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Determination of Zn–, Cu– and Mn–glycinate complexes in feed samples and *in-vitro* and *in-vivo* assays to assess their bioaccessibility in feed samples

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ABSTRACT

A method was developed for the quantification of Zn–, Cu– and Mn–glycinates in supplemented feed samples. The coupling of capillary electrophoresis (CE) with ICP MS detection after purification of the extract by ultrafiltration was shown to be efficient for the quantitative recovery of glycinates. The method developed was then applied to evaluate the bioaccessibility of glycinates using a sequential enzymolysis approach. The data obtained indicated a strong bioaccessibility of each element (79–94%). A new complex was also found to be formed during the digestion process. Bioavailability was then evaluated by analyzing plasma samples of horses supplemented with glycinates-rich feed. Intact glycinates could not be detected in plasma samples but a Cu-containing molecule was found more abundant after CuGly treatment.

1. Introduction

An adequate supply of trace elements in the diet is indispensable for the health and correct growth of farmed animals. The lack of Zn can lead to poor hair and feather development [1]. Cu takes part in many physiological functions such as iron metabolism, blood formation or immunity and protection of tissues from oxidative stress [2]. Mn is essential for osseous growth and reproduction [3]. The essential role of trace minerals has resulted in the creation of feed additives in which they are present under organic forms. The supplementation with organic sources, such as chelates or complexes with glycine (glycinates), is becoming more and more popular as they represent one of the most efficient sources in terms of bioavailability [4,5,6]. In practice, the partial or total substitution of inorganic trace minerals by chelated forms is frequently proposed in order to narrow the gap between the official limitations and the widespread high supplementation practices [7,8]. The introduction of organic trace elements into feeds is still based on inorganic forms recommendations at least in the European Union [9] and do not take into consideration the differences in bioavailability of these trace element sources. This is mainly due to the lack of analytical methods able to discriminate quantitatively the different organic and inorganic sources of trace minerals available on the market but also the lack of clear information on the reasons of their bioavailability differences. A better knowledge of the inclusion dose of identifiable organic trace elements should allow a reduction of their addition in feeds and, as a consequence, a decrease of their release into the environment without impacting animal performance.

Little research has been devoted to the speciation of glycinates in feed samples. To our knowledge, the only quantitative data reported in the literature concerned premix samples [10] and were obtained by CE–ICP MS. The transfer of this method to feed samples should take into account (i) the lower concentration of trace elements (by nearly a factor 1000 in comparison to premix samples) and (ii) the overall chemical composition of the feed sample, especially the presence of other ligands that can compete with glycine for trace elements binding.

In addition to the knowledge of the total concentration of glycinates in feed samples, an important parameter is their bioavailability. The latter refers to the fraction of a substance which reaches the systematic circulation (blood) from the gastro-intestinal tract and which is available to promote its action in the exposed organism [11]. The first step in assessment of bioavailability is the study of the bioaccessibility which indicates the maximum fraction of glycinates which is theoretically released from the feed sample in the gastro-intestinal tract, and thus becomes available for intestinal absorption [12]. *In-vitro*, one way to evaluate the digestion efficiency consists of simulating processes occurring in the stomach and the small intestine [13]. This approach was used to evaluate the Cd and Pb bioavailability in

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Table 1
Optimum CE separation conditions for ICP MS detection.

Runnng buffer	20 mM ammonium acetate buffer (pH 7.4)
Injection	Hydrodynamic: 50 mbar for 2 s
Volume injected	Around 2.3 nL
Voltage	+30 kV
Temperature	20 °C
Capillary	Fused silica capillary (55 cm, i.d. 50 μm)
Sheath liquid	20 mM ammonium acetate buffer (pH 7.4) containing 50 ng mL^{-1} Tl (self aspirated)

cocoa [14], or to study the bioaccessibility of Se in selenized yeast [15]. Such *in-vitro* enzymolysis can mimic the physico chemical phenomena occurring in the digestive tract of living organisms and they are simple, easy to control and inexpensive. However, even if gastro-intestinal extraction purports to simulate the release of chemicals from sample matrixes, there are three main problems with this method [16].

- The lack of pH stability and the time taken to increase pH from acidic to neutral can affect the kinetics of metal complexation with the matrix, and in turn may affect the estimation of the bioaccessibility.
- It is based on human and not animal physiology.
- There is a lack of Certified Reference Materials (CRM) to validate the different approaches.

Therefore *in-vivo* data from animal studies should be used to confirm the predictive values of the model. It could be achieved by dosing the target compounds in animal biological fluids such as plasma.

The objective of this work was therefore to develop a method for the determination of Zn–, Cu– and Mn–glycinates in feed samples and then to study their bioaccessibility and bioavailability in plasma of horses.

2. Experimental

2.1. Instrumentation

The ICP MS used was an Agilent 7500ce (Agilent, Tokyo, Japan). CE experiments were carried out with the Agilent system (Agilent, Waldbronn, Germany). CE was connected with the ICP MS by means of the CETAC CEI-100 interface (CETAC, Omaha, NE). Feed samples were shaken mechanically during extraction (KS 250 basic, Kika Labortechnik, Staufen, Germany) or incubated in a water bath (OLS200, Grant, UK) for the bioaccessibility study. They were then centrifuged (15 min, 3000 rpm) (Universal 16, Hettich, Germany).

2.2. Reagents, standards and materials

Analytical reagent grade chemicals purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) and water (18 MΩcm) obtained from a Milli-Q system (Millipore, Bedford, MA) were used throughout unless stated otherwise. The Cu–glycinate (CuGly): ($[Cu(C_2H_5NO_2)(H_2O)_2(SO_4)]_n$), Zn–glycinate (ZnGly):($[Zn(C_2H_5NO_2)(H_2O)_2(SO_4)]_n$) and Mn–glycinate (MnGly):($[Mn(SO_4)(C_2H_5NO_2)]_n$) complexes were obtained from Pancosma (Geneva, Switzerland). They were characterized by X-ray diffraction [17] and in solution by ESI QqTOF MS(/MS) [10]. Stock solutions of glycinates (1000 $\mu g.mL^{-1}$ as metal) were prepared in 10 mM ammonium acetate buffer (pH 7.4) and were further diluted. Capillary electrophoresis separations were carried out in a 50 μm i.d. fused silica capillary (Agilent).

2.3. CE–ICP MS conditions

CE separations were carried out using 20 mM ammonium acetate (pH 7.4) as electrolyte. A constant voltage of +30 kV was applied. Hydrodynamic injections were performed by applying a pressure of 50 mbar during 2 s. After running each sample, the capillary was regenerated using 0.1 M of KOH for 1 min, followed by 1 min of flushing with water and 1 min flushing with the electrolyte. During coupling with ICP MS, the sheath liquid was 20 mM ammonium acetate (pH 7.4) spiked with 50 $\mu g.mL^{-1}$ of Tl. The signal from ^{205}Tl was monitored continually, on the one hand to optimize measurement conditions (nebulizer gas flow, lens voltages, etc.) daily and on the other hand to act as an internal standard to check the stability of the nebulisation process during electrophoretic run. For quantitative purpose, the area of the glycinate peak was normalized by the intensity of ^{205}Tl. The detailed instrumental conditions are listed in Table 1.

2.4. Extraction of glycinates from feed samples

A feed sample was weighed (1 g) in triplicate and leached with 10 mL of 10 mM ammonium acetate (pH 7.4) for 1 h using an elliptic table. The supernatant obtained after centrifugation (3000 rpm, 5 min) was further ultrafiltered using a 10 kDa filter unit (4000 rpm, 30 min) prior to injection on the capillary.

2.5. In-vitro gastric and gastro-intestinal digestion of feed samples

The *in-vitro* enzymolysis procedure originally described by Crews et al. [18] was used. Enzymolysis was carried out in two steps, corresponding to the conditions present in the stomach and the intestine. A portion of 1 g of feed sample was incubated in a shaker water bath at 37 °C for 4 h with 5 mL of gastric juice (1% of pepsin in 0.15 M NaCl acidified with HCl to pH 2). Then, the digested samples were brought to neutral pH by adding 2 M $NaHCO_3$. Subsequently, 5 mL of intestinal juice (3% of pancreatin, 1.5% of amylase and 1% of bile salts in 0.15 M NaCl) was added. The samples were further incubated for 4 h at 37 °C. After extraction, the supernatant was separated by centrifugation (3000 rpm, 5 min) and further ultrafiltered using a 10 kDa filter unit (4000 rpm, 30 min) prior to injection on the capillary.

2.6. Horses supplementation

Sixteen horses, from three different locations and fed with different types of trace element supplements or none, were selected based on their age, exercise, etc. Four horses were fed with an unsupplemented diet, six horses were fed with a diet supplemented with glycinates and six horses were fed with a diet supplemented with a combination of inorganic trace elements and another chelated form (ITE). Blood samples were taken in lithium heparin sampling tubes by a Veterinary Practitioner from the jugular vein on two subsequent days, 3 h post feeding. The sampling of each horse was done at the same hour on these two days. The samples were kept refrigerated until centrifugation. The plasma obtained was deep frozen until analyzed. For each

horse, plasma samples were pooled, freeze-dried and dissolved in 1 mL of water. Depending on the sample, a preconcentration factor of *ca.* 3 could be achieved.

3. Results and discussion

3.1. CE–ICP MS determination of glycinates in feed samples

A method was previously developed for the quantification of glycinates in premix samples [10]. It was based on an extraction with 10 mM of ammonium acetate (pH 7.4) under mechanical shaking on an elliptic table during 1 h, followed by a dilution of the extract prior to analysis by CE–ICP MS. However, the concentration in feed samples is expected to be 1000 lower than in the premix samples. The literature method was therefore not sensitive enough and needed to be modified. A preconcentration step by lyophilisation was first investigated but the simultaneous preconcentration of the matrix led to a strong deterioration of the quality of the electropherograms. Therefore a removal of the matrix by size-exclusion HPLC, which is commonly used as a purification procedure of extracts containing non-covalent species [19,20], was attempted. In previous experiments [10], it was shown that the quantitative recovery of glycinates from a size-exclusion column could only be achieved for a high ammonium acetate buffer concentration (200 mM). Such a high concentration degraded the electrophoretic separation. This clean up procedure was therefore forsaken. Ultrafiltration was preferred even if it was not expected to remove the co-extracted salts from the extracts. An additional attempt to preconcentration was then made by increasing the injected volume in comparison with the volume injected for premix analysis [10]. However, a broadening of the glycinate peak was observed along with the increased peak height. The best compromise was to increase the injection time to 2 s leading to an injected volume of around 2.3 nL. Care was taken to clean regularly the system with 2% HNO_3 to keep the baseline as low as possible.

A feed sample supplemented by ZnGly, CuGly and MnGly was then analyzed. Typical CE–ICP MS electropherograms obtained are shown Fig. 1a. For Cu, two peaks were observed. Only the first one (migrating just before 4 min) increased after the addition of a CuGly standard. The second peak was also present in the unsupplemented feed sample extract and therefore may correspond to another Cu species naturally present in the feed sample. For Mn and Zn, the electropherograms showed a single peak absent in the unsupplemented feed sample extract. This peak increased after the addition of a standard of MnGly or ZnGly to the extract confirming that it corresponded to the glycinate complex.

The glycinate complexes were then quantified in the extract. Due to the matrix effect, the method of the standard addition was used. Table 2 summarizes the concentrations measured in comparison with the target values. Taking into account the possible inhomogeneity of the samples and the intrinsic analytical figures of merit of the CE–ICP MS coupling, the results show a good agreement between the target and measured concentrations. Those results prove additionally the stability of the glycinate complexes in solution in the presence of other ligands at neutral pH.

3.2. In-vitro bioaccessibility of glycinates in feed samples

Bioaccessibility of glycinates was assessed by simulating *in-vitro* gastric and gastro-intestinal digestion of enriched feed samples according to an approach developed elsewhere [18]. The results obtained in terms of total extracted trace element are shown in Table 2. The data confirm the previously shown high bioaccessibility of glycinate complexes [4,5,6]. Nevertheless a slight decrease in the recovery after the treatment with gastro-intestinal juice was observed. A similar phenomenon observed in a Cd and Pb bioaccessibility study in cocoa was attributed to the precipitation as a result of the pH increase [14].

The electropherograms obtained for the *in-vitro* gastric extracts are shown Fig. 1b. Peaks at 4.2 min, 2.7 min and 3 min for Cu, Mn and Zn, respectively, were detected. For the three elements, either a stronger tailing than in the case of aqueous extracts (Fig. 1a) or a second not well resolved peak was observed. These peaks increased after the addition of a glycinate, the first peak generally to a less extent than the tailing. Other peaks, when present, did not change after glycinates standard addition. The increase of at least two peaks after addition of a glycinate standard regardless of the metal indicates its presence in two chemical forms in the extract. Two hypotheses can be made to explain this phenomenon.

(i) The splitting of the peak could be due to a partial dissociation of the complex into inorganic metal because of the acidic matrix. This would be in agreement with previously obtained ESI MS analysis [10] that showed that glycinates were still detected at pH 2 in aqueous methanol but that their amount decreased while acidifying the solution. An addition of metal sulfates to the extract led to the increase in the first peak only. It confirms that the second part of the large peak corresponds to the glycinate complex. However, inorganic species are expected to elute after glycinates [10]. It suggests that the peak detected here corresponds to sulfates complexed with another ligand. This is in agreement with the results obtained by Tastet et al. [21] which showed the absence of sulfates and the presence of a new copper complex together with the glycinate complexes in both compartments of an Ussing chamber by CE–ESI MS.

(ii) The change from pH 2 of the gastric juice to pH 7.4 of the electrolyte would lead to the, at least partial, reconstitution of the complex inside the capillary. However, experiments by CE with parallel ICP MS and ESI MS detection showed that the glycine-free metal and the metal-free glycine migrated separated by more than 3 min. In the range of the migration times observed here, it suggests a short contact time between both species inside the capillary. The glycinate complexes are therefore unlikely to be reconstituted within the capillary. The reconstitution of a complex with another co-extracted ligand cannot be totally excluded if this ligand migrates closer to the inorganic metal ion.

In conclusion, the glycinate complexes seem to be partly degraded during the gastric digestion but the strong binding capacity of the feed samples led to the formation of a new complex. The low glycinate concentration in the extract together with the problems encountered during lyophilisation made the identification of this new complex by CE–ESI MS impossible.

The electropherograms obtained for the *in-vitro* gastro-intestinal extracts are shown in Fig. 1c. Contrary to the results obtained for the gastric extracts, they contained principally one peak which increased after an addition of a glycinate standard. As the glycinate complex was, at least partly, destroyed during the gastric digestion, it seems that after buffering of the solution, the complex was reconstituted. It suggests that at neutral pH, the formation of the glycinate is favored again in comparison to the new complex.

However, the stability of glycinate compounds does not indicate if they would be better absorbed or more efficient than any other source *in-vivo*. Therefore *in-vivo* data from animal studies should be used to confirm the predictive values of the model. Previous experimental data [22] showed that Zn-glycinate compound induced some increase in Zn peak serum response in horses after

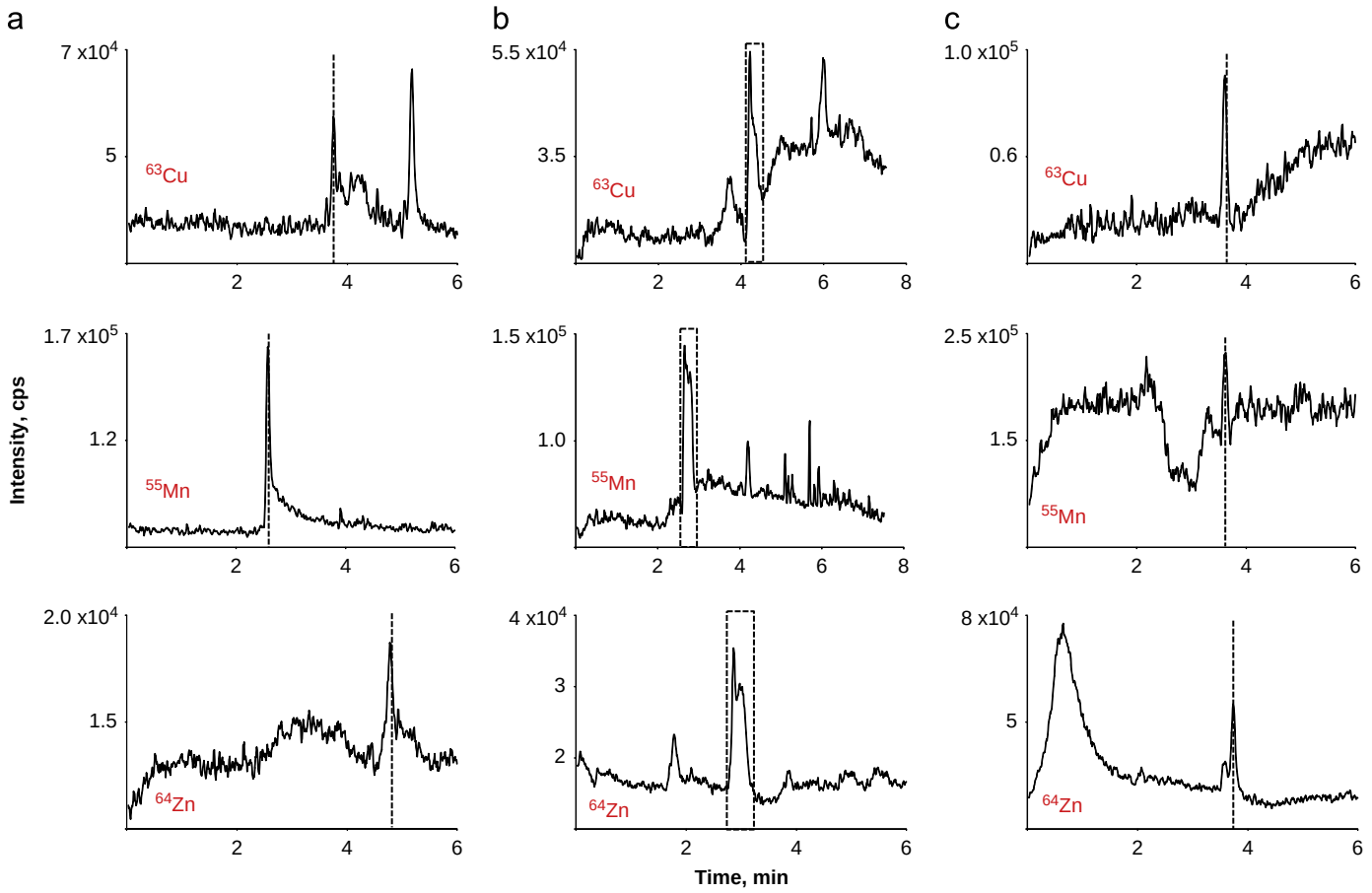

Fig. 1. Typical CE–ICP MS electropherograms obtained from: (a) a glycinate supplemented feed sample after an ammonium acetate extraction, (b) a glycinate supplemented feed sample after *in-vitro* gastric extraction and (c) a glycinate supplemented feed sample after *in-vitro* gastro-intestinal extraction. First line: ^{63}Cu, second line: ^{55}Mn, and third line: ^{64}Zn. The dotted line represents the peak(s) increasing after addition of a glycinates standard.

Table 2
Quantification and evaluation of the bioaccessibility of Zn–, Cu– and Mn-glycinates in enriched feed samples.

	C_{target} (mg/kg)	$C_{measured}$ (mg/kg)	Released by gastric juice (%)	Released by gastro-intestinal juice (%)
Cu	15	14 ± 3	94 ± 6	81 ± 5
Mn	15	14 ± 3	88 ± 17	79 ± 15
Zn	45	48 ± 3	95 ± 11	60 ± 7

Table 3
Total Cu, Mn and Zn concentrations in plasma horse samples.

	Control group (μg/kg) (n=4)	Glycinate group (μg/kg) (n=6)	ITE group (μg/kg) (n=6)
Cu	670 ± 15	678 ± 110	679 ± 140
Mn	1.8 ± 1.6	3.4 ± 2.1	2.9 ± 3.3
Zn	334 ± 80	526 ± 247	366 ± 51

feeding a single dose of 20 mg Zn/kg body weight compared to other sources. Horses were therefore selected in order to check if the observed better absorption could be related to product specific chemical through dosing the target compounds in animal biological fluids such as plasma.

3.3. *In-vivo bioavailbility of glycinates in horse plasma*

The total Cu, Mn and Zn concentrations obtained in the plasma samples are shown in Table 3. For Cu, there was a good correlation between the Cu ingested and the Cu concentration in plasma (Pearson correlation coefficient of 0.67). For Zn, the mean Zn concentrations in plasma were *ca.* 44% more important in the glycinates group than in the ITE group despite their higher Zn supplementation levels. The calculated Pearson correlation coefficient between ingested Zn and the Zn concentration in plasma was negative indicating that the level of supplementation was probably higher than the horse effective needs, leading to a regulation by the animal. For Mn, due to the low concentrations involved (quarter of the samples were below the detection limits), no correlation could be observed. The plasma samples were therefore investigated in terms of speciation only for Cu.

Electropherograms of preconcentrated plasma samples were then acquired using the conditions developed for feed samples (Fig. 2). A peak was detected in the range 8–10 min. This peak was more abundant in the glycinates group plasma samples than in the ITE group. Taking into account that the total Cu amount was very similar in both groups, the higher presence of this molecule may be related to the presence of CuGly in the diet.

The addition of the CuGly standard to the control group plasma sample led to a peak around 2 min splitted into two unresolved peaks showing that the glycinates were subject to degradation in the plasma matrix but also that the compound detected in the plasma samples was neither a glycinate nor a glycinate degradation product. The unambiguous identification of this peak would necessitate the coupling of CE with ESI MS/MS detection. However, due to its low concentration, this could not be achieved despite the use of state-of-the-art instrumentation. Nevertheless, the peak of Cu exactly matched the peak of Zn (no peak could be detected

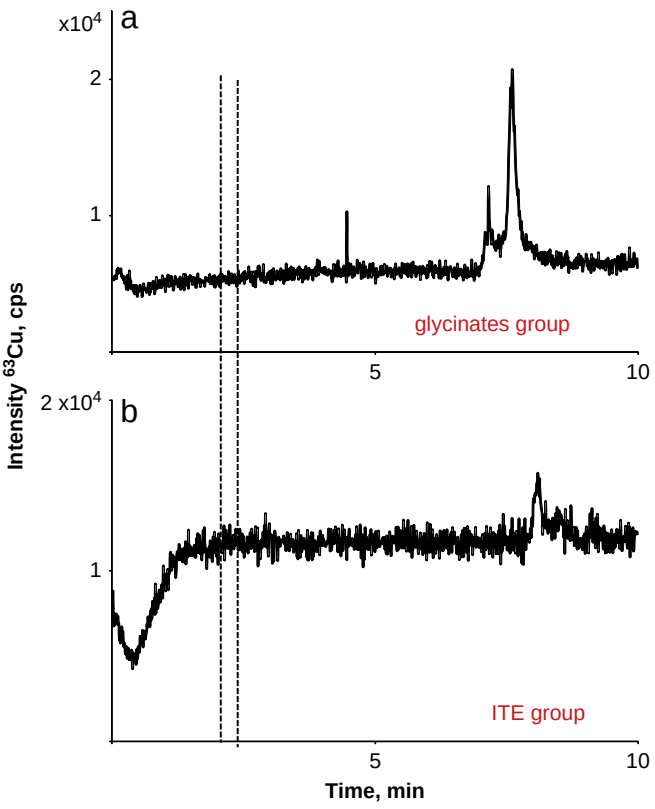

Fig. 2. Typical CE–ICP MS electropherograms obtained from (a) preconcentrated plasma samples from the glycinates group and (b) preconcentrated plasma samples from the ITE group. The dotted line represents the apparition of peaks after addition of a CuGly standard.

of Mn) in terms of migration time and shape. It suggests that this compound contained both Cu and Zn.

4. Conclusion

Following a sample clean up by ultrafiltration, the coupling of CE with ICP MS allows the quantitative determination of glycinates in feed samples. The method developed offers some new opportunities to feed producers to introduce glycinates with precision and to control the effective metal supplementation.

In-vitro gastric and gastro-intestinal digestions of those feed samples showed high bioavailability of the glycinates. The results also demonstrated that glycine might exchange with other exogenous or endogenous ligands present but is also able to recombine with glycine depending on the applied conditions. Further experiments would be necessary to identify the new complex put into evidence. *In-vivo* experiments based on the speciation analysis of plasma horse samples did not allow the detection of glycinates whatever the source at the sensitivity level of the CE–ICP MS coupling. However, at the supplied levels, a Cu-containing molecule was present in higher quantity in the glycinates group plasma samples indicating a possible modification of the metabolism of Cu in presence of CuGly.

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