced using *C. glutamicum* CGMCC 7.358 to be toxic by inhalation, irritant to the skin or eyes, or a dermal sensitiser due to the lack of data.

The product L-valine produced by fermentation using *C. glutamicum* CGMCC 7.358 is regarded as an efficacious source of the essential amino acid L-valine for non-ruminant nutrition. For the supplemental L-valine to be as efficacious in ruminants as in non-ruminant species, it requires protection against degradation in the rumen.

5. Documentation as provided to EFSA/Chronology

Date	Event
06/11/2019	Dossier received by EFSA. L-Valine produced using *C. glutamicum* CGMCC 7.358 for all animal species. Submitted by Welding GmbH
06/12/2019	Reception mandate from the European Commission
29/01/2020	Application validated by EFSA – Start of the scientific assessment
11/03/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: Characterisation of the additive, characterisation of the production microorganism, safety for the user*
20/04/2020	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
29/04/2020	Comments received from Member States
07/07/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
30/09/2020	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2014a. Scientific Opinion on the safety and efficacy of L-valine (ValAMINO®) produced by *Corynebacterium glutamicum* (DSM 25202) for all animal species, based on a dossier submitted by Evonik Industries AG. EFSA Journal 2014;12(7):3795, 14 pp. https://doi.org/10.2903/j.efsa.2014.3795

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Abbreviations

CAS	Chemical Abstracts Service
CFU	colony forming unit
CV	coefficient of variation
DM	dry matter
EINECS	European Inventory of Existing Commercial Chemical Substances
EURL	European Union Reference Laboratory
FCC	Food Chemical Codex
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
IEC-VIS	ion exchange chromatography coupled with post-column derivatisation and photometric detection
IUPAC	International Union of Pure and Applied Chemistry
LOD	limit of detection
LOQ	limit of quantification
MIC	minimum inhibitory concentration
OECD	Organisation for Economic co-operation and Development
PCB	polychlorinated biphenyl
PCDD/F	polychlorinated dibenzodioxin/dibenzofuran
RH	relative humidity
RSDr	Relative standard deviation for repeatability
RSDR	Relative standard deviation for reproducibility
TEQ	toxic equivalents
WGS	whole genome sequence
WHO	World Health Organisation

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for L-valine produced by fermentation *C. glutamicum* CGMCC 7.358 in animal feed

In the current application an authorisation is sought under Article 4(1) for L-valine produced by fermentation with *Corynebacterium glutamicum* CGMCC 7.358, 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 the Food Chemical Codex (FCC), where identification is based on infrared absorption.

For the quantification of L-valine in the feed additive, premixtures, feedingstuffs and water the Applicant submitted the ring-trial validated European Union method based on ion exchange chromatography coupled with post-column derivatisation and photometric detection (IEC-VIS). The method does not distinguish between the salts of amino acids and it cannot differentiate between enantiomers. The following performance characteristics were reported for the quantification of total valine in feed: a relative standard deviation for repeatability (RSDr) ranging from 1.7 to 3.8% and a relative standard deviation for reproducibility (RSDR) ranging from 8.8 to 16.1%. In addition, in the frame of the batch-to-batch analysis and for the stability studies of L-valine in the feed additive and water, the Applicant presented acceptable experimental data when applying the abovementioned European Union method.

In the frame of this authorisation the EURL recommends for official control (i) the "L-valine monograph" of the Food Chemical Codex (FCC) based on infrared absorption for the identification of L-valine in the feed additive, and (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.

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.