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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-Glutamic acid and Monosodium L-glutamate monohydrate produced using strain Corynebacterium glutamicum NITE BP-01681

(FAD-2020-0047; CRL/190030)

European Union Reference Laboratory
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Dossier related to:	**FAD-2020-0047 - CRL/190030**
Name of Product:	***L-Glutamic acid and Monosodium L-glutamate monohydrate produced using strain Corynebacterium glutamicum NITE BP-01681***
Active Agent (s):	***L-Glutamic acid*** ***Monosodium L-glutamate***
Rapporteur Laboratory:	**European Union Reference Laboratory for Feed Additives (EURL-FA)** **JRC Geel, Belgium**
Report prepared by:	**Zigmas Ezerskis**
Report checked by: Date:	**María José González de la Huebra** **12/02/2021**
Report approved by: Date:	**Christoph von Holst** **12/02/2021**

EXECUTIVE SUMMARY

In the current application an authorisation is sought under Article 4(1) for *L-glutamic acid* and *monosodium L-glutamate monohydrate* produced using the strain *Corynebacterium glutamicum* NITE BP-01681, under the categories/functional groups 2(b) 'sensory additives' / 'flavouring compounds' and 3c '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 additives* (*L-glutamic acid* and *monosodium L-glutamate monohydrate*) are white crystalline powders with a minimum purity (mass fraction) of 98 % (based on anhydrous weight). The *feed additives* are intended to be added directly into *feedingstuffs* or through *premixtures* and *water* for drinking. The Applicant proposed no minimum or maximum content of *L-glutamic acid* and *monosodium L-glutamate monohydrate* in *feedingstuffs* when used as nutritional additives, while inclusion levels of up to 10 g of *L-glutamic acid* and *monosodium L-glutamate monohydrate*/ kg *feedingstuffs* were suggested by the Applicant when used as sensory additives.

For the quantification of *glutamic acid* and *monosodium glutamate* in the *feed additives* and *premixtures* the Applicant submitted the ring-trial validated method EN ISO 17180:2013 dedicated for the determination of lysine, methionine and threonine in commercial amino acid products and premixtures containing more than 10 % of amino acid. The method 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 Applicant presented the results from validation and verification studies demonstrating the extension of scope of the above mentioned method for the determination of *glutamic acid* and *monosodium glutamate* in the *feed additives* and *premixtures*. The following performance characteristics were reported for the determination of *glutamic acid* and *monosodium glutamate* in the *feed additives* and *premixtures*: a relative standard deviation for *repeatability* (RSD_r) ranging from 0.3 to 5.3 %, a relative standard deviation for *intermediate precision* (RSD_{ip}) ranging from 0.3 to 5.6 % and a recovery rate (R_{Rec}) ranging from 97 to 103 %.

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated method EN ISO 17180:2013 based on IEC-VIS/FLD for the quantification of *glutamic acid* and *monosodium glutamate* in the *feed additives* and *premixtures*.

For the quantification of *glutamic acid* and *monosodium glutamate* content in *feedingstuffs* the Applicant submitted the ring-trial validated European Union (EU) method (Commission Regulation (EC) No 152/2009) based on IEC with photometric detection (VIS). This method,

designed for the analysis of amino acids in *premixtures* and *feedingstuffs*, 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 EU method was further ring-trial validated resulting in the EN ISO 13903:2005 method. The following performance characteristics were reported for the quantification of *glutamic acid* in feed at mass fractions ranging from 15.1 to 79.7 g/kg: RSD_r ranging from 0.9 to 2.7 % and a relative standard deviation for *reproducibility* (RSD_R) ranging from 4.7 to 9.1 %. Furthermore, a limit of quantification (LOQ) ranging from 30 to 350 mg/kg has been reported for the analysis of various amino acids, while a specific LOQ for *glutamic acid* (or *monosodium glutamate*) has not been indicated.

In addition, based on NRL's experience and following the previous reports for various amino acids, the EURL recently recommended in the frame of another evaluation report for *monosodium L-glutamate* (FAD-2018-0090) the above mentioned EU method for the quantification of *monosodium glutamate* in the *feed additive*.

Based on the overall available data, the EURL recommends for official control the ring-trial validated EU method, based on IEC-VIS to quantify *glutamic acid* and *monosodium glutamate* in the *feed additives*, *premixtures* and *feedingstuffs* (only when they are intended to be used as nutritional *feed additives*).

Since it is not known what will be the maximum recommended content authorised for *glutamic acid* and *monosodium glutamate* in *feedingstuffs* when they are used as sensory additives / flavouring compounds in the frame of the current dossier, the EURL is unable to recommend the EU method for the official control of *glutamic acid* and *monosodium glutamate* in *feedingstuffs* when they are intended to be used as sensory additives. However, the EU method is at least fit-for-purpose for the quantification of *glutamic acid* and *monosodium glutamate* in *feed* in the validated concentration range.

For the quantification of *glutamic acid* in *water* the Applicant submitted the ring-trial validated EN ISO 13903:2005 method, which is equivalent to above mentioned EU method. This method was successfully applied in the frame of the stability studies of *glutamic acid* in *water*. Hence, the EURL recommends for official control the above mentioned EU method based on IEC-VIS to quantify *glutamic acid* and *monosodium glutamate* in *water*.

For the identification of the *feed additives* the EURL recommends the “L-Glutamic acid” and "Monosodium L-glutamate" monographs of the Food Chemical Codex (FCC).

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-Glutamic acid, *monosodium L-glutamate monohydrate* produced using strain *Corynebacterium glutamicum* NITE BP-01681, sensory additives, flavouring compounds, 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-glutamic acid* and *monosodium L-glutamate monohydrate* produced using the strain *Corynebacterium glutamicum* NITE BP-01681, under the categories/functional groups 2(b) 'sensory additives' / 'flavouring compounds' and 3c '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]. Synthetically produced *glutamic acid* and *monosodium L-glutamate* are already authorised as sensory *feed additives* [3,4].

According to the Applicant, the *feed additives* (*L-glutamic acid* and *monosodium L-glutamate monohydrate*) are white crystalline powders with a minimum purity (mass fraction) of 98 % (based on anhydrous weight) [5,6].

The *feed additives* are produced by fermentation with a genetically modified strain of *Corynebacterium glutamicum* [7,8]. The production strain is deposited in the Japan National Institute of Technology and Evaluation (NITE) with deposition number NITE BP-01681 [9].

The *feed additives* are intended to be added directly into *feedingstuffs* or through *premixtures* and *water* for drinking [10]. The Applicant proposed no minimum or maximum content of *L-glutamic acid* and *monosodium L-glutamate monohydrate* in *feedingstuffs* when used as nutritional additives [10], while inclusion levels of up to 10 g of *L-glutamic acid* and *monosodium L-glutamate monohydrate*/ kg *feedingstuffs* were suggested by the Applicant when used as sensory additives [10]. However, according to the Regulation (EU) 2018/249, when *L-glutamic acid* and *monosodium L-glutamate monohydrate* are used as sensory additives / flavouring compounds, they are added through *premixtures* at a recommended maximum content of 25 mg /kg *feedingstuffs* [3].

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-glutamic acid* and *monosodium L-glutamate monohydrate* 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 *glutamic acid* and *monosodium glutamate* in the *feed additives* and *premixtures* the Applicant proposed [11] and submitted the ring-trial validated method EN ISO 17180:2013 dedicated for the determination of lysine, methionine and threonine in commercial amino acid products and premixtures containing more than 10 % of amino acid [12]. The method 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. As a consequence, *L-glutamic acid* and *monosodium L-glutamate* are detected as *glutamic acid*.

The Applicant presented validation and verification studies aiming to demonstrate the extension of scope of the above mentioned method for the determination of *glutamic acid* and *monosodium glutamate* in the *feed additives* [13] and *premixtures* [14] when applying a slightly modified standard operating procedure [13,14] of the above mentioned EN ISO 17180:2013 method [12].

According to the method, *glutamic acid* and *monosodium glutamate* (as free *glutamic acid*) are extracted from the *feed additives* and *premixtures* 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). *Glutamic acid* and *monosodium glutamate* 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 [12-14].

The performance characteristics reported in the frame of validation and verification studies of the EN ISO 17180:2013 method for the quantification of *glutamic acid* and *monosodium glutamate* in the *feed additives* and *premixtures* are listed in Table 1.

Table 1: The performance characteristics obtained in the frame of validation and verification studies of the EN ISO 17180:2013 method for the quantification of *glutamic acid* and *monosodium glutamate* in the *feed additives* and *premixtures*

	Feed additive				***Premixtures***			
	Glutamic acid		**Monosodium glutamate**		**Glutamic acid**		**Monosodium glutamate**	
	Val.	**Ver.**	**Val.**	**Ver.**	**Val.**	**Ver.**	**Val.**	**Ver.**
Content, % (w/w)	98		98		5		5	
RSD_r, %	1.7	1.0	0.5	0.3	1.9	2.9	5.2	4.6
RSD_{ip}, %	2.1	1.1	1.2	0.3	1.9	2.9	5.6	4.6
R_{Rec}, %	97	101	101	99	102	101	101	103
Reference	[13]				[14]			

Val. – validation; Ver. – verification; *RSD_r, RSD_R* – relative standard deviation for *repeatability* and *reproducibility,* respectively; R_{Rec} – *Recovery* rate.

Based on the performance characteristics available, the EURL recommends for official control the ring-trial validated method EN ISO 17180:2013 based on IEC-VIS/FLD for the quantification of *glutamic acid* and *monosodium glutamate* in the *feed additives* and *premixtures*.

For the quantification of the *glutamic acid* and *monosodium glutamate* content in *feedingstuffs* the Applicant proposed [11] and submitted the ring-trial validated European Union (EU) method [15]. This method applies for the determination of free (synthetic and natural) and total (peptide-bound and free) amino acids (including glutamic acid) an amino acid analyser or HPLC equipment with an ion exchange column (IEC). The method is intended for *premixtures* and *feedingstuffs*, 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. As a consequence, *L-glutamic acid* and *monosodium L-glutamate* are detected as *glutamic acid*.

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 free *glutamic acid* is determined after post-column derivatisation with ninhydrin by spectrophotometric detection at 570 nm (visible – VIS).

The procedure chosen for the determination of the *total* amino acids depends on the amino acids under investigation. *Glutamic acid* and *monosodium glutamate* 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 the oxidation reagent is decomposed with sodium disulfite. The oxidised or non-oxidised sample is hydrolysed with hydrochloric acid (6 mol/l) containing 1 g phenol/l for 23 h. The hydrolysate is adjusted to pH 2.2. The amino acids are separated by IEC and total *glutamic acid* is determined by post-column derivatisation with ninhydrin and photometric detection at 570 nm [15].

As a general rule, in order to avoid an overestimation of the added content of *glutamic acid* and *monosodium glutamate*, the procedure based on quantification of *free* amino acids in *premixtures* and *feedingstuffs* has to be followed. When the procedure for the quantification of *total* amino acids is applied on *premixtures* and *feedingstuffs* containing proteins, an overestimation of *glutamic acid* and *monosodium glutamate* content may occur.

The EU method was further ring-trial validated by twenty-three laboratories for the determination of total *glutamic acid* in *feed* and resulted in the equivalent standard method EN ISO 13903:2005 [16]. The reported performance characteristics are listed in Table 2.

While limits of quantification (LOQ) ranging from 30 to 350 mg/kg have been reported for the analysis of various amino acids, a specific LOQ for *glutamic acid* (or *monosodium glutamate*) has not been indicated [16].

In addition, based on NRL's experience and following the previous reports for various amino acids, the EURL recently recommended in the frame of the evaluation report [17] the above mentioned EU method for the quantification of *monosodium glutamate* in the *feed additive*, even though the original scope of the method is not covering *feed additives*.

Based on the overall available data, the EURL recommends for official control the ring-trial validated EU method, based on IEC-VIS, to quantify *glutamic acid* and *monosodium glutamate* in the *feed additives*, *premixtures* and *feedingstuffs* (only when they are intended to be used as nutritional *feed additives*).

Table 2: Method performance characteristics obtained in the frame of EN ISO 13903:2005 [16] for the determination of total *glutamic acid* in *feed*

Ring-Trial	Matrix	*Glutamic acid* content g/kg	RSD_r %	RSD_R %
[16]	Poultry meal	79.7	2.7	9.1
	Broiler finisher feed	32.5	1.6	7.0
	Broiler starter feed	40.4	1.8	8.4
	Corn	15.1	2.4	6.2
	Fishmeal	73.7	0.9	4.7

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

Since it is not known what will be the maximum recommended content authorised for *glutamic acid* and *monosodium glutamate* in *feedingstuffs* when they are used as sensory additives / flavouring compounds in the frame of the current dossier, the EURL is unable to recommend the EU method for the official control of *glutamic acid* and *monosodium glutamate* in *feedingstuffs* when they are intended to be used as sensory additives. However, the EU method is at least fit-for-purpose for the quantification of *glutamic acid* and *monosodium glutamate* in *feed* in the validated concentration range.

For the quantification of *glutamic acid* in *water* the Applicant submitted the ring-trial validated EN ISO 13903:2005 method [16] mentioned above. This method, equivalent to the above mentioned EU [15] method, is intended to be applied for *premixtures* and *feedingstuffs* only. The Applicant successfully applied the EN ISO 13903:2005 method (after minor adaptations of the protocol) in the frame of the stability studies of *glutamic acid* in *water* [18].

Hence, the EURL recommends for official control the above mentioned EU method based on IEC-VIS to quantify *glutamic acid* and *monosodium glutamate* 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)

For the identification of the *feed additives* the EURL recommends the "L-Glutamic acid" and "Monosodium L-glutamate" monographs of the Food Chemical Codex (FCC) where different tests including the one based on specific optical rotation are described [19,20].

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-Glutamic acid" and "Monosodium L-glutamate" monographs of the Food Chemical Codex (FCC) for the identification of *L-glutamic acid* and *monosodium L-glutamate monohydrate* in the *feed additives*; (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 *glutamic acid* and *monosodium glutamate* in the *feed additives* and *premixtures*; and (iii) the ring-trial validated European Union method based on IEC-VIS for the quantification of *glutamic acid* and *monosodium glutamate* in the *feed additives*, *premixtures*, *feedingstuffs* and *water* (the EU method is recommended for *feedingstuffs* and *water* only when *L-glutamic*

acid and *monosodium L-glutamate monohydrate* are intended to be used as nutritional *feed additives*).

Recommended text for the register entry (analytical method)

For the identification of *L-glutamic acid* and *monosodium L-glutamate monohydrate* in the *feed additives*:

- Food Chemical Codex monographs: "L-Glutamic acid" and "Monosodium L-glutamate"

For the quantification of *glutamic acid* and *monosodium glutamate* in the *feed additives*:

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

For the quantification of *glutamic acid* and *monosodium glutamate* in *premixtures*:

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

Only for L-glutamic acid and monosodium L-glutamate monohydrate authorised under the category/functional groups 3(c) 'nutritional additives'/'amino acids, their salts and analogues':

For the quantification of *glutamic acid* and *monosodium glutamate* in *feedingstuffs*:

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

For the quantification of *glutamic acid* and *monosodium glutamate* 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-glutamic acid* and *monosodium L-glutamate monohydrate* 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, Reference SANTE/E5: Forw. Appl. 1831/0042-2020

[2] *Application, Annex 1 – submission number 1591716287124-2630

[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] Commission Implementing Regulation (EU) 2018/1567 of 18 October 2018 correcting Implementing Regulation (EU) 2018/249 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

[5] *Technical dossier, Section II: 2.1.3. Qualitative and quantitative composition

[6] *Technical dossier, Section II: 2.1.5. Physical state of each form of the product

[7] *Technical dossier, Section II: 2.2.1.2. Microorganism: information on the production strain used to produce 'L-glutamic acid' and 'Monosodium glutamate monohydrate'

[8] *Technical dossier, supplement to section II: II.2. Characterisation of the microorganism

[9] *Technical dossier, supplement to section II: II.1. Assessment

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

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

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

[13] *Technical dossier, Section II – Annex II_66

[14] *Technical dossier, Section II – Annex II_68

[15] 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

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

[17] FAD-2018-0090, Monosodium L-glutamate produced by fermentation with Corynebacterium glutamicum KCCM80188, Ref. Ares(2019)4123960 - 28/06/2019 https://ec.europa.eu/jrc/sites/jrcsh/files/finrep_fad-2018-0090-msglutamate.pdf

[18] *Technical dossier, Section II – Annex II_71

[19] Food Chemical Codex monograph "L-Glutamic acid", FCC 7 (2010), p.439

[20] Food Chemical Codex monograph "monosodium L-glutamate", FCC 7 (2010), p.698

*Refers to Dossier no: FAD-2020-0047

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)
- Centro di referenza nazionale per la sorveglienza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Wien (AT)
- Wageningen Food Safety Research[1] (WFSR) (NL)
- Thüringer Landesanstalt für Landwirtschaft (TLL). Abteilung Untersuchungswesen. Jena (DE)
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- Instytut Zootechniki — Państwowy Instytut Badawczy, Krajowe Laboratorium Pasz, Lublin (PL)
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- Landwirtschaftliche Untersuchungs- und Forschungsanstalt (LUFA), Speyer (DE)

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