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Studies on the Bioavailability of Zinc in Rats Supplementated with Two Different Zinc-Methionine Compounds

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SUMMARY. The effects of zinc-methionine complexes with molar relation 1:1 and 1:2 have been examined as nutritional supplements in rats. The synthesis and characterization of two compounds were studied by elemental analysis and FTIR. The bioavailability effect was studied by zinc retention and its content in rat tissues in rats fed with different zinc-methionine complexes. The compound 1:1 was a cation complex of formula $[Zn(C_5H_{10}NO_2S)(H_2O)_2]^+$, very water soluble, while the compound 1:2 was a neutral complex of formule $[Zn(C_5H_{10}NO_2S]_2]$, only soluble in pH below 3. FTIR spectra of both complexes show strong absorption bands due to C-O stretching of the amino acid group (ranging from ν_s(C=O) 1638 cm^{-1} and ν_{as}(C-O) 1414 cm^{-1}, in ZnMet, to ν_s(C=O) 1608 cm^{-1} and ν_{as}(C-O) 1426 cm^{-1}, in Zn(Met)$_2$) shifted significantly with respect to the ones observed for the free methionine (ν_{as}(COO) 1582 cm^{-1} and ν_s(COO) 1415 cm^{-1},1720 cm^{-1}). The nutritional result in the zinc fecal elimination of the animals of the control group was significantly different ($P < 0.05$) to the ones observed for the animals treaties with zinc supplemented diets, though this was not observed during urinary elimination. This study indicates that the content of zinc in the feces collected in 14 days for all zinc diet were significantly different ($P<0.05$) from the control group (animals treaties with zinc practically absent). The retention of zinc in the groups treated with the methionine compounds was significantly higher than the ones fed with $ZnSO_4$ and ZnO diet. In conclusion, these data indicate that the use of zinc-methionine chelates is a valuable tool to increase bioavailability of zinc, however without significant differences between ZnMet and Zn(Met)$_2$.

RESUMEN. "Estudios sobre la Biodisponibilidad de Zinc en Ratas Suplementadas con Dos Diferentes Compuestos Zinc-Metionina". Se han estudiado los efectos de los complejos zinc-metionina con la relación molar 1:1 y 1:2 como suplementación alimentaria de zinc en ratones. La obtención y la caracterización de dos complejos de zinc fueron evaluadas por análisis elemental y FTIR. El efecto de la biodisponibilidad de zinc fue estudiado por la retención en el contenido del metal en los tejidos de ratones alimentados con diversas fuentes. El compuesto 1:1 era un complejo del catión de fórmula $[Zn(C_5H_{10}NO_2S)(H_2O)_2]^+$, muy soluble en agua, mientras que el compuesto 1:2 era un complejo neutro de fórmula $[Zn(C_5H_{10}NO_2S)_2]$, solamente soluble debajo de pH 3. Los espectros de FTIR de ambos complejos demostraron franjas de absorción fuertes debido al estiramiento del C-O del grupo del aminoácido (extendiéndose de ν_s(C=O) 1638 cm^{-1} y ν_{as}(C-O) 1414 cm^{-1}, en ZnMet, a ν_s(C=O) 1608 cm^{-1} y ν_{as}(C-O) 1426 cm^{-1}, en Zn(Met)$_2$, cambiados de posición perceptiblemente las frecuencias asimétricas aumentan y las frecuencias simétricas disminuyen con respecto a la metionina libre ν_s(COO) 1582 cm^{-1} y ν_{as}(COO) 1415 cm^{-1}, 1720 cm^{-1}. El resultado de la alimentación fue observado claramente en la eliminación fecal del zinc de los animales del grupo de control perceptiblemente diferente ($P < 0,05$) que los tratados de los animales con dietas suplementadas con zinc, pero no se observó en la eliminación urinaria. El estudio indica que el contenido del zinc en las heces recogidas en 14 días para toda la dieta del zinc era perceptiblemente diferente ($P < 0,05$) del grupo de control (tratamiento de los animales con prácticamente ausencia de zinc). La retención del zinc en los grupos a los que se administraron compuestos de metionina fue perceptiblemente más alta que la alimentación con dieta de $ZnSO_4$ y de ZnO. En conclusión, estos datos indican que el uso del los complejos zinc-metionina constituyen una herramienta valiosa a la biodisponibilidad del aumento del zinc, no obstante sin diferencias significativas entre ZnMet y la forma Zn(Met)$_2$.

INTRODUCTION

As zinc takes part in the metabolism of numerous enzymes [1,2], and it is involved in the major networks, i.e. the nervous [3], neuroendocrine and immune systems it is an essential element required for normal growth, and indispensable in the diet [4,5]. Artificial diets must possess the zinc content required by the animal metabolism for health maintenance and high weight gain rates [6]. However, essential elements

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must also be in an available form to be utilized by the organism. Chelates, a form of mineral bound to an organic substance (e.g. amino acids [7], protein [8], polysaccharide [9], etc.), are known to have higher availability than inorganic salts because of their relative stability. Metal chelates of metal transition ions, including zinc(II) and others essential elements for survival (ex: Fe^{2+}, Fe^{3+} Cu^{2+}), are widely used in animal feeding, as they appear to have higher intake than inorganic salts [10]. Although there are still inconsistencies in the availability of Zn element from organic sources and the mode of action remains largely obscure [11], there is some evidence to suggest that the chemical form of the metal ingested does indeed affect its bioavailability [7,12]. Among the compounds that have been studied the Zn-amino acid complexes are widely used in animal nutrition and it has been shown that zinc-methionine appear to induce a faster growth and a better resistance to various diseases in comparison with simple inorganic salts [12-14].

From the chemical point of view, the coordination form of the methionine to zinc is very clear in the literature [15], with donor sites NH2 and COO- participating on the metal coordination. This reaction can generate at least two compounds with stoichiometric 1:1 and 1:2, respectively [16]. The synthesis conditions are decisive in the number of ligands that are coordinate to the zinc ion. Abdel-Moem [17] demonstrated that pH affects the number of coordinated ligands. Although the pH control allows for the formation of two different compounds only the monocoordination complexes have been evaluated as alimentary supplement for animals.

To gain insight into bioavailability of different methionine zinc chelates, studies in rats have been developed by our research group. In this context, the present study deals with the synthesis and characterization of zinc-methionine complexes by elemental analysis and FTIR, and the *in vivo* effect of zinc-methionine complexes supplementation on rats. The *in vivo* investigations on bioavailability were realized through both retention and content of zinc in rat tissues fed with different sources.

MATERIAL AND METHODS

Chemicals

Reagents and solvents were purchased from commercial sources and were purified (when necessary) and dried before use by standard procedures. The Zn compounds were prepared by the modificated method previously described [17-19]. Chemical analysis of the basal diet was run using standard procedures of AOAC [20].

Abbreviations and/or acronyms used

Met, methionine; ZnMet, zinc methioninato hydrogensulfate; $Zn(Met)_2$, zinc bismethioninato; FTIR, fourier transform infrared spectrocopy; *P,* standard level of significance; DM,dry matter; SD, standard deviation.

Instrumental analyses

The elemental analysis was carried out with an Perkin-Elmer PE2400 CHN elemental analyzer. The sulfate ion content was established by gravimetric method of $BaSO_4$ [21]. Infrared spectra were recorded on a Bomem serie MB FTIR spectrophotometer from KBr pellets (4000-500 cm^{-1}). The zinc fed, tissue, feces, and urine quantitation were obtained on a CG AA 7000 BC atomic absorption spectrophotometer.

Synthesis of zinc(II) methioninate hydrogensulfate, ZnMet

The heptahydrated zinc sulfate ($ZnSO_4.7H_2O$, 1.00 g, 3.5 mmol) and methionine (0.52 g, 3.5 mmol) were dissolved in 10 ml of distilled water by the aid of gentle heat. After one hour the hot solution was treated with 300 ml of acetone, stirred vigorously and allowed to cool (-10 °C) by one hour. A white precipitate was formed, filtered, washed with acetone and dried in the vacuum at 100 °C for 2 hours. The weight of the material formed was 0.85 g and yielded 64%. It is a white crystalline compound with molecular formula $[Zn(C_5H_{55}NO_2S)(H_2O)_2]$ HSO_4 and very soluble in H_2O at neutral pH. Anal. Calcd for $[Zn(C_5H_{10}NO_2S)(H_2O)_2]$ HSO_4: C, 17.32; H, 4.36; N, 4.04; SO_4^{2-}, 27.70. Found: C, 17.77; H, 4.89; N, 4.39; SO_4^{2-}, 26.23. Infrared data: ν(C=O) 1638s cm^{-1}; ν(C-O) 1414s cm^{-1}; ν(SO) 1142-1103vs cm^{-1}.

Synthesis of zinc(II) bismethioninate, $Zn(Met)_2$

Methionine (1.04 g, 7.0 mmol) was dissolved in 8 ml of NaOH (1.0 mol/l) under stirring condition for 30 min at 25 °C and pH 10. In this solution was added a solution of heptahydrated zinc sulfate (1.00 g, 3.5 mmol) under stirring with the formation of a white precipitate. The product was filtered and washed several times with distilled water and dried in the vacuum at 100 °C for 2 hours. The weight of the material formed was 1.27 g and yielded 92%. It is a white crystalline compound with molecular for-

mula [$Zn(C_5H_{10}NO_2S)_2$], sparingly soluble in H_2O at neutral pH and soluble in H_2O at acid pH. Anal. Calcd for [$Zn(C_5H_{10}NO_2S)_2$]: C, 33.20; H, 5.57; N, 7.74; SO_4^{2-}, 26.55. Found: C, 33.01; H, 5.15; N, 7.22; SO42-, 25.70. Infrared data: ν(NH) 3314-3257m cm^{-1}; ν(C=O) 1608s cm^{-1}; ν(C-O) 1426s cm^{-1}.

Solubility of 1:2 Zn(met)$_2$ complex versus pH

Solubility of zinc compounds were measured at pH ranging from 2 to 9 in diluted solutions of HCl or NaOH. The HCl solution was used for pH range 2-6 and NaOH solution was used on pH 7 and 9. The compound was weighed (10 mg) and mixed with 20 ml of solution, adjustments were made with either diluted HCl or NaOH to reach the target pH. Mixtures were incubated at 37 °C for 12 h in a shaking water bath. Subsequent incubations were conducted to determine effects of pH and then filtered through Whatman 541 ashless filter paper. Filtrates were analyzed for metals after dilution with nitric acid solution (0.1mol/L). Filter papers were dry ashed at 500 °C, dissolved in 5 ml of 20% HCl, and diluted with deionized water before analysis for metal content. Zinc was quantified by atomic absorption spectroscopy. Metals in the filtrates were assumed to be soluble, and metals remaining on rinsed filters were assumed to be insoluble. Samples were analyzed in duplicate for each pH.

In vivo trial with rats

A basal diet was formulated to meet or exceed all nutrient requirements [22] for young rats male *(Rattus norvegicus)* averaging 250.0 g (Table 1). The basal diet contained 25 mg Zn kg-1 DM, while in the different treatments 150 mg Zn kg^{-1} DM. Diets were offered to the rats in the form of crumb. All the components reported in Table 1 were analyzed. Zinc concentration in the diets was obtained digesting feed samples with a mixture of concentrated nitric acid and hydrogen peroxide 30% (3:1) and warmed for 3 h.

Initially, thirty five animals were treated with the basal diet with practically absence of zinc for 7 days. After this period, the animals were divided into five groups (seven animals each) and treated with the following experimental diets: *i.* Basal diet with residual Zn; *ii.* Basal diet supplemented with commercial $ZnSO_4.7H_2O$; *iii.* Basal diet supplemented with commercial ZnO; *iv.* Basal diet supplemented with ZnMet; *v.* Basal diet supplemented with $Zn(Met)_2$. Animals were kept individually in metabolic cages that allowed separated collection of urine and feces. Diets were fed *ad libitum* for 14 days and feed intake was recorded. Deionized water was provided *ad libitum*. Urine and feces were collected daily. Subsequently, animals were sacrificed by cervical dislocation and femur, spleen, muscle, heart, kidneys, liver and feces masses were determined and volumes of blood. The urine were measured and then freeze-dried. Tissue and feces samples from each animal were dried in an oven at 70 °C for 24 h and later weighed. All collected samples were then digested and analyzed and the zinc concentration in the mineralized samples was measured using an atomic absorption spectrophotometer.

Item	Amount (%)
Maize meal	40.00
Soyabean meal	18.21
Corn gluten meal	35.44
Dicalcium phosphate	3.30
Salt	0.79
Mineral premix [a]	1.96
Mineral premix [b]	0.30
Chemical composition	
Crude protein, %	22.0
Ether extract, %	4.0
Crude fiber, %	6.0
Ash, %	8.0

Table 1. Ingredient composition of basal diet for rats. [a] Supplied (mg/kg in diet): iron, 180.0; copper, 30.0; manganese, 110.0; iodine, 2.0; selenium, 2.0. [b] Supplied indiet: Vitamine A, 30000 UI; riboflavin, 13 mg; pantothenic acid, 50 mg; niacin, 180.0 mg; Vitamine B12, 66.0 μc; Vitamine D, 6000.0 mg; Vitamine E, 110.0 mg.

Data mineral tissue and fece concentration results were analyzed by one-way ANOVA. The differences among means of groups were analyzed using the Tukey's test. Diferences were statiscally significant at $P < 0.05$.

RESULTS AND DISCUSSION

Characterization of the Methionine Chelates

The prepared compounds in this study are the product of the reaction between the Zn^{2+} ion and the methionine amino acid in aqueous solution in two different pH. In pH controlled between neutral or acid conditions the formed compound was 1:1 zinc and methionine cation complex with each zinc ion becoming com-

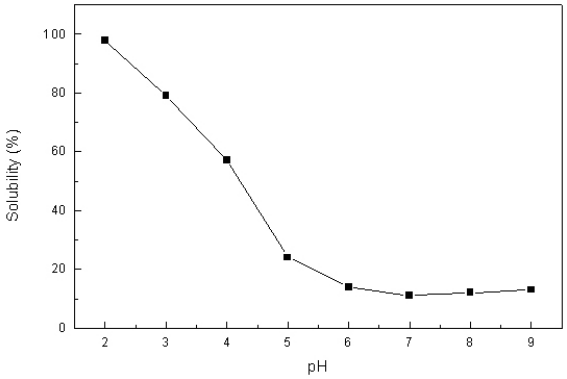

Figure 1. Solubility in % of 1:2 complex zinc methionine in different pH values.

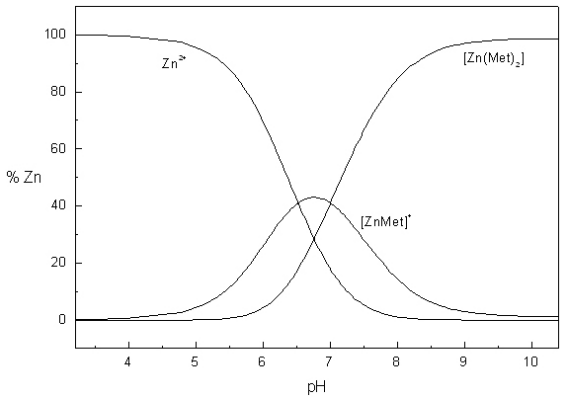

Figure 2. Distribution diagram of the Zn^{2+}/Met system with protonation constant of the ligand (log β_1 = 2.28) and constants for the Zn^{2+}/Met system (Zn^{2+}/Met: β_1 = 4,38 e β_2 = 8,40) [18].

plexed with one methionine molecule, with molecular formula $[Zn(C_5H_{10}NO_2S)(H_2O)_2]^+$, ZnMet, which is very soluble in water. In basic pH, the resulting product was a 1:2 zinc and methionine neutral complex with formula $[Zn(C_5H_{10}NO_2S)_2]$, $Zn(Met)_2$. This later compound is insoluble in water, but it is soluble in pH below 3, as observed in the solubility curve in Fig. 1 [17]. That shows that the ligand number methionine bonded to the zinc ion depends on the pH reaction conditions. This results agree with the representative distribution diagram of the Zn^{2+}/Met system that is reported in Figure 2, using formation constants (Zn^{2+}/Met: β_1 = 4,38 and β_2 = 8,40) previously described [23]. In pH < 6.8 the formation of ZnMet prevails while in higher pH $Zn(Met)_2$ appears in larger extension.

In both compounds, the methionine molecule should coordinate to the zinc cation through one oxygen atom from the carboxylic group and though nitrogen atom from the amine group, producing penta-atomic chelation rings. In this regard, the FTIR spectra of both complexes show strong absorption bands due to C-O stretching of the amino acid group (ranging from ν_{as}(C=O) 1638 cm^{-1} and ν_s(C-O) 1414 cm^{-1}, in ZnMet, to ν_{as}(C=O) 1608 cm^{-1} and ν_s(C-O) 1426 cm^{-1}, in $Zn(Met)_2$), significantly shifted to lower frequencies with respect to free methionine (ν(C-O) 1720 cm^{-1}), as expected for coordination. As expected, IR data for the amino acid group is well comparable to those reported for the corresponding amino acid complexes [24].

Supplemental evaluation of rats with zinc

The results of the zinc balance found in the Table 2 show that the animal supplemented with different sources of zinc presented a zinc intake appropriated in the diet, between 27.4 and 33.8 mg/rat, while the group control tries low levels of zinc in the diet. This result was clearly observed in the zinc fecal elimination of the animals of the control group significantly different ($P < 0.05$) to the animals treaties with zinc supplemented diets. Though it was not observed during urinary elimination.

Urine samples, collected in 14 days revealed no significant difference ($P>0.05$) in urinary zinc loss in the treated animals with different sources as well as in control group, indicating that is not the main elimination route for zinc when oral administered. This result agree with the works of House [25] which observed that zinc is mainly absorbed and eliminated in rats on intestine.

Nevertheless, our work indicates that the content of zinc in the feces collected in 14 days

Sources	Feed intake (g/rat)	Zinc intake (mg/rat)	Zinc fecal eliminated (mg/rat)	Zinc urine eliminated (mg/rat)	Zinc retained (% Zn intake)
Control	180 ± 15.5	4.50 ± 0.4 [a]	2.6 ± 0.2 [a]	1.12 ± 0.02	17.3 ± 2.6 [a]
ZnSO4	182 ± 10.8	27.4 ± 1.6 [b]	7.8 ± 0.2 [b]	1.26 ± 0.02	66.9 ± 5.5 [b]
ZnO	205 ± 10.7	30.8 ± 1.6 [b]	8.4 ± 0.2 [b]	1.13 ± 0.02	69.1 ± 5.2 [b]
ZnMet	230 ± 7.2	34.5 ± 1.1 [b]	7.2 ± 0.2 [b]	1.19 ± 0.02	75.7 ± 4.3 [c]
$Zn(Met)_2$	225 ± 6.6	33.8 ± 1.0 [b]	6.7 ± 0.2 [b]	1.16 ± 0.02	76.7 ± 4.3 [c]

Table 2. Feed intake and retention in rats fed with different sources of zinc after 14 days (averange of 7 animals ± SD). [a] Different letters within the same column indicate a significant difference ($P < 0.05$).

Sources	Femur (mg/kg DM)	Spleen (mg/kg DM)	Muscle (mg/kg DM)	Heart (mg/kg DM)	Kidneys (mg/kg DM)	Liver (mg/kg DM)	Blood (µg/dl)
Control	153.4 ± 11.1 [a]	42.1 ± 6.6	24.1 ± 7.2 [a]	29.2 ± 5.1	34.2 ± 4.0	37.1 ± 3.8 [a]	165.0 ± 59.1 [a]
ZnSO4	166.8 ± 13.7 [b]	42.3 ± 8.9	38.0 ± 9.6 [b]	23.7 ± 5.1	30.8 ± 2.5	41.2 ± 6.0 [b]	200.3 ± 69.9 [b]
ZnO	169.1 ± 16.5 [b]	40.8 ± 7.7	28.0 ± 6.5 [b]	29.2 ± 1.3	26.6 ± 3.6	42.0 ± 2.5 [b]	197.9 ± 25.3 [b]
ZnMet	197.9 ± 9.40 [b]	35.5 ± 2.5	33.7 ± 4.9 [b]	30.9 ± 1.8	31.6 ± 3.3	47.9 ± 4.5 [c]	219.0 ± 39.2 [b]
$Zn(Met)_2$	201.3 ± 10.9 [b]	44.6 ± 4.2	34.7 ± 6.7 [b]	27.1 ± 4.5	31.7 ± 4.4	49.4 ± 4.9 [c]	217.2 ± 34.4 [b]

Table 3. Zinc content of femur, spleen, muscle, heart, kidneys, liver and blood in rats after 14 days (averange of 7 animals ± SD). Different letters within the same column indicate a significant difference (P<0.05).

for all zinc diet were significantly different (P < 0.05) from the control group (animals treaties with practically absence of zinc). The retention of zinc in the groups treated with the methionine compounds was significantly higher than the ones fed with $ZnSO_4$ and ZnO diet. These results agree with the coefficients of retention previously measured according to a similar method in zinc compounds with methionine analogue [26]. However, the results show that there is no significant difference (P > 0.05) among rats fed with ZnMet or with $Zn(Met)_2$, even considering the different water solubilities for each complexes.

These results seem to show a higher bioavailability of zinc organic source compared to zinc inorganic, confirming in the rat what had already been observed in chicks [27] and pigs [28] when zinc chelates were fed. On the contrary, some authors reported no difference between bioavailability of inorganic and organic sources of zinc in cattle [29] and pigs [30]. In our study, zinc bioavailability of zinc methionine was much higher than that of zinc sulfate and oxide (+8.2%; P<0.05), although has not shown significant difference (P > 0.05) among the ZnMet and $Zn(Met)_2$ compounds.

Zinc in femur, muscle, liver and blood were influenced by this treatment when compared with the group control (P < 0.05) without distinguishing among the zinc sources, except for liver which was show significant difference (P < 0.05) for the methionine compounds (Table 3). In fact, some works have been showing that 90% of corporal zinc are concentrated in the bones, muscle, liver and skin [31]. Zinc in spleen, kidneys, muscle and heart was not influenced by this treatment (P>0.05). However, it was observed a small improvement in the contents of zinc in the femur, blood and liver.

In conclusion, these data indicate that the use of zinc-methionine chelates is a valuable tool to increase bioavailability of zinc in rats, however without significant differences among ZnMet and $Zn(Met)_2$ form.

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REFERENCES

1. Lipscomb, W.N. & N. Sträter (1996) *Chem. Rev.* **96**: 2375-433.
2. Coleman, J.E. (1998) *Curr. Opin. Chem. Biol.* **2**: 222-34.
3. Willians, R.J., J.P.E. Spencer, F.M. Goni & C.A. Rice-Evans (2004) *Neurosc. Lett.* **371**: 106-10.
4. Salhab, A.S., S.M. Zmeili, M.N. Gharaibeh, H.H. Elayan, E. Sallam, & S.A. Deleq (1999) *Int. J. Pharm.* **178**: 171-81.
5. Yamaguchi, M. (1998) *J. Trace Elem. Exp. Med.* **11**: 119-35.
6. National Research Council (1994) *"Nutrient Requirements of Poultry"*. 9th Rev. ed., National Academy of Sciences, Washington, DC.
7. Guo, R., P.R. Henry, R.A. Holwerda, J. Cao, R.C. Littell, R.D. Miles & C.B. Ammerman (2001) *J. Anim. Sci.* **79**: 1132-41.
8. Eckert, G.E., L.W. Greene, G.E. Carstens & W.S. Ramsey (1999) *J. Anim. Sci.* **77**: 244-9.
9. Kennedy, D.W., W.M. Craig & L.L. Southern (1993) *J. Anim. Sci.* **71**: 1281-7.
10. Predieri, G., M. Tegoni, E. Cinti, G. Leonardi & S. Ferruzza (2003) *J. Inorg. Biochem.* **95**: 221-4.

11. Beutler, K.T., O. Pankewycz & D.L. Brautigan (1998) *Biol. Trac. Elem. Res.* **61**: 19-31.
12. Kratzer, F.H., P. Vohra (1986) *"Chelates in Nutrition",* CRC Press Inc, Boca Raton, FL.
13. Wedekind, K.J., A.E. Hortin & D.H. Baker (1992) *J. Anim. Sci.* **70**: 178-87.
14. Cao, J., P.R. Henry, S.R. Davis, R.J. Cousins, R.D. Miles, R.C. Littell & C.B. Ammernan (2002) *Anim. Feed Sc. Technol.* **101**: 161-70.
15. Wilson, R.B., P. de Meester & D.J. Hodgson (1977) *Inorg. Chem.* **16**: 1498-502.
16. Berthon, G. (1995) *Pure & Appl. Chem.* **67**: 1117-240.
17. Abdel-Monem, M.M., Zinpro Corp (1976) International patent US3941818.
18. Abdel-Monem, M.M., Zinpro Corp (1977) International patent GB1474409.
19. Souza, A.R., R.N. Ferreira, L.C. Faria & L.T. Silva (2003) Brazillian patent PI 030995-7.
20. Association of Official Analytical Chemists (1990) "Official Methods of Analysis of the Association of Official Analytical Chemists", 15th edition. AOAC, Washington DC, USA.
21. Jeffery, G.H., J. Bassett, J. Mendham & R.C. Denney (1992) "VOGEL Análise Química Quantitativa". LTC-Editora, 5ª. Ed., Rio de Janeiro
22. National Research Council (1995) *"Nutrient Requirements of Laboratory Animals. A report of the Committee on Animal Nutrition".* Washington, D.C.: National Academy Press.
23. Smith, R.M. & A.E. Martell (1997) NIST *Critically selected stability constants of metal complexes database - version 3.0.* Texas A&M University.
24. Nakamoto, K., (1986) *"Infrared and Raman of Inorganic Coordinated Compunds"* (John Wiley&Sons, New York), pp. 233.
25. House, W.A. (1999) *Field Crops Res.* **60**: 115-41.
26. Predieri, G., L. Elviri, M. Tegoni, I. Zagnoni, E. Cinti, G. Biagi, S. Ferruzza & G. Leonardi (2005) *J. Inorg. Biochem.* **99**: 627-36.
27. Wedekind, K.J. & D.H. Baker (1990) *J. Anim. Sci.* **68**: 684-689
28. Hahn, J.D. & D.H. Baker (1993) *J. Anim. Sci.* **71**: 3020-4.
29. Rojas, L.X., L.R. McDowell, F.G. Martin, N.S. Wilkinson, A.B. Johnson & C.A. Njeru (1996) *J. Trace Elem. Med. Biol.* **10**: 205-9.
30. Swinkels, J.W., E.T. Kornegay, W. Zhou, M.D. Lindemann, K.E., Webb, & M.W. Verstegen (1996) *J. Anim. Sci.* **74**: 2420-30.
31. Vallee, B.L. & K.H. Falchuk, (1993) *Physiol. Rev.* **73**: 79-118.