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Safety and efficacy of a feed additive consisting of copper 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 copper chelate of ethylenediamine (Copper-EDA-Cl) as a feed additive for all animal species. The EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP Panel) identified several issues related to the data provided concerning the chemical characteristics of the additive and, based on the information provided from an *in vitro* dissociation study, considered it unlikely that the additive consists only of copper mono-chelate of EDA but of several coexisting (copper) species. Therefore, in the absence of adequate experimental data and owing to the uncertainties identified, the Panel cannot conclude on its identity and characterisation of the additive. The FEEDAP Panel concludes that the additive is safe for chickens for fattening and reared for laying/breeding but cannot conclude on the safety for other animal species/categories. The FEEDAP Panel cannot conclude on the safety of the additive for the consumer or the environment. The FEEDAP Panel concludes that handling the additive poses a risk to users by inhalation. The additive should be considered as non-irritant for the skin but corrosive for the eyes and a skin sensitiser. The Panel notes the uncertainties on the genotoxicity potential of the additive that might have an impact on the conclusions on the safety for the user. The FEEDAP Panel concludes that the additive is efficacious in providing copper to meet the nutritional requirements of this trace element in all animal species.

Keywords: nutritional additives, compounds of trace elements, copper chelate of ethylenediamine, CuEDA, safety, efficacy

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1. Introduction

1.1. Background and Terms of Reference

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

The European Commission received a request from Zinpro Animal Nutrition (Europe), Inc.[2] for authorisation of the product copper 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 15 March 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 copper chelate of ethylenediamine, when used under the proposed conditions of use (see Section 3.1.5).

1.2. Additional information

Copper chelate of ethylenediamine (EDA) is intended to be used as a source of copper in all animal species. The additive has not been previously authorised as a 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 dossier[3] in support of the authorisation request for the use of copper chelate of EDA 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 copper chelate of EDA 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 copper chelate of EDA is in line with the principles laid down in Regulation (EC) No 429/2008[5] and the relevant guidance documents: Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008), 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 studies concerning the safety of use of the additive for users/workers (EFSA FEEDAP Panel, 2012b), 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

[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-2018-0089.
[4] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2018-0089-cueda.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.

the target species (EFSA FEEDAP Panel, 2017b) and Guidance on the assessment of the safety of feed additives for the consumer (EFSA FEEDAP Panel, 2017c).

3. Assessment

The additive under assessment is copper chelate of ethylenediamine,[6] and will be referred from here onwards as Copper-EDA-Cl. It is intended to be used as a nutritional additive (functional group: compounds of trace elements) for all animal species and categories.[7]

3.1. Characterisation

3.1.1. Manufacturing process

[redacted]

3.1.2. Identity of the additive

Five batches of the product were analysed for copper, EDA, moisture and chloride. The average content of copper was about 24.4% (24.1–24.9%), EDA 25.1% (23.6—25.9%), chloride 45.2% (44.9–45.6%) and moisture 1.18% (1.1–1.2%);[10] in addition to these data, the applicant provided the content of bound-water which was on average 4.6% (2.5–6.4%).[11]

The applicant provided experimental data to support the amount of chelated and free copper in the additive. Five batches of the additive were analysed; the amount of bound copper averaged to 97.5% (range: 96.9–98.8%).[12]

Based on the available information and knowledge, the applicant made an attempt[13] to provide a chemical description of the additive under assessment. The following characteristics were provided for Copper-EDA-Cl:

- IUPAC Name, Chloro-ethane-(1- ammonium-2-amine)-copper (II) chloride monohydrate.
- Molecular weight of 249.03 g/mol
- Chemical formula $C_2H_{11}Cl_3N_2OCu$.
- The compound is not identified by a Chemical Abstracts Service (CAS) number.

The structural formula, as provided by the applicant, (Figure 1) describes the copper ion (Cu^{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 25.5% copper, 24.5% EDA and 42.7% chloride.

[6] Other names used in the dossier: 'Copper chelate of EDA', 'Copper chelate of ethylenediamine', 'Copper-EDA', 'Copper-Ethylenediamine', 'Copper-Ethylenediamine complex', 'CuEDA' and 'Cu-EDA'. Technical Dossier/Supplementary information (April 2020).
[7] Technical dossier/Section II/2.2.1.
[8] Technical dossier/Section II/2.3.
[9] Technical dossier/Section II/Annexes II-28–29.
[10] Technical dossier/Section II/Annexes II-1—II-5.
[11] Technical Dossier/Supplementary information (September 2020)/Annex: Cooper EDA Identity clarification.
[12] Technical dossier/Supplementary information (April 2020)/1. Experimental report To determine the quantity of chelated copper in the feed additive- CuEDA.pdf. The applicant further indicated that a specification on the level of chelation is not intended to accompany this product.
[13] Technical Dossier/Supplementary information (September 2020)/Annex: Copper EDA Identity clarification.

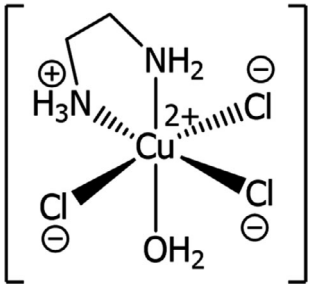

Figure 1: Structural formula of copper chelate of EDA, as provided by the applicant

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 copper for the formation of a coordinate bond unlikely, (ii) the theoretical composition, calculated from the proposed structural formula, showed deviations for chloride, copper 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.[14]

On the other hand, the existence of different copper chelates with EDA, including the mono-, bis- and tris(ethylenediamine) copper (II) complexes has been widely reported in the literature (Bennet et al., 1990) and the occurrence of the mono- and bis (EDA) copper (II) complexes was also shown in an *in vitro* dissociation study (see Section 3.2.2.1). [redacted]

Considering all the above, the FEEDAP Panel is therefore unable to confirm the identity of the additive. The remaining analysis 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: 0.21–0.31 mg/kg, lead: 2.66–4.2 mg/kg, mercury: < 0.05 mg/kg), arsenic: < 0.1 mg/kg and fluorine: < 1.5 mg/kg were reported.[15,16] The levels of dioxins and the sum of dioxins and dioxin-like-PCBs were 0.27–0.35 ng WHO-PCDD/F-TEQ/kg and 0.54–0.69 ng WHO-PCDD/F-PCB-TEQ/kg, respectively.[17] The concentrations of the undesirable substances analysed comply with the limits set in Directive 2002/32/EC for compounds of trace elements[18] or, if not mentioned in the Directive, do not represent a concern. The nickel content of the additive (analysis of three batches) showed an average of 2.03 mg/kg (range 1.70–2.65).[19]

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.[20] Levels of aflatoxin B1 and ochratoxin A analysed in three batches were below the limit of detection (LOD < 0.1 μg/kg).[21]

[14] The applicant provided the infrared absorption spectra for three lots of the additive to identify the NH2 peaks, showing peaks at three wavelengths (1,635.6, 1,628.0 and 1,628.1 cm^{-1}); Technical dossier/Section II/2.2.2.1.
[15] Technical dossier/Section II/Annexes II-10—II-14.
[16] Technical Dossier/Supplementary information (April 2020)/Where the symbol '<' is used, the figure corresponds to the limit of detection of the analytical method.
[17] Technical dossier/Section II/Annexes II-15—II-19.
[18] 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.
[19] Technical Dossier/Supplementary information (April 2020).
[20] Technical dossier/Section II/Annexes II-20–II-22.
[21] Technical dossier/Section II/Annexes II-23–25.

3.1.3. Physical properties of the additive

The additive is a powder with a bulk density of 1,013 kg/m^3(average of three batches).[22] 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 acetate;[23] however, no supporting data was made available.

Particle size distribution was studied in three batches of the additive (laser-diffraction);[22] particles below 10, 50 and 100 μm were on average 10.2% (range: 10.06–10.25%), 36.6% (range: 36.35–36.70%), and 51.7% (range: 51.49–51.82%), respectively.

Dusting potential was analysed in three batches of the additive by the Stauber–Heubach method in the same three batches as the particles size distribution; four measures were taken on each batch.[22] The results showed a dusting potential ranging from 6.3 to 7.3 g/m^3 air.[22] The applicant provided data on the copper content of the dust; the average copper content was 281 mg Cu/kg dust (range 276–286 mg Cu/kg).[22]

3.1.4. Stability and homogeneity

A broiler vitamin/mineral premixture and a mash and pelleted feed containing the additive were stored for six months (at room temperature). At the end of the experiment, the content of copper in the premixture was 98% that of initially measured; in the mash and pelleted feed this value was 97% and 93%, respectively.

The capacity of the additive to homogeneously distribute in a premixture and complete feed (mash and pelleted) for chickens for fattening was investigated.[24] The copper content was analysed in ten subsamples each. The coefficient of variation (CV) of the copper concentration in the premixture (mean 4.407 mg/kg) was 7.2%. The CV of the mash feed (mean copper content: 32 mg/kg) was up to 4.8%; on the same feed after pelleting (mean copper content: 29 mg/kg) the CV was up to 4.7%.

3.1.5. Conditions of use

The additive is intended to be used in feed – via a premixture – for all animal species up to a maximum total copper content in feed of 15 mg/kg complete feed (bovines before the start of rumination and ovines), 30 mg/kg complete feed (other bovines), 35 mg/kg complete feed (caprines), 50 mg/kg complete feed (crustaceans), 150 mg/kg (piglets suckling and weaned up to 4 weeks after weaning) and 100 mg/kg complete feed (piglets from 5th week after weaning up to 8 weeks after weaning), and 25 mg/kg (other species).

3.2. Safety

3.2.1. Metabolic studies

No data concerning the metabolic fate of Copper-EDA-Cl have been 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 of the dissociation of the additive in gastric-ruminal fluids.[25]

[redacted]

[22] Technical dossier/Section II/Annex II-26.
[23] Technical dossier/Section II/Annex II-30.
[24] Technical dossier/Section II/Annex II-27.
[25] Technical Dossier/Supplementary information (September 2020)/Annex 'CuEDA dissociation analysis report rev 2- Confidential'.

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.

3.2.1.1. Residue study

In the tolerance study for chickens for fattening (see Section 3.2.3), copper and EDA deposition in liver, kidneys, muscle (breast) and skin/fat were measured after feeding the additive for 35–37 days at 15, 25 and 200 mg Cu/kg complete feed. Measurements were taken in 12 birds per treatment. Copper deposition was compared to that resulting from the administration in the same conditions copper sulfate at 8, 15, 25 and 200 mg Cu/kg complete feed.

Copper deposition

Total copper was analysed in tissues after microwave digestion of samples with nitric acid at 230°C using an inductively coupled plasma-mass spectrometry (ICP-MS) method validated in-house with a LOD of 0.13 mg/kg and a limit of quantification (LOQ) of 0.38 mg/kg.[26] The results on copper deposition are reported in Table 1.

[26] Technical Dossier/Supplementary information (May 2020).

Table 1: Copper deposition (mg/kg wet tissue) in tissues from chickens for fattening fed Copper-EDA-Cl or copper sulfate for 37 days at different levels

Source	Added Cu mg/kg feed	Total Cu mg/kg	Liver	Muscle	Skin/Fat	Kidneys	Bone
Control	0*	8	0.352[b]	0.031	0.034[b]	0.255	0.118
Cu-EDA-Cl	7	15	0.338[b]	0.034	0.035[b]	0.259	0.121
	17	25	0.324[b]	0.030	0.039[b]	0.255	0.113
	192	200	0.431[a]	0.034	0.079[a]	0.261	0.124
Cu sulfate	7	15	0.317[b]	0.031	0.035[b]	0.258	0.122
	17	25	0.319[b]	0.031	0.038[b]	0.257	0.115
	192	200	0.432[a]	0.032	0.086[a]	0.267	0.133

*: After the copper content was determined in the basal feed, supplemental copper from copper sulfate was added to reach an intended concentration of 8 mg Cu/kg in the basal diet for the starter and grower phases.
a,b: Different letter superscripts in the same column indicate significant differences ($p < 0.05$).

No significant differences in copper contents of kidney, muscle or bone of all groups were observed. Copper contents of liver and skin/fat corresponding to the lowest and middle levels of organic or inorganic copper were similar. Copper contents in the liver and skin/fat at the highest doses of organic and inorganic copper were similar to each other and significantly higher when compared to the lower levels.

Total EDA in tissues was analysed after extraction with diluted hydrochloric acid and dissolution of extracts with acetonitrile containing 0.1% formic acid via liquid chromatography–mass spectrometry (LC–MS);[27,28] the analytical method was validated in-house with LOD of 5 μg/L and LOQ of 10 μg/L (corresponding to 0.8 mg/kg chicken tissue extract considering a dilution of × 80). The results provided by the applicant were not consistent with the LOQ described in the validation report[29] and therefore, the applicant was invited to re-submit the complete raw data on EDA residues considering the LOQ of 0.8 mg/kg. However, these data were not provided and consequently the FEEDAP Panel was not in the position to conclude on EDA residues in tissues.

Moreover, no data on residues of copper and EDA in the tissues and products (milk, egg) of other target animal species administered the additive were made available.[30]

3.2.2. Toxicological studies

The applicant provided information supporting the toxicological profile of the additive.

With the exception of genotoxicity studies, for which the item tested was Copper-EDA-Cl, the applicant provided separated data on copper toxicity and ethylenediamine dihydrochloride (EDA·2HCl) toxicity.

The toxicology of copper has been reviewed by Ellingsen et al. (2015) and by the FEEDAP Panel (EFSA FEEDAP Panel, 2015). To the knowledge of the FEEDAP Panel, there are no new relevant toxicology studies on copper that could modify the previous review. Under normal circumstances, copper homoeostasis ensures that copper overload in humans does not occur. An excess of copper has been recorded and shown to cause problems only under certain specific conditions, notably genetic disorders such as Wilson disease (EFSA NDA Panel, 2015). The primary target of copper toxicity is the hepatocytes and copper excess impairs liver function (European Commission, 2003). The Scientific Committee on Food (SCF), based on adverse effects on liver, set a tolerable upper intake level (UL) of 5 mg Cu/day for adults and 1 mg/day for toddlers (1–3 years of age) (European Commission, 2003).

3.2.2.1. Genotoxicity studies

The dossier includes two genotoxicity studies (bacterial reverse mutation assay and *in vitro* mammalian cell micronucleus test) performed with Copper-EDA-Cl.

- Bacterial reverse gene mutation assay

[27] Technical Dossier/Supplementary Information May 2020.
[28] Severe analytical deficiencies were identified in the submitted data set preventing a thorough assessment of the presence of residues in tissues.
[29] The applicant indicated directly in the study report an LOQ of 5 mg/kg but the FEEDAP Panel considered valid the LOQ reported in the laboratory's report of the validation of the analytical method (i.e. 0.8 mg/kg). Technical Dossier/Supplementary information (September 2020).
[30] Technical Dossier/Supplementary information (March 2020).

In order to investigate the potential of Copper-EDA-Cl (Cu 22.3%, purity unknown, see Appendix 4) to induce gene mutations in bacteria, the Ames test was performed according to OECD Test Guideline (TG) 471[31] and following Good Laboratory Practice (GLP) in *Salmonella* Typhimurium strains [redacted] No increase in the mean number of revertant colonies was observed at any tested concentration in any tester strains with or without S9-mix. The Panel concluded that Copper-EDA-Cl did not induce gene mutations in bacteria under the experimental conditions employed in this study.

- *In vitro* mammalian cell micronucleus test

An *in vitro* micronucleus test was performed according to OECD TG 487[33] and following GLP to evaluate the potential of Copper-EDA-Cl (Cu 22.3%, purity unknown, see Appendix 5) to induce chromosome damage in TK6 lymphoblastoid human cells in the absence and presence of metabolic activation.[34]

The compound was tested at concentrations ranging from 2.5 to 80 μg/mL; maximum concentrations were limited by cytotoxicity, measured as relative population doubling. A short treatment (3 + 24 h of recovery) with and without S9-mix and a continuous treatment (27 + 0 h recovery) without S9-mix were the experimental conditions applied. Appropriate positive and negative control chemicals were used and the results obtained confirmed that the experimental system was sensitive and valid. No significant increase of micronucleated cells was induced by treatment with Copper-EDA-Cl compared to concurrent vehicle controls in the absence of metabolic activation. A statistically significant increase of micronucleated cells was observed at the highest concentration tested after short treatment in the presence of S9-mix, associated with 54% cytotoxicity. The increase was dose-related and above the range of negative historical controls. The Panel is aware that cautions should be applied when evaluating the biological relevance of a result observed at highly toxic concentration and considered these results inconclusive.

3.2.2.2. Subchronic oral toxicity study in rats

In a non-GLP study,[35] Fischer 344 rats (10 animals/sex per group) were fed EDA·2HCl at 0, 50, 250, 1,000 mg/kg/day (equivalent to 0, 23, 113 and 452 EDA mg/kg body weight (bw) per day) for 90 days. Investigated toxicity parameters were body weights, 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 (ALP), 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 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 in 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 in the red blood cell counts, and a slight increase in mean corpuscular volume were observed in both sexes at 1,000 mg/kg bw per day. Additionally, in females, a slight decrease in haematocrit and haemoglobin and a slight increase in mean corpuscular haemoglobin were reported.

[31] OECD Guideline Version of 1997. Available online: https://www.oecd.org/chemicalsafety/risk-assessment/1948418.pdf
[32] Technical dossier/Section III/Annex III_3_2 CERB OECD 471_Ames_Cu-EDA_Oct 2017.pdf.
[33] OECD Guideline Version of 2014. Available online: https://www.oecd-ilibrary.org/environment/test-no-487-in-vitro-mammalian-cell-micronucleus-test_9789264224438-en
[34] Technical dossier/Section III/Annex III_3_3 CERB OECD 487_In vitro Mammalian cell_Cu-EDA_Oct 2017.pdf.
[35] Technical dossier/Section III/References/13_Hermansky et al_1999.pdf.

While these changes were dose-related, the small magnitude of these changes is not considered of toxicological relevance or of enough adversity to describe a clinical state of anaemia.

A statistically significant serum glucose level reduction and an increase of ALP, 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 gross lesions associated with the treatment. The histopathological examination showed an increase in hepatocellular pleomorphism (i.e. cytomegaly, nucleomegaly and multinucleated cells) and occasional mild hepatocellular degeneration at 1,000 mg/kg bw per day.

A no observed adverse effect level (NOAEL) of 250 EDA·2HCl mg/kg bw per day (equivalent to 113 EDA mg/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 and histopathologically examined.

3.2.2.3. Chronic oral toxicity studies

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).[36] Two separate untreated control groups were used. The number of animals of the groups were 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 weights, food and water consumption, a limited number of haematological[37] and clinical biochemistry[38] 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 was 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 female 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.

[36] Technical dossier/Section III/Annex: 13_Hermansky et al_1999.pdf.

[37] Red and white blood cell counts, haemoglobin, mean corpuscular volume, haematocrit, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration.

[38] Glucose, urea nitrogen, total protein, albumin, creatinine, bilirubin (conjugated and total) aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and sorbitol dehydrogenase.

From this study, a NOAEL of 20 mg EDA·2HCl/kg bw per day (equivalent to 9 EDA mg/kg bw per day) was identified by the authors based on reduced survival in females at 100 mg/kg bw (Hermansky et al., 1999).

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 histopathologically examined and lack of ophthalmological measurements and recording of neurological observations.

3.2.2.4. Reproductive toxicity studies

Two studies were assessed.

Study 1

In a non-GLP two-generation reproduction study Fischer 344 rats were fed in diet EDA-2HCl at levels of 0, 50, 150 and 500 EDA-2HCl mg/kg bw per day (equivalent to 0, 23, 68 and 226 EDA mg/kg bw per day).[39] 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 per dose, 10 control rats/sex), F1 adults (10 rats/sex per dose, 20 control rats/sex), and F2 weanlings (5 rats/sex per 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 haematopoietic 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.

A NOAEL for reproduction of 500 EDA·2HCl mg/kg bw per day (equivalent to 226 EDA mg/kg bw per day) – the highest level tested – was identified by the authors of the study.

A NOAEL for parental toxicity was 150 EDA·2HCl mg/kg bw per day (equivalent to 68 EDA mg/kg bw per day), based on reduced body weight gain and liver histopathological effects in both sexes at 500 mg/kg bw level (Yang et al., 1984 as cited in Hermansky et al, 1999).

The Panel notes that this study was not compliant and not performed under the relevant OECD Guideline (Test Guideline 416: Two-Generation Reproduction Toxicity). The main deviations from the current Guidance are (i) no pathological investigation was performed in F0 males; (ii) no sperm parameters were investigated; however, no reproductive apical effect was observed that could be ascribable to effects on sperms; (iii) 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.

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 EDA·2HCl mg/kg bw per day (equivalent to 0, 23, 113 and 452 EDA mg/kg bw per day) (DePass et al., 1987).[40] Twenty animals per each treatment

[39] Technical dossier/Section III/Reference 13_Hermansky et al_1999.pdf.
[40] Technical dossier/Section III/15_DePass at al_1987.docx.

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 foetuses 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.

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 dose of 1,000 mg/kg bw per day.

To investigate whether the above observed fetal effects could be ascribed to poor nutrition, a follow-up study was conducted in the same laboratory. Two control groups were used; one control group with *ad libitum* access to diet 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 a NOAEL for maternal toxicity was 50 EDA-2HCl mg/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, a NOAEL of 250 EDA·2HCl mg/kg bw per day (equivalent to 113 EDA mg/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 41: 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.

3.2.2.5. Other toxicological studies

The applicant provided a report in which the neurotoxicity of EDA was addressed (WHO, 1999).[41] 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).

3.2.2.6. Conclusions on the toxicological studies

The results obtained in the genotoxicity studies with Copper-EDA-Cl showed no induction of gene mutations in bacteria and inconclusive data for the induction of chromosome damage. Due to the limitations of the micronucleus test, the FEEDAP Panel cannot conclude on the genotoxic potential of the additive. The toxicological profile of copper is well established and the Panel does not expect any concern from the copper content of Copper-EDA-Cl. Human intake levels of copper below the UL are not associated with any concern for the consumer.

From the toxicological studies submitted with the EDA component of the additive, the Panel identified a lowest NOAEL of 9 mg EDA/kg bw per 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.

[41] Technical Dossier/Section III/Reference 24.

3.2.3. Safety for the target species

The applicant provided a tolerance study with Copper-EDA-Cl in chickens for fattening [redacted]

[redacted]

[redacted]	[redacted]	[redacted]	[redacted]	
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	[redacted]	[redacted]	[redacted]	[redacted]
	[redacted]	[redacted]	[redacted]	[redacted]

[redacted]

[redacted] The results are summarised in Table 3.

[42] Technical dossier/Section III/Annex III_3_1.
[43] Due to the capacity of laboratory to process samples, the necropsies took place over 3 days upon termination of the study (until day 37).
[44] Haemoglobin, haematocrit, white blood cells (WBC), heterophils, eosinophils, basophils, monocytes, lymphocytes.
[45] Sodium, potassium, chloride, phosphorus, calcium, copper, uric acid, aspartate aminotransferase (AST), alamine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), glutamate dehydrogenase (GLDH), lactate dehydrogenase (LDH), creatinine kinase (CK), total proteins, albumin, globulin, glucose, amylase, cholesterol, triglycerides, total bilirubin.

Table 3: Description of the performance parameters from the tolerance study in chickens for fattening (1–35 days)[(a)]

Source of added copper	Total Cu (mg/kg)	Feed intake (kg)	Final weight (g)	Weight gain (g)	Feed/gain ratio	Mortality (%)
Control group	8	3.72[a]	2,431	2,387	1.56	4.17
Copper-EDA-Cl	15	3.48[bc]	2,330	2,289	1.52	1.39
	25	3.61[ab]	2,410	2,366	1.53	2.78
	200	3.39[c]	2,288	2,245	1.51	2.78
Copper sulfate	15	3.64[ab]	2,366	2,321	1.57	8.33
	25	3.71[a]	2,442	2,399	1.55	2.78
	200	3.48[bc]	2,386	2,345	1.49	1.39

a, b: values in the same columns with different superscripts are significant at $p \leq 0.05$.
(a): Technical dossier/Supplementary information April 2020/1. FeEDA Signed final T&E report. (appendix 11 end of the Appendix 208 and following).

Mortality was in the average 3.4%, and no differences between treatments were identified. The treatment had a significant negative effect on feed intake in the groups receiving Copper-EDA-Cl at 15 and 200 total copper per kg complete feed and those with 200 mg total copper from copper sulfate. ANOVA could not identify significant differences in body weight and body weight gain at $p < 0.05$, but a tendency ($p < 0.08$ and < 0.09, respectively). Using a Fisher LSD test these endpoints were significantly lower ($p < 0.05$) in the Copper-EDA-Cl group receiving 200 mg total copper per kg feed compared to control group. No statistical differences were observed for the other endpoints.

The additive is safe up to a level of 25 mg total copper/kg feed (maximum authorised level for chickens for fattening), no margin of safety can be established.

3.2.3.1. Conclusions on safety for the target species

Based on a tolerance study in chicken for fattening in which birds tolerated up to 25 mg total copper/kg feed (the maximum authorised level of copper for chickens), the Panel concludes that Copper-EDA-Cl is safe for chickens for fattening at the proposed conditions of use. This conclusion is extended/extrapolated to all poultry species for fattening and this conclusion can be extended to chickens reared for laying/breeding.

Considering that the additive under assessment is a chelate compound of a trace element with a xenobiotic substance (EDA), the Panel considers that in order to extrapolate the safety to all animals species, tolerance studies in pigs, cows and salmonids would be required. In the absence of these studies, the Panel is not in a position to conclude on the safety of Copper-EDA-Cl for species/ categories other than chickens for fattening/reared for laying/breeding.

The Panel notes the uncertainties on the genotoxicity potential of the additive that might have an impact on the conclusions on the safety for the target species in particular for long living and reproduction animals.

3.2.4. 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 inconclusive outcome of one of the genotoxicity studies provided, (v) the absence of toxicological studies (except genotoxicity) with the Iron-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.

3.2.5. Safety for the user

3.2.5.1. 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 7.3 g/m^3 air; see above Section 3.1.3), an estimation of the copper inhalation exposure was performed.

Taking into consideration the copper concentration in the dust (average concentration of 281 mg Cu/kg dust), a release of 2.05 mg Cu/m^3 can be expected when handling the additive. Considering the

potential number of particles of respirable size of the dust, the copper concentration in the respirable dust would be of 0.57 mg/m^3.[46] The estimated value is above the internationally accepted proposed thresholds for copper (copper occupational exposure limit 0.01 mg/m^3 (European Commission, 2014)). Consequently, concerns are identified regarding inhalation exposure due to the copper 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 Copper-EDA-Cl could be dissociated in the lungs, and owing to the well-known irritation properties of EDA (ECHA, 2018a,b), the FEEDAP Panel concludes that the additive poses a risk to users upon inhalation.

Concerning nickel, the additive contains up to 2.65 mg Ni/kg. The dusting potential of the product amounted to 7.3 g/m^3, corresponding to approximately 0.02 mg Ni/m^3, which is below the occupational exposure limit (OEL) for the inhalable fraction of water-soluble nickel (0.03 mg Ni/m^3; ECHA, 2018a,b). However due to the sole presence of nickel in the additive, it 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.

3.2.5.2. Effects on the skin and eyes

An acute skin irritation assay was performed to determine any irritant property and/or degree of corrosion of Copper-EDA-Cl in the rabbit following a single semi-occluded application to intact skin according to OECD TG 404 (April 24, 2002).[47] No other cutaneous lesions were found. Under the experimental conditions adopted, Copper-EDA was found to be non-irritant for the skin of the rabbit.

An acute eye irritation assay was performed to determine any irritant property and/or degree of corrosion of Copper-EDA-Cl following a single ocular instillation in the rabbit according to OECD Guideline No. 405 (October 2012).[48] Twenty-four and 72 h after instillation, corneal opacity was present on an area greater than one-quarter of the cornea, associated with purulent discharge and signs of pain (such as reduced spontaneous locomotor activity and eye closed) in spite of the analgesic procedure applied. Under the experimental conditions adopted, Copper-EDA 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 2.65 mg/kg; given its well-known sensitisation potential (European Commission, 2011; ECHA, 2018a,b) and in the absence of skin sensitisation studies the additive should be classified as a skin sensitiser.

3.2.5.3. 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 non-irritant for the skin but corrosive for the eyes and a skin and respiratory sensitiser.

The Panel notes the uncertainties on the genotoxicity potential of the additive that might have an impact on the conclusions on the safety for the user.

3.2.6. 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 copper sources, including chelates with amino acids or glycine from previous FEEDAP Panel opinion (2015), ERA of EDA performed by 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.

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).

[46] Assuming that the dust contains only particles of diameter (< 50 μm), its respirable fraction could be determined as 27.9% (10.2 of 36.6), the copper content in the dust would be 0.57 mg/m^3 (27.9% × 2.051).
[47] Technical dossier/Section III/Annex III_3_4.
[48] Technical dossier/Section III/Annex III_3_5.

The applicant provided a combined tolerance/efficacy study in chickens for fattening[49] (see Sections 3.2.1.2 and 3.2.3). The experimental groups in the study are shown in Table 2. In this trial, copper concentration in edible tissues/organs and bones was measured.

There was no difference in the copper deposition in tissues, organs and bones between the additive and the inorganic (standard) source of copper at the same levels (see Table 1). Copper-EDA-Cl was as effective as copper sulfate in increasing copper concentrations in the liver and the skin/fat when added at 200 mg total copper per kg complete feed. The Panel notes that for poultry copper accumulation in liver starts at high copper intakes (50 times the requirements) and therefore the effects can only be seen at doses higher than the use levels.

3.3.1. Conclusions on efficacy

The FEEDAP Panel concludes that the additive is efficacious in providing copper to meet the nutritional requirements of this trace element in chickens for fattening. This conclusion can be extended to all animal species.

3.4. Post-market monitoring

The FEEDAP Panel considers that there is no need for specific requirements for a post-market monitoring plan other than those established in the Feed Hygiene Regulation[50] and Good Manufacturing Practice.

4. Conclusions

In the absence of adequate experimental data and owing to the uncertainties identified in characterisation of the additive, the Panel cannot conclude on the identity and characterisation.

The FEEDAP Panel concludes that the additive is safe for chickens for fattening and reared for laying/breeding at the maximum authorised level of total copper, but cannot conclude on the safety for other animal species/categories.

The FEEDAP Panel cannot conclude on the safety of the additive for the consumer or the environment.

The FEEDAP Panel concludes that handling the additive poses a risk to users by inhalation. The additive should be considered as non-irritant for the skin but corrosive for the eyes and skin and respiratory sensitiser. The Panel notes the uncertainties on the genotoxicity potential of the additive that might have an impact on the conclusions on the safety for the user.

The FEEDAP Panel concludes that the additive is efficacious in providing copper to meet the nutritional requirements of this trace element in all animal species.

Chronology

Date	Event
13/12/2018	Dossier received by EFSA
01/02/2019	Reception mandate from the European Commission
15/03/2019	Application validated by EFSA – Start of the scientific assessment
15/05/2019	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended. *Issues: characterisation, safety for the consumer*
15/06/2019	Comments received from Member States
15/06/2019	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
07/06/2019	Clarification teleconference during Risk Assessment with the applicant according to the "EFSA's Catalogue of support initiatives during the life-cycle of applications for regulated products"
23/04/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
23/04/2020	Reception of supplementary information from the applicant - Scientific assessment re-started

[49] Technical dossier/Section IV/Annex IV_4_1 and IV_4_2.

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

Date	Event
17/07/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 – Scientific assessment suspended *Issues: characterisation, safety for the consumer*
21/09/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
25/09/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
17/03/2021	Opinion adopted by the FEEDAP Panel. End of the Scientific assessment

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Abbreviations

AAS	atomic absorption spectrometry
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
AST	aspartate aminotransferase
bw	body weight
CAS	Chemical Abstracts Service
CFU	colony forming unit
Copper-EDA-Cl	copper chelate of ethylenediamine
CV	coefficient of variation
ECHA	European Chemicals Agency
EDA	ethylenediamine
EURL	European Union Reference Laboratory
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
FOB	functional observational battery
FSA	UK Food Standards Agency
GABA	gamma-aminobutyric acid
GD	gestation days
GLP	Good Laboratory Practice
HILIC	hydrophilic interaction chromatography
ICP-AES	inductively coupled plasma-atomic emission spectrometry
ICP-MS	inductively coupled plasma-mass spectrometry
IUPAC	International Union of Pure and Applied Chemistry
LC–MS	liquid chromatography coupled to mass spectrometry
LC–MS/MS	liquid chromatography coupled to mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
NOAEL	no observed adverse effect level
OEL	occupational exposure limit
PCB	polychlorinated biphenyl
PCDD/F	polychlorinated dibenzo-*p*-dioxin and dibenzofuran
TLV	threshold limit value
UL	tolerable upper intake level
WHO	World Health Organization

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

In the current application authorisation is sought under Article 4(1) for copper as an *copper chelate of ethylenediamine* preparation 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.

Copper chelate of ethylenediamine is a solid preparation for supplementing copper with a minimum content of 23% (w/w) of *copper* and 23% (w/w) of *ethylenediamine (EDA).*

The *feed additive* is intended to be incorporated into premixtures and feedingstuffs according to the maximum levels of *total copper* in *feedingstuffs* which range from 15 to 150 mg/kg depending on the animal species/category, as established by Regulation (EU) 2018/1039.

For the quantification of *total copper* in the *feed additive*, *premixtures* and *feedingstuffs* the Applicant submitted the internationally recognised ring-trial validated CEN method EN 15621 based on inductively coupled plasma-atomic emission spectrometry (ICP-AES) after pressure digestion. This method together with the CEN method EN 15510 based on ICPAES after ashing or wet digestion 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 previous copper dossiers.

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 copper* in the *feed additive*, *premixtures* and *feedingstuffs*; ii) EN 15510 and EN 17053 for the quantification of *total copper* in *premixtures* and *feedingstuffs*; and iii) the Community method (Commission Regulation (EC) No 152/2009 – Annex IV-C) for the quantification of *total copper* 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 (LC-MS/MS) detection using a hydrophilic interaction chromatography (HILIC) stationary phase. This method was previously evaluated by the EURL in the frame of the other *ethylenediamine chelate* dossiers for the characterisation of the ligand in the *feed additive* and it was considered as fit-for-purpose.

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.