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Safety and efficacy of a feed additive consisting of manganese 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 manganese chelate of ethylenediamine (Manganese-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 manganese mono-chelate of EDA, but of several coexisting (manganese) 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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Keywords: nutritional additives, compounds of trace elements, manganese chelate of ethylenediamine, MnEDA, safety, efficacy

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Table of contents

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 manganese 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 30 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 manganese chelate of ethylenediamine, when used under the proposed conditions of use (see Section 3.1.5).

1.2. Additional information

Manganese chelate of ethylenediamine is intended to be used as a source of manganese 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 dossier[3] in support of the authorisation request for the use of manganese 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 manganese 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 manganese chelate of ethylenediamine is in line with the principles laid down in Regulation (EC) No 429/2008[5] and the relevant guidance documents: 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 use of the additive for users/workers (EFSA FEEDAP Panel, 2012), Guidance

[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-2018-0067.
[4] The full report is available on the EURL website: https://ec.europa.eu/jrc/sites/jrcsh/files/finrep-fad-2018-0067-mn-eda.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.

on the assessment of the efficacy of feed additives (EFSA FEEDAP Panel, 2018) and Technical Guidance for assessing the safety of feed additives for the environment (EFSA, 2008).

3. Assessment

The additive under assessment is manganese chelate of ethylenediamine (EDA),[6] and will be referred from here onwards as Manganese-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 manganese chloride and ethylenediamine (EDA) to form Manganese-EDA-Cl. [redacted][7,8]

3.1.2. Identity and characterisation of the additive

Five batches of the product were analysed for manganese, EDA, moisture and chloride. The average content of manganese was about 21.7% (21.1–22.2%), EDA 22.2% (21.6–22.6%), chloride 49.1% (48.0–49.7%) and moisture 0.2%[9]; in addition to these data, the applicant provided the content of bound-water which was on average 6.5% (5.4–8.1%).[10]

The applicant provided experimental data to support the amount of chelated and free manganese in the additive. Five batches of the additive were analysed; the amount of bound manganese averaged to 97.3% (range: 96.1–99.1%).[11]

Based on the available information and knowledge, the applicant made an attempt[12] to provide a chemical description of the additive under assessment. The following characteristics were provided for Manganese-EDA-Cl:

- IUPAC Name, Chloro-ethane-(1- ammonium-2-amine)-manganese (II) chloride monohydrate
- Molecular Weight, 240.42 g/mol
- Chemical Formula, $C_2H_{11}Cl_3MnN_2O$
- The compound is not identified by a Chemical Abstracts Service (CAS) number

The structural formula, as provided by the applicant (Figure 1), describes the manganese ion (Mn^{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 22.9% manganese, 25.4% EDA and 44.2% chloride.

[6] Other names used in the dossier: 'Manganese chelate of EDA', 'Manganese chelate of ethylenediamine', 'Manganese-EDA', 'Manganese-Ethylenediamine', 'Manganese-Ethylenediamine complex', 'MnEDA', 'Mn-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 to –5.
[10] Supplementary information (September 20). The FEEDAP Panel notes that these data were provided without a certificate of analysis.
[11] 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.
[12] Technical Dossier/Supplementary information (September 2020)/Annex: Manganese EDA Identity clarification.

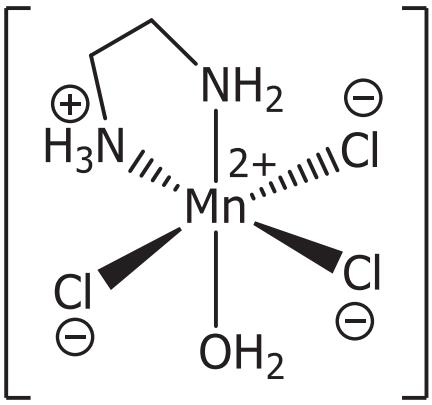

Figure 1: Structural formula of manganese chelate of ethylenediamine, as provided by the applicant

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 manganese for the formation of a coordinate bond unlikely, ii) the theoretical composition, calculated from the proposed structural formula, showed deviations for manganese, EDA and chloride, 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.[13] No evidence was provided to demonstrate that i) the additive is a monochelate of manganese with EDA, ii) chloride ions act (at least in part) as ligands rather than as counterions and iii) manganese is hexa- rather than tetra-coordinated.

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

Considering all the above, the FEEDAP Panel is therefore 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: 0.19–0.24 mg/kg, lead: 2.3–3.2 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 0.099–0.625 ng WHO-PCDD/F-TEQ/kg and 0.105–0.704 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 2.74 mg/kg (range 2.70–2.79).[19]

[13] The applicant provided the infra-red absorption spectra for three lots of the additive to identify the NH_2 peaks, showing peaks at three wavelengths (1633.7, 1633.8 and 1634.7 cm^{-1}); Technical dossier/Section II/2.2.2.1.
[14] Manganese species measured in the dissociation study (see Section 3.2.2.1): Mn^{2+}, $[Mn(EDA)]^{2+}$ $[Mn(EDA)_2]^{2+}$ $[Mn(OH)]^{+}$ and $[Mn_2(OH)_3]^{+}$.
[15] Technical dossier/Section II/Annexes II-10–14.
[16] Technical Dossier/Supplementary information (February 2019)/Where the symbol '<' is used, the figure corresponds to the limit of detection of the analytical method.
[17] Technical dossier/Section II/Annexes II-15–19.
[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 (February 2019).

Three batches of the additive were analysed for microbial contamination. Counts of *Enterobacteriaceae*, moulds and yeasts were < 10 colony-forming units (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]

3.1.3. Physical properties of the additive

The additive is a powder with a bulk density of 1,007 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)[24]; particles below 10, 50 and 100 μm were on average 8.0, 30.2 and 43.5%, respectively.

Dusting potential was analysed by the Stauber-Heubach method in the same three batches as the particle size distribution; five measures were taken on each batch.[24] The results showed a dusting potential ranging from 23.1 to 25.8 g/m^3 air. The applicant provided data on the manganese content of the dust measured in the same dust samples (total of 15 subsamples); the average manganese content was 208 mg Mn/kg dust (range 201–213 mg Mn/kg).[24]

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 and the poultry feed (mash and pelleted) containing the additive were stored for 6 months at room temperature. At the end of the storage, the content of manganese in the premixture was 93.4% of that initially measured; in the mash and pelleted feed, this value was about 100% and 89.3%, respectively.

The capacity for homogeneous distribution of the additive in premixtures (vitamin–mineral) and complete feed (mash and pelleted) for chickens for fattening was investigated[25]; the manganese content was analysed in 10 subsamples each. The coefficient of variation (CV) of the manganese concentration in the premixture (mean 22,840 mg Mn/kg) was 4.0%. The CV in the mash feed (mean manganese content: 154 mg/kg) was 4.2% and in the same feed after pelleting (mean manganese content: 150 mg/kg) was 4.9%.

3.1.5. Conditions of use

The additive is intended to be used in feed – via a premixture – for all animal species. It should be used up to a maximum total manganese in feed of 100 mg/kg (fish) and 150 mg/kg (other species and categories).

3.2. Safety

3.2.1. Safety for the target species

The applicant provided a tolerance study with Manganese-EDA-Cl in chickens for fattening with duration of 35 days.[26,27] 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 uncertainties since EDA was found in the analysis of tissues and organs of animals from groups not supplemented with the additive, but with manganese sulfate monohydrate; in the absence of an adequate explanation, the FEEDAP Panel has serious reservations on the acceptability of this trial.

[20] Technical dossier/Section II/Annex II-20.
[21] Technical dossier/Section II/Annexes II-23–25.
[22] Technical dossier/Section II/2.2.2.1.
[23] Technical dossier/Section II/Annex II-30.
[24] Technical dossier/Section II/Annex II-26.
[25] Technical dossier/Section II/Annex II-27.
[26] Technical dossier/Section III/Annex III_3_1.
[27] Due to the capacity of laboratory to process samples, the necropsies took place over 3 days upon termination of the study (until day 37).

In the absence of adequate studies, the FEEDAP Panel cannot conclude on the safety of the additive Manganese-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 Manganese-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.[28,29]

The *in vitro* study shows that the additive is extensively dissociated at pH below 6.8, whilst at higher pH, there is the coexistence of several manganese-containing species and free Mn^{2+}. 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.

3.2.2.2. 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 manganese and EDA in the tissues and products (milk, egg) of other target species administered the additive were made available.[28]

[28] Technical Dossier/Supplementary information (April 2020).
[29] Technical Dossier/Supplementary information (September 2020)/Annex 'MnEDA dissociation analysis report rev 2- Confidential'.

3.2.2.3. Toxicological studies

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

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

In previous scientific opinions, the FEEDAP Panel reviewed the relevant literature and considered several previous toxicological assessments of manganese (see e.g. EFSA FEEDAP Panel, 2016 and references therein). In summary, manganese is a well-known occupational toxicant upon inhalation exposure; however, there are relatively limited data available on oral toxicity in laboratory animals and humans. Whereas manganese is an essential trace element and the usual intake levels of dietary manganese do not appear to be associated with any adverse health effects, high oral exposures are associated with severe adverse neurological effects in humans. The putative pathways of neurological damage (e.g. dopamine oxidation) have been identified, but the available evidence is not robust enough to derive a tolerable upper intake level (UL). Due to the lack of a UL, it is therefore advisable that oral exposure to manganese should not increase over the background dietary intake.

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 Manganese-EDA-Cl (Mn 23%, purity unknown) to induce gene mutations in bacteria, the Ames test was performed according to OECD Test Guideline (TG) 471[30] and following Good Laboratory Practice (GLP) in *Salmonella* Typhimurium strains TA98, TA100, TA1535, TA1537 and TA102.[31] Manganese-EDA-Cl was tested at five concentration levels ranging from 50 to 5,000 μg/plate in two independent experiments performed in the presence and absence of metabolic activation (S9-mix). 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 and toxicity were not observed.

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 FEEDAP Panel concludes that Manganese-EDA-Cl did not induce gene mutations in bacteria under the experimental conditions employed in this study.

3.2.2.3.1.2. In vitro *micronucleus test*

An *in vitro* micronucleus test was performed according to OECD TG 487[32] and following GLP to evaluate the potential of Manganese-EDA-Cl (Mn 23%, purity unknown) to induce chromosome damage in TK6 lymphoblastoid human cells in the absence and presence of metabolic activation.[33]

The compound was tested at concentrations ranging from 2.5 to 80 μg/mL; maximum concentration was limited by solubility or 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 Manganese-EDA-Cl compared to concurrent vehicle controls in the presence of metabolic activation. A statistically significant increase of micronucleated cells was observed at 60 μg/mL after short treatment in the absence of S9-mix, associated with almost 60% cytotoxicity. The FEEDAP Panel notes that cautions should be applied when evaluating the biological relevance of positive results observed in the presence of concurrent severe cytotoxicity. No increase of micronucleus frequency was induced by Manganese-EDA-Cl after continuous treatment without metabolic activation. The Panel concludes that the test item did not induce micronuclei in mammalian cells under the experimental conditions employed in this study.

[30] OECD Guideline Version of 1997. Available online: https://www.oecd.org/chemicalsafety/risk-assessment/1948418.pdf
[31] Technical dossier/Section III/Annex III_3_2.
[32] OECD Guideline Version of 2014. Available online: https://www.oecd-ilibrary.org/environment/test-no-487-in-vitro-mammalian-cell-micronucleus-test_9789264224438-en
[33] Technical dossier/Section III/Annex III_3_3.

3.2.2.3.2. Subchronic oral toxicity study in rats

In a non-GLP study, Fischer 344 rats (10 animals/sex per group) were fed EDA·2HCl at 0, 50, 250, 1,000 mg/kg body weight (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.

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 1,000 mg/kg bw per day.

A no observed adverse effect level (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).[34] Two separated 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

[34] Technical Dossier/Section III/Reference34.

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 of 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.

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).[35] 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 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.

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

[35] Technical Dossier/Section III/Reference39.

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).[36] 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.

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 EDA·2HCl mg/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).[37] 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 manganese and EDA separately is questionable for the safety assessment of the additive.

Regarding manganese, there are relatively limited data available on oral toxicity in laboratory animals and humans. Whereas manganese is an essential trace element and the usual intake levels of

[36] Technical Dossier/Section III/Reference36.
[37] Technical Dossier/Section III/Reference71.

dietary manganese do not appear to be associated with any adverse health effects, high oral exposures are associated with severe adverse effects.

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 and (iv) the absence of toxicological studies (excluding genotoxicity) with the Manganese-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.

3.2.3. 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 25.8 g/m^3 air; see Section 3.1.3), an estimation of the manganese inhalation exposure was performed.

Taking into consideration the manganese concentration in the dust (average concentration of 208 mg Mn/kg dust), a release of 5.4 mg Mn/m^3 can be expected when handling the additive. Considering the potential amount of particles of respirable size of the dust, the manganese concentration in the respirable dust would be of 1.4 mg Mn/m^3.[38] The Health and Safety Executive (HSE) of the UK and the Occupational Safety and Health Administration (OSHA) of the US have set an occupational exposure standard for dust from manganese and its compounds (HSE, 2003; OSHA, 2007) of 5 mg Mn/m^3. In the 2013 edition of its threshold limit value (TLV) and Biological Exposure Indices publication, the American Conference of Governmental Industrial Hygienists reduced the TLV for inhalable manganese particles to 0.1 mg/m^3. The corresponding value for respirable fractions of manganese was set to 0.02 mg/m^3. The new TLVs do not distinguish between the form of manganese found in welding fume and other forms of manganese and are thus relevant for the additive under consideration. The estimated manganese exposure exceeds the TLV for respirable particles of about 70 times. Consequently, the FEEDAP Panel considers that the additive poses a risk to users upon inhalation.

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 Manganese-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 upon inhalation by the users.

Concerning nickel, the additive contains up to 2.79 mg Ni/kg. The dusting potential of the product amounted to 25.8 g/m^3, corresponding to 0.072 mg Ni/m^3,[39] which is above the occupational exposure limit (OEL) for the inhalable fraction of water-soluble nickel (0.01 mg Ni/m^3; European Commission, 2011). Therefore, the handling of the additive poses a risk to the users by inhalation due to its nickel content.

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 eyes and skin

An acute skin irritation GLP study performed according to the OECD Guideline No. 404 was submitted.[40] Under the experimental conditions adopted, Manganese-EDA-Cl was found to be non-

[38] Assuming that the dust contains only particles of < 50 μm diameter, its respirable fraction could be determined as 26.5% (8 of 30.2), the manganese content in the dust would be 1.5 mg/m^3 (26.5 × 5.5 mg/m^3 per 100).

[39] 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).

[40] Technical dossier/Section III/Annex III_3_4.

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 the OECD Guideline No. 405 was submitted.[41] Under the experimental conditions adopted, Manganese-EDA-Cl caused serious damages to the eye of the rabbit and is considered corrosive to eyes.

Furthermore, the nickel content of the additive is up to 2.79 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 the handling of the additive poses a risk to users by inhalation. The additive should be considered as corrosive to eyes and a 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 manganese sources, including chelates with amino acids or glycine from previous FEEDAP Panel opinions (e.g. EFSA FEEDAP Panel, 2016), ERA of EDA performed by tshe 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).

The applicant provided a combined tolerance/residue/efficacy study in chickens for fattening[42] 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 Manganese-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 Regulation[43] 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 studies submitted.

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

Documentation provided to EFSA/Chronology

Date	Event
11/09/2018	Dossier received by EFSA. Manganese chelate of ethylenediamine for all animal species. Submitted by Zinpro Animal Nutrition (Europe), Inc.
18/09/2018	Reception mandate from the European Commission
30/10/2018	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. *Issues: characterisation, safety for the consumer*

[41] Technical dossier/Section III/Annex III_3_5.
[42] Technical dossier/Section IV/Annex IV_4_1 and IV_4_2.
[43] 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
25/01/2019	Reception of the Evaluation report of the European Union Reference Laboratory for Feed Additives
30/01/2019	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. *Issues: safety*
20/02/2019	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. *Issues: safety for consumers, safety for the environment*
07/06/2019	Clarification teleconference during Risk Assessment
04/03/2020	Reception of supplementary information from the applicant - Scientific assessment re-started
16/07/2020	Request of supplementary information to the applicant in line with Article 8(1)(2) of Regulation (EC) No 1831/2003 (Addendum) – Scientific assessment suspended. *Issues: characterisation, safety for consumers*
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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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
GLP	Good Laboratory Practice
HILIC	hydrophilic interaction chromatography
HSE	Health and Safety Executive
LOD	limit of detection
NOAEL	no observed adverse effect level
OSHA	Occupational Safety and Health Administration
OEL	occupational exposure limit
RSDr	standard deviation for *repeatability*
RSDR	relative standard deviation for *reproducibility*
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 manganese chelate of ethylenediamine

In the current application, authorisation is sought under Article 4(1) for *manganese 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.

Manganese chelate of ethylenediamine is a solid preparation with a minimum content of 21% (w/w) of *manganese* and 21% (w/w) of *ethylenediamine (EDA).*

The *feed additive* is intended to be incorporated into *premixtures* and *feedingstuffs.* In addition, the Applicant proposed maximum levels of *total manganese* in *feedingstuffs* complying with the limits set in Regulations (EC) No 1334/2003 and (EU) 2017/1490: 100 mg/kg for fish; and 150 mg/kg for other species.

For the quantification of *total manganese* 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 Manganese 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).

The following performance characteristics were reported for the five above-mentioned CEN methods in the frame of the ring-trial validation studies for quantification of total *manganese* content ranging from 12 to 13200 mg/kg in matrices of the scope: a relative standard deviation for *repeatability* (RSDr) ranging from 1% to 6.4%; and a relative standard deviation for *reproducibility* (RSDR) ranging from 3.4% to 19.8%.

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 manganese* in the *feed additive, premixtures* and *feedingstuffs;* ii) EN 15510 and EN 17053 for the quantification of *total manganese* in *premixtures* and *feedingstuffs;* and iii) the Community method (Commission Regulation (EC) No 152/2009 – Annex IV-C) for the quantification of *total manganese* 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.