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Safety and efficacy of a feed additive consisting of zinc chelate of ethylenediamine for all animal species (Zinpro Animal Nutrition (Europe), Inc.)

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Abstract

Following a request from the European Commission, EFSA was asked to deliver a scientific opinion on the safety and efficacy of zinc chelate of ethylenediamine (Zinc-EDA-Cl) as feed additive for all animal species. The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) identified several issues related to the data provided concerning the chemical characteristics of the additive. Based on the information provided, the FEEDAP Panel considered unlikely that the additive consists only of zinc mono-chelate of EDA, but of several coexisting (zinc) species; therefore, the FEEDAP Panel was unable to confirm the identity of the additive. The FEEDAP Panel could not evaluate the safety for target species, consumer and environment and the efficacy of the additive owing to the uncertainties and limitations identified in the studies submitted. Concerning the safety of the additive for the users, the Panel considered that handling the additive poses a risk to users by inhalation. The additive should be considered as corrosive to eyes and a skin sensitiser.

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1. Introduction

1.1. Background and Terms of Reference

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 Zinpro Animal Nutrition (Europe), Inc.² for authorisation of the product zinc chelate of ethylenediamine, when used as a feed additive for all animal species (category: nutritional additives; functional group: compounds of trace elements).

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 19 October 2018.

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 zinc chelate of ethylenediamine, when used under the proposed conditions of use (see Section 3.1.5).

1.2. Additional information

Zinc chelate of ethylenediamine is intended to be used as a source of zinc in all animal species. The additive has not been previously authorised as feed additive in the European Union (EU).

2. Data and methodologies

2.1. Data

The present assessment is based on data submitted by the applicant in the form of a technical dossier³ in support of the authorisation request for the use of zinc chelate of ethylenediamine 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 and other scientific reports 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 zinc chelate of ethylenediamine in animal feed. The Executive Summary of the EURL report can be found in Annex A.⁴

2.2. Methodologies

The approach followed by the FEEDAP Panel to assess the safety and the efficacy of zinc chelate of ethylenediamine is in line with the principles laid down in Regulation (EC) No 429/2008⁵ and the relevant guidance documents: Guidance for the preparation of dossiers for nutritional additives (EFSA FEEDAP Panel 2012a), Technical guidance Tolerance and efficacy studies in target animals (EFSA FEEDAP Panel, 2011), Guidance on the identity, characterisation and conditions of use of feed additives (EFSA FEEDAP Panel, 2017a), 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

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 Zinpro Animal Nutrition (Europe), Inc. Akkerdistel 2E. 5831 PJ. Boxmeer. The Netherlands.

³ FEED dossier reference: FAD-2018-0063.

⁴ The full report is available on the EURL website: <https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2018-0063-zneda.pdf>

⁵ 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.

use of the additive for users/workers (EFSA FEEDAP Panel, 2012b) and Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008).

3. Assessment

The additive under assessment is zinc chelate of ethylenediamine (EDA),⁶ and will be referred from here onwards as Zinc-EDA-Cl. It is intended to be used as a nutritional additive (functional group: compounds of trace elements) for all animal species and categories.

3.1. Characterisation

3.1.1. Manufacturing process

The product is produced by complexing zinc chloride and ethylenediamine (EDA) to form Zinc-EDA-Cl.

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^{7,8}

3.1.2. Identity and characterisation of the additive

Five batches of the product were analysed for zinc, EDA, moisture and chloride. The average content of zinc was about 30.6% (30.1–30.9%), EDA 26.9% (26.5–27.3%), chloride 42.4% (41.7–43.5%) and moisture 0.6% (0.5–0.7%).⁹

The applicant provided experimental data to support the amount of chelated and free zinc in the additive. Five batches of the additive were analysed; the amount of bound zinc averaged to 96.8% (range: 96.6–97.5%).¹⁰

Based on the available information and knowledge, the applicant made an attempt¹¹ to provide a chemical description of the additive under assessment. The following characteristics were provided for Zinc-EDA-Cl:

- IUPAC Name, Chloro-ethane-(1-ammonium-2-amine)-zinc (II) chloride monohydrate.
- Molecular Weight, 250.86 g/mol.
- Chemical Formula, $C_2H_{11}Cl_3N_2OZn$.
- The compound is not identified by a Chemical Abstracts Service (CAS) number.

The structural formula, as proposed by the applicant (Figure 1), describes the zinc ion (Zn^{2+}) as hexa-coordinated by two nitrogen atoms of a single EDA molecule, one of the two being protonated, one water molecule and three chloride ions, resulting in a neutral compound. The theoretical composition, based on the proposed structural formula, would be 26.1% zinc, 24.4% EDA, 42.4% chloride, 6.47% bound water and 0.7% moisture.

⁶ Other names used in the dossier: 'Zinc chelate of EDA', 'Zinc chelate of ethylenediamine', 'Zinc-EDA', 'Zinc-Ethylenediamine', 'Zinc-Ethylenediamine complex', 'ZnEDA' and 'Zn-EDA'. Technical Dossier/Supplementary information (February 2019).

⁷ Technical dossier/Section II/2.3.

⁸ Technical dossier/Section II/Annexes II-28–29.

⁹ Technical dossier/Section II/Annexes II-1–5.

¹⁰ Technical Dossier/Supplementary information (February 2019)/Annex 1. The applicant further indicated that a specification on the level of chelation is not intended to accompany this product.

¹¹ Technical Dossier/Supplementary information (September 2020)/Annex: Zinc EDA Identity clarification.

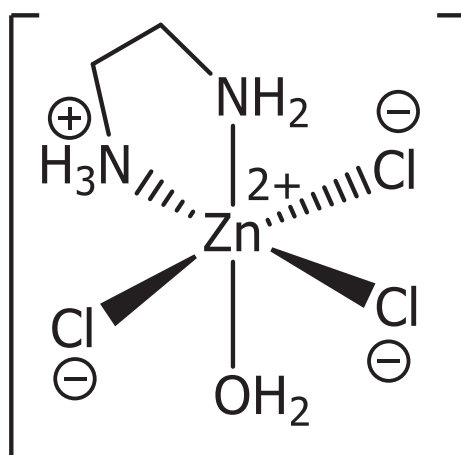


Figure 1: Structural formula of zinc chelate of ethylenediamine, as provided by the applicant

The FEEDAP Panel identified the following issues related to the proposed structural formula: (i) the protonated nature of one of the nitrogen atoms in the EDA ligand makes the donation of the pair of electrons to zinc for the formation of a coordinate bond unlikely, (ii) the theoretical composition, calculated from the proposed structural formula, showed deviations for zinc and EDA, when compared to the analytical data and (iii) the structural formula, as proposed by the applicant, would not match with the IUPAC name, particularly concerning the number and the role of chloride ions (as ligands or counterions). Moreover, the FEEDAP Panel has reservations on the soundness of the proposed IUPAC name.

The FEEDAP Panel further notes that no supporting evidence was provided to substantiate the proposed structural formula, with the exception of infra-red analyses of the compound, without any description of the analytical conditions and a proper interpretation.¹² No evidence was provided to demonstrate that (i) the additive is a monochelate of zinc with EDA, (ii) chloride ions act (at least in part) as ligands rather than as counterions and (iii) zinc is hexa- rather than tetra-coordinated.

On the other hand, the existence of different zinc chelates with EDA, including the mono-, bis- and tris(EDA)zinc(II) complexes has been widely reported in the literature (Bennett et al., 1990)

Considering all the above, the FEEDAP Panel is unable to confirm the identity of the additive. The remaining analyses provided to support the characterisation of the additive are described in the paragraphs below.

Five batches of the additive were analysed for undesirable substances. Levels of heavy metals (cadmium: 0.18–0.31 mg/kg, lead: 2.54–3.12 mg/kg, mercury: < 0.05 mg/kg), arsenic: < 0.1 mg/kg) and fluorine: < 1.5–5 mg/kg) were reported.^{14,15} The levels of polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/F) and the sum of PCDD/F and dioxin-like polychlorinated biphenyls (PCBs) were 0.10–0.14 ng WHO-PCDD/F-TEQ/kg and 0.11–0.15 ng WHO-PCDD/F-PCB-TEQ/kg, respectively.¹⁶ The concentrations of the undesirable substances analysed comply with the limits set in Directive 2002/32/EC for compounds of trace elements¹⁷ or, if not mentioned in the Directive, do not represent a concern. The

¹² The applicant provided the infra-red absorption spectra for three lots of the additive to identify the NH₂ peaks, showing peaks at three wavelengths (1,622.1, 1,622.1 and 1,624.3 cm⁻¹); Technical dossier/Section II/2.2.2.1.

¹³ Zinc species measured in the dissociation study (see Section 3.2.2.1): Zn²⁺, [Zn(OH)₂], [Zn(EDA)]²⁺, [Zn(EDA)₂]²⁺, [Zn(OH)]⁺ and [Zn(EDA)₂OH]⁺.

¹⁴ Technical dossier/Section II/Annexes II-10–14.

¹⁵ Technical Dossier/Supplementary information (February 2019)/Where the symbol '<' is used, the figure corresponds to the limit of detection of the analytical method.

¹⁶ Technical dossier/Section II/Annexes II-15–19.

¹⁷ Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. OJ L 140, 30.5.2002, p. 10.

nickel content of the additive (analysis of three batches) showed an average of 2.44 mg/kg (range 2.10–2.75).¹⁸

Three batches of the additive were analysed for microbiological contamination. Counts of *Enterobacteriaceae*, moulds and yeasts were < 10 cfu/g and *Salmonella* was not detected in a 25 g sample.¹⁹ Levels of aflatoxin B1 and ochratoxin A analysed in three batches were below the limit of detection (LOD; < 0.1 µg/kg).²⁰

3.1.3. Physical properties of the additive

The additive is a powder with a bulk density of 930 kg/m³ (average of three batches).²¹ The applicant declared that the product is soluble in water and slightly soluble in methyl alcohol and in ethyl alcohol, whilst it is practically insoluble in ethyl acetate²²; however, no supporting data was made available.

Particle size distribution was studied in three batches (laser diffraction)²¹; particles below 10, 50 and 100 µm were on average 4.9, 20.7 and 33.0%, respectively.

Dusting potential was analysed by the Stauber-Heubach method in the same three batches as the particle size distribution; four measures were taken on each batch.²¹ The results showed a dusting potential ranging from 0.83 to 0.93 g/m³ air.²¹ The applicant provided data on the zinc content of the dust measured in the same samples (total of 12 subsamples); the average zinc content was 278 mg Zn/kg dust (range 259–319 mg Zn/kg).²¹

3.1.4. Stability and homogeneity

Although for compounds of trace elements (including chelates) stability studies are generally not required, the applicant provided information on the stability of the additive in premixtures and feed (mash and pelleted).

The premixture containing the additive was stored for 6 months, whilst the poultry feed (mash and pelleted) for 3 months at room temperature. At the end of the experiment, the content of zinc in the premixture was 92.7% that of initially measured; in the mash and pelleted feed, this value was 99.2% and 96.2%, respectively.

The capacity for homogeneous distribution of the additive in a premixture (vitamin–mineral) and complete feed (mash and pelleted) for chickens for fattening was investigated²³; the zinc content was analysed in 10 subsamples each. The coefficient of variation (CV) of the zinc concentration in the premixture (mean 21,154 mg/kg) was 3.1%. The CV of the mash feed was up to 5.7% and in the same feed after pelleting (mean zinc content: 157 mg/kg),²⁴ the CV was up to 2.1%.

3.1.5. Conditions of use

The additive is intended to be used in feed – directly or via a premixture or complementary feed – for all animal species. It should be used up to a maximum total zinc content in feed of 200 mg/kg (dogs and cats), 180 mg/kg (salmonids and milk replacers for calves), 150 mg/kg (piglets, sows, rabbits and all fish other than salmonids) and 120 mg/kg (other species and categories).

3.2. Safety

3.2.1. Safety for the target species

The applicant provided a study with Zinc-EDA-Cl in chickens for fattening with duration of 35 days.^{25,26} This study was designed to support safety for target species and efficacy of the additive, as well as to provide data for the residues' evaluation. The evaluation of the residues casted substantial

¹⁸ Technical Dossier/Supplementary information (February 2019).

¹⁹ Technical dossier/Section II/Annex II-20.

²⁰ Technical dossier/Section II/Annexes II-23–25.

²¹ Technical dossier/Section II/Annex II-26.

²² Technical dossier/Section II/Annex II-30.

²³ Technical dossier/Section II/Annex II-27.

²⁴ The FEEDAP Panel notes that the levels of zinc reached in feed are higher than the maximum permitted for chickens for fattening.

²⁵ Technical dossier/Section III/Annex III_3_1.

²⁶ Due to the capacity of laboratory to process samples, the necropsies took place over 3 days upon termination of the study (until day 37).

uncertainties since EDA was found in the analysis of tissues and organs of animals from groups not supplemented with the additive, but with zinc sulfate monohydrate; in the absence of an adequate explanation, the FEEDAP Panel has serious reservations on the acceptability of this trial. In addition, the Panel notes that overall mortality of the study was high (9.5%) and animals had to be treated with antibiotics.

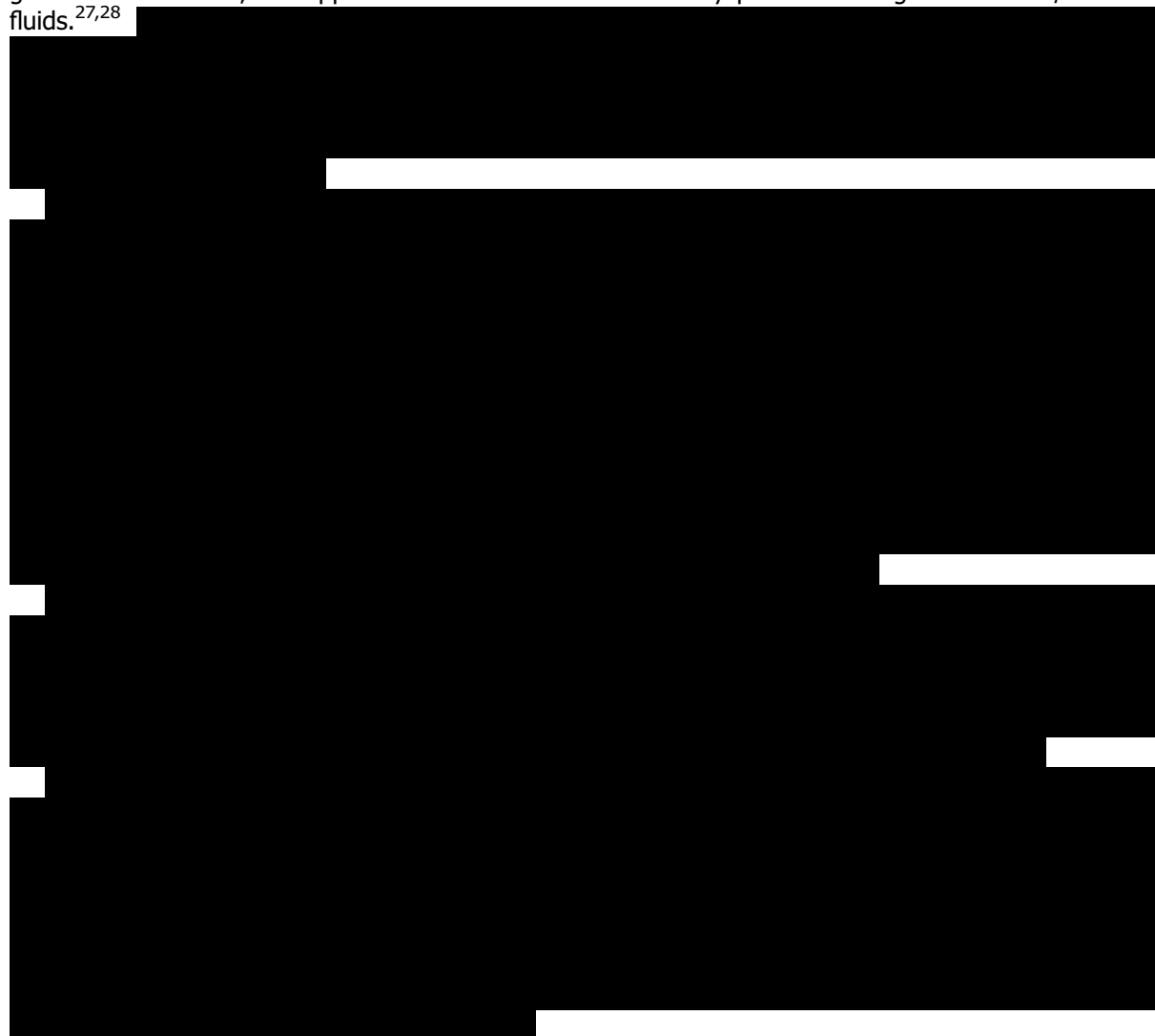
In the absence of adequate studies, the FEEDAP Panel cannot conclude on the safety of the additive Zinc-EDA-Cl for the target species.

3.2.2. Safety for the consumer

3.2.2.1. Metabolic studies

In the original dossier, no data concerning the metabolic fate of Zinc-EDA-Cl were submitted.

Upon the FEEDAP Panel's request of data on the potential dissociation of the additive in the gastrointestinal tract, the applicant submitted an *in vitro* study performed in gastro-ruminal/intestinal fluids.^{27,28}



The *in vitro* study shows that the additive is extensively dissociated at pH below 4.5, whilst at higher pH, there is the coexistence of several zinc-containing species and free Zn²⁺. However, due to the uncertainties related to the identity of the additive, and the limitations identified in the methodology of the dissociation study, a final conclusion from the study, including an extrapolation to the *in vivo* conditions, could not be drawn.

²⁷ Technical Dossier/Supplementary information (March 2020).

²⁸ Technical Dossier/Supplementary information (September 2020)/Annex 'ZnEDA dissociation analysis report rev 2- Confidential'.

3.2.2.2. Residue studies

From the study in chickens for fattening, the residue deposition could not be assessed (see Section 3.2.1).

No data on the residues of zinc and EDA in the tissues and products (milk, egg) of other target species administered the additive were made available.²⁷

3.2.2.3. Toxicological studies

The applicant provided limited information supporting the toxicological profile of the additive. Only genotoxicity studies were performed with Zinc-EDA-Cl. No other toxicological studies were provided with the additive under assessment.

The applicant provided separated data on zinc toxicity and ethylenediamine dihydrochloride (EDA-2HCl) toxicity, under the assumption that the additive would be extensively dissociated in the gastro-intestinal tract.

Zinc toxicity has been extensively described by the Scientific Committee on Food (SCF) (European Commission, 2003), by Sandstead (2015) and in EFSA FEEDAP opinions (e.g. EFSA FEEDAP Panel, 2015); no substantial differences between inorganic and organic zinc compounds are expected concerning zinc toxicity. Depressed copper uptake with associated copper deficiency is the most sensitive and well-characterised effect of chronic excess of zinc intake in humans and animals. Accordingly, a tolerable upper intake level (UL) for zinc of 25 mg/day in adults is derived from a human no observed adverse effect level (NOAEL) of 50 mg/day in adults for altered copper status and an uncertainty factor of 2 to allow for observed variability within the general population (European Commission, 2003).

3.2.2.3.1. Genotoxicity studies

3.2.2.3.1.1. Bacterial reverse gene mutation assay

In order to investigate the potential of Zinc-EDA-Cl (purity 100%) to induce gene mutations in bacteria, the Ames test was performed according to OECD Test Guideline 471²⁹ and following Good Laboratory Practice (GLP) in *Salmonella* Typhimurium strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* tester strain WP2 uvrA.³⁰

In two independent experiments, Zinc-EDA-Cl was tested at least at five concentration levels ranging from 100 to 5,000 µg/plate, applying the plate incorporation method in the presence and absence of metabolic activation. Appropriate positive and negative controls were evaluated concurrently. All positive control chemicals induced significant increases in revertant colony numbers, confirming the sensitivity of the tests and the efficacy of the S9-mix. Precipitate was observed at 3,000 or 5,000 µg/plate, while toxicity was reported at 3,000 µg/plate and above. No increase in the mean number of revertant colonies was observed at any tested concentration in any tester strains with or without S9-mix, with the exception of TA100 and TA1535 strains. In the first and second experiment, TA100 strain showed an increase in the number of revertant colonies up to 1.9-fold the negative control values in the absence and presence of metabolic activation. The increases were mostly observed at the highest concentration in association with toxicity, as measured by the reduction of background lawn.

A third experiment performed with TA100 showed an increase in the number of revertant colonies up to twofold the concurrent vehicle control both in the absence and presence of S9-mix.

In all the three experiments, the increased values of revertant colonies were within the historical negative control ranges. In the first experiment, TA1535 showed increases up to 2.7-fold the concurrent negative controls in the presence and absence of metabolic activation. The increases were not reproducible in the second experiment, not dose-related and fell within the historical negative control ranges; the FEEDAP Panel considers the increases not biologically relevant.

3.2.2.3.1.2. In vitro micronucleus test

An *in vitro* micronucleus assay was performed according to OECD TG 487 and following GLP to evaluate the potential of Zinc-EDA-Cl (Zn 29.4%, purity 100%) to induce chromosome damage in Chinese Hamster Ovary (CHO) cells in the absence and presence of metabolic activation.³¹ In a preliminary cytotoxicity test, precipitation was observed at the conclusion of the treatment period in

²⁹ OECD Guideline Version of 1997. Available online: <https://www.oecd.org/chemicalsafety/risk-assessment/1948418.pdf>

³⁰ Technical dossier/Section III/Annex III_3_3.

³¹ Technical dossier/Section III/Annex III_3_4.

the presence of S9-mix at 246.5 µg/mL and above. Cytotoxicity ($\geq 55\%$) was induced by Zinc-EDA-Cl treatment in the absence of S9-mix at 73.95 µg/mL and above, and at 2,465 µg/mL in the presence of S9-mix. Based on these results, three concentrations were selected for the analysis of micronuclei in binucleate cells for each experimental condition applied. Short treatment in the absence of metabolic activation was performed at 50, 85, 150 µg/mL of Zinc-EDA-Cl; 52% cytotoxicity was observed at the high-dose level, while statistically significant increase in the frequency of micronucleated binucleated (MNBN) cells was detected at the low- and high-dose levels ($p \leq 0.01$, not dose-related). The increased values of Zinc-EDA-Cl induced MNBN cells were within the range of the historical negative controls.

A short treatment in the presence of metabolic activation was performed at 5, 10, 20 µg/mL of Zinc-EDA-Cl; cytotoxicity at the high concentration was 51% relative to the vehicle control; statistically significant increase of MNBN cells was reported at all concentrations tested ($p < 0.05$); the increase was not dose-related ($p > 0.05$, Cochran–Armitage test) and the values of Zinc-EDA-Cl-induced MNBN cells were within the historical vehicle control range. Concentrations selected for continuous treatment in the absence of S9-mix were 10, 30, 50 µg/mL; significant cytotoxicity (53%) was observed at the high concentration tested. The frequency of MNBN cells detected after treatment with Zinc-EDA-Cl was comparable to the value observed in the concurrent negative control.

3.2.2.3.1.3. *In vivo micronucleus test*

A micronucleus test was performed in bone marrow cells from Hsd:ICR (CD-1) mice according to OECD TG 474²⁹ to evaluate the potential of Zinc-EDA-Cl (Zn 29.4%, purity unknown) to induce chromosomal damage.³² The test item was administered by oral gavage at 100, 200 and 400 mg/kg in male mice and 125, 250, 500 mg/kg bw in female mice. The highest dose levels corresponded to the maximum tolerated dose (MTD) identified in a preliminary toxicity study, showing mortality at 1,000 mg/kg bw and, thus, indicating systemic availability of the test item. Two thousand polychromatic erythrocytes (PCEs) were scored for each animal for the analysis of micronuclei. Positive and negative control values of micronucleus frequency were within the historical control ranges of the laboratory confirming the sensitivity of the assay. No toxicity was observed in the bone marrow at the MTDs. The frequency of micronuclei was comparable between treated and negative control groups.

The FEEDAP Panel concludes that Zinc-EDA-Cl did not induce chromosome damage in the experimental conditions employed in this study.

3.2.2.3.2. *Subchronic oral toxicity study in rats*

In a non-GLP study, Fischer 344 rats (10 animals/sex/group) were fed EDA-2HCl at 0, 50, 250, 1,000 mg/kg bw per day (equivalent to 0, 23, 113 and 452 mg EDA/kg bw per day) for 90 days (Yang et al., 1983). Investigated toxicity parameters were body weight, food and water consumption, haematology parameters and a limited number of clinical chemistry parameters (glucose, urea nitrogen, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, total protein, albumin and creatinine). At necropsy, organ weights were recorded for brain, liver, kidneys, spleen, heart, adrenals and testes. Tissues were collected and subjected to microscopic evaluation.

Marked significant decreases in body weight gain were observed in animals of both sexes, and food consumption in females, at 1,000 mg/kg bw per day. A dose-related water consumption decrease was observed in females, but given that this effect was minimal (about 1.95 mL water per rat and day) it was considered to be of no toxicological relevance.

In males and females at 1,000 mg/kg bw per day, a significant decrease in absolute and relative liver weights was observed. In addition, males at this level showed a statistically significant decrease of absolute and relative spleen weights. Other statistically significant organ weights changes were reported in both sexes, however, were not considered toxicologically relevant because of the lack of dose response and/or values were similar to one of the two concurrent controls or were considered a result of the marked body weight gain reduction.

A slight decrease of the red blood cell counts and a slight increased mean corpuscular volume were observed in both sexes at 1,000 mg/kg bw per day. Additionally, in females, a slight decrease of haematocrit and haemoglobin and a slight increase in mean corpuscular haemoglobin were reported. While these changes were dose-related, given their small magnitude they are not considered of toxicological relevance or of enough adversity to describe a clinical state of anaemia.

³² Technical dossier/Section III/Annex III_3_5.

A statistically significant serum glucose level reduction and an increase of alkaline phosphatase activity, AST and ALT activities were reported in both sexes at 1,000 mg/kg bw per day. These findings suggest the probability of an EDA-related effect on the liver of the animals.

A statistically significant lower urine pH was observed in both sexes at 1,000 mg/kg bw per day. This effect may be explained by the known effect of EDA-2HCl as an urine acidifier in human and veterinary medicine. This would also explain the absence of triple phosphate crystals in urine due to an increase of their solubility.

There were no treatment-related gross lesions. The histopathological examination showed an increase in hepatocellular pleomorphism (i.e. cytomegaly, nucleomegaly and multinucleated cells) and occasional mild hepatocellular degeneration at 1000 mg/kg bw per day.

An NOAEL of 250 mg EDA-2HCl/kg bw per day (equivalent to 113 mg EDA/kg bw per day) was identified by the authors, based on reduced body weight gain in both sexes, food and water consumption in females, histopathological effects in liver in both sexes and tracheitis in males observed at 1,000 mg/kg bw per day.

The FEEDAP Panel notes that this study was not GLP-compliant and not performed under the relevant OECD Guideline (Test Guideline 408: Repeated Dose 90-day Oral Toxicity Study in Rodents). Deviations from the regulatory test guideline protocol included the lack of ophthalmological and functional observational battery (FOB) measurements, limited number of haematological and clinical biochemistry parameters measured and a limited number of organs weighed.

3.2.2.3.3. Chronic oral toxicity study

In a non-GLP study performed with Fischer 344 rats, EDA-2HCl was fed at 0, 20, 100 or 350 mg/kg bw per day (equivalent to 9, 45 and 158 mg EDA/kg bw per day) for 2 years (Hermansky et al., 1999).³³ Two separate untreated control groups were used. The number of animals of the dosed groups was 100 animals/sex for the low and the mid levels, and 120 animals/sex for the high level. Interim sacrifices were at 6, 12 and 18 months and the terminal sacrifice was at 24 months. Investigated toxicity parameters were body weight, food and water consumption, a limited number of haematological and clinical biochemistry parameters. A complete urine analysis was conducted in all animals. The evaluation of organ weights was limited to brain, liver, kidneys, spleen, heart, adrenals and testes; the histopathological examination was conducted in a wider range of tissues of all groups.

Most toxic responses were observed at the 12-month sacrifice and thereafter. Reduced body weight gain was observed in males at 350 mg/kg bw per day throughout most of the study and in females at 350 mg/kg bw per day after approximately 18 months. Significant increased mortality was observed in both sexes at 350 mg/kg bw per day and in females at 100 mg/kg bw per day. Most of the deaths occurred after 20 months exposure. The authors indicated that the cause of the decreased survival was unclear but probably ascribable to increased chronic nephropathy.

Erythrocyte counts, haemoglobin concentrations and haematocrit values were generally decreased in males at 350 mg/kg bw per day. Increased urine volume and decreased urine specific gravity were observed in both sexes at 350 mg/kg bw per day in the last half of the study, suggesting a possible alteration in kidney function; these changes reached only significance in males. Yet, altered urine volume and specific gravity persisted to termination in females only, even if significant differences were not detected.

Absolute and relative kidney weights were slightly increased in females at 350 mg/kg bw per day during the second half of the study. Absolute and relative liver weights were slightly increased in females (several measurement intervals) and relative liver weights in males at 350 mg/kg bw per day at 24 months. Hepatocellular pleomorphism was observed in both sexes at 350 mg/kg bw per day. In females hepatocellular pleomorphism incidence increase was reported starting from month 12 while in males at terminal sacrifice. Rhinitis and tracheitis increased in both sexes at 350 mg/kg bw per day.

From this study, an NOAEL of 20 mg EDA-2HCl/kg bw per day (equivalent to 9 mg EDA/kg bw per day) was identified by the authors based on reduced survival in females at 100 mg/kg bw.

The FEEDAP Panel notes that this study was not GLP-compliant and not performed under the relevant OECD Guideline (Test Guideline 452: Chronic Toxicity Studies). Deviations from regulatory test guideline protocol included the following: lack of detailed clinical observations, limited number of haematological and clinical biochemistry parameters measured, limited number of organs weighed and lack of ophthalmological measurements and recording of neurological observations.

³³ Technical Dossier/Section III/Reference32.

3.2.2.3.4. *Reproduction toxicity studies*

Two studies were assessed.

3.2.2.3.4.1. *Study 1*

In a non-GLP two-generation reproduction study, Fischer 344 male and female rats were fed EDA-2HCl at levels of 0, 50, 150 or 500 mg EDA-2HCl/kg bw per day (equivalent to 0, 23, 68 and 226 EDA mg/kg bw per day) (Yang et al., 1984).³⁴ Parameters examined included indices of fertility, gestation of dams, gestation survival, survival of pups, number of pups born alive and number of pups weaned per litter. Furthermore, observations were made on mortality, and body weight of the adult rats in F0 and F1 generation. Necropsies were performed on F1 weanlings (5 rats/sex/dose, 10 control rats/sex), F1 adults (10 rats/sex/dose, 20 control rats/sex) and F2 weanlings (5 rats/sex/dose, 10 control rats/sex). Organ weights were recorded for the liver, kidneys, spleen, heart, brain, adrenals and testes, for all sacrificed rats. A complete gross necropsy examination was conducted on all sacrificed animals. Tissues (high dose and control groups; target organs and lesions for all levels) were histologically examined providing an evaluation of the endocrine, cardiovascular, respiratory, gastrointestinal, reproductive, nervous, musculoskeletal and hematopoietic systems.

No treatment-related mortalities were observed. A statistically significant body weight gain reduction was reported in F0 and F1 adult animals at 500 mg/kg bw per day. A minor body weight gain reduction was reported in F0 females at 150 mg/kg bw per day but given the small magnitude of change, this finding was not considered of toxicological relevance.

A statistically significant decrease of absolute liver weight was observed in F1 adult males at 500 mg/kg bw per day, and a significant increase of absolute and relative kidney weights was observed in F1 adult females at 150 and 500 mg/kg bw per day. In the absence of histopathological correlates, changes of kidney weight are considered of low toxicological significance. A statistically significant increased incidence of hepatocellular pleomorphism was observed in F1 adult animals at 500 mg/kg bw per day.

No treatment-related effects on reproduction parameters were reported.

An NOAEL for reproduction of 500 mg EDA-2HCl/kg bw per day (equivalent to 226 mg EDA/kg bw per day) – the highest level tested – was identified by the authors of the study. An NOAEL for parental toxicity was 150 mg EDA-2HCl/kg bw per day (equivalent to 68 mg EDA/kg bw per day), based on reduced body weight gain and liver histopathological effects in both sexes at 500 mg/kg bw level.

The FEEDAP Panel notes that this study was not GLP-compliant and not performed under the relevant OECD Guideline (Test Guideline 416: Two-Generation Reproduction Toxicity). Deviations from regulatory test guideline protocol included the following: a) no pathological investigation was performed in F0 males; b) no sperm parameters were investigated however, no reproductive apical effect was observed that could be ascribable to effects on sperms; and c) weights of the following organs were not recorded: uterus, ovaries, prostate, seminal vesicles, pituitary and thyroids; however, it seems that the histopathology investigation was performed to evaluate endocrine, cardiovascular, respiratory, gastrointestinal, reproductive, nervous, musculoskeletal and haematopoietic systems.

3.2.2.3.4.2. *Study 2*

In a non-GLP developmental toxicity study, EDA-2HCl was fed to Fischer 344 rats on gestation days (GD) 6 through 15 at levels of 0, 50, 250 and 1,000 mg/kg bw per day (equivalent to 0, 23, 113 and 452 EDA mg/kg bw per day) (DePass et al., 1987).³⁵ Twenty animals per each treatment group were used and 40 served as control timed-pregnant. Food consumption and maternal body weight were measured at several intervals during gestation. On GD 21, the fetuses were delivered by caesarean section, and the standard endpoints for teratogenicity were evaluated.

In animals at 1,000 mg/kg bw per day, a statistically significant body weight loss was reported during GD 6–11 and thereafter body weight gain remained significantly reduced until sacrifice when compared to controls. In animals at 250 mg/kg bw per day, body weight gain was significantly reduced during the exposure period (GD 6–15); thereafter, animals gained weight but remained significantly lower than controls until sacrifice. Food consumption was generally significantly lower than controls during the exposure period in animals at 250 and 1,000 mg/kg bw per day.

³⁴ Technical Dossier/Section III/Reference35.

³⁵ Technical Dossier/Section III/Reference33.

Toxicity effects on fetuses were reduced body weight and crown-rump length, increase of litter incidence with resorptions, skeletal variations and missing or shortened innominate arteries at the highest level of 1,000 mg/kg bw per day.

To investigate whether the above observed fetal effects could be ascribed to poor nutrition or eventually due to palatability, a follow-up study was conducted in the same laboratory. Two control groups were used: one control group with ad libitum access to diet without the test compound and a pair-feeding control to the EDA group. A third group was fed EDA·2HCl at a level of 1,000 mg/kg bw per day. Results showed that all developmental effects observed in the main study were attributable to EDA·2HCl, and not to food restriction, except for missing innominate arteries.

The authors set an NOAEL for maternal toxicity of 50 mg EDA·2HCl/kg bw per day (equivalent to 23 EDA mg/kg bw per day), based on reduced food intake and body weight gain at the level of 250 mg EDA·2HCl/kg bw per day. For developmental toxicity, an NOAEL of 250 mg EDA·2HCl/kg bw per day (equivalent to 113 mg EDA/kg bw per day) was identified, based on fetal weight and crown-rump length reduction, and increased incidences of litter resorptions, skeletal variations and shortened innominate arteries at 1,000 mg EDA·2HCl/kg bw per day. The authors of the study concluded that EDA·2HCl is not teratogenic in Fischer 344 rats.

The FEEDAP Panel notes that this study was not GLP-compliant and not performed under the relevant OECD Guideline (Test Guideline 414: Prenatal developmental Toxicity). Deviations from regulatory test guideline protocol included the following: no observations for potential clinical signs of toxicity were performed on pregnant animals.

3.2.2.3.5. Other toxicological studies

The applicant provided a report in which the neurotoxicity of EDA was addressed (WHO, 1999).³⁶ From the studies described, it was suggested EDA to be a neurotoxic agent, particularly in neonates and in disease states where the blood–brain barrier is incomplete or altered. The potency of this mechanism of action appears to be comparable to that exerted by the gamma-aminobutyric acid (GABA).

3.2.2.3.6. Conclusion of toxicological studies

Data from genotoxicity studies performed with the additive did not raise safety concerns. No other toxicological studies were made available with the additive under assessment. The FEEDAP Panel notes that, in the absence of evidence that the additive completely dissociates in the gastrointestinal tract, the relevance of the toxicological studies performed with zinc and EDA separately is questionable for the safety assessment of the additive.

The toxicological profile of zinc is established and the FEEDAP Panel does not expect any concern from the zinc of Zinc-EDA-Cl. Human intake levels of zinc below the UL are not associated with any concern for the consumer.

From the studies submitted with the EDA component of the additive, the FEEDAP Panel identified a lowest NOAEL of 9 mg EDA/kg bw and day based on the rate of mortality observed from a chronic toxicity study conducted in rats fed with EDA·2HCl. However, the Panel identified several limitations in the completeness of the available data (e.g. ophthalmological measurements and functional observational battery are missing). Moreover, the FEEDAP Panel notes that the neurotoxicity of EDA has been suggested. Therefore, owing to the limitations and uncertainties above described, the FEEDAP Panel is not in the position to assess the toxicity of the EDA component of the additive.

3.2.2.4. Conclusions on safety for the consumer

Considering (i) the overall uncertainty related to the identity of the additive, (ii) the uncertainty related to the fate of the additive, (iii) the absence of reliable residue data in tissues and products, (iv) the absence of toxicological studies (excluding genotoxicity) with the Zinc-EDA-Cl and the limitations and uncertainties of the toxicological studies for EDA, the FEEDAP Panel cannot conclude on the safety of the additive for the consumer.

³⁶ Technical Dossier/Section III/Reference71.

3.2.3. Safety for the user

3.2.3.1. Effects on the respiratory system

No specific inhalation toxicity studies for the product under assessment were provided by the applicant. However, owing to the dusting potential of the additive (up to 930 mg/m³ air; see Section 3.1.3), an estimation of the zinc inhalation exposure was performed.

Taking into consideration the zinc concentration in the dust (average concentration of 278 mg Zn/kg dust), a release of 0.258 mg Zn/m³ can be expected when handling the additive. Considering the potential amount of particles of respirable size of the dust, the zinc concentration in the respirable dust would be of 0.061 mg Zn/m³.³⁷ The estimated value is below the internationally accepted proposed thresholds for zinc (2 mg/m³) set by the American Conference of Governmental Industrial Hygienists as threshold limit value (TLV) (ACGIH, 2003), respectively. Consequently, no concerns are identified regarding inhalation exposure due to the zinc content of the additive.

Uncertainty remains on the effect of the chelate compound in the respiratory system, due to lack of evidence on the fate of the compound in the respiratory tract. However, considering that Zinc-EDA-Cl could be dissociated in the lungs, and owing to the well-known irritation properties of ethylenediamine (ECHA, 2018), the FEEDAP Panel concludes that the additive poses a risk to users upon inhalation.

Concerning nickel, the additive contains up to 2.75 mg Ni/kg. The dusting potential of the product amounted to 930 mg/m³, corresponding to 0.003 mg Ni/m³,³⁸ which is below the occupational exposure limit (OEL) for the inhalable fraction of water-soluble nickel (0.01 mg Ni/m³; European Commission, 2011). However, due to the sole presence of nickel in the additive, given the well-known sensitising properties of nickel, Zinc-EDA-Cl should be considered as a respiratory sensitiser.

Thus, regarding the effects of the additive on the respiratory system, the FEEDAP Panel considers that handling the additive poses a risk to users by inhalation.

3.2.3.2. Effects on the skin and eyes

An acute skin irritation GLP study performed according to the OECD Guideline No. 404 was submitted.³⁹ Under the experimental conditions adopted, Zinc-EDA-Cl was found to be non-irritant for the skin of the rabbit. However, owing to the EDA component of the additive, it should be considered a skin sensitiser (WHO, 1999).

An acute eye irritation GLP study performed according to OECD Guideline No. 405 was submitted.⁴⁰ The rabbit was exposed to a single ocular instillation of the test item (Zinc-EDA-Cl). Under the experimental conditions adopted, Zinc-EDA-Cl caused irreversible damage to the eye of the rabbit and is considered corrosive to eyes.

Furthermore, the nickel content of the additive is up to 2.75 mg/kg; given its well-known sensitisation potential (European Commission, 2011), the additive should be classified as a skin sensitiser.

3.2.3.3. Conclusions on safety for the user

The FEEDAP Panel concludes that handling the additive poses a risk to users by inhalation. The additive should be considered as corrosive to eyes and skin sensitiser.

3.2.4. Safety for the environment

Considering that (i) the data provided in the technical dossier supporting the environmental safety of the additive were not adequate for the assessment (i.e. references to the outcome of environment risk assessment (ERA) on other inorganic and organic zinc sources, including chelates with amino acids or glycine from previous FEEDAP Panel opinions (e.g. EFSA FEEDAP Panel, 2015), ERA of EDA performed by the WHO (1999)) and (ii) the overall uncertainty in the identity of the additive and in its metabolic fate, the FEEDAP Panel cannot conclude on the safety of the additive for the environment.

³⁷ Assuming that the dust contains only particles of < 50 µm diameter, its respirable fraction could be determined as 23.7% (4.9 of 20.7), the zinc content in the dust would be 0.061 mg/m³ (23.7 × 0.258 mg Zn/m³ per 100).

³⁸ Assuming an equivalent distribution of nickel in the dust than that in the additive. (No information of the content of nickel in dust was available).

³⁹ Technical dossier/Section III/Annex III_3_6.

⁴⁰ Technical dossier/Section III/Annex III_3_7.

3.3. Efficacy

For demonstration of the efficacy of nutritional additives, one study in a single animal species or category, including laboratory animals, is generally considered sufficient (EFSA FEEDAP Panel, 2011).

The applicant provided a combined tolerance/residue/efficacy study in chickens for fattening⁴¹ that was not considered as valid (see Section 3.2.1). In the absence of a proper study in the target species, the Panel cannot conclude on the efficacy of Zinc-EDA-Cl.

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 FEEDAP Panel could not confirm the identity of the additive.

The safety for target species, consumer and environment and the efficacy of the additive could not be assessed owing to the uncertainties and limitations identified in the available data.

Handling the additive poses a risk to users by inhalation. The additive should be considered as corrosive to eyes and a skin sensitiser.

5. Documentation provided to EFSA/Chronology

Date	Event
22/08/2018	Dossier received by EFSA. Zinc chelate of ethylenediamine for all animal species. Submitted by Zinpro Animal Nutrition (Europe), Inc.
07/09/2018	Reception mandate from the European Commission
19/10/2018	Application validated by EFSA – Start of the scientific assessment
21/12/2018	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</i>
14/01/2019	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
19/01/2019	Comments received from Member States
01/02/2019	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 (Addendum) – Scientific assessment suspended. <i>Issues: safety</i>
18/02/2019	Reception of supplementary information from the applicant - Scientific assessment re-started
08/05/2019	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 (Addendum) – Scientific assessment suspended. <i>Issues: safety for consumers, safety for the environment</i>
07/06/2019	Clarification teleconference during Risk Assessment
04/03/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
29/06/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 (Addendum) – Scientific assessment suspended. <i>Issues: characterisation, safety for consumers</i>
15/09/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
10/02/2021	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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⁴¹ Technical dossier/Section IV/Annex IV_4_1 and IV_4_2.

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

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Abbreviations

AAS	atomic absorption spectrometry
ATR-FTIR	Attenuated Total Reflectance Fourier Transform Infra-Red
Bw	body weight
CFU	colony-forming units
EURL	European Union Reference Laboratory
FOB	functional observational battery
GLP	Good Laboratory Practice

HILIC	hydrophilic interaction chromatography
HSE	Health and Safety Executive
LOD	limit of detection
MNBN	micronucleated binucleated
MTD	maximum tolerated dose
NOAEL	no observed adverse effect level
OSHA	Occupational Safety and Health Administration
OEL	occupational exposure limit
PCEs	polychromatic erythrocytes
RSDr	standard deviation for <i>repeatability</i>
RSDR	relative standard deviation for <i>reproducibility</i>
SCF	Scientific Committee on Food
TLV	threshold limit value

Annex A – Executive Summary of the Evaluation Report of the European Union Reference Laboratory for Feed Additives on the Method(s) of Analysis for zinc chelate of ethylenediamine

In the current application, authorisation is sought under Article 4(1) for *zinc chelate of ethylenediamine* under the category/functional group (3b) 'nutritional additives'/compounds of trace elements', according to the classification system of Annex I of Regulation (EC) No 1831/2003. Specifically, authorisation is sought for the use of the *feed additive* for all categories and species.

Zinc chelate of ethylenediamine is a solid preparation with a minimum content of 26% (w/w) of zinc and 26% (w/w) of *ethylenediamine (EDA)*. The *feed additive* is intended to be incorporated into *premixtures* and *feedingstuffs*. In addition, the maximum levels of *total zinc* in the *feedingstuffs* ranging from 120 to 200 mg/kg depending on the animal species/category, are established by Regulation (EU) 2016/1095.

For the quantification of *total zinc* in the *feed additive*, *premixtures* and *feedingstuffs*, the Applicant submitted the internationally recognised ring-trial validated CEN method EN 15621 based on ICP-AES after pressure digestion. This method together with the CEN method: EN 15510 based on inductively coupled plasma atomic emission spectrometry (ICP-AES) and the Community method based on atomic absorption spectrometry which was further ring-trial validated by the UK Food Standards Agency (FSA), were previously evaluated and recommended by the EURL in the frame of the Zinc group dossier.

In addition, the EURL is aware of two ring-trial validated methods, namely: ISO 6869 based on atomic absorption spectrometry (AAS) and EN 17053 based on inductively coupled plasma mass spectrometry (ICP-MS).

Based on the acceptable method performance characteristics available, the EURL recommends for official control the five ring-trial validated methods: i) EN 15621 and ISO 6869 for the quantification of *total zinc* in the *feed additive*, *premixtures* and *feedingstuffs*; ii) EN 15510 and EN 17053 for the quantification of *total zinc* in *premixtures* and *feedingstuffs*; and iii) the Community method (Commission Regulation (EC) No 152/2009 – Annex IV-C) for the quantification of *total zinc* in *feedingstuffs*.

For the quantification of *ethylenediamine* in the *feed additive*, the Applicant submitted a single-laboratory validated method based on high-performance liquid chromatography coupled to mass spectrometry detection (LC-MS/MS) using hydrophilic interaction chromatography (HILIC) stationary phase.

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.