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A Multi-Model Assessment of Copper(II)-Humic Acid Binding: Insights from Ion-Selective Electrode Data and a Critical Comparison of Discrete and Continuous Binding, and Computational Chemical Equilibrium MINTEQA Models

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Abstract

Speciation of Cu^{2+} with Humic acid (HA) was studied by Cu-Ion Selective Electrode (ISE) potentiometry. Free Cu^{2+} concentration was measured directly from the Cu-ISE titration data, and the bound Cu^{2+} was calculated from the mass balance of total copper added $[Cu^{2+}]_t$. The titration data were analyzed and compared with six data reduction models: Scatchard, Ružić, Buffle Y function (BYF), Klotz and Hunston (KH), Discrete Binding Model (DBM), Continuous Binding Model (CBM), and the Computational Chemical Equilibrium Model (MINTEQA). All models yielded complexing capacity of HA and the binding constant, K. The models were primarily validated by residual plots. Only CBM gave statistically validated values for two binding sites. The effective molecular weight of HA and the binding constant of metal with protonated ligand HA were determined only by BYF. It is clear from this study that all models except KH and CBM are valid for 1:1 complex within a limited portion of the data, while CBM, a non-linear model, is valid for the entire data, therefore, yields a robust estimate of the parameters and their uncertainties for two binding sites of HA in CuHA complex. The model predictions are further validated by MINTEQA, a chemical reaction model firmly based on the reaction stoichiometry and thermodynamics.

Keywords: Copper complexation/speciation, Humic acid, ISE, multi-model data evaluation, MINTEQA

I. Introduction

The organic complexation and speciation of trace metals like Cu, Cd, and Zn is of great environmental health significance because it controls their bioavailability and toxicity.[1–4] Copper(II) is a critical water quality regulating element, essential for living systems at trace level but toxic at higher concentrations, depending on factors like pH.[5],[6] Humic acids (HAs), on the other hand, constituting about 50% of all dissolved organic matter in natural waters,[7] contain numerous binding sites (e.g., hydroxyl, carboxylic, amino) that form strong complexes with metal ions.[8] These metal-humic complexes could be either soluble, potentially contaminating groundwater, or insoluble, reducing metal bioavailability.[6],[9]

In the past decades, research has been focused on determining physicochemical parameters like the binding constant (K) and ligand concentration (L) for these interactions. However, reported K values show wide variations due to differing methodologies and HA sources.[10] Data is typically collected via electroanalytical methods like stripping voltammetry or ion-specific electrode (ISE) potentiometry,[11] but complex data requires specialized treatment procedures for accurate interpretation.[12] While different methods can yield similar K values within an order of magnitude, calculated ligand concentrations may vary significantly.[13] Studies have also shown that binding constants can decrease with increasing metal-to-ligand ratios,[14] and models like MINTEQA are used to predict speciation.[15] Assuming the formation of ML_1 (metal-first ligand site) and ML_2 (metal-second ligand site) complexes has been found to yield better results in data analysis.[15-17] Here, the ligand (HA) is assumed to have two binding sites (L_1 and L_2) are independent and chemically distinct from each other.

In this study, we investigated Cu^{2+}-HA complexation at pH 5 using Cu-ISE (Ion Selective Electrode) titrations and six data reduction models: Scatchard[18], Ruzic,[19] Buffle Y Function (BYF)[16], Klotz and Hunston (KH)[20, 21] Discrete Binding Model (DBM), Continuous Binding Model (CBM)[16,17], and MINTEQA[22] to calculate the binding constant and complexation capacity. Only the KH model yielded the effective molecular weight of HA and its proton binding constant to Cu-HA. These models were validated by residual plots and other numerical fitting criteria.

II. Experimental

Materials and Methods

Reagents

Humic acid with 20% ash content and Fluka certified molecular weight (600-1000) (Fluka-Aldrich, Germany) was used in this work. Cupric nitrate ($Cu(NO_3)_2.3H_2O$) (E. Merck) with 99.5% purity was used as the standard (in 1% HNO_3) to calibrate Cu-Ion-selective electrode (Cu-ISE). Orion ionic strength adjuster, KNO_3 (ATI-Orion, USA) (Original stock solution. 5 M KNO_3) was used for all potentiometric measurements. All other reagents (HNO_3, NaOH, KNO_3) were of AnalaR grade purity (99.5%).

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Milli-Q 18 MΩ water (Barnstead System, USA) was used for reagent preparation and rinsing of volumetric wares and Cu-ISE. The glassware, titration cell and high-density polyethylene and polypropylene wares were cleaned in acid bath at least 24 hours, first washed with distilled deionized water, then finally with Milli-Q water.

Preparation of Humic acid (HA) solution

A standard solution of Humic acid (HA) (29.5 mg L^{-1}) was prepared in Milli-Q water in a Pyrex flask (250 mL) after filtering through 0.45μM acetate membrane filter (Millipore, USA), with Millipore pressurized filtering setup and stored in dark at room temperature (26^o C). Analytical standards of Cu^{2+} solution were prepared in 0.1M KNO_3 after appropriate dilution of the standard stock solution and preserved at 4^o C in a refrigerator until use.

Methodology and Instrumentation

Cupric ion-selective electrode (Cu-ISE) potentiometry was applied in this study. An Orion digital Ion Analyzer Model 720 A equipped with auto temperature controller (ATC) (Orion, MA, USA), Cu-ISE (Orion Model 94-29), Double junction reference electrode (Ag/AgCl) (3 M KCl) (Orion Model 90-02) and a Ross Combination pH electrode (Orion Model 8102 BNC) were used in all potentiometric measurements. The crystal membrane of Cu-ISE was polished with Al_2O_3 polishing strip (Orion Cat No. 948201) before each titration, rinsed with Milli-Q water and preserved in 0.1M KNO_3 before use. The titration cell (100 mL) was made of Pyrex glass with a plastic cover with holes for electrode entry. A magnetic stirrer with a Teflon coated bar magnet was used to equilibrate the test solutions. During titration the cell was covered with aluminum foil to avoid electrode response to any stray light. The standard solutions and the titrant were dispensed with Eppendorf micropipettes and a Microburette (5 mL). All measurements were done at ambient temperature 30.7 ^{o}C.

Calibration of Cu^{2+} Ion-selective electrode (Cu-ISE)

The Cu-ISE was calibrated in a test solution of 25 mL taken in a 100 mL Pyrex titration cell, at pH 5 and ionic strength I = 0.1M KNO_3. Cu^{2+} standard solution (1mM) was used to calibrate the electrode, covering the range of 0.1– 200 μM. After each addition of the titrant, the solution was equilibrated for 1-2 min and after 1-min stop time, potentials were recorded to an accuracy of ± 0.1 mV. The electrode was calibrated afresh each day for new measurements at the experimental pH. The calibration equation: E (mV) = $(-31.1_3 \pm 0.2)$ *log* $[Cu^{2+}]$ + (304.5 ±0.9), n = 23, r^2 =0.9994, where the standard errors were calculated with 95% CL and random residuals.

Titration of Humic acid (HA) with Cu^{2+}

The methodology of complexometric titration of Humic acids (HA) for chemical speciation by Cu-ISE potentiometry was established in an earlier work from this laboratory.[17] Briefly, all Cu-ISE titrations of HA standard (29.5 mg/L) with Cu^{2+} standard solution (1 mM) were carried out in a test solution of 25 mL taken in a 100 mL Pyrex titration cell, at pH 5 and ionic strength 0.1M KNO_3. Dissolved oxygen and other dissolved gases were removed by purging with 99.987 % pure N_2 while stirring for about 25 min. During titration, positive pressure of N_2 was maintained. Therefore, no ambient CO_2 was present. After each addition of the titrant, the test solution was equilibrated by stirring for about 1-2 min and after 1 min of stop time, potentials were recorded to an accuracy of ± 0.1 mV. During titration, pH was maintained at 5.0 with 0.1M NaOH. The concentration range of the titrant was 2.4–138 μM Cu^{2+}. Free Cu^{2+} concentration was directly measured from the calibration equation. The complexation capacity (CuL or ML) was calculated from the mass balance. The results are shown in Table 1.

Table 1. Calculated values of total, free, and bound Cu^{2+} from the titration of a known concentrations of Humic acid (L) and measured by Cu-ISE. Temp 30.7^{o}C. I = 0.1 M KNO_3, pH = 5.0. The experimental data was used for the computation of binding constants, binding capacity, and other parameters outlined later. Concentrations were calculated with a precision of ± 0.2 μM.

Total $[Cu^{2+}]$, μM	**$[Cu^{2+}]$, μM**	**[CuL], μM**
2.4	0.1	2.3
3.2	0.2	3.0
4.0	0.4	3.6
4.8	0.6	4.2
5.6	0.8	4.7
6.8	0.9	5.8
7.9	1.3	6.7
9.9	1.6	8.3
11.9	2.4	9.5
15.7	4.0	11.8
19.6	5.8	13.9
23.4	6.9	16.5
31.0	10.5	20.5
38.5	14.1	24.3
56.6	25.1	31.5
74.1	33.1	40.9
90.9	43.7	47.3
107.1	52.5	54.6
122.8	61.7	61.1
137.9	70.8	67.1

III. Results and Discussion

In this section we evaluate and critically compare the six models- Scatchard, Ruzic, Buffle Y Function (BYF), Klotz and Hunston (KH) discrete, Continuous Binding Model (CBM), and MINTEQA to calculate and validate the ligand binding constants (K_1 and K_2) and the binding capacities [L]. We consider here binding of a free metal ion, M (Cu^{2+} in this case) with *humic acid (HA) having two independent and chemically distinct binding sites with molar_capacities L_1 and L_2* in aqueous media. The following equilibrium and mass balance equations can be expressed as:

$$M + L_1 \rightleftharpoons ML_1; \qquad K_1 = [ML_1]/([M]\,[L_1]) \qquad (1)$$

$$M + L_2 \rightleftharpoons ML_2; \qquad K_2 = [ML_2]/([M]\,[L_2]) \qquad (2)$$

$$M_t = [M] + [ML_1] + [ML_2] \qquad (3)$$

$$L_t = [L_1] + [L_2] \qquad (4)$$

$$[ML]_t = [ML_1] + [ML_2] \qquad (5)$$

where, M_t, L_t, and $[ML]_t$ are the total concentrations of metal, ligands, and metal-ligand complexes, respectively. Equations 1-5 can be used to find the binding constants K_1 and K_2 and their binding capacities, L_1 and L_2, respectively. Table 2 shows the various models used to find these constants. Majority of the models were used to find K_1 and L_1 from a limited range of experimental data. To extract K and L, we need the total concentration of M_t and the free metal ion concentration, M, where the bound M as ML_t can be found by equations 3 and 5. Experimentally, finding free metal ion concentrations (M^{n+}) was not simple, until the availability of modern ion selective electrodes. This study involves the measurement of free Cu^{2+} in presence of HA over a wide range of total Cu^{2+} ion concentrations. Thus, it is possible to critically test the models in Table 2 for their validity. As shown in Table 2, most of the models consider one ligand site, L_1, as the dominant site. We have tested these models with the data shown in Table 1 and the final results of the parameters are shown in Table 3.

Table 2. Metal binding equations for six models described in the text.

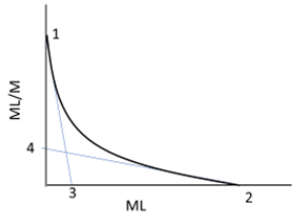
Models	Equations	Scope and limits
Scatchard: Linearized Discrete Ligand Model	$ML_1/M = K_1.L_1 - K_1.ML_1$ $ML_1 = M_t - M$	Linearized graphical method for 1:1 complex: Limiting model. Error inherent in fitting to a non-linear data, equal weights of all data.
Ružić	$M/ML_1 = M/L_1 + 1/(K_1.L_1)$	Linearized and transformed parameters. Model fits a limited data range.
Buffle Y function, BYF	$(L_t/M_t)(\alpha/(\alpha-1)) = (Mw/K_1)(1+(H^{+x}/\beta_1)(\alpha/M_t))$ $\alpha = M_t/M$ $\beta_1 = ML_1.Y\,H^{+x}/(M.L_1H_x^{+})$	Linear 1:1-Limiting model. Model allows estimation of molecular weight, M_w, and β_1 of protonated L_1 or $L_1.H_x$.
Klotz and Hunston (KH) - Discrete, non-linear, L_1 only	Refer to Figure on the right Intercept 1= $K_1.L_1$ Intercept 2= L_1 Intercept 3= $(K_1.L_1)^2/(K_1^2.L_1)$ Intercept 4= $L_1^2/(L_1/K_1)$	ML_1 complex, limiting model reduces to Scatchard 
KH-Discrete Non-linear L_1 and L_2	Intercept 1= $K_1.L_1 + K_2.L_2$ Intercept 2= L_1+L_2 Intercept 3= $(K_1.L_1+K_2.L_2)^2/(K_1^2.L_1+K_2.L_2)$ Intercept 4= $(L_1+L_2)^2/(L_1/K_1+L_2/K_2)$	ML_1 and ML_2 complexes due to the presence of two chemically independent sites, L_1 and L_2
Continuous Binding	$[ML]_t = (L_1.K_1.[M])/(1+K_1.[M]) + (L_2.K_2.[M])/(1+K_2.[M]) + ..$	Non-linear. Uses all data and able to estimate several independent binding equilibria.

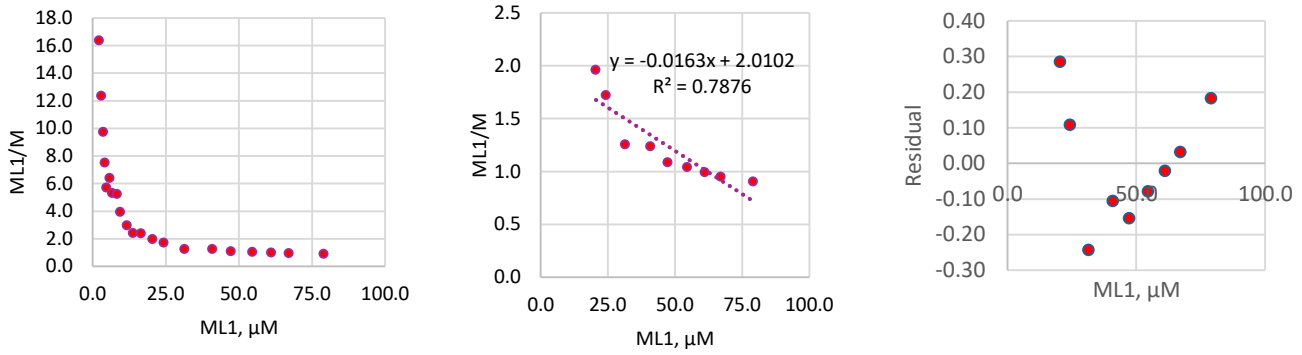
Table 3. Values of K, L, and other parameters form binding models in Table 2.

Models	Binding Constants, K_1, K_2, M^{-1}	Binding Capacity L_1, L_2, µM	Correlation Coefficient, R^2	Comments
Scatchard (limited data) Linear fit, ([Cu^{2+}] >10 µM),	$K_1 = (1.6_3 \pm 0.6) \times 10^4$	123.3 ± 20	0.787	NL, NRR, poor fit
Scatchard (all data) MCMC, non-linear fit	$K_1 = (2.3 \pm 0.01) \times 10^5$	$72.82 \pm 1._6$	0.887	NL, NRR, poor fit
Scatchard ([Cu^{2+}] >10 µM), limited, MCMC, non-linear fit	$K_1 = (1.6 \pm 1.) \times 10^6$	$98.2 \pm 3._8$	0.981	L, NRR
Ružić ([Cu^{2+}] >10 µM), linear fit, limited	$K_1 = (4.1 \pm 1.) \times 10^7$	2.34 ± 0.5	0.924	L, NRR
Ružić (all data) MCMC, non-linear fit	$K_1 = (1.3 \pm 0.1_3) \times 10^4$	$107.5 \pm 4._3$	0.992	NL, NRR
Ružić ([Cu^{2+}] >10 µM), MCMC, non-linear fit, limited	$K_1 = (1.4 \pm 0.2) \times 10^6$	104.7 ± 7.	0.988	L, NRR
Buffle Y Function (BYF) Initial slope, limited data,	$K_1 = (5.45 \pm 0.2) \times 10^6$ MW= 534 g/mol x= 0.5 (power of H+) β_1= 49.4 /µM	229.4 ± 5	0.9939	L, R
Discrete Binding Model (DBM) Linearized one site, limited	$K_1 = (1.0 \pm 0.2) \times 10^5$	229.7 ± 10	0.9879	NRR
Discrete Binding Model (DBM) Non-linear, two sites, limited data	$K_1 = (8.0 \pm 0.3) \times 10^4$	L_1: 229.6 ± 0.3	0.9824	RR
	$K_2 = (3. \pm 0.02) \times 10^6$	L_2: 2.02 ± 0.04	0.989	RR
Continuous Binding Model (CBM)- One site, hyperbolic *linearly transformed*	$K_1 = (1.3 \pm 0.1) \times 10^6$	75.1 ± 2.	0.9993	NL, NRR
Continuous Binding Model (CBM)- One site, hyperbolic *non-linear fitting*	$K_1 = (2.95 \pm 0.4) \times 10^3$	232.8 ± 6.	0.9994	NL, NRR
Continuous Binding Model (CBM)- Non-linear, two sites, all data, SOLVER non-linear fit	$K_1 = (3.0 \pm 0.2) \times 10^3$	L_1: 298.1 ± 2	0.9992	R, best fit
	$K_2 = (9.3 \pm 0.4) \times 10^5$	L_2: 10.7 ± 0.3		

Notes: L: linear or linearized, NL: Non-linear, R: Random residual, NRR: Non-random residual.
Residual = Model predicted value – Experimental value

Scatchard model

The Scatchard Model assumes a simple system with only one type of binding site. The analysis involves a linear fit of $[ML_1]/[M]$ versus $[ML_1]$. As seen in the Figure 1 below, the data points clearly form a curve, not a straight line. Forcing a linear fit onto this curved data (R^2=0.7876) is inappropriate and produces unreliable parameters. The inadequacy of the Scatchard Model is confirmed by its residual plot. This non-random (V-shaped) pattern is clear evidence that the linear model is a poor fit. Therefore, a non-linear fit by using the Markov Chain Monte Carlo (MCMC) method was used to fit the data.[23] MCMC is a computational technique used to determine the probability distribution of model parameters. Instead of just finding a single "best-fit" line, it explores all plausible lines that could fit the data. Even with this data reduction technique, the residual plot (Figure 1c) is not random for a robust estimate of the parameters and their uncertainties.

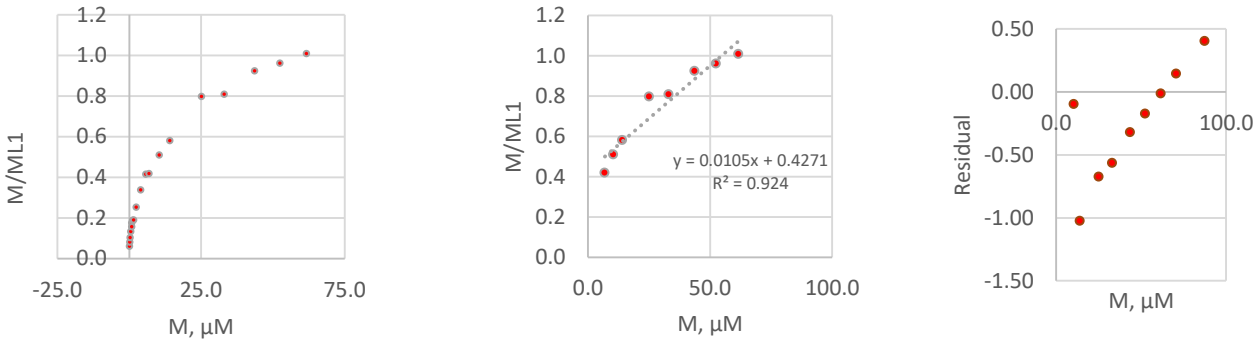


(a) (b) (c)

Fig. 1. (a) Data plot using Scatchard equation, (b) Linear section to obtain K_1 and L_1, (c) Non-random residual plot with a V-shape.

Ružić model

Like Scatchard, the Ružić Model is a graphical method that use linearized equations to determine a system's complexing capacity (ML_1) and binding constant (K_1) from experimental data. It assumes a continuous distribution of binding affinities and linearizes the data using the equation shown in Table 2. Here, a plot of M/ML_1 vs. M yields a slope $1/L_1$ and an intercept $1/(K_1. L_1)$ with $R^2 = 0.924$. The Ružić Model, like the Scatchard Model is also non-linear for the experimental data even with the limited data when M>10 µM. The poor data fitting is also characterized by the non-random residual plot (Figure 2c). With MCMC non-linear data fitting procedure, the results are comparable to that of Scatchard, but yielded a non-random residual plot (not shown). These results indicate that the system is non-linear, and may have more than a single binding site. Also, the data weighting factors are uncertain in linearized and transformed equations. Most importantly, Table 3 also shows that data fitting technique can alter the parameter values significantly.



(a) (b) (c)

Fig. 2. (a) Non-linear Ružić plot, (b) Limited linear section (M=10-70 µM) to obtain K_1 and L_1, (c) Non-random residual plot with a straight-line shape.

Buffle Y Function (BYF)

Another model equation based on the Buffle Y Function (BYF) is the left-hand side of equation 6 written as:

$$(L_t /M_t)(\alpha/ (\alpha -1)) = (Mw/K_1) (1+ (H^{+x} / \beta_1) (\alpha/M_t)) \quad (6)$$

$$\alpha = M_t/M \quad (7)$$

$$\beta_1 = \quad ML_1. H^{+x} / (M.L_1H^{+}_x) \quad (8)$$

where, L_t is the total concentration of ligand L_1 in µM, Mw is the average molecular weight of the ligand in g/mol, H^{+x} is the proton concentration with an exponent x, and β_1 is the binding constant of metal with protonated ligand. Other parameters are described earlier. Equation 6 is valid only in the initial portion of the curve in Figure 3 (a). Figure 3(b) shows the fit of equation 6 and the residual plot is shown in Figure 3(c). The best fit parameters are listed in Table 3 with excellent R^2 0.9939. The BYF shows random residual for a limited data fit. BYF is the only model that yielded the molecular weight of the ligand, Mw 534 g/mol, which is within the range 400-1000 g/mol given by the manufacturer. The value β_1= 4.9 x 10^5 M^{-1} and log (β_1/H^+) = 6.7 indicates the monoprotic (x=1) HA has a moderately strong H^+ affinity at pH 5. These later results are in fair agreement with that of the Buffle's study of Cu^{2+} - HA system.[16]

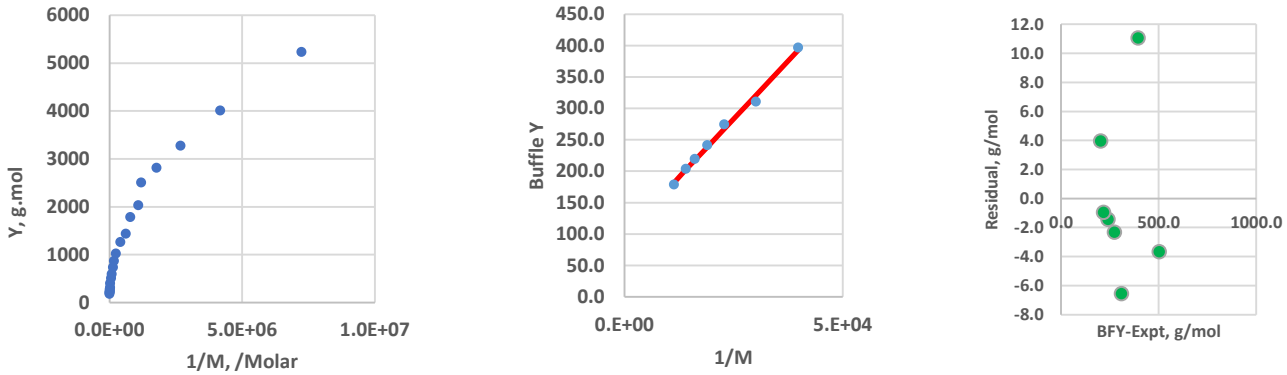


Fig. 3. (a) Plot of Buffle Y function, (b) Limited linear section (M=10-90 µM) to obtain K_1 and L_1, (c) Random residual plot

Klotz and Hunston (KH) discrete Model

The KH model is a graphical model based on equations shown in Table 2. This is a general discrete model which can be extended to i^{th} ligand. Here, the intercepts of limited initial and final data for a plot of ML/M vs. ML are related to four equations for the parameters K_1, L_1, K_2 and L_2. It shows that intercept 2 is equal to L_1 and K_1 is related to intercept 1. For two sites, four simultaneous equations have to be solved. The final results for one and two site models are shown in Table 3. The consistency of the model is proven by fair agreements of K_1 and L_1 from the two models. The results show that K_2 is an order of magnitude higher than K_1, and more than 99% of binding sites are L_1. This is indicative of high-affinity L_2 sites (phenolic type) saturate at low metal concentrations and low-affinity L_1 sites dominate at high concentrations. Thermodynamically, weaker and non-specific binding sites (-COOH type) in humic substances provide high capacity enabling buffering, detoxification, and metal transport over a wide concentration range.[24] Strong binding (Cu^{2+} to phenolic sites) is enthalpically driven ($\Delta H < 0$), while weak binding (to carboxylates) has smaller ΔH but less negative ΔS, therefore, entropically favored for multiple binding sites.[25] It shows values for strong sites: log K_2 ~ 6–9, L_2 ~ 0.5–1.5 mmol/g, and weak sites: log K_1 ~ 3–5, L_1 ~ 4–6 mmol/g. Our values are in the range of lower limits (log K 4.9 -6.5).

Continuous Binding Model (CBM)

CBM can be used to extract equilibrium parameters from multiple independent binding sites and uses all the experimental data for M and ML_t. The CBM equation is shown in Table 2. For one site, L_1, the CBM can be transformed to a hyperbolic equation, which can be linearized or a direct non-linear fitting can be used. For two sites, L_1 and L_2, optimized parameter values for K_1, K_2, L_1, and L_2 can be obtained by using MS Excel SOLVER. In SOLVER the sum of the squares of the residuals (SSQR) and the standard deviation, Σ_i $([ML]_{t,expt} - [ML]_{t,calc})^2$ / $n)^{1/2}$, are minimized until the values of the two parameters, $K_i.L_i$ and K_i (i= 1 or 2), are optimized. Table 3 shows the best fit values. It shows that K_1 and L_1 values obtained with two methods are different, although the fitting R^2 exceed 0.999 for both. Figure 4 shows the two fitting results and the residual plot for the ML vs. M fitting. The residuals are non-random with a clear pattern. The overriding conclusion from this exercise is that linearization of a non-linear equation does not guarantee reliable parameter values when the residual has a non-random shape.

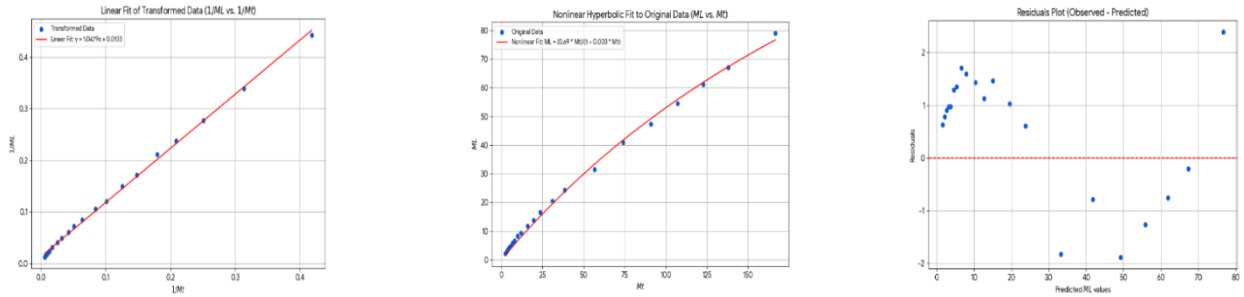
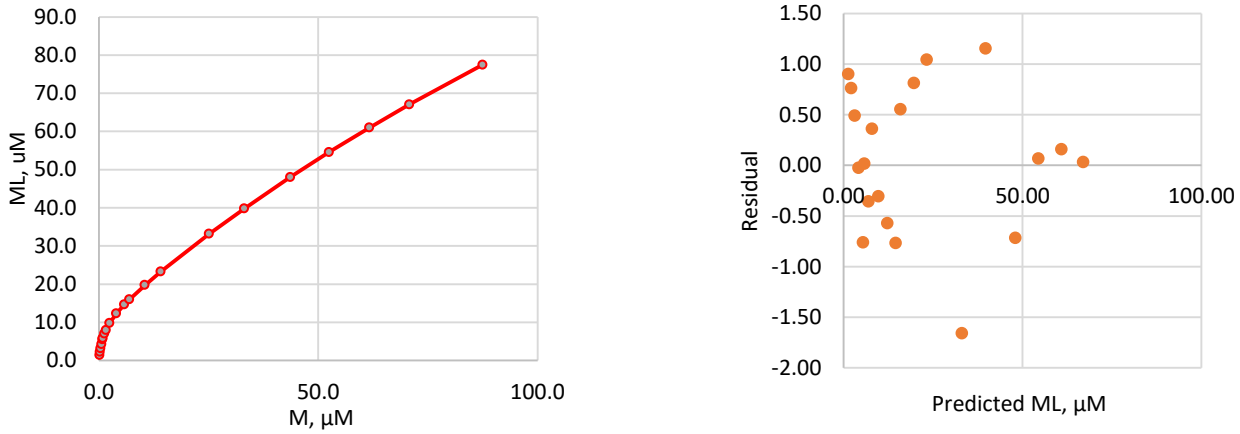


Fig. 4. (a) Fit of the linearized hyperbolic equation, 1/ML vs. 1/M, (b) CBM: ML vs. M fitted to one site hyperbolic equation with non-linear SOLVER, (c) Residual plot of data in b shows non-random shape.

Therefore, a two site CBM should be considered. Table 3 shows the best values for K_1, K_2, L_1, and L_2 obtained with SOLVER for the two site CBM. Figure 5 shows the fitted data and the random residual confirming the validity of this model without a systemic bias. Results in Table 3 show that 96.5% of the sites have lower binding constant compared to that of 3.5% with a higher binding constant.



(a) (b)

Fig. 5. (a) Fit of two site CBM. Dots are experimental and line is the SOLVER fitted data, (b) Residual plot shows random distribution.

The KH-DBM and CBM yields K_1, K_2, L_1, and L_2. However, the results are significantly different in K_1 and K_2 values by nearly an order of magnitude, while the values for L_1 and L_2 are comparable. While binding parameters obtained from KH-DBM used only the intercept values, which are sensitive to extrapolation of initial and terminal data points, the CBM model used all data points, and, therefore, inherently more accurate.

MINTEQA Model

Computational speciation by Minimization of Total Equilibrium Activity (MINTEQA) is based on known reactions of the metal ions, M (Cu^{2+}) in solution in presence of all other ligands (HA) and ions, ionic strength, pH, and temperature. Unlike other models, MINTEQA is entirely based on reaction stoichiometry and a thermodynamic data base for the reactions. Here, we used the U.S. EPA model MINTEQA2. The open source program, Visual MINTEQA 3.1, and extensive documentation can be found elsewhere.[26] The total ion (or component) concentrations in MINTEQA is based on the experimental values. The input DOM (dissolved HA) is 309 µM, which is the sum of L_1 and L_2 obtained from CBM, and its MW 534 g/mol is obtained from BYF model to have about 50% organic carbon as mentioned by the manufacturer of HA.

Table 4 shows that 85.4% of total Cu^{2+} is bound to DOM and 13% Cu^{2+} is free. Only 2.4 % of the total DOM is bound to Cu^{2+}. The later increases with the increase of total Cu^{2+} or M_t. To simulate the experimental data, we ran MINTEQA with different M_t, keeping other components fixed to find the distribution of M and ML. Figure 6a shows the experimental ML (Cu(DOM)) vs. M_t (Cu^{2+}-total) and those calculated by MINTEQA and CBM. The species distribution is shown in Figure 6b. The agreement among experimental ML, MINTEQA and CBM calculated ML are excellent over 80% of the data at lower M_t. The species distribution shows that the system is non-linear with two approximately linear portions in the low and high total Cu^{2+} concentrations as indicated by all models with one ligand site.

Table 4. Distribution of species from components obtained from MINTEQA calculation. Input: Total [Cu^{2+}] = 2.4 µM, DOM = 308.8 µM, [K^+] = 0.1 M, [NO_3^-] =0.1 M, pH =5, Temp =30.7 ^oC, and free from dissolved CO_2(g).

Component	% of total concentration	Species name
DOM (L_1+L_2)	76.3	DOM
	21.3	H DOM
	2.4	Cu DOM
Cu^{2+}	13.1	Cu^{2+}
	0.011	$Cu(NO_3)_2$ (aq)
	85.4	Cu DOM
	0.026	$CuOH^+$
	1.43	$CuNO_3^+$
K^+	96.7	K^+
	3.3	KNO_3 (aq)
NO_3^{-1}	96.7	NO_3^{-1}
	3.3	KNO_3 (aq)

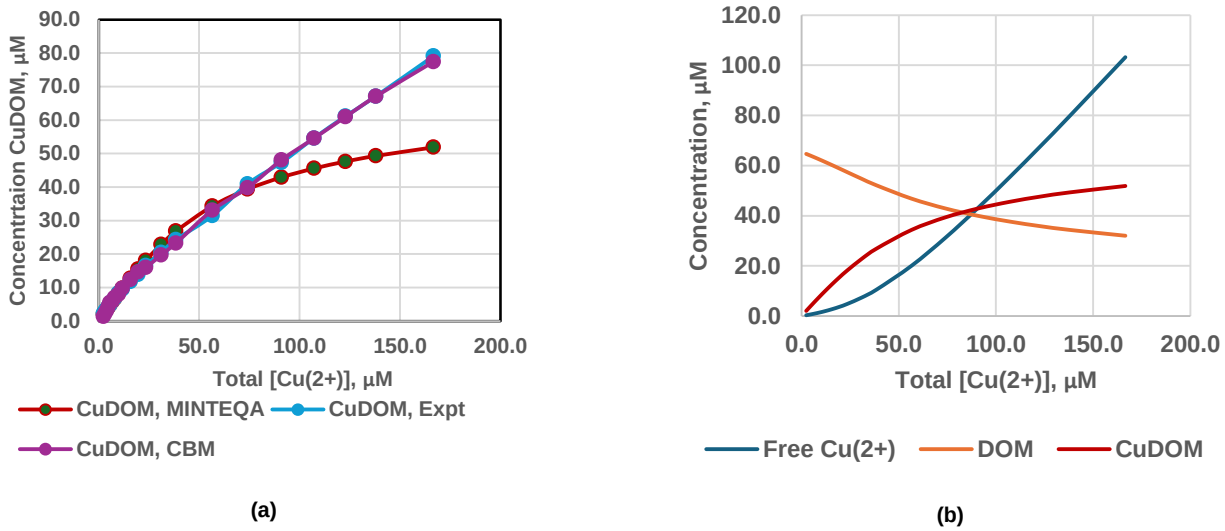


Fig. 6. (a) Comparison of experimental ML (or Cu(DOM)) vs. M_t (Cu^{2+}-total) and those calculated by MINTEQA and CBM Models. (b) Species distribution vs. M_t (Cu^{2+}-total).

IV. Conclusions

Rigorous validation of physicochemical models is essential for accurately describing the complex interactions between free and bound metals in the presence of natural dissolved organic matter (or DOM) like humic and fulvic acids. While high correlation coefficients (R^2 >0.99) are often cited as proof of a good fit, this research confirms that such metrics can be misleading. The ultimate criterion of an unbiased model and its validity is the randomness of its pattern-free residual plot, which serves as the only true diagnostic for experimental data.

Historically, linearized models such as the Scatchard, Ružić and other discrete binding models were instrumental, yet they are inherently limited to the linear section of the data (<20% data). As demonstrated in this work, these models frequently fail the critical residual test over a broad range of concentrations, typically producing distinct, non-random patterns. This systematic deviation reveals that their underlying assumptions—such as a single class of binding sites or a simplified affinity distribution—are insufficient to capture the multiple binding sites of DOM. While these models may appear to fit a limited portion of the data, they provide an incomplete and potentially biased understanding of the system's overall binding capacity and affinity. A simple one site, two-parameter linear model is fundamentally inadequate for describing a complex system with two distinct and chemically independent binding sites.

With the advent of modern computational techniques, solving complex, multiparameter non-linear models is not only feasible but necessary. This research shows that a non-linear continuous binding model (CBM) successfully overcomes the limitations of its predecessors. The CBM provides an excellent fit to the *entire* dataset, and most importantly, it yields a randomly distributed residual plot, confirming its statistical validity and lack of systemic bias. The model predictions are further validated by MINTEQA, a chemical reaction model firmly based on the reaction stoichiometry and thermodynamics.

The significance of this finding extends beyond superior statistical performance. The parameters obtained from the CBM are physically and chemically meaningful, offering a more profound insight into the binding mechanism. This detailed characterization is crucial for accurately predicting the speciation, bioavailability, and environmental fate of heavy metals, thereby providing a more robust foundation for ecological risk assessment and environmental remediation strategies.

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