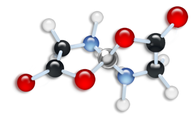
Development of a Novel Method to Quantitate the Amount of Unbound Metal in Copper Glycinate Solutions

Jennifer Hartle and R. Charles Thompson
Albion Advanced Nutrition, Clearfield, Utah USA



Abstract

Copper is a micronutrient which participates in many biochemical reactions. In recent years there has been a trend in the nutritional market toward more accurate quantitation and representation of active compounds present in nutritional supplements. This method has been designed to analyze the amount of unbound metal in copper glycinate in solution. Copper glycinate solutions were introduced to a strong cation exchange column where the unbound copper was separated from the ligand bound metal. It was observed that the copper bound to glycine could be separated effectively from unbound copper using different buffered solvent systems. Varying copper concentrations, metal to ligand ratios as well as pH of the solutions all had an effect on the amount of bound copper in solution. This poster discusses the findings of this method and possible implications of this research.

Introduction

The use of mineral amino acid chelates as a source of mineral nutrition has been widely practiced for many years. The uses of amino acid chelates vary greatly, and have been successfully utilized for plant nutrition[1], animal feed[2], and in human nutritional[3] supplements. With the use of Fourier Transforming Infrared spectroscopy (FTIR), we can substantiate the bonding model of amino acid chelates by looking at changes in IR bands that correspond to both the carboxyl and amine portion of the amino acid before and after bonding to minerals (metal atoms)[4]. This allows us to successfully quantify[5] the amount of bound metal in a solid state, but because FTIR is sensitive to moisture it becomes difficult or impossible to quantify the amount of unbound metal present when an amino acid chelate is dissolved into solution. Here we present a novel approach to the determination of the unbound metal ions in solution by using cation exchange solid phase extraction technology. Using a strong cation exchange resin to carry out the separation, and Inductively Coupled Plasma-Optical Emmission Spectroscopy (ICP-OES) for the detection of metal ions we have developed a method for determining the amount of unbound metal in solution for a given metal amino acid chelate. This experiment focused on copper glycinate to establish the optimum buffer composition, strength, and pH in which to carry out the separation of bound and unbound metal when a chelated metal amino acid is dissolved into solution. Our goal is that, once optimized, this procedure will work well to determine the unbound metal in various metal amino acid chelate solutions.

Scope

This project was initiated to determine the amount of unbound/bound metal in copper glycinate solutions. This can be difficult to determine because the analytical measurement of chelated metal in solution is difficult to distinguish from unbound metal. The measurement must be done without changing the equilibrium of bound to unbound metal in solution. Several methods were evaluated for this purpose, and eventually ion exchange chromatography was selected. Differences in pH as well as metal to ligand (M:L) ratios were found to have an effect on the amount of unbound copper in solution[6]. These differences were evaluated in the work presented in this poster.

Procedure

Overall procedure

Samples were prepared and separated by ion exchange chromatography. The cation exchange columns used were Oasis 6cc MCX LP extraction columns (Waters Corporation). All fractions were then quantitatively analyzed by ICP-OES for copper content and qualitatively analyzed by High Performance Liquid Chromatography (HPLC) for glycine, with ninhydrin used as a secondary confirmation, when necessary.

The cation exchange column[7] separated the bound copper from the unbound copper. Cu EDTA, $CuSO_4$ and glycine were used as positive and negative controls. Cu EDTA was used as a 100% bound copper control, $CuSO_4$ was used as a 100% unbound control and the glycine control was used to ensure that no ligand bound to the column. Three buffers were used to carry out the fractionation of bound and unbound copper in solution. One was used to charge the column, a second buffer was used to elute bound copper and third buffer was used to elute unbound copper. All glycine was eluted into the first fraction, as confirmed by HPLC analysis[8]. For the titration of copper bisglycinate and subsequent analysis of bound copper, glycine elution was confirmed by ninhydrin[9].

Titration Procedure

One liter of 0.05M copper glycinate at a metal to ligand molar ratio of 1:2 was titrated with 2N NaOH from an initial pH of 3 to a final pH of 12, with data points at key intervals sampled and fractionated using a NaCOOH buffer system and tested for unbound Cu^{+2} ions. More samples were taken at the inflection point where the pH change was the greatest in order to accurately predict the trend for unbound copper. This replicated a fractionation of species diagram, predicted by a mathematical modeling program developed by Hancock and Martell[10]. It can be clearly seen in Figures 1 and 2 that the overall trend line matches the theoretical prediction. It can also be deduced from a study of the diagrams that the pH values correspond well with the predicted values for bound copper.

Column Procedure

A separate column was used for each sample. The column was initially charged with 15 mL of buffer 1. The column was then equilibrated with 10 mL of buffer 2. At this point, 0.5 mL of sample was introduced to the column. The bound copper was eluted into two fractions with buffer 2: a 10 mL and a 5 mL fraction. These fractions were labeled fraction 1 and fraction 2, respectively. The unbound copper was eluted into an additional fraction with 10 mL of buffer 3. This fraction was labeled fraction 3.

Buffer and Sample Preparation

Six solvent systems were evaluated: NaCOOH, Na_2SO_4, NH_4COOH, $(NH_4)_2SO_4$, $MgSO_4$ and $Mg(COOH)_2$. A set of three buffers was prepared for each set.

The monovalent buffers were prepared in the following manner; buffer 1 was made at a pH of 7 with an ionic strength of 500 mM for the cation concentration. Buffer 2 was prepared at a pH of 7 with an ionic strength of 10 mM for the cation concentration. Buffer 3 was prepared at a pH of 3 with an ionic strength of 500 mM cation concentration.

The Mg buffers were prepared at half the ionic strength of the Na^{+1} and NH_4^{+1} buffer systems. The pH of the buffers was the same. The concentrations were halved to match the total cation charge present in the monovalent systems.

The samples and copper controls were all prepared at 0.5 M Cu^{+2} concentration. The Cu EDTA had an M:L molar ratio of 1:1. The glycine control was prepared as 0.10 M glycine with no pH adjustment.

The samples for the M:L molar ratio evaluation were adjusted to pH 7 with 1 N NaOH, with the exception of the copper glycinate with a molar ratio of 1:1 M:L. This was adjusted up to a pH of 5 because a precipitate formed above a pH of approximately 5.35.

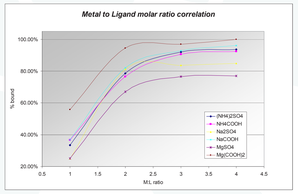


Figure 1. Metal to ligand correlation

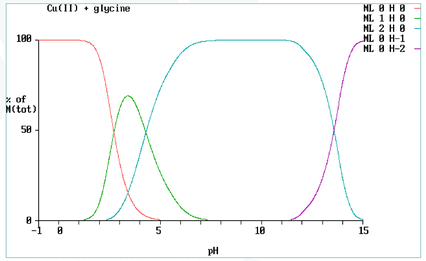


Figure 3. Species Diagram of 0.05M copper bisglycinate

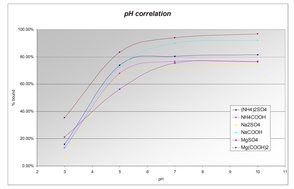


Figure 2. pH correlation

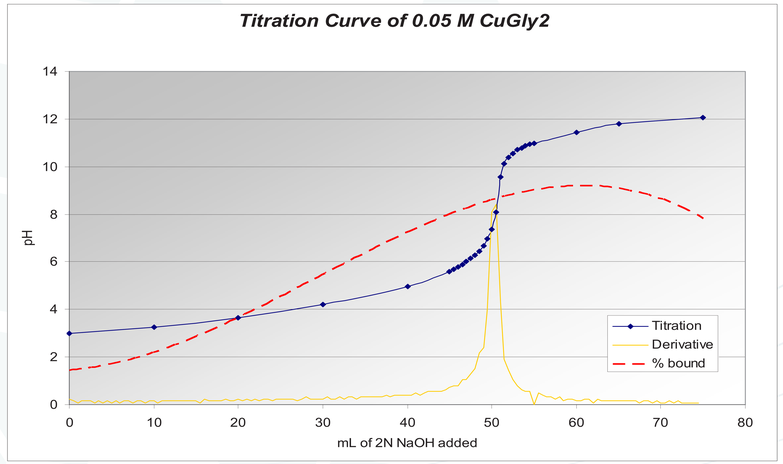


Figure 5. Titration curve and % bound experiment

% Bound Cu

M:L trial set	*pH*	*$(NH_4)_2SO_4$*	*NH_4COOH*	*Na_2SO_4*	*NaCOOH*	*$MgSO_4$*	*$Mg(COOH)_2$*
Cu Gly (1:4)	7	93.60%	92.33%	84.88%	96.10%	76.88%	100.00%
Cu Gly (1:3)	7	92.00%	90.62%	83.69%	92.80%	76.55%	96.90%
Cu Gly (1:2)	7	78.60%	76.48%	81.12%	82.00%	66.97%	94.40%
Cu Gly (1:1)	5	33.50%	36.63%	24.87%	36.80%	25.08%	55.80%
pH trial set	*pH*	*$(NH_4)_2SO_4$*	*NH_4COOH*	*Na_2SO_4*	*NaCOOH*	*$MgSO_4$*	*$Mg(COOH)_2$*
Cu Gly (1:2)	10	81.59%	76.28%	74.86%	92.10%	76.64%	96.70%
Cu Gly (1:2)	7	80.46%	76.66%	71.89%	90.00%	75.52%	94.10%
Cu Gly (1:2)	5	73.80%	67.83%	65.21%	72.50%	56.33%	83.30%
Cu Gly (1:2)	3	15.93%	13.16%	5.55%	13.70%	21.30%	35.30%
Control set	*pH*	*$(NH_4)_2SO_4$*	*NH_4COOH*	*Na_2SO_4*	*NaCOOH*	*$MgSO_4$*	*$Mg(COOH)_2$*
Glycine	n/a	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
$CuSO_4$	n/a	0.00%	0.00%	0.00%	0.00%	3.45%	8.60%
Cu EDTA	7	100.00%	99.68%	97.99%	100.00%	99.65%	99.30%

Table 1. % Bound Copper (n/a signifies no change to pH was made)

Samples			All buffer systems			Monovalent anion buffer systems		
		pH	Average	Std. Dev.	rsd	Average	Std. Dev.	rsd
M:L	Cu Gly (1:4)	7	90.63%	0.084	9.25%	91.73%	0.0482	5.26%
	Cu Gly (1:3)	7	88.76%	0.074	8.30%	89.78%	0.0416	4.63%
	Cu Gly (1:2)	7	79.93%	0.089	11.14%	79.55%	0.0250	3.15%
	Cu Gly (1:1)	5	35.45%	0.113	31.93%	32.95%	0.0560	16.98%
pH	Cu Gly (1:2)	10	83.03%	0.092	11.10%	81.21%	0.0782	9.63%
	Cu Gly (1:2)	7	81.44%	0.088	10.76%	79.75%	0.0768	9.63%
	Cu Gly (1:2)	5	69.83%	0.091	13.00%	69.84%	0.0401	5.74%
	Cu Gly (1:2)	3	17.49%	0.101	57.74%	12.08%	0.0452	37.40%
Controls	Glycine	n/a	0.00%	0.000		0.00%	0.0000	0.00%
	CuSO4	n/a	2.01%	0.035	174.84%	0.00%	0.0000	0.00%
	Cu EDTA	7	99.44%	0.008	0.76%	99.42%	0.0096	0.97%

Table 2. Statistical Comparisons

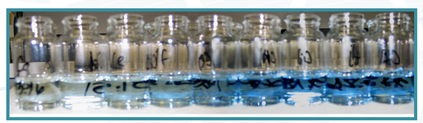


Figure 4. Visual observations of bound copper

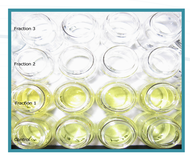


Figure 6. Ninhydrin confirmation of glycine in fraction 1. The controls are on the bottom, followed by fractions 1, 2 and 3 going up.

Results

Several buffer systems were evaluated: NaCOOH, Na_2SO_4, NH_4COOH, $(NH_4)_2SO_4$, $MgSO_4$ and $Mg(COOH)_2$. In each buffer system, the M:L molar ratio was evaluated, as well as the effect of pH on a 1:2 Cu to glycine molar ratio. The buffers were chosen to determine if the procedure was robust and repeatable using differing anions and cations. An Mg buffer set was chosen to assess the differences in monovalent and divalent cation exchange. Overall, two monovalent cations, a divalent cation, a monovalent anion and a divalent anion were evaluated.

The system appeared to be fairly robust between differing monovalent solvent systems. (See Figures 1 and 2, Table 1 and 2.) With each monovalent buffer set, the glycine was consistently eluted in only the first fraction. The results from the control fractionation demonstrated that the system worked well. However, the divalent buffer systems had different results. Much higher percent bound copper was calculated in the divalent systems. Also, there was Cu^{+2} present in fraction 2 of the $CuSO_4$ control. This could indicate that the Mg^{+2} eluted some Cu^{+2} ions prematurely into fraction 2, which would make them appear to be bound to glycine.

The statistical data in Table 2 showed the correlation between the monovalent buffers systems was much better than the correlations calculated when all buffer systems were included.
As the pH and the ligand in the M:L increased, the copper was bound to a higher degree (see Figures 1 and 2).

This is to be expected, as one can deduce from the species diagram (Figure 3).

In all samples tested, the glycine was found exclusively in fraction 1 as confirmed by HPLC and/or ninhydrin testing.
The effect of pH and percent bound copper can also be examined visually. As the pH increases, the color of the copper glycinate solutions change from a light blue to a deep royal blue. In these fractions (figure 4), this can be observed as the solutions change from clear/ light blue to a darker color of blue.

A titration curve of $CuGly_2$ was performed. The amount of bound copper was determined, using the NaCOOH buffer system, at each measurement point in the $CuGly_2$ titration. The results can be seen in Figure 5.

Because of the number of samples generated by the titration, glycine analysis was confirmed using ninhydrin. When ninhydrin combines with a chelate, the resulting color is yellow instead of purple[11]. This is visually apparent in the samples shown in Figure 6. The controls are positioned on the bottom row, with additional rows representing fractions 1, 2 and 3 going up.

Conclusion

It has been demonstrated that the amount of unbound metal in a copper glycinate solution can be measured. This experimental method warrants further research in the analysis of unbound metal ions in solution. Many future experiments with respect to the analysis of unbound metal ion in amino acid chelates as well as other organic ligands could be predicted by the data presented here. Future research will be conducted to evaluate the use of these procedures with other metals and amino acids. Through the use of this methodology, a diagram of the theoretical fraction of species could be modeled with empirical data. Another direction for future research could include the development of a direct analytical method for determining stability constants for metal amino acid chelates as well as other organometallic compounds.

Acknowledgements

This work was fully funded by Albion Advanced Nutrition. We appreciate their continuing support and backing of this project. We would like to thank Debra Carey for her artistic assistance in arranging and putting together this poster. We would also like to thank Dr. Robert Hancock for his mathematical species modeling program and his guidance and education in the field of chelation.

Citations

1. Lester, G.E., Jifon, J.L, Rogers, G. *Supplemental Foliar Potassium Applications during Muskmelon Fruit Development Can Improve Fruit Quality, Ascorbic Acid, and Beta-carotene Contents*. Journal of the American Society for Horticultural Science. 130(4), 649-653, 2005.

2. Ashmead, H.D., Ashmead, S.D., Samford, R.A. *Effects of Metal Amino Acid Chelates on Milk Production, Reproduction, and Body Condition in Holstein First Calf Heifers.* International Journal of Applied Research in Veterinary Medicine. Vol. 2, No. 4, 2004.

3. Bovell-Benjamin, A.C., Viteri, F.E., Allen, L.H. *Iron absorption from ferrous bisglycinate and ferrous trisglycinate in whole maize is regulated by iron status*. American Journal of Clinical Nutrition 71, 2000., Ashmead, H.D., Graff, D.J., Ashmead, H.H. *Intestinal absorption of metal ions and chelates*. (IL: Charles C. Thomas) 1985.

4. Ashmead, S.D. *FT-IR Characterization of Metal Amino Acid Chelates: Zinc Bisglycinate Model*. AOAC 117th International meeting, 2003.

5. Hartle, J.W., Ashmead, S.D. *Single Laboratory Validation of the Quantification of Chelation in Metal Glycinate Chelates Through the Use of FT-IR Analysis.* AOAC 118th International meeting, 2004., Hartle, J., Ashmead, D., *Bonds important for amino acid chelates*. Feedstuffs, Issue 37, Volume 78, September 11, 2006., Hartle, J., Ericson, C., Ashmead, S. *Process for determining the percent of chelation in a dry mixture*. US Patent 7,144,737. December 5, 2006.

6. Murphy, C.B., Martell A.E. *Metal Chelates of Glycine and Glycine Peptides.* Journal of Biological Chemistry. 226:37-50.

7. Oasis® Applications Notebook, Waters Corporation.

8. Waters AccQtag™ Chemistry Package, Waters Corporation.

9. Skoog, D.A., West, D.M., Holler, F.J. *Fundamentals of Analytical Chemistry, 7th edition.* Saunders College Publishing, New York, 1996, pp. 723.

10. Hancock, R. D., Martell, A. E., *Species: Mathematical Modeling Program*.

11. Ericson, C., Ashmead, S.D. *A Novel Approach in Confirming Dietary Amino Acid Chelates by Utilization of Ninhydrin*, AOAC 118th International meeting, 2004.