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Safety and efficacy of L-cystine produced using *Pantoea ananatis* strain NITE BP-02525 for all animal species

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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-cystine produced using *Pantoea ananatis* NITE BP-02525 when used as a nutritional additive (amino acid) or as a sensory additive (flavouring compound) in feed and water for drinking for all animal species. L-Cystine is a dispensable sulfur-containing amino acid, naturally occurring in proteins of plants and animals. The amino acid L-cystine produced by fermentation with *P. ananatis* NITE BP-02525 is safe for all animal species, if the requirements for sulfur-containing amino acids are respected. The maximum amount of L-cystine that can be safely added to the diet will depend on the levels of other sulfur amino acids. This conclusion would also cover its use as a sensory additive. The use of L-cystine produced by fermentation with *P. ananatis* NITE BP-02525 in animal nutrition raises no safety concerns to consumers of animal products. The additive under assessment is considered slightly irritating by inhalation, not irritating to the skin or eyes and is not a skin sensitiser. There is no risk for persons handling the additive from the exposure to endotoxins by inhalation. The use of the L-cystine produced by fermentation with *P. ananatis* NITE BP-02525 as a feed additive does not represent a risk to the environment. L-Cystine is considered efficacious in partially meeting the requirements of sulfur-containing amino acids in all animal species. For the supplemental L-cystine to be as efficacious in ruminants as in non-ruminant species, it would require protection against degradation in the rumen. It is also considered efficacious as a feed flavouring compound under the proposed conditions of use.

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1. Introduction

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

Regulation (EC) No 1831/2003¹ 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 Ajinomoto Animal Nutrition Europe² for authorisation of the product L-cystine, when used as a feed additive for all animal species (category: nutritional additive; functional group: amino acids, their salts and analogues; and category: sensory additive; functional group: flavouring compounds).

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 2 July 2019.

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-cystine produced using a genetically modified strain of *Pantoea ananatis* (NITE BP-02525), when used under the proposed conditions of use (see Section 3.1.4).

1.2. Additional information

L-Cystine (minimum 98%) produced by fermentation using *P. ananatis* NITE BP-02525 is the object of the present assessment. It has not been previously authorised as a feed additive in the European Union (EU).

L-Cystine (minimum 98.5%) produced by hydrolysis of natural keratin from poultry feathers is authorised as a nutritional additive in the EU register of feed additives.³

L-Cystine (FL No 17.006) is authorised as food flavouring.⁴

L-Cystine is authorised for use in food for nutritional purposes,⁵ and for use in cosmetics.⁶ No maximum residue limits are required for cysteine when used as a veterinary medicinal product.⁷

The FEEDAP Panel published an opinion on L-cystine for all animal species produced (EFSA FEEDAP Panel, 2013a) and another on L-cysteine HCl monohydrate as a flavouring additive for pets (EFSA FEEDAP Panel, 2013b).

The Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Food (AFC) of EFSA delivered an opinion on the safety of L-cystine as food flavouring in 2010 (EFSA AFC Panel, 2010).

The European Pharmacopoeia contains a monograph for L-cysteine (monograph 01/2017:0998).

The Norwegian Scientific Committee of Food Safety (VKM, 2015) assessed the safety of L-cysteine and L-cystine in food supplements.

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 Ajinomoto Animal Nutrition Europe. Rue Guersant 32, 75017, Paris Cedex 17, France.

³ Commission Implementing Regulation (EU) No 1006/2013 of 18 October 2013 concerning the authorization of L-cystine as a feed additive for all animal species. OJ L 279, 19.10.2013, p.59.

⁴ Commission implementing regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. OJ L 267, 2.10.2012, p 1.

⁵ Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009. OJ L 181, 29.6.2013, p. 35–56.

⁶ Commission Decision 2006/257/EC of 9 February 2006 amending Decision 96/335/EC establishing an inventory and a common nomenclature of ingredients employed in cosmetic products. OJ L 97/1, 5.4.2006. p. 598.

⁷ Commission Regulation (EC) No 37/2010 of 22 December 2009, on pharmacologically active substances and their classification regarding maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. OJ L 15, 20.1.2010, pp. 1.

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier⁸ in support of the authorisation request for the use of L-cystine produced using a genetically modified strain of *P. ananatis* (NITE BP-02525) as a feed additive.

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, peer-reviewed scientific papers, other scientific reports and experts' knowledge, 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-cystine produced using a genetically modified strain of *P. ananatis* (NITE BP-02525) in animal feed. The Executive Summary of the EURL report can be found in Annex A.⁹

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of L-cystine produced using a genetically modified strain of *P. ananatis* (NITE BP-02525) is in line with the principles laid down in Regulation (EC) No 429/2008¹⁰ and the relevant guidance documents: Guidance on studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012), 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 the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018b) and Guidance for assessing the safety of feed additives for the environment (EFSA FEEDAP Panel, 2019).

3. Assessment

The product subject of this application is K-cystine produced by fermentation with a genetically modified strain of *P. ananatis* (NITE BP-02525). It is intended to be used as a nutritional additive, under the functional group 'amino acids, their salts and analogues' and as a sensory additive under functional group 'favouring compounds' in feed or in water for drinking for all animal species and categories.

3.1. Characterisation

The batches of the additive tested in this application were obtained using industrial pilot equipment (industrial pilot is defined as the ultimate phase of the manufacturing process development before routine industrial implementation). Hence, the product from these batches is considered to be representative of the additive that is intended to be placed on the EU market.¹¹

3.1.1. Characterisation of the production microorganism

The additive is produced by a genetically modified strain of *P. ananatis*, which is deposited in the Japanese National Institute of Technology and Evaluation with deposition number NITE BP-02525.¹² The parental strain was identified as *P. ananatis* [REDACTED]

The susceptibility of the production strain to the antibiotics listed in the guidance on the characterisation of microorganisms used as feed additives or as production organisms for

⁸ FEED dossier reference: FAD-2019-0026.

⁹ The full report is available on the EURL website: <https://ec.europa.eu/jrc/en/eurl/feed-additives/evaluation-reports>

¹⁰ Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorisation of feed additives. OJ L 133, 22.5.2008, p. 1.

¹¹ Technical dossier/Section II.2.1.3.1.

¹² Technical dossier/Section II/Annexes Supp II/Annex Supp II.1.

'Enterobacteriaceae' was tested by the broth microdilution method (EFSA FEEDAP Panel, 2018a).

The whole genome sequence (WGS) of the production strain was interrogated for the presence of antimicrobial resistance genes (AMRs)

No virulence factors were identified. The genome interrogation of the production strain did not identify any toxigenic or pathogenic factor.

3.1.1.1. Information relating to the genetically modified microorganism

²⁰ Technical dossier/Section II/Annex Supp II.18 and II.19.

[REDACTED]

The absence of all full-length antibiotic resistance genes used during the genetic modification from the final production strain was demonstrated [REDACTED]

3.1.2. Characterisation of the product/active substance

L-Cystine is a dimeric amino acid synthesised through an oxidative process from two cysteine molecules, which become linked by a disulfide bond. L-Cystine (International Union of Pure and Applied Chemistry (IUPAC) name (2*R*)-2-amino-3-[[[(2*R*)-2-amino-2-carboxyethyl]disulfanyl]propanoic acid; Chemical Abstracts Service (CAS) number 56-89-3) has the molecular formula $C_6H_{12}N_2O_4S_2$; its molecular weight is 240.3 g/mol, and its molecular structure is given in Figure 1.

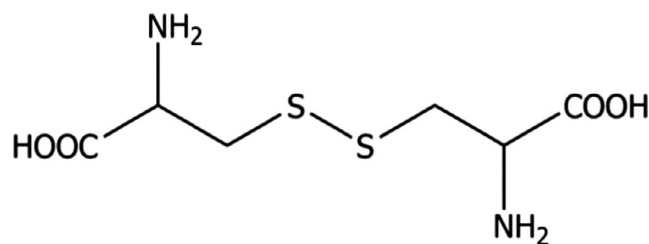


Figure 1: Molecular structure of L-cystine

The product contains by specification $\geq 98\%$ L-cystine. The analysis of five batches showed an average content of cystine of 99.6% ([REDACTED]) on as is basis.²¹ Moisture was on average 0.02% ([REDACTED]). Ashes analysed in three batches ranged 0.01–0.02%. Other constituents detected were sodium ([REDACTED]) and calcium ([REDACTED]).

The specific optical rotation of the additive was determined in five batches and was on average -222.4° with a very narrow range (-223 to -222°).²² Those values fall within the reference interval set in the European Pharmacopeia for this substance (-224 to -218°) and confirm the presence of the L-enantiomer.

Impurities

Three batches of the additive were analysed for impurities. Heavy metals (cadmium, lead and mercury), arsenic, fluorine, melamine and hydrogen cyanide were measured and all values were below the limit of quantification (LOQ).²³ Values for dioxins (polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/F)) amounted to 0.021 ng WHO-PCDD/F-TEQ/kg, and the sum of dioxins and dioxin-like polychlorinated biphenyls (DL-PCBs) was 0.031 ng WHO-PCDD/F-DL-PCB-TEQ/kg. Non-DL-PCBs ranged between 0.017 and 0.022 $\mu\text{g}/\text{kg}$.²⁴

The amount of the above-mentioned impurities does not raise safety concerns.

As regards mycotoxins (aflatoxins B1, B2, G1, G2, zearalenone, deoxynivalenol, ochratoxin A, T2 toxin, HT2 toxin, fumonisins B1, B2 and B3), their concentrations in three batches analysed were below the LOQ.²⁵

Microbial contamination of five batches of the additive was tested. Total microbial count at 30°C and suspected *Bacillus cereus* at 30°C were < 100 colony forming units (CFU)/g except in one batch that had ≤ 400 CFU/g; suspected coliforms, Enterobacteriaceae, *Staphylococcus coagulase* positive, yeasts and moulds were < 10 CFU/g; *Salmonella* spp. was absent in 25 g samples.²⁶

Pesticides (organophosphorus, organochlorine and pyrethroids) were analysed in three batches and found below the LOQ.²⁷

The potential antimicrobial activity of the additive was tested (three batches) in accordance of the guidance on characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018a). [REDACTED]

Endotoxin activity was measured in three batches of the additive [REDACTED]

[REDACTED] No endotoxin activity was detected in one batch, and the endotoxin activity of the other two batches was 19 and 54 EU/g additive.

No viable cells of the production strain were found [REDACTED]

No recombinant DNA was detected [REDACTED]

²¹ Technical dossier/Section II/Annex II.4. Analytical method based on ISO 17180 expanded to cysteine.

²² Technical dossier/Section II/Annex II.4.

²³ Technical dossier/Section II/Annex II.5. LOQ in mg/kg were 0.005 for mercury, 0.01 for cadmium, 0.05 for lead and melamine, 0.1 for arsenic, 1.5 for hydrogen cyanide and 20 for fluorine.

²⁴ Technical dossier/Section II/Annex II.5.

²⁵ Technical dossier/Section II/Annex II.5. LOD in $\mu\text{g}/\text{kg}$ were 0.1 for aflatoxins B1, B2 and G1; 0.2 for aflatoxin G2 and ochratoxin A; 20 for zearalenone, T2 toxin, HT2 toxin and fumonisins B1, B2 and B3; and 50 for deoxynivalenol.

²⁶ Technical dossier/Section II/Annex II. 8 for analytical results and Annexes II.19 to II.25 for analytical methods.

²⁷ Technical dossier/Section II/Annex II.5. LOQ ranged from 0.002 to 0.05 mg/kg for organochlorine and pyrethroid pesticides (depending on the pesticide considered); and from 0.01 to 0.03 mg/kg for organophosphorus pesticides (depending on the pesticide considered)

²⁸ Technical dossier/Section II/Annex II.7.

²⁹ Technical dossier/Section II/Annex II.29.

Physicochemical characteristics

The additive is a white crystalline powder having a solubility in water at 25°C of 0.112%, a pH (measured in three batches at 0.01% dilution) of 5.5–5.8.²² Packed bulk density ranged from 864 to 944 kg/m³.²⁹

The dusting potential was measured (Stauber–Heubach method) in three batches of the additive and the values ranged from 0.55 to 0.88 g/m³.²⁹ The particle size distribution of the dust of the batch showing higher dusting potential was measured: 20% of particles had a diameter < 10 µm and 100% had a diameter < 53 µm.

Particle size distribution of the additive was measured in three batches by laser diffraction. None of the batches had particles < 10 µm diameter (v/w); only one had particles of < 50 µm diameter and those represented 1.9%; and the range of particles < 100 µm diameter was 0.7–7.5%.³²

Stability and homogeneity

The shelf life of the additive (three batches) was tested when stored in sealed nylon-polyethylene plastic bags at 25 and 40°C for 1 year. Losses observed ranged from 1 to 5% at 25 °C and from 2 to 4% at 40°C.³³

The stability of the additive (three batches) in premixtures was tested in a series of [REDACTED] premixtures (for piglets, for gestating sows and for chickens for fattening, one batch each) [REDACTED], when supplemented at 5% (piglets and sows) or at 7% (chickens).³⁴ Choline chloride was present in the three premixtures at 0.8%, 1.6% and 2%, respectively. The premixtures were stored in sealed plastic bags at 25 or 40°C for 6 months. In the premixture for piglets, the losses observed at 25°C were 21% and those observed at 40°C 7%. In the premixture of gestating sows, losses observed at 25°C were 4% and no losses were observed at 40°C. In the premixture for chickens for fattening no losses were observed at 25°C and those observed at 40°C were 2%.

The stability of three batches of the additive was tested in three pelleted compound feeds (one batch each): for piglets, for gestating sows and for chickens for fattening.³⁵ The compound feed for piglets was based on wheat, soya and barley, and was supplemented at 0.38%; that of sows was based on barley, wheat and maize and was supplemented at 0.23%; the one of chicken for fattening was based on wheat and maize and was supplemented at 0.3%. The compound feed samples were stored in sealed plastic bags at 25 or 40°C for 3 months. No losses were observed in the feed for piglets. The compound feed for gestating sows showed losses of 3% at 25°C and of 4% at 40°C. The compound feed for chickens for fattening showed a loss of 7% at 25°C and no loss at 40°C. The stability during feed processing was not reported but by comparing the concentration of cystine in mash feed with that of pelleted feed at the start of the stability test,³⁶ it has been estimated that the piglet feed lost 10% cystine during feed processing and no losses occurred in the other two compound feeds.

The capacity of the additive to distribute homogeneously in the premixtures described above was tested by analysing 10 subsamples of each premixture. The coefficients of variation (CVs) were 4, 2 and 1%, respectively.³⁴

The capacity of the additive to distribute evenly in the compound feed for gestating sows and for chickens for fattening described above was tested by analysing 10 subsamples (9 sub-samples in the chicken feed). The CVs were 5% in both cases.³⁵

The stability of one batch of the additive in sterilised water for drinking was tested at 25 or at 40°C when stored (packaging not described) for 30 days.³⁷ No losses were observed at 25 or 40°C in water solution.

3.1.3. Manufacturing process

L-Cystine is produced by fermentation using *P. ananatis* strain NITE BP-02525. [REDACTED]

³² Technical dossier/Section II/Annex II.6.

³³ Technical dossier/Section II/Annex II.52.

³⁴ Technical dossier/Section II/Annex II.54.

³⁵ Technical dossier/Section II/Annex II.56.

³⁶ Technical dossier/Section II/Annex II.56, Appendix D.

³⁷ Technical dossier/Section II/Annex II.59.

[REDACTED]

The applicant stated that no antibiotics are used during the fermentation process.

3.1.4. Physico-chemical incompatibilities

No physico-chemical incompatibilities in feed are expected with other additives, medicinal products or feed materials.

3.1.5. Conditions of use

The application is for the use of L-cystine as a nutritional additive and as a flavouring compound, for use in premixtures, complete feed, complementary feed and water for drinking for all animal species.

No proposed inclusion levels are provided for its use as a nutritional additive (amino acid), as the optimal daily allowance in quantitative terms depends on the species, the physiological state of the animal, the performance level and the environmental conditions, as well as the amino acid composition of the unsupplemented diet.

The recommended use level as flavouring compound is of 25 mg/kg complete feed.

3.2. Safety

3.2.1. Safety of the production organism

The production strain NITE BP-02525 belongs to a species known to be phytopathogenic and associated with human infections. [REDACTED]

[REDACTED]. None of the introduced modifications raise a safety concern. There are no [REDACTED] antibiotic resistance genes in the production strain remaining from the genetic modification process. The production strain and its DNA were not detected in the additive. Therefore, the additive does not pose any safety concern regarding the genetic modification of the production strain.

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

The metabolic fate of L-cystine, the requirements of sulfur-containing amino acids, potential adverse effects of excesses of L-cysteine in animal diets, L-cystine tolerance in comparison to methionine and its relative toxicity in comparison to cysteine had been reviewed in a previous opinion (EFSA FEEDAP Panel, 2013a,b).

The additive is highly purified (> 99% cystine and < 1% unidentified matter on a dry matter basis). Concerns from the use of the additive would not derive from L-cystine which is considered safe but may arise from the residues of the fermentation process/production strain remaining in the final product. Regarding the production strain, none of the introduced modifications raise a safety concern. There are no [REDACTED] antimicrobial resistance genes in the production strain remaining from the genetic modification process. The production strain and its DNA were not detected in the final product. Consequently, no safety concerns for the environment arise regarding the production strain. The additive is considered safe for all animal species and categories, if the requirements for sulfur-containing amino acids are respected. The requirement for total sulfur-containing amino acids in feed range between 0.35 and 0.85% and for L-methionine between 0.15 and 0.40%, depending on animal species, genetics and the sex and physiological state of the animal. Due to the risk of nutritional imbalances and hygienic reasons, associated to the use of amino acids via water for drinking (EFSA FEEDAP Panel, 2010), the FEEDAP Panel has concerns on the safety of the use of L-cystine via water for drinking.

Since the levels proposed for the use of L-cystine as a flavouring compound (25 mg/kg complete feed) are substantially lower than the animal requirements, the FEEDAP Panel considers L-cystine produced using *P. ananatis* NITE BP-02525 safe when used as a feed flavouring compound.

The composition of tissues and products of animal origin will not be affected by the use of L-cystine in animal nutrition. The use of L-cystine originating from *P. ananatis* NITE BP-02525 in animal nutrition is considered safe for the consumer.

L-Cystine is a physiological and natural component of proteins in animals and plants. When supplemented to feed, it will be incorporated into proteins of tissues and/or products of animal origin and any potential excess will be catabolised and excreted as urea/uric acid, sulfur and carbon dioxide.

The use of L-cystine in animal nutrition would not lead to any localised increase of its concentration in the environment. The use of amino acids in water for drinking, when given in addition to complete diets with a well-balanced amino acid profile, would disturb the nitrogen balance and increase nitrogen excretion via urine. It is concluded that the use of this product as a feed additive does not represent a risk to the environment.

Consequently, the use of L-cystine produced using *P. ananatis* NITE BP-02525 in animal nutrition according to the proposed conditions of use is considered safe for the target species, the consumer and the environment.

3.2.3. Safety for user

The applicant provided an acute inhalation toxicity test, an eye irritation test, a skin irritation test and a dermal sensitisation test all performed with L-cystine produced by the strain under assessment (*P. ananatis* NITE BP-02525).

3.2.3.1. Effects on the respiratory system

Dusting potential (measured in three batches) was up to 0.88 g/m³ and the particle size distribution of the dust of the batch showing higher dusting potential had 20% of particles with a diameter < 10 µm and 100% of particles with a diameter < 53 µm. Therefore, workers may be exposed by inhalation.

In an acute inhalation toxicity study in accordance with the Organisation for Economic Co-operation and Development (OECD) Guideline 403, a group of 10 Crl:WI(Han) strain rats (5 males and 5 females) were exposed to a concentration of 5.3 mg L-cystine/L air for 4 h (nose only exposure system).³⁸ The signs observed (irregular respiration in 4 males and 2 females) disappeared within 2 hours after exposure except in a male in which was resolved the second day post-exposure. No mortality occurred and at necropsy, macroscopic lesions consisted of red discolouration and/or red spots (indicating small haemorrhages that might be a sign of respiratory irritation) in the lungs (3 males and 3 females), thymus (2 males, 2 females) and mandibular lymph nodes (3 males and 5 females). One male had pale lungs. The lethal concentration that would kill 50% of the rat population (LC₅₀) for acute inhalation toxicity after 4 h exposure is considered to be > 5.3 mg/L (g/m³).

Users can suffer from occupational respiratory disease depending on the level of endotoxins in air and dust (Rylander, 1999; Thorn, 2001). The scenario used to estimate the exposure of persons handling the additive to endotoxins in the dust, based on the EFSA guidance on user safety (EFSA FEEDAP Panel, 2012), is described in Appendix A. The threshold for the quantity of inhaled endotoxins per working day is 900 IU, derived from the provisional occupational exposure limits given by the Dutch Expert Committee on Occupational Safety (Health Council of the Netherlands, 2010) and the UK Health and Safety Executive (HSE, 2013). Based on calculations of the content of endotoxins in dust, exposure by inhalation would be 26 IU per eight-hour working day, indicating no risk of exposure to endotoxins for people handling the additive (see Appendix A).

3.2.3.2. Effects on skin and eyes

In an *in vitro* isolated chicken eye (ICE) assay in accordance with OECD Guideline 438, 30 mg of L-cystine (99.9% purity) were applied to adult chicken corneas for 10 s.³⁹ As the index for the test item was 'no effect', no classification is required.

In an *in vitro* skin irritation study using reconstructed human epidermis model (EpiDermTM) in accordance with OECD Guideline 439,⁴⁰ the test item was considered not irritant for the skin.

In a *in vivo* skin sensitisation study (local lymph node assay in mouse) in accordance with OECD Guideline 429, L-cystine (99.9% purity) at doses of 25, 50 and 100% caused no signs of local irritation or systemic toxicity.⁴¹ The additive was classified as no skin sensitiser.

3.2.3.3. Conclusions on safety for the user

The additive under assessment is considered slightly irritating by inhalation, not irritating to the skin or eyes and is not a skin sensitiser. There is no risk for persons handling the additive from the exposure to endotoxins by inhalation.

³⁸ Technical dossier/Section III/Annex III.45.

³⁹ Technical dossier/Section III/Annex III.49.

⁴⁰ Technical dossier/Section III/Annex III.50.

⁴¹ Technical dossier/Section III/Annex III.51.

3.3. Efficacy

L-Cystine is naturally occurring in proteins of plants and animals. The nutritional role of the amino acid L-cystine is well established in the scientific literature. The dispensability of this amino acid, its relationship with the metabolism of methionine and its relative toxicity with methionine and cysteine have been reviewed in a previous opinion (EFSA FEEDAP Panel, 2013a,b).

L-Cysteine and its oxidation product L-cystine can satisfy approximately 50% of the need for total sulfur amino acids (SAAs). Depending on animal species, genetics and the sex and physiological state of the animal, the requirement for total SAAs and methionine in feed, according to the National Research Council (NRC), ranges between 0.35% and 0.85% and between 0.15% and 0.40%, respectively.

In general, the product L-cystine is considered efficacious in partially meeting the requirements of sulfur-containing amino acids in all animal species. For the supplemental L-cystine to be as efficacious in ruminants as in non-ruminant species, it would require protection against degradation in the rumen.

Since L-cystine is used in food as a flavouring compound (FL No 17.006), and its function in feed is essentially the same as that in food, no further demonstration of efficacy is necessary.

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 Regulation⁴² and Good Manufacturing Practice.

4. Conclusions

The production strain *P. ananatis* NITE BP-02525 and its DNA were not detected in the additive. Therefore, the additive does not pose any safety concern regarding the genetic modification of the production strain.

The amino acid L-cystine produced by fermentation with *P. ananatis* NITE BP-02525 is safe for all animal species, if the requirements for sulfur-containing amino acids are respected. The maximum amount of L-cystine that can be safely added to the diet will depend on the levels of other sulfur amino acids. This conclusion would also cover its use as a sensory additive.

The use of L-cystine produced by fermentation with *P. ananatis* NITE BP-02525 in animal nutrition raises no safety concerns to consumers of animal products.

The additive under assessment is considered slightly irritating by inhalation, not irritating to the skin or eyes and is not a skin sensitiser. There is no risk for persons handling the additive from the exposure to endotoxins by inhalation.

The use of the L-cystine produced by fermentation with *P. ananatis* NITE BP-02525 as a feed additive does not represent a risk to the environment.

L-Cystine is considered efficacious in partially meeting the requirements of sulfur-containing amino acids in all animal species. For the supplemental L-cystine to be as efficacious in ruminants as in non-ruminant species, it would require protection against degradation in the rumen. It is also considered efficacious as a feed flavouring compound under the proposed conditions of use.

Documentation as provided to EFSA/Chronology

Date	Event
04/05/2019	Dossier received by EFSA. L-Cystine produced using strain NITE BP-02525. Submitted by Ajinomoto Animal Nutrition Europe.
06/05/2019	Reception mandate from the European Commission
02/07/2019	Application validated by EFSA – Start of the scientific assessment
10/10/2019	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. <i>Issues: Characterisation of the additive</i>
10/10/2019	Comments received from Member States
05/11/2019	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives

⁴² Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 laying down requirements for feed hygiene. OJ L 35, 8.2.2005, p. 1.

Date	Event
18/11/2019	Reception of supplementary information from the applicant - Scientific assessment re-started
28/01/2020	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

AFC	EFSA Panel on Food Additives, Flavourings, Processing Aids and Materials in contact with Food
AMR	antimicrobial resistance genes
CAS	Chemical Abstracts Service
CFU	colony forming unit
CV	coefficient of variation
DL-PCB	dioxin-like polychlorinated biphenyls
DM	dry matter
EURL	European Union Reference Laboratory
FCC	Food Chemical Codex
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
HSE	Health and Safety Executive
IEC-VIS/FLD	ion-exchange chromatography coupled to visible or fluorescence detection
IUPAC	International Union of Pure and Applied Chemistry
LC ₅₀	lethal concentration, median
LOD	limit of detection
LOQ	limit of quantification
MIC	minimum inhibitory concentration
NRC	National Research Council
OECD	Organisation for Economic Co-operation and Development
PCDD/F	polychlorinated dibenzo- <i>p</i> -dioxins and dibenzofurans
RH	relative humidity
RSDip	relative standard deviation for intermediate precision
RSDr	relative standard deviation for repeatability
SAA	sulfur amino acid
VKM	Norwegian Scientific Committee for Food Safety
WGS	whole genome sequence
WHO	World Health Organization

Appendix A – Safety for the user

The effects of the endotoxin inhalation and the exposure limits have been described in a previous opinion (EFSA FEEDAP Panel, 2015).

Calculation of maximum acceptable levels of exposure from feed additives

The likely exposure time according to EFSA guidance (EFSA FEEDAP Panel, 2012) for additives added in premixtures assumes a maximum of 40 periods of exposure per day, each comprising 20 s, equal to 800 s per day. With an uncertainty factor of 2, maximum inhalation exposure would occur for $2 \times 800 = 1,600$ s (0.444 h per day). Again, assuming a respiration volume of 1.25 m³/h, the inhalation volume providing exposure to potentially endotoxin-containing dust would be $0.444 \times 1.25 = 0.556$ m³ per day. This volume should contain no more than 900 IU endotoxin, so the dust formed from the product should contain no more than $900/0.556 = 1,619$ IU/m³.

Calculation of endotoxin content of dust

Two key measurements are required to evaluate the potential respiratory hazard associated with endotoxin content of the product (the dusting potential of the product, expressed in g/m³; the endotoxin activity of the dust, determined by the *Limulus amoebocyte lysate* assay (expressed in IU/g)). If data for the dust are not available, the content of endotoxins of the product can be used instead. If the content of endotoxins of the relevant additive is 'a' IU/g and the dusting potential is 'b' g/m³, then the content of endotoxins of the dust, 'c' IU/m³, is obtained by the simple multiplication $a \times b$. This resulting value is further used for calculation of potential inhalation exposure by users to endotoxin from the additive under assessment (Table A.1) (EFSA FEEDAP Panel, 2012).

Table A.1: Estimation of user exposure to endotoxins from the additive L-cystine produced by *P. ananatis* NITE BP-02525 including consideration of using filter half mask (FF P2 or FF P3)⁴³ as a preventive measure

Calculation	Identifier	Description	Amount	Source
	a	Endotoxin content IU/g product	54	Technical dossier
	b	Dusting potential (g/m ³)	0.879	Technical dossier
$a \times b$	c	Endotoxin content in the air (IU/m ³)	47.47	
	d	Number of premixture batches made/working day	40	EFSA FEEDAP Panel (2012)
	e	Time of exposure (s)/production of one batch	20	EFSA FEEDAP Panel (2012)
$d \times e$	f	Total duration of daily exposure/worker (s)	800	
	g	Uncertainty factor	2	EFSA FEEDAP Panel (2012)
$f \times g$	h	Refined total duration of daily exposure (s)	1,600	
$h/3\ 600$	i	Refined total duration of daily exposure (h)	0.44	
	j	Inhaled air (m ³)/eight-hour working day	10	EFSA FEEDAP Panel (2012)
$j/8 \times i$	k	Inhaled air during exposure (m ³)	0.56	
$c \times k$	l	Endotoxin inhaled (IU) during exposure/eight-hour working day	26.37	
	m	Health-based recommended exposure limit of endotoxin (IU/m ³)/eight-hour working day	90	Health Council of the Netherlands (2010)
$m \times j$	n	Health-based recommended exposure limit of total endotoxin exposure (IU)/eight-hour working day	900	
//10		Endotoxins inhaled (IU)/eight-hour working day reduced by filter half mask FF P2 (reduction factor 10)	3	
//20		Endotoxins inhaled (IU)/eight-hour working day reduced by filter half mask FF P3 (reduction factor 20)	1	

⁴³ Filtering face piece or filtering half mask according to European standard EN 149. They are graded from 1 to 3 depending on their filtering capacity.

Annex A – Executive summary of the evaluation report on the analytical methods for detection of L-cystine produced using strain NITE BP-02525 derived from *Pantoea ananatis*

In the current application authorisation is sought under Article 4(1) for L-cystine using the bacteria strain NITE BP-02525 derived from *Pantoea ananatis*, 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-cystine has a minimum purity (mass fraction) of 98%. As nutritional feed additive, the amino acid is intended to be added directly into feedingstuffs or through premixtures and water for drinking. As sensory feed additive, L-cystine 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-cystine in feedingstuffs. However, when authorised as sensory additive, it is recommended to use the amino acid at a level of 25 mg/kg complete feed.

For the quantification of L-cystine in the feed additive and premixtures the Applicant submitted the ring-trial validated method EN ISO 17180:2013 specifically designed for the determination of 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 abovementioned ISO method for the determination of L-cystine in the feed additive and premixtures. The following performance characteristics are reported: a relative standard deviation for repeatability (RSDr) ranging from 0.6 to 2.0%, a relative standard deviation for intermediate precision (RSDip) ranging from 0.7 to 2.7% and a recovery rate from 94 to 108%. In addition, the EURL identified the "L-cystine monograph" of the Food Chemical Codex (FCC) for the identification of L-cystine in the feed additive.

For the quantification of L-cystine in feedingstuffs the Applicant submitted the ring-trial validated European Union 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, the amino acid enantiomers and between cystine and cysteine (both compounds are determined together and indicated as cyst(e)ine). 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 cyst(e)ine: a RSDr ranging from 1.7 to 4.6% and a RSDR ranging from 8.8 to 19%. Furthermore, a limit of quantification of 350 mg/kg feedingstuffs was derived for total cyst(e)ine. However, when intended as sensory feed additive, the Applicant recommended a maximum content of L-cystine in feedingstuffs of 25 mg/kg. Therefore, the EURL is unable to recommend the European Union method for the official control of this product in feedingstuffs when intended as flavouring compound.

In the frame of the stability studies the Applicant presented experimental data obtained analysing the feed additive in water according to EN ISO 13903:2005 thus demonstrating its applicability for the determination of L-cystine in water.

In the frame of this authorisation the EURL recommends for official control (i) the "L-cystine monograph" of the Food Chemical Codex (FCC) based on infrared absorption for the identification of L-cystine 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 L-cystine in the feed additive and premixtures; (iii) the ring-trial validated European Union method based on IEC-VIS for the quantification of L-cystine, intended as nutritional additive, in premixtures and feedingstuffs; and (iv) the ring-trial validated EN ISO 13903:2005 method based on IEC-VIS for the quantification of L-cystine 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.