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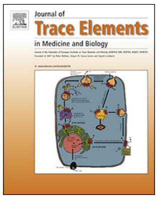

Technical note

Accurate quantification of metal-glycinates-sulphate complexes and free metals in feed by capillary electrophoresis inductively coupled plasma mass spectrometry

Catherine Ionescu[a], Peter Grill[b], Heidi Witte[b], Christian Boigues[a], Alexandra Blanchard[a], Bärbel Löffler[b], Bernhard Michalke[b,*]

[a] Pancosma SA, voie des Traz 6, 1218 Le Grand Saconnex, Switzerland
[b] Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH), Research Unit: Analytical BioGeoChemistry, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany

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ABSTRACT

Traceability of metal-glycinate-sulphate complexes (Metal-GLY) in feed requires specific analysis to differentiate complexes from inorganic forms. A previously described method focused on the quantification of Metal-GLY at one single concentration but not on the quantification of free metal ion forms. The objective of this work was to extend the method to quantify both Metal-GLY and free metal ion forms of various metals at low inclusion levels. A 50/50 w/w mix of corn flour and soybean meal was used as feed. Copper-glycinate(Cu-GLY), Manganese-glycinate (Mn-GLY) and Zinc-glycinate (Zn-GLY) complexes (provided by Pancosma SA) were used for in-feed inclusions. The feed metal background concentrations and species repartitions were assessed. Cu-GLY was spiked on feed at levels matching 5, 15 and 45 mg/kg, corresponding to metal concentrations of 1.2, 3.6 and 10.8 mg/kg. Mn-GLY and Zn-GLY were spiked at 15, 45 and 100 mg/kg, corresponding to 3.3, 9.9, 22 mg/kg Mn and 3.9, 11.7, 26mg/kg Zn, respectively. The water soluble fraction of un-supplemented feed contained 0.06 mg/kg Cu, 0.05 mg/kg Mn and 0.12 mg/kg Zn, with 69.5% of Cu, 33.2% of Mn and 24.3% of Zn being present under free metal ions but 30.4% of Cu being present under Cu-GLY, 66.82% of Mn and 75.7% of Zn being present under Mn-GLY and Zn-GLY, respectively. The supplemented feeds at the 3 tested doses, from the lowest to the highest inclusion levels, contained in total respectively: 1.1, 3.05 and 9.06 mg/kg Cu; 2.99, 8.9 and 18.2 mg/kg Mn; 3.72, 10.9 and 23.4 mg/kg Zn. The M-GLY species recovered by analysis within the different supplemented feeds ranged from 76.26 to 89.32% for Cu-GLY, form 94.5 to 98.51% for Mn-GLY and from 76.05 to 98.96% for Zn-GLY. These results showed that CE-ICP-MS technique can be used to quantify low doses and to measure metal-species repartition between Metal-GLY and free metal ions, when included in feeds. For the first time, this study highlighted that the raw materials used contain Metal-GLY compounds. This raises the question of the occurrence of these compounds within the different raw materials used in feed production that could dramatically affect the way to supplement minerals in animal feed.

1. Introduction

Large numbers of trace mineral species are available to supplement feed for livestock. In the European Union (EU), feed producers can use 34 legally defined sources of Cu, Mn and Zn [1]. Products are either classified as inorganic trace minerals (including mainly metal-sulphates, -oxides and -carbonates) or as organic trace minerals. This second class corresponds to transition metals complexed with organic ligands. Organic trace minerals (OTM) represent 56% of the authorised sources in EU and represented a worldwide market value over 500 million USD in 2017 [2]. The OTM made with glycine, have shown a better bioavailability and complementary effects in animals compared to inorganic sources [3–6]. The positive outcomes of feeding OTM create interest for the feed industry to differentiate and quantify these

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* Corresponding author at: Helmholtz Zentrum München, Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH), Ingolstädter Landstr. 1, 85764 Neuherberg, Germany.

E-mail addresses: catherine.ionescu@pancosma.ch (C. Ionescu), grill@helmholtz-muenchen.de (P. Grill), heidi.witte@helmholtz-muenchen.de (H. Witte), christian.boigues@pancosma.ch (C. Boigues), alexandra.blanchard@pancosma.ch (A. Blanchard), baerbel.loeffler@helmholtz-muenchen.de (B. Löffler), bernhard.michalke@helmholtz-muenchen.de (B. Michalke).

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Table 1
Supplemented metal levels from Cu-GLY, Mn-GLY and Zn-GLY for A, B and C doses in feeds (in mg/kg).

DOSES OF SUPPLEMENTATION	CU FROM CU-GLY (mg/kg)	MN FROM MN-GLY (mg/kg)	ZN FROM ZN-GLY (mg/kg)
Dose A	1.2	3.3	3.9
Dose B	3.6	9.9	11.7
Dose C	10.8	22	26

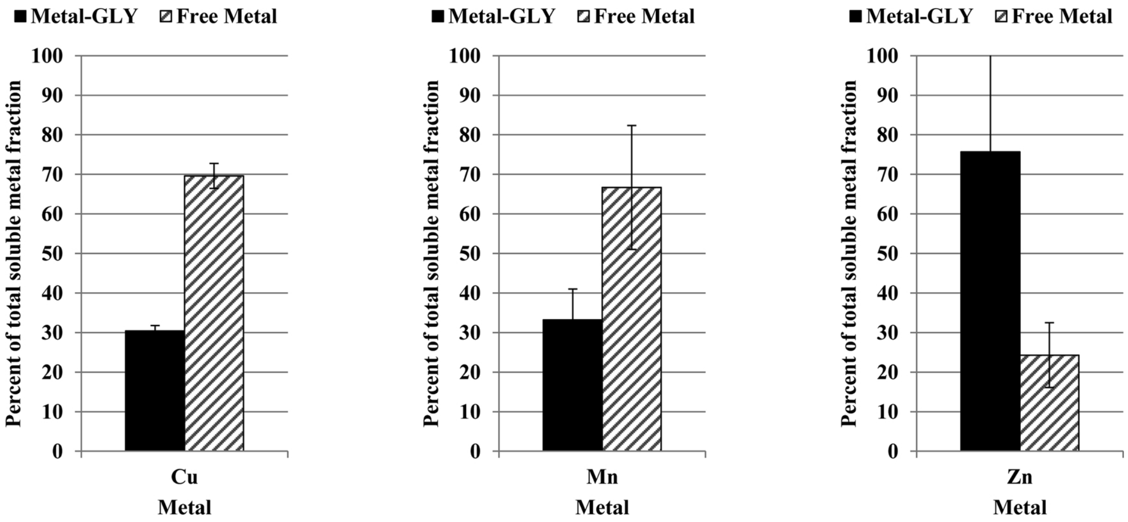


Fig. 1. Repartition of free-metal ion and metal-glycinate (Metal-GLY) species in un-supplemented feed presented as percentages of total soluble metal fraction.

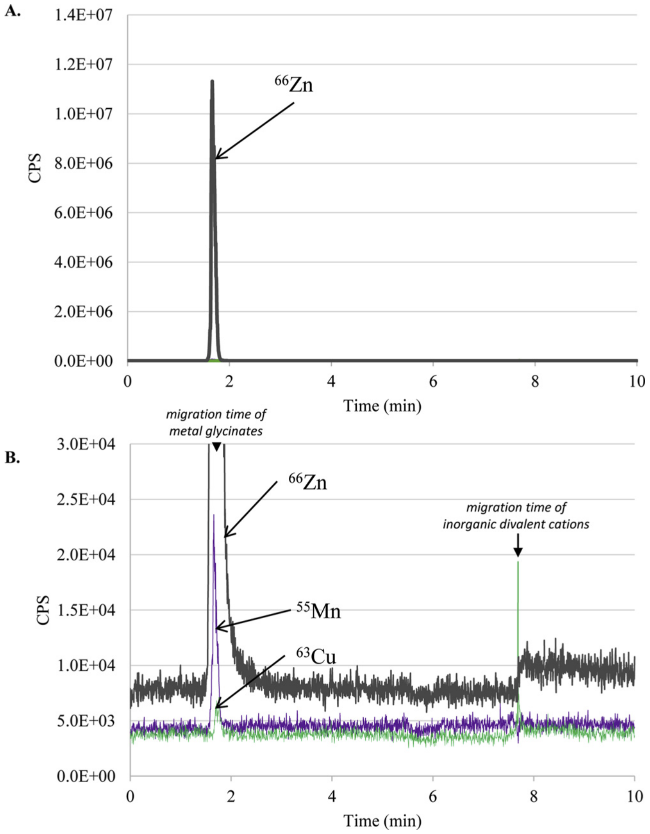


Fig. 2. A. Typical electropherogram obtained from (dose C) Zn-GLY supplemented feed extracts and B. Zoom of a typical electropherogram obtained from (dose C) Zn-GLY supplemented feed extracts to enable observation of Mn and Cu metal peaks. The zoom also shows the position of inorganic divalent metals – in this example seen the inorganic Cu - well separated from metal glycinyates.

metal species in animal diets. This should aim for analytical capability enabling accurate control of supplemented levels in feed and adjustments of trace mineral supplementation to their most effective doses.

Analytical methods used for feed trace minerals quantifications are generally based on Atomic Absorption Spectrometry (AAS) or Inductively Coupled Plasma – Atomic Emission Spectrometry (ICP-AES) [7]. However, if these techniques enable total metal quantification, it does not discriminate metal species that is needed for feed assessment.

A specific analytical method has been previously developed by Vacchina et al. [8] for the quantification of some metal-glycinate-sulphate (Metal-GLY) in feed using the hyphenation of Capillary Electrophoresis with Inductively Coupled Plasma Mass Spectrometry (CE-ICP-MS). This technique allowed quantification of a dose of Metal-GLY but did not measure free metal levels. The objective of this study was to extend the previous method to free metal ions and Metal-GLY in parallel. The analyses were performed in both un-supplemented and supplemented feeds at three low inclusion doses of Metal-GLY to assess the accuracy of the method.

2. Material and methods

2.1. Samples

The feeds used were composed of a mix of 50/50 (w/w) of corn flour and soybean meal.

The Metal-GLY complexes used included: Copper-glycinate (Cu-GLY): ($[Cu(C_2H_5NO_2)(H_2O)_2(SO_4)]_n$) with minimum 24% Cu, Manganese-glycinate (Mn-GLY): ($[Mn(C_2H_5NO_2)(SO_4)]_n$) with minimum 22% Mn and Zinc-glycinate (Zn-GLY): ($[Zn(C_2H_5NO_2)(H_2O)_2(SO_4)]_n$) with minimum 26% Zn (Pancosma SA, Geneva). The products used had been previously characterized under their powder form by X-ray diffraction [9] and in solutions by Electrospray Ionization Quadrupole Time Of Flight double Mass Spectrometry (ESIQqTOF MS/MS) [10].

Table 2
Analysed concentrations of Cu, Mn, Zn from metal-glycinates after their in-feed supplementation under dose A (1.2 mg/kg Cu, 3.3 mg/kg Mn, 3.9 mg/kg Zn), dose B (3.6 mg/kg Cu, 9.9 mg/kg Mn, 11.7 mg/kg Zn) and dose C (10.8 mg/kg Cu, 22 mg/kg Mn, 26 mg/kg Zn).

	DOSES OF SUPPLEMENTATION	CU FROM CU-GLY SUPPLEMENTED FEED	MN FROM MN-GLY SUPPLEMENTED FEED	ZN FROM ZN-GLY SUPPLEMENTED FEED
ANALYSED METAL CONCENTRATIONS (PPM)	Dose A	1.1	2.99	3.72
	Dose B	3.05	8.9	10.9
	Dose C	9.06	18.2	23.4 Declaration of interest conflict: none

2.2. Inclusion levels

The Metal-GLY products were added in feed at 3 defined doses hereafter presented as doses A, B and C. The A doses corresponded to levels of 5 mg/kg of Cu-GLY and 15 mg/kg of Mn-GLY or Zn-GLY. The B doses matched levels of 15 mg/kg of Cu-GLY and 45 mg/kg of Mn-GLY or Zn-GLY. The C doses included concentrations of 45 mg/kg of Cu-GLY and 100 mg/kg of Mn-GLY or Zn-GLY.

The corresponding supplemented metal concentrations are presented in Table 1.

2.3. Total element determination after digestion

For total element concentration determination, solid feed samples were properly weighed (150 mg) into quartz vessels. Subsequently, 1 mL HNO_3, suprapure, subboiling distilled (Merck, Darmstadt) was added. The vessels were closed and introduced into a pressure digestion system (Seif, Unterschleissheim) for 10 h at 170 ^{0}C. The resulting clear solution was filled up exactly to 10 mL with Milli-Q® H_2O and was then ready for element determination

The values from feed digests were used for comparison with concentrations after water extraction and calculation of extraction efficiency.

2.4. Extraction of water soluble metal fractions including metal-glycinates from feed samples

Feed samples were exactly weighed into quartz vials (1 g), filled up with 100 mL Milli-Q® water and shaken for dissolution at room temperature (controlled at 22 °C) for 1 h. The suspension was subsequently filtered and the filtrate used for CE-ICP-MS and ICP-AES analysis.

2.5. Method parameter for analyses

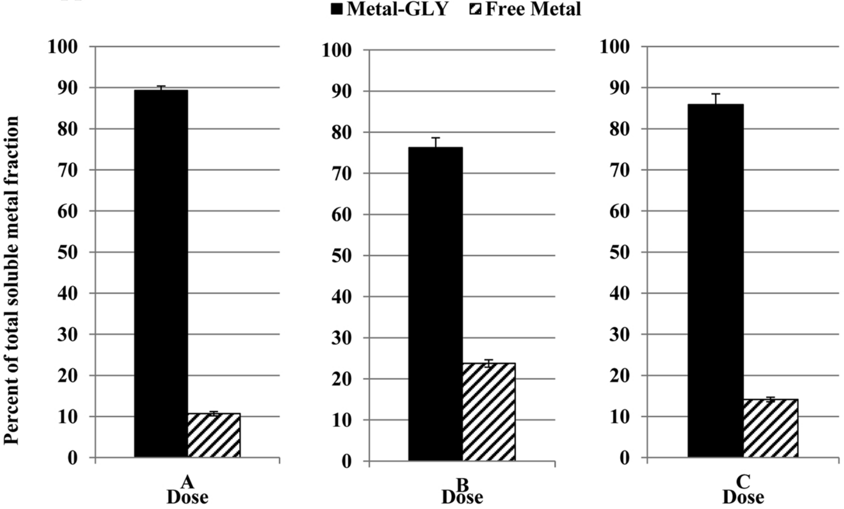
Total element concentrations in digests and the filtrates of water extracts were determined using an ICP-AES „Arcos“system (Spectro-Ametek, Kleve, Germany). In preliminary investigations the extracts were also digested and element concentrations determined (n = 2). The results were compared to measurements of undigested extracts. The measured spectral element lines were: Cu: 324.754 nm; Mn: 257.611 nm; Zn: 213.856 nm. The differentiation between Metal-GLY and free metal ions was realized with CE-ICP-MS. The CE-capillary (85 cm x50 μm ID, CS-Chromatographie Service GmbH, Langerwehe, Germany) was installed in a *PrinCe-760* CE (Prince, Emmen, The Netherlands) and directly hyphenated to ICP-MS (NexIon, Sciex, Toronto, Canada) with a special CE-ICP-interface described by Michalke, 2004 and Michalke et al., 2019 [11,12]. The separation method used 5 mM NH_4Ac, pH 4 as background electrolyte and 25 kV as separation voltage. The NexIon 300 D ICP-MS was used as on-line detector for CE.

ICP-MS parameter were:

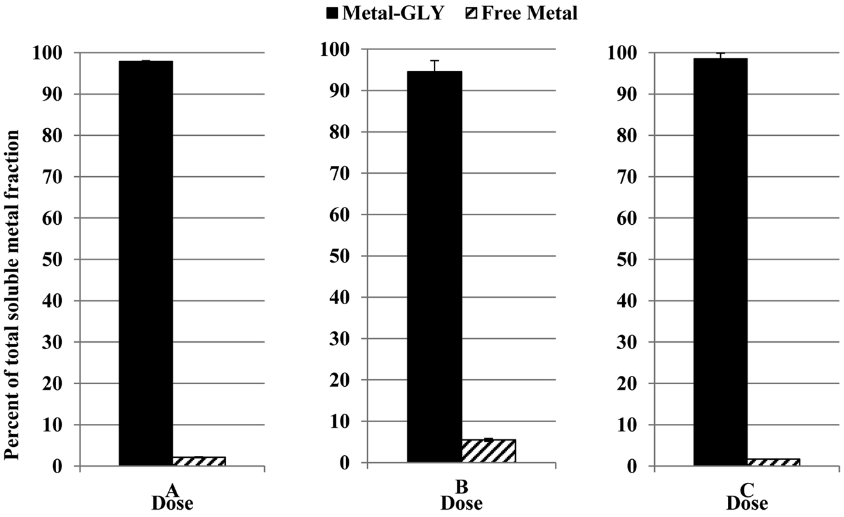
An NexIon 300 D ICP-MS, (Perkin Elmer, Rodgau-Jügesheim, Germany), was employed for on-line determination of isotopes: ^{55}Mn, ^{63}Cu, ^{66}Zn. The RF power was set to 1200 W, the outer gas was 15 L Ar /min. The nebulizer gas was optimized and finally set to 0.96 L Ar/min. The dwell time was 80 ms during CZE coupling. The system operated in standard mode with band pass (q) 0.25.

In the beginning of experiments both, ^{63}Cu and ^{65}Cu were monitored for elucidating a possible $[^{23}Na^{40}Ar]^+$ interference on ^{63}Cu and – concerning isotope abundances ^{63}Cu: 69.17%; ^{65}Cu 30.83%) – probably worse LOD for ^{65}Cu. During primary experiments it turned out that Na-ions reached ICP-MS after 13.5 min and resulted only in a slight increase of the ^{63}Cu baseline at that late migration time. Since the electrophoretic peaks of Cu-glycinate or inorganic Cu were not interfered, but the response, i.e. LOD with ^{63}Cu was superior to ^{65}Cu, only ^{63}Cu was monitored in further experiments.

a. Copper



b. Manganese



c. Zinc

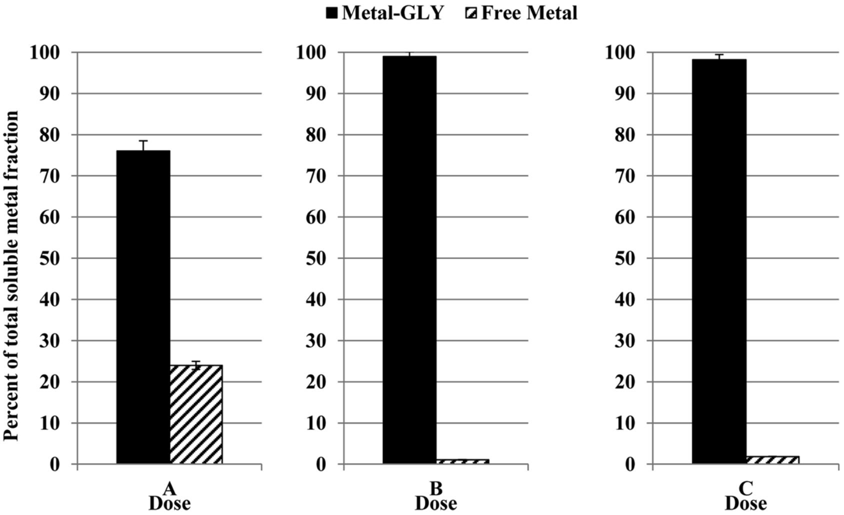


Fig. 3. a. Repartition of free-metal ions and metal-glycinate (Metal-GLY) species in supplemented feed presented as percentages of total soluble metal fraction per metal for supplementation doses A, B and C for copper. b. Repartition of free-metal ions and metal-glycinate (Metal-GLY) species in supplemented feed presented as percentages of total soluble metal fraction per metal for supplementation doses A, B and C for manganese. c. Repartition of free-metal ions and metal-glycinate (Metal-GLY) species in supplemented feed presented as percentages of total soluble metal fraction per metal for supplementation doses A, B and C for zinc.

3. Results and discussion

3.1. Total element concentrations after digestion: un-supplemented feed

The digestion of feed samples resulted in concentrations of 13.1 mg Cu/kg, 30.0 mg Mn/kg and 47.7 mg Zn/kg. The water soluble metal fraction showed levels for Cu = 60 µg/L (corresponding to 5.84 mg/kg, based on the exact weight (1.015 g) used in 100 mL extraction solution), Mn = 55 µg/L (corresponding to 5.4 mg/kg) and Zn = 127 µg/L (corresponding to 12.5 mg/kg) in un-supplemented feed extracts. We calculated extraction rates of 44.7%, (Cu), 18.0% (Mn) and 26.2% (Zn).

The comparison of digested extracts and non-digested extracts showed practically identical results. Based on the digested extract samples, set as 100%, we found in the non-digested extract Cu 99.04 ± 0.64%, Mn 97.68 ± 1.2% and Zn 102.11 ± 0.82%.

Limits of detection (3σ) and quantification (10σ) were determined with CE-ICP-MS in feed extracts. We found the following Lod / LoQ values given in µg/L: Cu: 1.0 / 3.0; Mn: 2.9 / 9.6; Zn: 4.4 / 14.8.

Fig. 1 demonstrates the distribution of metals between Metal-GLY and free metal ions from un-supplemented feed samples. While Cu was mainly present as a free metal ion (almost 70%), Cu-GLY represented 30% of the chemical species in the soluble fraction. Mn-GLY chemical species represented 33% of the Mn present and the Zn-GLY chemical species represented 75.7% of the total Zn in the soluble fraction. The respective peaks per metal were quantified and their sums compared to the total metal concentrations (= 100%) determined in extracts for mass balances during CE-ICP-MS (n = 4). Recoveries were 104.3 ± 10.8% (Cu), 94.4 ± 11.4% (Mn), 97.4 ± 10.2% (Zn).

As the feed contained two raw materials, namely corn flour and soybean meal, it was impossible to identify the exact source of the Metal-GLY found in the feed. When looking at Zn based compounds, the presence of zinc complexed forms have already been identified in maize silage and identified as low molecular weight complexes [13]. Flis et al. (2016) have identified various organic forms in circulating *Pisum sativum* fluids including Zn-bis-glutaminates, Zn bis-histidinates, Zn bis-citrates and Zn-nicotinaminates [14]. Copper histidine forms have been previously identified in rice (*Oryza sativa* L.) root xylem [15]. These results showed for the first time, the presence of Metal-GLY species in feed in absence of supplementation aside from free metal ions. Variation of measurements per sample ranged from 0 to 3.1 % (except 1 determination varied by 5.9%), resulting in a mean precision of 2%. This precision data confirm the reliability of the CE-ICP-MS method for Metal-GLY species and free metal ions. The relatively short analysis time of ca. 8 min allows the application to even big sample size with quick analysis. The analytical conditions used have enabled an improved performance compared to the analytical method developed by Vacchina et al. [8]. This analytical method now allows the industry to consider the presence of organic sources in un-supplemented feed (here Metal-GLY) to adjust the supplementation differentially per metal species.

3.2. Supplemented feeds

The Fig. 2A and B showed a typical example of an electropherogram obtained from the extracts of feed supplemented with Zn-GLY. The Zn-peak of high intensity present in Fig. 2A is representative of Zn bound as Zn-GLY, monitored at 1.67 min. When enlarging the electropherogram, (Fig. 2B) other metals can also be observed in a much lower intensity.

Metal-GLY concentrations were quantified in supplemented feeds and results obtained are presented in Table 2. The analysed Metal-GLY levels were close to the theoretical inclusion in feed.

The proportions between free-metal and Metal-GLY compounds for each dose are presented in Fig. 3. At the three inclusion levels, the fraction of Metal-GLY was always higher than 76% showing the high stability of the Metal-GLY in feed and during the used extraction process. In the Zn-GLY supplemented feeds, at the 2 highest doses, Zn-GLY presented over 98% of the Zn soluble fraction. However, at the lowest inclusion rate, Zn-GLY only represented 76% of the soluble fraction, questioning slightly weaker metal-complexes stability at low doses and/or the higher variability of the analytical technique. For the Cu-GLY supplemented feeds, Cu-GLY represented between 76 and 89% of the Cu soluble fraction but the results obtained looked to be independent of the dose tested, indicating either a poorer stability of the metal-complex in the set condition or a higher variability of the analytical technique for Cu at the tested dose. The Mn-GLY represented more than 94% of the total soluble fraction at the 3 tested doses showing its high stability under the tested conditions.

However from these analyses, at these inclusion levels, analytic variability is observed. Nevertheless, the accuracy is still of high quality.

When comparing the different percentage fractions of metal-GLY from Cu, Zn and Mn at their different inclusion concentrations it can be concluded that increased metal-GLY supplementation (probably even saturation) may improve the complex stability during the analyses.

4. Conclusion

CE-ICP-MS allowed, for the first time, to highlight the presence of metal-glycinates in un-supplemented feed. The analytical method proved to be a valuable, fast and reproducible tool to analyse metal glycinate complexes aside from their respective free metal ions both in feed and metal-glycinates supplemented feed. The analysed concentrations for metal-glycinate were found to be close to the theoretical included levels in feed. At high (saturated) supplementation, complex stability of metal glycinates seems to be improved during the analyses. The technique used provides an accurate and relevant method to assess the metal fraction with advanced bioavailability in feed. This is a major concern for the feed industry to adjust metal supplementation and meet the animal needs while preserving the environmental impact by a decrease of supplementation through the use of more bioavailable trace mineral chemical species.

Declaration of Competing Interest

None.

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