

CHARACTERIZATION OF THE ACID-BASE PROPERTIES OF SELECTED HUMIC
SUBSTANCES AND MODEL COMPOUNDS

by

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Characterization of the Acid-Base Properties of Selected Humic Substances and Model
Compounds

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at George Mason University

by

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DEDICATION

To my loving husband Ed, my two wonderful children Matthew and Max, and my family in Korea, especially, my mom. At the age 62, she went back to middle school where she was forced to drop out due to financial difficulty and has shown me a great example not to give up on learning and continued education.

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LIST OF ABBREVIATIONS AND SYMBOLS

Acid Concentrations at the ith Addition of Titrant	C_{ai}
Activity Coefficient	γ
Aldrich Humic Acid.....	AHA
Apparent Acidity Constant for the jth Site	K_{ja}^{app}
Base Concentrations at the ith Addition of Titrant	C_{bi}
Black Chernozemic Soil Humic Acid.....	CHA
Change in Enthalpy.....	ΔH
Change in Entropy	ΔS
Change in Gibbs Free Energy	ΔG
Charge of the Ion	z
Contaminants of Emerging Concern.....	CECs
Deionized Distilled Water.....	DDW
Equilibrium Constant, Acidity Constant or Acid Dissociation Constant	K_a
European Union	EU
Fully Optimized Continuous pK_a Spectral Model	FOCUS
Fulvic Acid.....	FA
Gas Constant	R
Humic Acid.....	HA
Humic Substance	HS
International Humic Substances Society	IHSS
Ion Size Parameter	α
Ionic Strength (M, mol/L)	μ
Measure of Acid Strength (= $-\log K_a$)	pK_a
Nonideal Competitive Adsorption Donnan Model	NICA-Donnan model
Number of Binding Sites	m
Offset Term.....	S_0
Pharmaceuticals and Personal Care Products	PPCPs
Podzolic Soil Fulvic Acid	PFA
Purified Aldrich Humic Acid.....	PHA
Site Density in M (mol/L) of Sorbent	$[B_j]$
Stockholm Humic Model.....	SHM
Surface Charge Excess.....	Q
Temperature in Kelvin	T
The (Dimensionless) Dielectric Constant of Water	ϵ
United States Environmental Protection Agency	US EPA
Upper Mississippi River Nom	MIS
Water Autoprotolysis Constant or Water Autoionization Constant	K_w

ABSTRACT

CHARACTERIZATION OF THE ACID-BASE PROPERTIES OF SELECTED HUMIC SUBSTANCES AND MODEL COMPOUNDS

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George Mason University, 2019

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Natural organic matter (NOM) such as humic substances (HS) found in soil, sediment and water have diverse composition and molecular structural complexity. HS has significant influence on the heavy metals in terrestrial and aquatic environments due to its ability to bind the metal ions. Among various functional groups on the surface of HS, carboxylic acids and phenols are the most important ionizable sites present in HS. Determination of the surface density of acidic functional groups and the magnitude of pK_a of carboxylic and phenolic functional groups is a first step in characterizing acidity, ion exchange capacity, and charge accumulation properties in humic substances (HS). Potentiometric titration was used to determine the fundamental acidic properties of natural HS and selected model HS to better assess the role structure plays in carboxyl and phenol group dissociation. Acid-base titration data from the HSs were fitted to the fully optimized continuous pK_a spectral (FOCUS) model to determine the surface charge

excess, K_a and pK_a values of carboxylic and phenolic group on each selected HS. FOCUS model is an empirical model and provides the mean pK_a value determined from a Gaussian model for the peak shapes. Each HS was assumed as a mixture of monoprotic acids in the model. In this study, the pK_a values found to be in the range of 3.890 ± 0.018 to 4.881 ± 0.036 for carboxylic group and 8.712 ± 0.017 to 9.738 ± 0.071 for phenolic group. These results are in good agreement with literatures.

In order to evaluate the influence of structural variations in carboxylic and phenolic group dissociation, simple benzofuran (C_8H_6O) derivatives, a common structural motif in HS were titrated as well. In this work, it was observed that pK_a value increases as the carboxylic group is located close to the oxygen on furan ring. Temperature effect on carboxylic group on a model compound was also observed that pK_a value went up as the temperature increased.

INTRODUCTION

Humic Substances (HS) are natural macromolecular organic acids derived from the diagenesis of living matter representing a major component of the earth's carbon cycle in terrestrial and aquatic environments. HS are also known to be one of the promising trends of green chemistry as the renewable, economically advantageous and ecologically safe sources of raw materials for obtaining chemical products (Janoš et al., 2008). HS representing the bulk of organic matter (OM) in soils and sediment particles in the environment are divided into three broad categories, including humin, humic acid (HA) and fluvic acid (FA). A typical scheme for distinguishing humic fractions is illustrated in Fig. 1. OM is defined as a grouping of carbon containing compounds which have originated from living beings and deposited on or within the earth's structural components (Pettit, 2004). Humin derived from biopolymers (lignin and cellulose) and polyphenol condensation are found in soil that is insoluble in water and considered macro organic substances because their molecular weights (MW) range from approximately 100,000 to 10,000,000 (Pettit, 2004). Humic acids (HAs) consist of that fraction of HS that are precipitated from aqueous solution when the pH is decreased below 2. HAs comprise a mixture of weak aliphatic (carbon chains) and aromatic (carbon rings) organic acids which are not soluble in water under acid conditions but are soluble in water under alkaline conditions. The molecular size of HAs range from approximately 10,000 to

100,000, which is smaller than humin and larger than fulvic acids (Pettit, 2004). Fulvic acids (FAs), which molecular weight range from approximately 1,000 to 10,000, are a mixture of weak aliphatic and aromatic organic acids that are, unlike humin and HA, soluble in water at all pH conditions (acidic, neutral and alkaline). FAs have many carboxylic and phenolic groups. This makes FAs much more chemically reactive with high ion exchange capacities derived from a larger number of carboxylic groups present relative to other HS (Pettit, 2004). FAs generally end up in surface water the most among due to their small size and their solubility thus corresponds to 80-90% of the aquatic HSs. FAs are the most effective carbon containing chelating compounds known due to their small size and their solubility thus corresponds to 80-90% of the aquatic humic substances (AHA).

Aquatic humic substances (AHA) are often termed dissolved organic matter (DOM). Thus HA and FA are considered part of the DOM. DOM in water is usually expressed by concentration of reduced carbon, dissolved organic carbon (DOC). DOC is a measurement of the amount of organic carbon in water that can be passed through a filter, commonly 0.45 μm in nominal pore diameter. The concentration of organic matter in the colloids (expressed as C) remaining with the bulk water (kg OC/L).

The acidic functional group contents and proton binding affinities of HS significantly affect the acid-base buffering capacity of soils and have a marked influence on the complexation and speciation of cations in soil solid and liquid phases. Thus, HS can control the biological availability, physicochemical behavior, and environmental fate of macro- and micronutrients, toxic metal ions, and xenobiotic organic cations (e.g.,

pesticides such as paraquat, diquat, and s-triazines) (Plaza et al., 2005b). Among various functional groups, carboxylic acids and phenols are the most important ionizable sites present in HS and are likely to determine many of the properties of proton and metal ion binding exerted by these materials in natural waters, soils and sediments (Masini et al., 1998). The key to understand cation binding to HAs, for example Ca^{2+} , rests in the ability to quantify the acid dissociation constants of the acidic groups and their surface coverage (e.g., equivalents per gram).

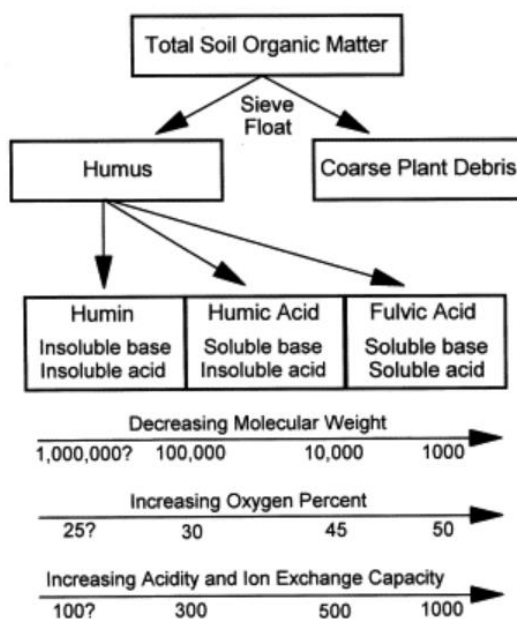


Figure 1. Chemical fractions of soil organic matter with typical compositional characteristics of the humic fractions (after Oades, 1989)

Conceptual 2D and 3D structures of HS are shown in Figs. 2 and 3, respectively. The major groups observed in Fig 2 are carboxyl groups, phenol groups, aldehyde groups

and amine groups. Ionized carboxyl groups (acidic group), which predominate in surface waters, profoundly increases water solubility. The pK_a for carboxyl group is approximately between 4 and 6. Phenol groups (acidic group), which have a pK_a close to 9, increase the alcohol K_a by delocalizing charge through the aromatic ring, this making the -OH group more acidic. Aldehyde groups (neutral group) also contribute to increasing water solubility. Amine groups (basic group) are proton acceptors and form cations in neutral water. Amine groups greatly increase water solubility as well by forming hydrogen bonds.

It is thought that HA structures have micelle like structure referred to as pseudomicelles. Like a micelle, HA has a hydrophobic interior and a more hydrophilic surface, giving it distinct solubilizing powers for nonpolar solutes (Fig. 3). HAs are also known to be excellent ligands for metal ions such as magnesium in solution serving as metal storage and transport (von Wandruszka, 2000). HS are known to be a dispersed solute in aqueous environment. Dispersed solute is not completely dissolved, becoming invisible and distributed evenly throughout the surrounding fluid like sugar. A dispersed solute or substance forms heterogenous mixture called a colloid in which a dispersed (solute-like) substance is distributed throughout a dispersing (solvent-like) substance. The particles are larger than simple molecules but too small to settle out (Silberberg, 2012).

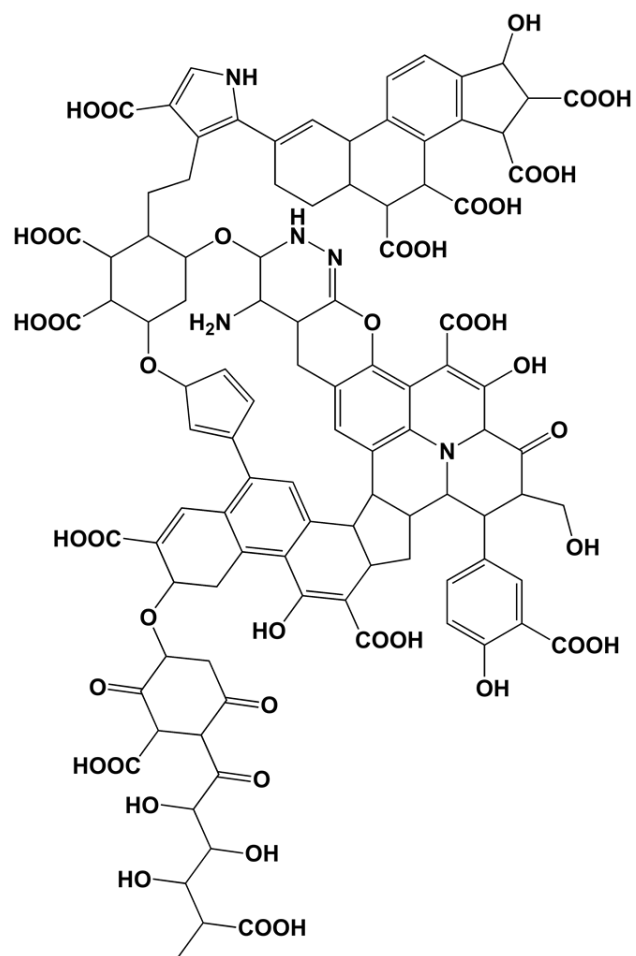


Figure 2. Empirical humic acid structure

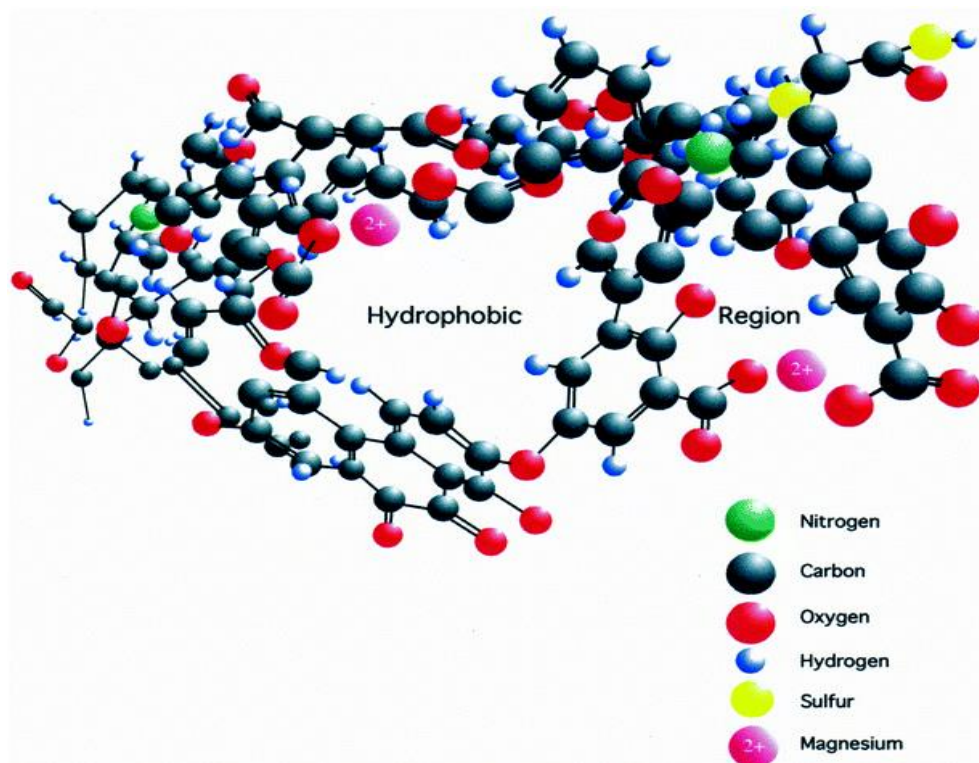


Figure 3. Portion of the proposed "type" structure of humic acid in 3D (Wandruszka, 2000)

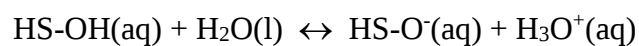
Typical HS are dissociated in water as shown in the equilibrium equations described below.

Carboxylic acid dissociation



$$K_a (\text{carboxylic}) = (\{\text{HS-COO}^-\} \{\text{H}_3\text{O}^+\}) / \{\text{HS-COOH}\}$$

Phenolic acid dissociation



$$K_a (\text{phenolic}) = (\{\text{HS-O}^-\} \{\text{H}_3\text{O}^+\}) / \{\text{HS-OH}\}$$

The dissociation constant K_a and, thus, pK_a ($-\log K_a$) values of HS can be determined using potentiometric titration.

Potentiometric titration is one method of analysis in which the endpoint of the titration is monitored with an indicator electrode that records the change of the potential as a function of the amount (usually the volume) of the added titrant of exactly known concentration. Potentiometric titrations are especially versatile because indicator electrodes suitable for the study of almost every chemical reaction used in titrimetry are readily available (Hulanicki et al., 2013).

Acid–base potentiometric titration is a method of studying proton equilibria and determining dissociation constants (Khil’ko et al., 2011) in which pH is monitored continuously as increments of acid or base are added, combined with thermodynamic binding models to describe the shapes of the titration curves can provide reliable, detailed, and valuable information about the acid-base properties of HA in terms of site density and proton-binding affinity. The dissociation constants of the acid groups of HS are important for describing reactivity, flocculation behavior, physicochemical properties in dispersed systems, and in evaluating their binding interactions with aqueous cations in solution. To study acid-base properties, ionic dissociation constants were determined empirically. Experimentally, pH values were determined by potentiometric titration and the obtained titration data was fitted into non-linear method to calculate the surface charge excess, yielding K_a and pK_a .

In particular, the recently developed nonideal competitive adsorption (NICA)-Donnan model can successfully account for the extremely heterogeneous mixture of

acidic functional groups involved in proton binding and the various ionic strength effects arising from the polyelectrolytic nature (Plaza et al., 2005b). The work of Vidali et al. (2009) experimentally demonstrated the basis of calculations with the aid of the Donnan model that the degree of proton binding depends on the HS concentration, predicting that the lower the concentrations of HS in suspension will yield a higher degree of proton binding (Khil'ko et al., 2011) as is seen with acids in solution.

Another traditional method is also available that determines pK_a value of HS called Ca-acetate and $Ba(OH)_2$ exchange method is used to determine carboxylic group acidity and phenolic group acidity respectively. Using this method, the concentration of phenolic groups can be calculated as the difference between total and carboxylic acidity (Stevenson, 1982).

Study Design and Objectives

The K_a and pK_a ($-\log K_a$) constants are commonly referred to as acidity constants or acid dissociation constants for the dominant titratable acidic functional groups in selected humic substances. In the titration of dispersed HA with aqueous NaOH (KOH was used for this research), equivalence points were determined from the values of pH at the inflection of carboxylic (pH ~ 8.0) and phenolic (pH ~ 10.5) titrations. An example of the profile of humic acid (HA) titrations is illustrated in Fig. 4.

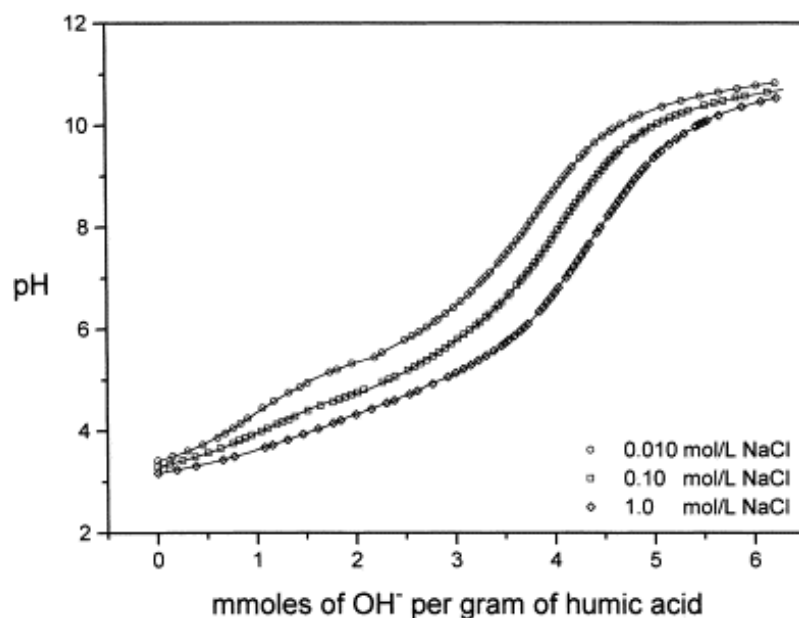


Figure 4. Experimental (----) and calculated by nonlinear regression titration curves of 50.00 ml of 0.685 g/L of Aldrich humic acid suspension with 0.1 mol/L NaOH in ionic medium of 0.010, 0.10, and 1.0 mol/L NaCl (Masini et al., 1998).

The study objective was to characterize the acid dissociation properties of humic substances and relate the acidic properties to chemical structure. A total four HS were

evaluated, including purified Aldrich humic acid (PHA), Podzolic soil fulvic acid (PFA), Black Chernozemic soil humic acid (CHA) and upper Mississippi River NOM (MIS), were titrated between pH 3.0 and pH 10.5 and evaluated for predominant acid dissociation constants (K_a 's) of carboxyl and phenolic acids present. Determination of the charge excess using collected raw titration data was fitted to a non-linear method for fitting (Fully Optimized Continuous - FOCUS pK_a Spectrum-Model) to determine the surface charge excess, K_a and pK_a values using "Solver" in Excel. The surface density of acidic functional groups represented as surface charge excess and the magnitude of pK_a 's of carboxylic and phenolic groups is a first step in characterizing acidity, ion exchange capacity, and charge accumulation properties in humic substances. In this research, potentiometric titration was used to determine the fundamental acidic properties of natural humic substances and model humic compounds selected to determine the role chemical structure plays in carboxyl and phenol dissociation. The model compounds were benzofuran derivatives that are structural motifs (Fig. 5 and Table. 2) common in HS.

Model Compounds

The use of model compounds aids in the interpretation of HS acid dissociation because they are simpler molecules and can be selectively purchased as needed. The K_a values of the model compounds were evaluated accurately and precisely, thus useful to determine the effect of structural positions of the acidic groups in dissociation. The model compounds, carboxylic and phenolic benzofuran derivatives were selected to address the following features of chemical structure:

- Proximity of carboxyl or phenol group to the benzofuran ring oxygen,
- Proximity of multiple carboxyl groups to each other,
- Proximity of carboxyl groups to phenolic groups,
- Influence of phenyl versus furan ring on carboxyl dissociation, and
- Influence of temperature changes on functional groups on Benzofuran ring

It was not expected that the model compounds would predict the K_a 's of the natural humic substances, rather they yield insight on how the position and electronic environment of the carboxyl and phenol groups effect the magnitude of dissociation constants in humic substances, such that this can be related to the highly variable structure of humic acids.

Benzofuran is composed with O-heterocyclic and acyl (especially carboxylic) functional groups that are common structural elements of HS. The Benzofuran model compounds (Fig. 5) provide a detail evaluation of this acidic properties of this particular motif in HS. Benzofuran is considered as an important class of heterocyclic compounds and is present in numerous pharmaceuticals (Yeung, 2012). Many of the clinically

approved drugs are synthetic and naturally occurring substituted benzofuran derivatives containing mono and fused benzofuran ring in conjunction with other heterocycles (Nevagi et al., 2015). Due to benzofuran's structural features common in natural humic substances as well as its medicinal importance from researchers and scientists, benzofuran derivatives were used for this search.

Numerous publications have been reported on benzofuran derivatives to have antimicrobial, enzyme inhibition, enzyme activation, receptor agonist and antagonist, anti-inflammatory, anticancer, antiviral, antitubercular, antioxidant, anticonvulsant, anti-alzheimer, complement system inhibitors, anti-ulcerogenic, ischemic cell death inhibitors and dopamine uptake inhibitor activities (Nevagi et al., 2015). A study on a Psychiatric Drug, benzodiazepine anxiolytic drug (oxazepam) reported that Environmental pollution effluents and remain biochemically active in aquatic systems (Brodin et al., 2013). US EPA also monitors contaminants of emerging concern (CECs), including pharmaceuticals and personal care products (PPCPs), that are increasingly being detected at low levels in surface water, and there is concern that these compounds may have an impact on aquatic life ("Contaminants of Emerging Concern including Pharmaceuticals and Personal Care Products," n.d.). European Union (EU) has added three CECs to the watch list along with certain pharmaceutically-active compounds (Tiedeken et al., 2017).

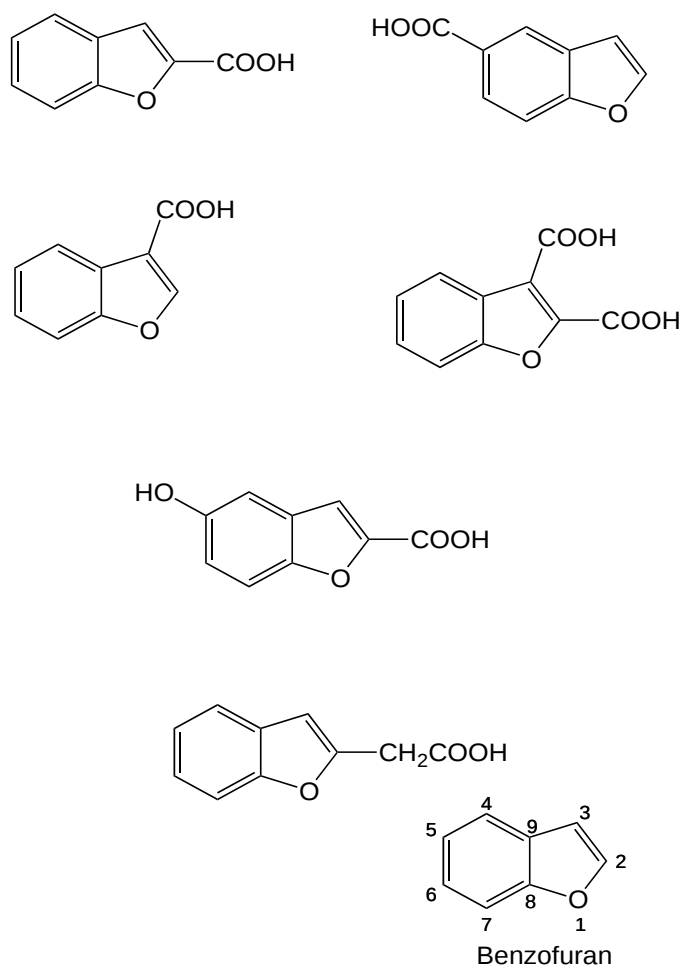


Figure 5. Examples of Benzofuran model compounds - selected carboxylic and phenolic benzofuran derivatives

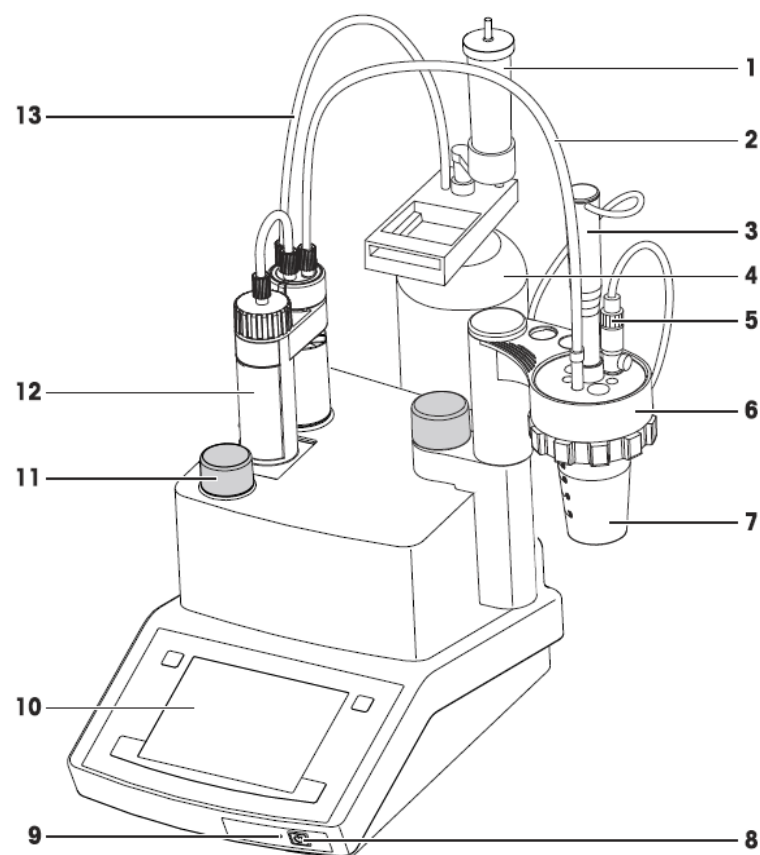
MATERIALS AND METHODS

Apparatus and reagents

Fully automated potentiometric titrations were carried out using a Mettler Toledo Compact Titrator G10S equipped with a pH electrode Mettler DG-115-SC (Mettler-Toledo, Columbus, Ohio) that was calibrated with standard buffers at pH 4.00, 7.00, and 9.22. A 1-L digital general-purpose water bath manufactured by Polyscience (Niles, Illinois) was used to maintain thermostatic environment for titration. The G10S Compact Titrator is ideal for precise potentiometric titration with very simple operation.

Potentiometric titration measured the concentration dependent potential (mV) of a solution (or analyte) against a reference potential of sensor such as Mettler DG-115-SC which was used for this work while many different types of sensors can be used for a titration. The Mettler DG-115-SC sensor is designed for aqueous acid/base titrations of low conductivity or food samples; therefore, DG-115-SC works best for low concentration of acid-base titration such as humic substance titrations. DG-115-SC is a glass pH electrode with a fixed ground-glass sleeve junction for aqueous media a measures pH 1-14 in the temperature range 0-100 °C. For precise titration it is important to make sure that reference electrolyte (KCl 3 mol/L for DG-115-SC) level must be higher than the solution (or analyte) level to allow electrolyte to leak into sample and solvent with gravity. It was critical to ensure the reference junction is immersed in the

sample solution for proper measurements and titrations. When the reference junction was not fully immersed during HS titration, it resulted in inconsistent titration data and curves and those data could not be used. The G10S titrator (Fig. 6) operation was simple and safe. First, the titrator was set up as shown in Fig 6. The titrant was filled (KOH 0.1 M for this work) in the titrant bottle (4) and the titrant was automatically filled up in the 20-ml burette (12) through suction tube (13).



1	Titrant bottle drying tube	8	Power button
2	Dispensing tube	9	Indicator light (LED)
3	Compact stirrer	10	Touchscreen
4	Titrant bottle	11	Burette arrestment knob
5	Electrode	12	Burette
6	Titration stand	13	Suction tube
7	Titration vessel		

Figure 6. Diagram of Mettler Toledo G10S Titrator (G10S user manual).

Before performing titration, the burette was rinsed to remove gases in the burette and dispensing tube (2). The titration vessel (7) was filled with analyte (50-mL humic substance solution or model compound solution for this research) to the titration stand (6) equipped with compact stirrer (3), the electrode (5) (DG-115-SC for this titration),

dispensing tube (2) and N₂ purging tube (not shown in Fig. 6). Once a titration starts, the titrant is dispensed into the titration vessel. During titration, the compact stirrer was purged with N₂ gas throughout the titration. Titration data was recorded and saved in the USB plugged into the titrator's USB port. Before running a titration, calibration of a pH sensor (electrode) with buffers was performed. Sensor Calibration is necessary for direct measurements or titrations that require set end point potentials for recognition. For this work pH 4.00, 7.00, and 9.22 buffers were used for linear calibration as recommend in the manual.

There were a few more things to consider for each titration. Standardization of the titrant was always the first step and the titer (correction to nominal concentration) was determined to calculate exact concentration of titrant before performing the HS titration. Titer was determined using a precisely known quantity (mg) of high purity (99%) potassium hydrogen phthalate (KHP) as the primary standard. Once the titer is obtained the actual concentration of the titrant was calculated using the eq. 1

Equation 1

$$t = \frac{c(x, \text{actual})}{c(x, \text{nominal})}$$

where t refers to titer and c to concentration. It is also important to prevent any titrant to react with CO₂ (carbonic acid) from the room atmosphere. This can be done by attaching a drying tube (1) containing an absorbent (e.g. NaOH on a granular carrier) to the titrant bottle. In order to provide thermostatic environment during titration, a 10-liter general purpose water bath manufactured by Polyscience was used. This water bath has working temperature range 5 to 100 °C and ±0.1 °C of temperature stability.

During titration, the titration vessel (7) was immersed in the water bath making sure the water level was higher than the analyte (solution) to maintain a constant temperature. N₂ gas for purging was supplied using a thin tube connected to N₂ gas tank (Fig. 7). Nitrogen gas was used to avoid CO₂ acidification because the interaction with atmospheric CO₂ in the air can possibly alter the pH during the titration. N₂ gas was also used to remove dissolved oxygen and to avoid oxygen contamination of the analyte.

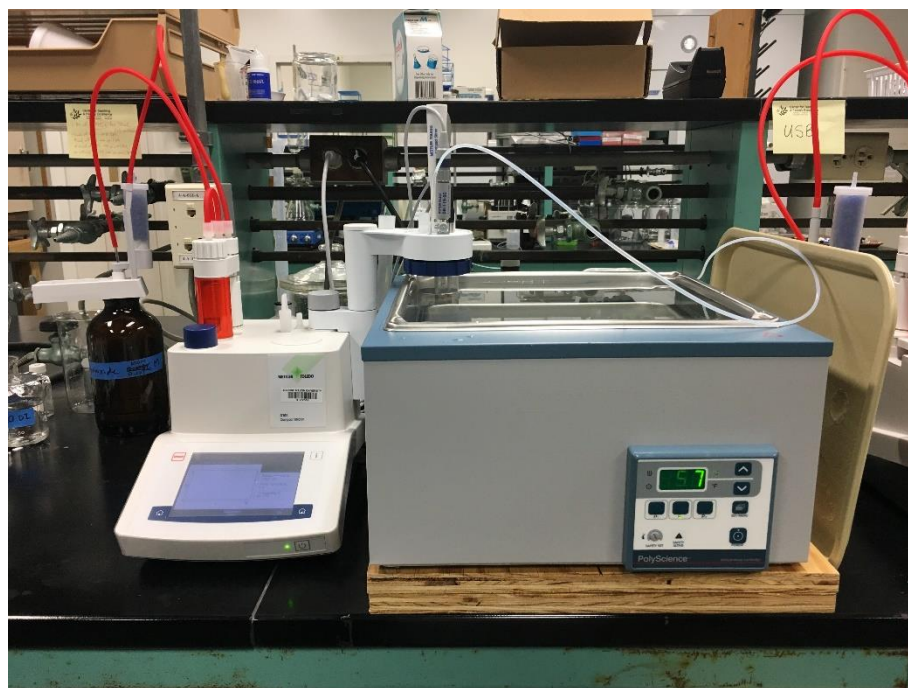


Figure 7. HS Titrator and water bath.

The humic substances used in this work were analytical grade humic acid purchased from Sigma-Aldrich Chemical (Milwaukee, WI), fulvic acid derived Podzolic soil from Eastern Canada (Schnitzer and Khan, 1972) (referred as PFA hereafter), humic acid found on black chernozemic soil of Alberta Canada (Schnitzer and Khan, 1972)

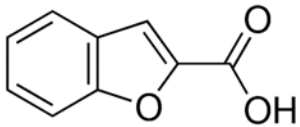
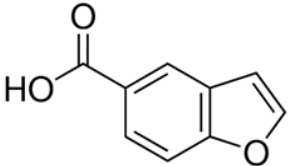
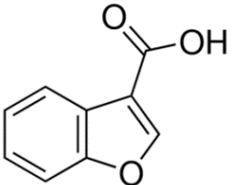
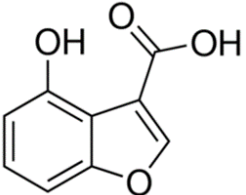
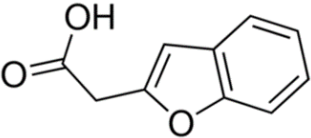
(referred as CHA hereafter) and Upper Mississippi River natural organic matter (NOM) (referred as MIS hereafter) purchased from the International Humic Substances Society (IHSS) (Table 1). Aldrich humic acid (AHA) purchased from Sigma-Aldrich was purified for better titration result by eliminating impurities. Purification was done by washing off impurities with deionized distilled water (DDW) multiple times after acidification of the AHA with HNO₃. The HS properties are illustrated in Table 1.

Table 1. Humic Substances Properties (Source 1-("International Humic Substances Society (IHSS),", n.d.), 2 – (Schnitzer and Khan, 1972), 3 – (Song et al., 2010))

Full Name	Acronym	Solubility	Element (%)				
			C	H	N	S	O
Upper Mississippi River NOM (1R110N) ¹ – Aquatic derived	MIS	Soluble	49.98	4.61	2.36	2.62	41.4
Fulvic Acid from Podzolic soil of Eastern Canada ² - soil derived	PFA	Soluble	45.7	5.43	2.1	1.9	44.8
Humic Acid from Black chernozemic soil of Alberta Canada ² - soil derived	CHA	insoluble	56.2	4.7	3.2	0.8	35.5
Purified Aldrich Humic Acid ³ – Aquatic derived	PHA	insoluble	57.6	5.11	0.87		34.2

Five commercially available benzofuran derivatives were purchased from Sigma-Aldrich. The benzofuran model compounds used for this research were benzofuran-2-carboxylic acid, 4-hydroxy-1-benzofuran-3-carboxylic acid, 2-benzofuranacetic acid, 1-benzofuran-3-carboxylic acid and benzofuran-5-carboxylic acid (Table 2). The preparation and the titrations are described below.

Table 2. Selected Benzofuran Model Compounds

	Chemical Structure	Compound Name
1		Benzofuran-2-carboxylic acid
2		Benzofuran-5-carboxylic acid
3		1-Benzofuran-3-carboxylic acid
4		4-Hydroxy-1-benzofuran-3-carboxylic acid
5		2-Benzofuranacetic acid

Sample preparation

Aldrich humic acid 1000.90 mg was extracted for 24 hours with 0.1 M KOH. Solid, undissolved HA was separated from the solution using centrifugation (rpm), upon which the supernatant contained dissolved humic acid. To centrifuge, 50 mL sterile polypropylene centrifuge tubes were used, and they were filled to approximately 45 mL of solution. Nitric acid (HNO_3 6 M) was added to the supernatant suspension until a pH of 1 was reached to re-precipitate the purified humic acid. The suspension was centrifuged for 30 min, and the residue extensively washed with deionized distilled water (DDW). Washing with DDW and centrifugation was

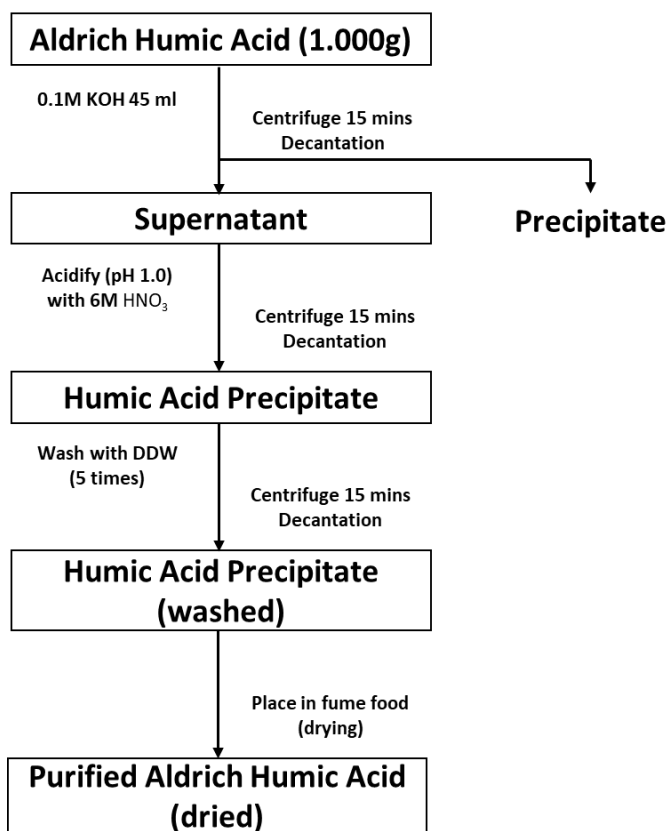


Figure 8. Aldrich Humic Acid Purification

repeated 10 times. After the last centrifugation, precipitated HA was placed a small beaker fully covered with a ventilating filter paper and place the beaker in the ventilator for 2~3 days to dry the HA sample. The completely dried HA after purification (referred as PHA hereafter) was used for titration.

Titration procedures

Each HS solution was prepared in 50-mL aliquots for titration. The HS solution (50 mL) made-up in 0.100M KOH at 1 mg/mL was used to make a titration mixture, which was diluted to 0.3 mg/ml for titration. The stock solution was prepared 24h before the titration for the best equilibration time. Each titration mixture was prepared with 15 ml of 1 mg/mL HS, 0.010M KNO₃ ionic strength reagent, 15 ml of 0.100 M HNO₃ and deionized distilled water (DDW) in a 100-mL plastic titrator vessel. Every HS titration mixture was adjusted to pH 3.0 ± 0.1 with 0.100 M HNO₃ for titrations with 0.100 M KOH. The HS mixture was titrated with 0.100 M (KHP standardized) HNO₃ up to pH 10.5 with 0.100 M KOH. The ionic strength of KOH titrant was matched with that of HS mixture (0.010 KNO₃). All the titrations were performed in the vessels designed for the G10S. Each titration solution was purged with N₂ for 15 minutes before titration and continuously throughout the entire titration. Each titration was performed with 0.005-0.01 mL increments of titrant (0.100 M KOH) from pH 3.0 to 10.5 to yield 100-300 data points. The volume of titrant added and pH (when stable for 30 s with a drift of no more than ± 0.02 mV) was automatically recorded by G10S after each addition of titrant. A blank titration was carried out with DDW at the appropriate KNO₃ ionic strength (i.e., no HA added).

The same titration procedure was conducted in a walk-in cold room at 6 °C to evaluate temperature effects in the thermodynamic acid dissociation constants, which yielded entropy (ΔS) and enthalpy (ΔH) estimates in relation to the K_a's. Enthalpy change, ΔH is defined as the heat absorbed or released when a reaction

occurs at constant pressure and entropy and ΔS is defined as the change in the “disorder” of a substance. Temperature experiments at 6 °C, 25 °C and 35 °C for the aquatic HS mixture allowed for the determination of the enthalpy of dissociation (ΔH_{dis}) using the standard Gibbs free-energy equation ($\Delta G_{\text{dis}} = \Delta H_{\text{dis}} - T\Delta S_{\text{dis}}$). ΔG , the change in Gibbs free energy for any process at constant temperature is related to the change in enthalpy, ΔH , and entropy, ΔS . T is temperature in Kelvins. For example, a process is spontaneous (thermodynamically favorable) if ΔG is negative (Harris, 2010). Three replicate titrations were performed on each HS sample. A fourth replicate titration was performed when there is unusual behavior of pH or a malfunction of the equipment. It was initially planned on performing titration at 5 °C, 25 °C and 40 °C to study the significant effect of temperature on thermodynamic property of HS, however, due to technical difficulties, the target temperatures were modified as available. The titration was performed in the cold room performed at 6 °C because the cold room could maintain only 6 °C (± 0.3) consistently. In addition, maintaining consistent 40 °C (± 0.3) using the Polyscience thermostatic water bath wasn’t possible. It was available to maintain 35 °C (± 0.3) in the laboratory using the water bath instead.

For the titration of the benzofuran derivatives, 0.002 M solutions of each compound were prepared in DDW at 35 °C for 2 hours. According to the solubility data obtained from EPISuite (*Environmental Protection Agency (EPA), EPISuite*, n.d.), the estimated water solubility from Log K_{ow} (Table. 3) is higher than 0.002M at 25 °C (K_{ow} Octanol-water partition coefficient). However, the model compounds were not easily dissolved at 25 °C in this experiment. To dissolve the model compounds, DDW was

heated up to 35 °C and the model compound was dissolved in the DDW for 2 hours. After the model compound was completely dissolved, the model compound solution was quickly cooled to 6 °C or 25 °C for titration. Purging with the N₂ gas was continued throughout the entire titration. Each model compound solution was titrated at I = 0.01M, where I is a total ionic strength, by adding KNO₃ with titrant 0.100 M of KOH. Temperature experiments at 6 °C, 25 °C and 35 °C were performed using Benzofuran-2-carboxylic acid for thermodynamic activity analysis for H⁺ dissociation from the carboxylic group.

Table 3. Water Solubility Estimates from Log K_{ow} of Benzofuran model compounds (Source EPISuite)

CAS No.	Model compound	Estimated Water solubility, mg/L at, 25 °C	Estimated Water solubility, M at 25 °C
496-41-3	Benzofuran-2-carboxylic acid	585.3	0.00361
90721-27-0	Benzofuran-5-carboxylic acid	573.7	0.00354
26537-68-8	1-Benzofuran-3-carboxylic acid	573.7	0.00354
112678-09-8	4-Hydroxy-1-benzofuran-3-carboxylic acid	4712	0.02645
62119-70-4	2-Benzofuranacetic acid	2941	0.01669

A constant ionic strength (I) of 0.01 M KNO₃ was used in all experiments and calculations because it provided a constant total ionic strength throughout the titration experiment. It is verified for the ionic strength did not cause any significant change in the shape of the curves for the vermicompost humic acid even though the electrostatic effects

tend to be minimized with the increase in the ionic strength (Masini et al., 1998 and Andres et al., 1987).

Data Analysis

The raw titration data were obtained using G10S auto titrator and the recorded data were evaluated using Excel for the calculation of the dissociation constants. The G10S recorded along with every entry of added titration volume (0.1 M KOH for this study) the pH, first-derivative value, temperature and pH change. Based on normal assumptions (von Wandruszka, 2000), HS is modeled as an impermeable spherical structure with a surface where interactions occur (Kinniburgh et al., 1999 and Tipping, 1998), exhibiting hydrophobic and rigid character, a non-linear method was used for the characterization of proton and metal reactivity on the surface of bacteriogenic iron oxides (BIOS) (Martinez, 2005). BIOS, abundant in surface water and groundwater system, is composed of a highly porous matrix of ferrihydrite intermixed with intact and fragmented *Gallionella ferruginea* cells which has functional group acids and protonated surface binding sites (Lovley, 2000 and Martinez, 2005). The surface reactivity of bacterial cells were suitably described with chemical equilibrium models of acid-base titration data in terms of a sum of monoprotic acids (Smith and Ferris, 2001). A distribution of pK_a values for each type of reactive site may be observed as a result of steric and electronic interactions (Martinez et al., 2002) because the surface of bacterial cells is a three-dimensional cross-linked structure (Actor and American Society for Microbiology, 1988).

The non-linear method for fitting was used to determine the surface charge excess, K_a and pK_a values using Solver within Excel. Those values were assessed using a fully optimized continuous pK_a spectral model (FOCUS) which employs the discrete

binding model to describe the dissociation character of the HS and this model was chosen because of availability and ease of use. FOCUS optimization provided pK_a spectra for the dissociated hydrogen ion fraction upon minimization of the error between the measured and calculated charge excess quantities (Martinez, 2005). This model considers 4 binding sites with the one attributed to carboxylic acid groups, the one to phenolic acid and two for other functional groups. The mechanisms of deprotonation and protonation reactions on the surface using the raw acid base titration data for the i th addition of titrant can be written as below:

$$Q_{\text{mas},i} = C_{\text{bi}} - C_{\text{ai}} + [H^+]_{\text{bulk},i} - [OH]_{\text{bulk},i} \quad \text{Equation 2}$$

where $Q_{\text{mas},i}$ represents the experimental net surface charge excess. C_{ai} correspond to the acid concentrations at the i th addition of titrant and C_{bi} correspond the base concentrations at the i th addition of titrant. The bulk hydrogen ion, $[H^+]_{\text{bulk},i}$ and the bulk hydroxide ion, $[OH]_{\text{bulk},i}$ calculated from pH measurements and $K_w = 1.01 \times 10^{-14}$ at 25°C. $\{H^+\}$ was measured with a pH electrode and $[H^+]$ was converted to concentration through the relationship $[H^+]_{\text{bulk},i} = \{H^+\}/\gamma_H$ and $[OH^-]_{\text{bulk},i} = K_w/[H^+]_{\text{bulk},i}$ using γ_H value for the proton activity coefficient, where $\{H^+\}$ is activity of H^+ and γ is activity coefficient (Harris, 2010). When determining K_a values of organic acids (eg. Carboxylic acid), bulk hydrogen ion activity is commonly measured using γ_H (Schwarzenbach et al., 2017).

To obtain γ_H at 6 °C and 35 °C which are not provided by Harris, 2010 were calculated using the temperature dependent form of the extended Debye-Hückel equation (Harris, 2010)

Equation 3

$$\log \gamma = \frac{(-1.825 \times 10^6)(\epsilon T)^{-\frac{3}{2}} z^2 \sqrt{\mu}}{1 + \frac{\alpha \sqrt{\mu}}{2.00 \sqrt{\epsilon T}}}$$

Where ϵ is the (dimensionless) dielectric constant of water, T is temperature in Kelvin, z is the charge of the ion, μ is ionic strength (M, mol/L), and α is the ion size parameter (pm) which is 900 for H^+ ion. The dependence of ϵ on temperature is

Equation 4

$$\epsilon = 79.755 e^{(-4.6 \times 10^{-3})(T - 293.15)}$$

The calculated γ_H values corresponded to an ionic strength of 0.01 M and at 6 °C, 25 °C and 35 °C were 0.915, 0.912 and 0.911 respectively.

Equation 5

$$Q_{\text{calc}, i} = \sum_{j=1}^m \left(\frac{K_{aj}^{\text{app}}}{K_{aj}^{\text{app}} + [H]_{\text{bulk}, i}} \right) \times [B_j] + S_0$$

Where $Q_{\text{calc}, i}$ represents the calculated net surface charge excess, K_{ja}^{app} is the apparent acidity constant for the jth site, m refers to the number of binding sites, $[B_j]$ is the site density in M (mol/L) of sorbent and S_0 is offset term that is necessary in order to account for positive charges on the surface (Brassard and others, 1990; Martinez and others 2002). For a triprotic acid, as an example, Eq. 5 extracts three K_a values, K_1 , K_2 and K_3

and three binding site concentrations, [B₁], [B₂] and [B₃]. The nonlinear optimizations program called SOLVER as available in Excel spreadsheet can extract these parameters.

Determination of ΔH^0 and ΔS^0 of H⁺ dissociation was done using standard Gibbs energy equations (Harris, 2010) and modified van't Hoff equation (Schwarzenbach et al., 2017).

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad \text{Equation 6}$$

$$\Delta G^0 = -RT * \ln K_a \quad \text{Equation 7}$$

$$\ln K_a = -\frac{\Delta H}{R} * \frac{1}{T} + C \quad \text{Equation 8}$$

By measuring K_a value at various temperatures (3 different temperatures for this work, 6 °C, 25 °C and 35 °C), ΔH can be obtained from linear regression of $\ln K_a$ versus $1/T$ (Eq. 8). ΔH , then can be calculated from the slope ($-\Delta H/R$) of the linear regression where R is gas constant, R=8.314 J/mol-K and C is intercept. Using the ΔH value from the from linear regression of $\ln K_a$ versus $1/T$ and ΔG^0 value using Eq. 7, ΔS can be obtained after pugging ΔG and ΔH into Eq. 6.

Based on the standard error values, a favorable reaction during acid dissociation was determined for each HS and the model compound. It was assumed in these calculations that ΔH and ΔS were not strongly temperature dependent.

The pK_a values for the benzofuran model compounds were directly obtained from titration data generated by G10S which is more precise than calculation for monoprotic

substance except for 4-hydroxy-1-benzofuran-3-carboxylic acid which is diprotic having both carboxylic and phenolic group. For this compound simple diprotic calculation was used as written in Harris, 2010.

RESULTS AND DISCUSSION

Acid dissociation in humic substances

Titration curves of all HS samples were conducted from an initial pH of 2.9 to 3.1 to a maximum pH of 10.5. Typical of multi-protic organic acids, the titration curves were featureless with no clear equivalence point inflection was observed as expected regardless of the type of humic substance analyzed in this study. The shape of the titration curves generally showed similarities to that of a weak diprotic acid curve with a buffer region around pH 5.5 to 7.5 for most of the samples considered. In this study, the raw titration data showed differing degrees of curvature depending on the type of HS (Figs. 9, 11, 12 and 13).

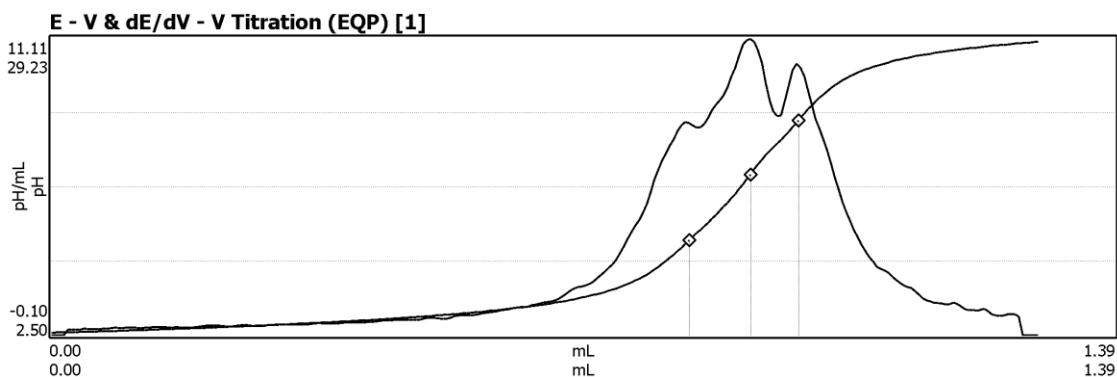


Figure 9. Titration curve and 1st derivative curve for 0.3 mg/ml raw Aldrich humic acid (AHA) titrated with 0.1M KOH at 25 °C. 3 Equilibrium points at pH 5.249, 7.134 and 8.687 are observed.

There was a significant difference observed between pK_a values for unpurified Aldrich HA (AHA) and purified Aldrich HA (PHA) titrated at 25 °C, as shown in Table 5 and Fig. 10. AHA is a crude and impure form of humic acid, therefore, a purification step was added for more accurate titration results. After purification, the Aldrich HA pK_a value increased significantly, which means acidity in the solution decreased. This observation is also supported in Fig. 10, where the AHA had a higher charge excess than PHA during the titration. The raw AHA ($pK_a = 3.89 \pm 0.0$) might possess many other functional groups or substituents that affect lowering carboxylic group's pK_a value such as -F, -Cl, -OR or NO_2 known to result inductive effect near carboxylic groups and many of them might have been removed during the purification process.

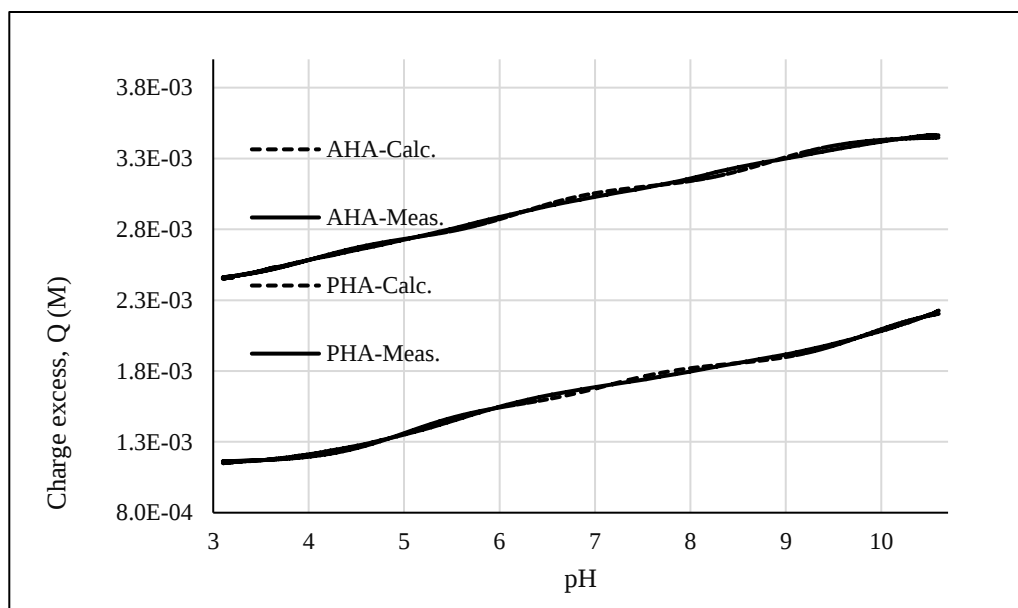


Figure 10. Charge excess (M) comparison between unpurified Aldrich HA (AHA) and purified Aldrich HA (PHA) for measured and calculated excess charge (Q).

It is also possible that other impurities that are more acidic than carboxylic group were washed off after the purification and only carboxylic groups were available for proton dissociation.

Net surface charge excess, Q , was determined from acid base titrations assuming that electroneutrality can be defined as the difference between known added amounts of acid and base and measured proton concentration (Smith and Ferris, 2001; Stumm and Morgan, 1996). Nevertheless, after washing off impurities from AHA (Fig. 9), more clean and concise titration graphs of PHA (Fig. 11) were obtained that minimized data error caused by impurities and other factors. Fig. 10 shows charge excess curves of HS for both measured and calculated Q . Charge excess curves plotted using Q values calculated from eq. 1 and eq. 4, respectively as a function of bulk pH include the effects of electrostatic interference and may be used to determine apparent dissociation constants (Martinez et al., 2002). Measured Q was obtained using direct titration data and FOCUS was used to calculate apparent acidity constants. Bulk pH was determined as a function of γ_H (proton activity coefficient) value calculated using extended Debye-Hückel equation (Eq. 3). Solver function equipped in excel enabled to generate calculated Q . Solver is a numerical least squares data fitting technique based on simplex or other optimization techniques. It compares the experimental data with that of the calculated values by the theoretical equation (FOCUS model) with initial values for the parameters. The parameter values are optimized by the Solver program until the sum of the squares of the residual (SSQR) between the calculated and experimental values are minimum.

Humic acids and fulvic acids showed quite different first-derivative curves (Fig.

9, 11, 12 and 13). In three humic acids (HA), AHA (Fig. 19), PHA (Fig. 11) and CHA (Fig. 12), two or more of significant inflection points within the titration curve were observed in the first derivative curves. Below pH 4.5~5.0, chemical equilibrium was established rapidly in all HA titrations. Above pH 4.5~5.0, the addition of KOH titrant resulted in a slow downward drift in pH, followed by a rapid increase in pH. This phenomenon visually showed clear buffer regions and unbuffered pH ranges in HA titration. In both AHA (raw Aldrich humic acid) and PHA, two distinct buffered regions were observed whereas only one buffered region was observed in CHA (Fig. 12) in direct reading on the raw titration curve. Based on the observation of the HA's titration data, it can be assumed that impurities in CHA affected the lack of distinct inflection point or other existing functional groups affected the equilibrium. It was observed that impurities affected magnitude of buffer region and K_a value. Both raw Aldrich HA (AHA) and purified HA (PHA) were titrated and it showed that impurity affected the titration curve (Fig. 9 and 11) as well as charge excess curve (Fig. 10). Purification of HS seems to provide concise titration curve that can be better data to use for analysis; however, with the limited availability of commercial aquatic HS isolates used in this research (<1 g). In the titration data, there were one or more of significant buffered regions observed in HA whereas FA (Fig. 13) gradually showed gradual increase and decrease with slight fluctuation in the first derivative curve along with the addition of the titrant (Fig. 12).

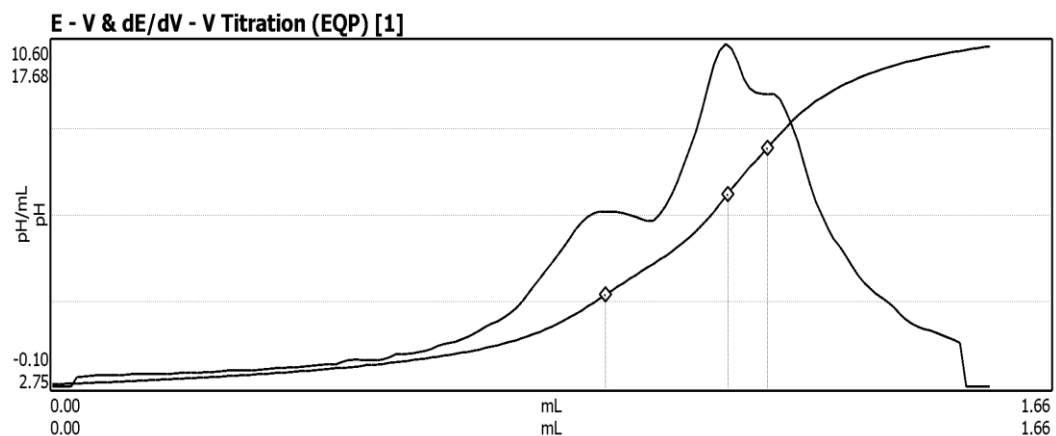


Figure 11. Titration curve and 1st derivative curve for 0.3 mg/ml purified Aldrich humic acid (PHA) titrated with 0.1M KOH at 25 °C. 3 Equilibrium points at pH 4.868, 7.139 and 8.183 were observed.

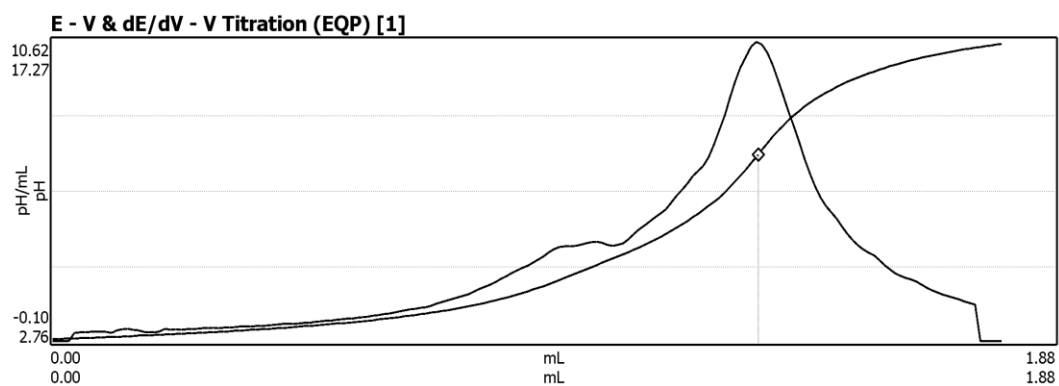


Figure 12. Titration curve and 1st derivative curve for 0.3 mg/ml Black Chernozemic soil Humic Acid (CHA) titrated with 0.1M KOH at 25 °C. One equilibrium point at pH 7.621 is observed.

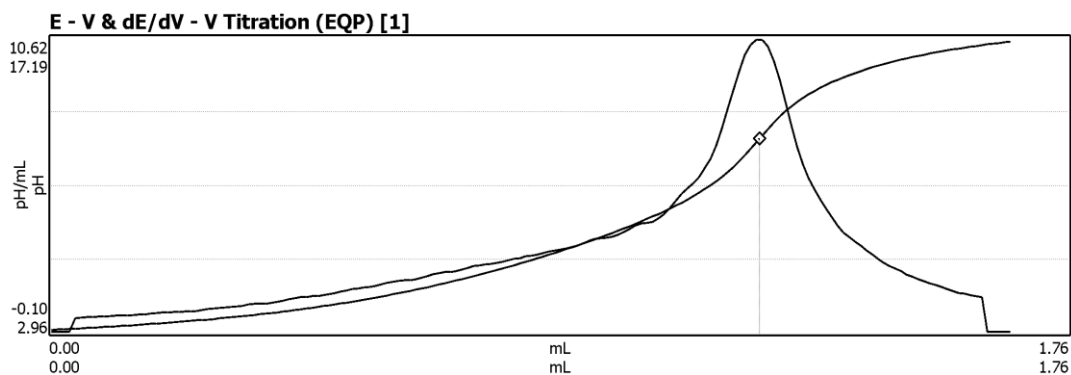


Figure 13. Titration curve and 1st derivative curve for 0.3 mg/ml Podzolic soil Fulvic Acid (PFA) titrated with 0.1M KOH at 25 °C. One significant equilibrium point at pH 7.984 is observed.

In this study, FOCUS pK_a model assumed to enable deconvolution of the mixed functional groups on the HS to remove overlapping binding site density contributions from the HS and recover the reactive surface heterogeneity as it was applied to the study that quantifies the surface chemical heterogeneity of bacteriogenic iron oxides (BIOS) and its end-members (2-line ferrihydrite and intermixed intact and Acid-base titration data) from the HS were fitted using a fully optimized continuous pK_a spectral (FOCUS) model in Excel spreadsheet. The FOCUS model is an empirical model and provides the mean pK_a value determined from a Gaussian model for the peak shapes. In this study, the FOCUS pK_a model assumed to enable deconvolution fragmented bacteria) (Martinez et al., 2003). FOCUS model was employed to this HS study because of the similarity in shapes between a bacterial cell and the empirical shape of HS and the property of a strong binding capacity for metals. The shapes are like sphere that possess ionizable functional groups on the surface and hydrophobic region inside (Fig. 3).

Using the FOCUS model, it was possible to obtain pK_a values for carboxylic and phenolic functional group by running “Solver” in Excel based on the charge excess on the protonated binding sites. The FOCUS model was designed to record multiple dissociation sites. Martinez et al. (2003) used this FOCUS model to record four individual pK_a values of a bacterial cells, and these values were found to be consistent with the ranges of 2 to 6, 5.6 to 7.2 and 9 to 11 for carboxyl, phosphate and amine functional groups reported for intact and fragmented bacterial cells (Martinez et al., 2003).

In this study, FOCUS model was programed to record four discrete dissociation sites, which is called tetraprotic model. Diprotic and triprotic models were also tested first since finding the optimal pK_a values of carboxylic group and phenolic groups were primary objective of this research; however, those two latter models failed to extract pK_a values consistent with those found in the literature for other HS. Examples of the solver result tables in details are shown in Appendix A.

Using tetraprotic model, it was expected that Solver would extract pK_a values for all four binding sites, however, only three distinct sites were observed (Table 4). The 4th binding site wasn’t considered important because of the extremely low site density, which means that the amount of dissociated H^+ was too little to measure pK_a value. All pK_a values derived through FOCUS are illustrated in Table 4 including standard deviations.

Table 4. All pK_a values derived through FOCUS.

Humic Sample	pK _{a1}	pK _{a2}	pK _{a3}
MIS	4.14 ± 0.02	5.98 ± 0.03	9.54 ± 0.01
PFA	4.17 ± 0.02	6.07 ± 0.02	9.59 ± 0.01
CHA	4.31 ± 0.08	6.21 ± 0.09	9.55 ± 0.05
PHA	4.88 ± 0.04	7.04 ± 0.08	9.69 ± 0.04
AHA	3.89 ± 0.02	6.11 ± 0.02	8.71 ± 0.02

Among the three extracted pK_a values, only two pK_a values were used for data analysis, including one for the carboxylic group (pK_{a1} 3.89~4.88) and one for phenolic group (pK_{a3} 8.71~9.69), because they can be unequivocally assigned based on the known structure of HS. The third pK_a value (pK_{a2} 5.98~7.04) was also extracted; however, it could not be correlated with any particular functional group other than a carboxylic or phenolic group based on the known structure of HS. In addition, a third pK_{a2} 5.98~7.04 dissociation site has not been reported previously in the literature for HS. The pK_{a2} values are too large for carboxyl and too small for phenolic dissociations, thus, these dissociation sites are unknown. But, this particular pK_a dissociation of 5.98~7.04 does represent an intriguing future direction of investigation.

According to literature K_a (Acid Dissociation Constants in Appendix G of Harris, 2010), most aromatic carboxylic groups' K_a values lie between 2.185 and 5.408 as 2-nitrobenzoic acid and phthalic acid, respectively. pK_a value of aromatic phenolic groups are between 7.15 and 13.7 as 4-nitrophenol and as salicylic acid, respectively. In addition, many other literature sources have also shown that carboxylic and phenolic groups on HS have pK_as within 2.80 - 4.94 for carboxylic acids and pK_as 7.15 - 9.80, for phenolic groups, which will be in more detail discussed below.

The measured pH with G10S and calculated using FOCUS model results showed different pK_a values depending on the HS (Table 5); however, the all the obtained pK_a values lie within acceptable tolerances among the same functional groups. The pK_a values were found to be in the range of 3.89 ± 0.02 to 4.88 ± 0.04 for carboxylic group and 8.71 ± 0.02 to 9.74 ± 0.07 for phenolic groups. In previous works on humic acids of different origin, carboxylic and phenolic groups have shown pK_a s within 2.80 - 4.94 for carboxylic group and pK_a s 7.15 - 9.80, respectively, for phenolic groups (D. Gondar et al., 2006, Dora Gondar et al., 2006, Kinniburgh et al., 1999, Christl et al., 2001, Benedetti et al., 1996, Christl and Kretzschmar, 2001, Milne et al., 2003, Plaza et al., 2005a, Janoš et al., 2008, Weber et al., 2006 and Robertson and Leckie, 1999). The pK_a values obtained from this work are in good agreement with the pK_a value of previous works on humic acids of different origin.

As shown in Fig 14, pK_a value for each functional group distributed within somewhat close range from 3.89 to 4.32 for carboxylic group while PHA's pK_a value is more than 0.5 higher than other HS titrated in this research. However, as the pK_a value of carboxylic group goes up, that of phenolic group goes up as well. The overall order of acidity is $AHA > MIS > PFA > CHA > PFA$, which indicate that the order of pK_a values is inversely proportional to acidity.

Table 5. Summary of pK_a and K_a values of selected HS titrated at 25 °C (SD denotes standard deviation; the pK_a is the mean pK_a value determined from Eq. 5 (FOCUS) model for the peak shapes)

Humic Sample	pK _a values				K _a values	
	Carboxylic group (-COOH)	SD	Phenolic group (-OH)	SD	Carboxylic group (-COOH)	Phenolic group (-OH)
MIS	4.14	0.02	9.54	0.01	7.23E-05	2.90E-10
PFA	4.17	0.02	9.59	0.02	6.75E-05	2.57E-10
CHA	4.31	0.08	9.55	0.06	4.88E-05	2.81E-10
PHA	4.88	0.04	9.69	0.04	1.32E-05	2.02E-10
AHA	3.90	0.02	8.71	0.02	1.29E-04	1.94E-09

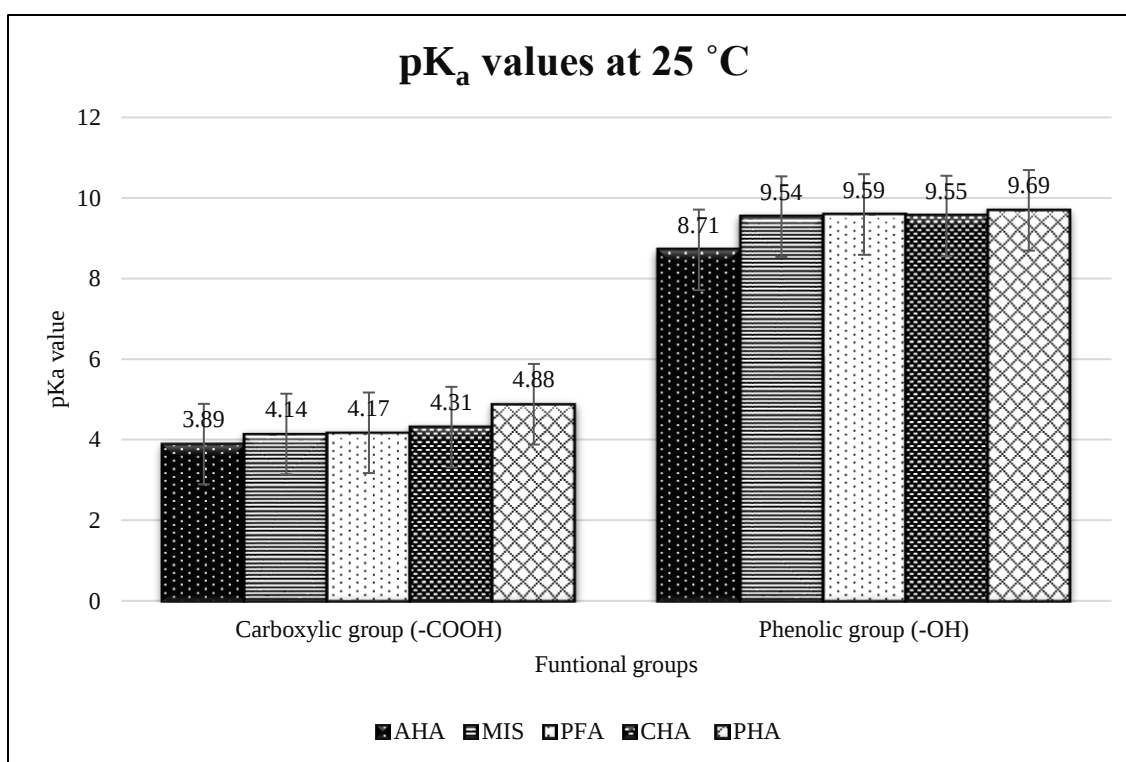


Figure 14. pK_a value comparison of selected HS at 25 °C

Among those five HSs used for this research, MIS and PFA were appeared to have many similarities in pK_a values for both carboxylic (4.14 and 4.17) and phenolic (9.53 and 9.59) group, titration curve, charge excess curve and physical appearance in color changes during titration.

As shown in Fig. 15, charge excess curve of MIS and PFA samples look very similar to each other. PFA curve lies slightly higher than MIS, but they look identical in curvature if those two curves are superimposed. All three HS analyte color remained dark brown suspension throughout the entire work from preparation to titration. Both MIS and PFA changed its color to clear yellow after addition of 0.1M of HNO_3 solution the stock brown or black color is characteristic of higher molecular weight species where the lower molecular weight components tend to be light brown or yellow solution. Based on the observation and the obtained pK_a value, it is suggested that MIS has similar characteristic to a fluvic acid, PFA. This is in good agreement with a dark brown or black color that is characteristic of higher molecular weight species where the lower molecular weight components tend to be light brown or yellow (Tinsley, 2004).

As addressed earlier, pK_a values obtained in this study are in good agreement with those from previous works done by various researchers. Many of them evaluated pK_a values with the modified Henderson-Hasselbalch model (Khil'ko et al., 2011, Andjelkovic et al., 2006, Ritchie and Perdue, 2003 and Zhang et al., 2013) or nonideal competitive adsorption (NICA)-Donnan model (Vidali et al., 2009, Plaza et al., 2005b and Milne et al., 2001). Milne et al. evaluated and obtained pK_a values using NICA-

Donnan model. For Milne's research, all the available published and unpublished data were used to evaluate pK_a values of FA and HA.

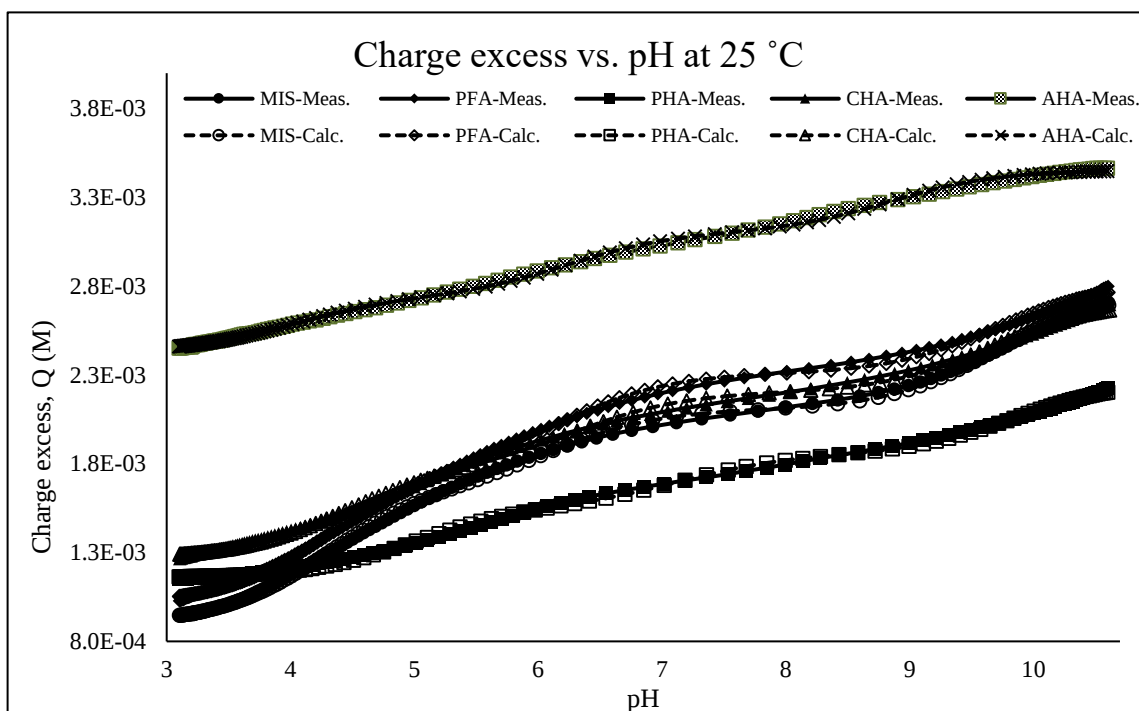


Figure 15. Selected HSs Charge excess along with pH changes at 25 °C.

All the collected data of 25 FA and 24 HA, were fitted individually to the NICA-Donnan model. The obtained mean pK_a values of carboxylic group and phenolic groups are FA 2.65 and 8.60 and HA 3.09 and 7.98, respectively. Another study also showed average pK_a of 3.5 and 7.5 using NICA-Donnan model. Those values are quite lower than the pK_a values obtained from this work using FOCUS model which are 4.14 - 4.17 carboxylic group and 9.54 - 9.59 for and phenolic group. In Milne's work, AHA and PHA were also included that pK_a value of AHA were 2.87 and 8.00 and PHA 3.76 and 8.07 for carboxylic and phenolic group, respectively whereas pK_a values from this study

are PHA 4.88 and 9.69 and AHA 3.89 and 8.71 obtained FOCUS model. pK_a values using FOCUS model are clearly higher than those of NICA-Donnan model even with or the consideration of ionic strength and the ionic strength for each HS is not provided in the article. However, the ionic strength did not cause any significant change in the shape of the curves so the ionic strength effect on the normalized curves is expected to be minimal (Baidoo et al., 2014).

A previous study showed that pK_a values depend on ionic strength, however, the maximum difference in pK_a was 0.5 between 1.0 M and 0.01 M (Masini et al., 1998). Baidoo et al., employed Stockholm humic model (SHM) to find out how protonation behavior of tropical and high molecular weight humic acids (HA) differs from humic moieties of other origin. The average pK_a of 4.39 and 8.37 were obtained respectively which is good agreement with the pK_a values obtained from this study on HS fitted to FOCUS model. pK_a values estimated with modified Henderson-Hasselbalch model showed good agreement with pK_a values with FOCUS model. Previous studies estimated pK_a values of carboxylic group and phenolic groups are FA 3.81 and 9.77 and HA 4.38 and 9.72, respectively with 14 HS samples (Ritchie and Perdue, 2003) and another study showed carboxylic group average pK_a value of 4.34 with 5 HAs (Zhang et al., 2013). Even though estimated pK_a values with NICA-Donnan model and Focus model showed difference in pK_a values, the differences are not significant and those pK_a values lie within the known pK_a ranges for carboxylic and phenolic functional groups. pK_a values estimated with Modified Henderson-Hasselbalch model support that FOCUS model which successfully evaluated and determined intrinsic bacterial surface acidity and pK_a

values of functional groups on the surface is another reliable method for HS evaluation (Martinez et al., 2002, 2003 and 2005).

The extent to which the reactive sites on each HS contribute to the surface reactivity could be assessed by determining the percent concentrations of the total normalized group density corresponding to each binding site type, as a function of acidity. Comparison of the each functional group contributions would be indicative of the nature of the HS surface reactivity (Martinez et al., 2003).

The total binding site density, $[B_T]$, from the HS sample data was calculated as the sum of the individual site densities determined from the area under the Gaussian distribution for each pK_a peak, as summarized in Tables 6 and 7.

Table 6. Summary of individual pK_a values and binding site density (B_T) values and pK_a values for each site.

Sample	Functional group	pK_a	Site Density (M)	Site Density ($\mu\text{mol/mg}$)
PHA	- COOH	4.88 ± 0.04	3.95E-04	0.40
	- OH	9.69 ± 0.04	4.02E-04	0.40
CHA	- COOH	4.31 ± 0.08	5.12E-04	0.51
	- OH	9.55 ± 0.06	5.35E-04	0.54
PFA	- COOH	4.17 ± 0.02	7.69E-04	0.77
	- OH	9.59 ± 0.02	5.19E-04	0.52
MIS	- COOH	4.14 ± 0.02	7.67E-04	0.77
	- OH	9.54 ± 0.01	6.53E-04	0.65

* The pK_a value plus or minus the width ((2σ)) of the mean pK_a value determined from a Gaussian model for the peak shapes

Table 7. Summary of Site density values ($\mu\text{mol}/\text{mg}$) for each functional group

Sample	B ₁ (-COOH)	B ₂ (Neutral)	B ₃ (-OH)	Total B _T
PHA	0.40	0.28	0.40	1.08
CHA	0.51	0.43	0.54	1.48
PFA	0.77	0.54	0.52	1.83
MIS	0.77	0.43	0.65	1.85

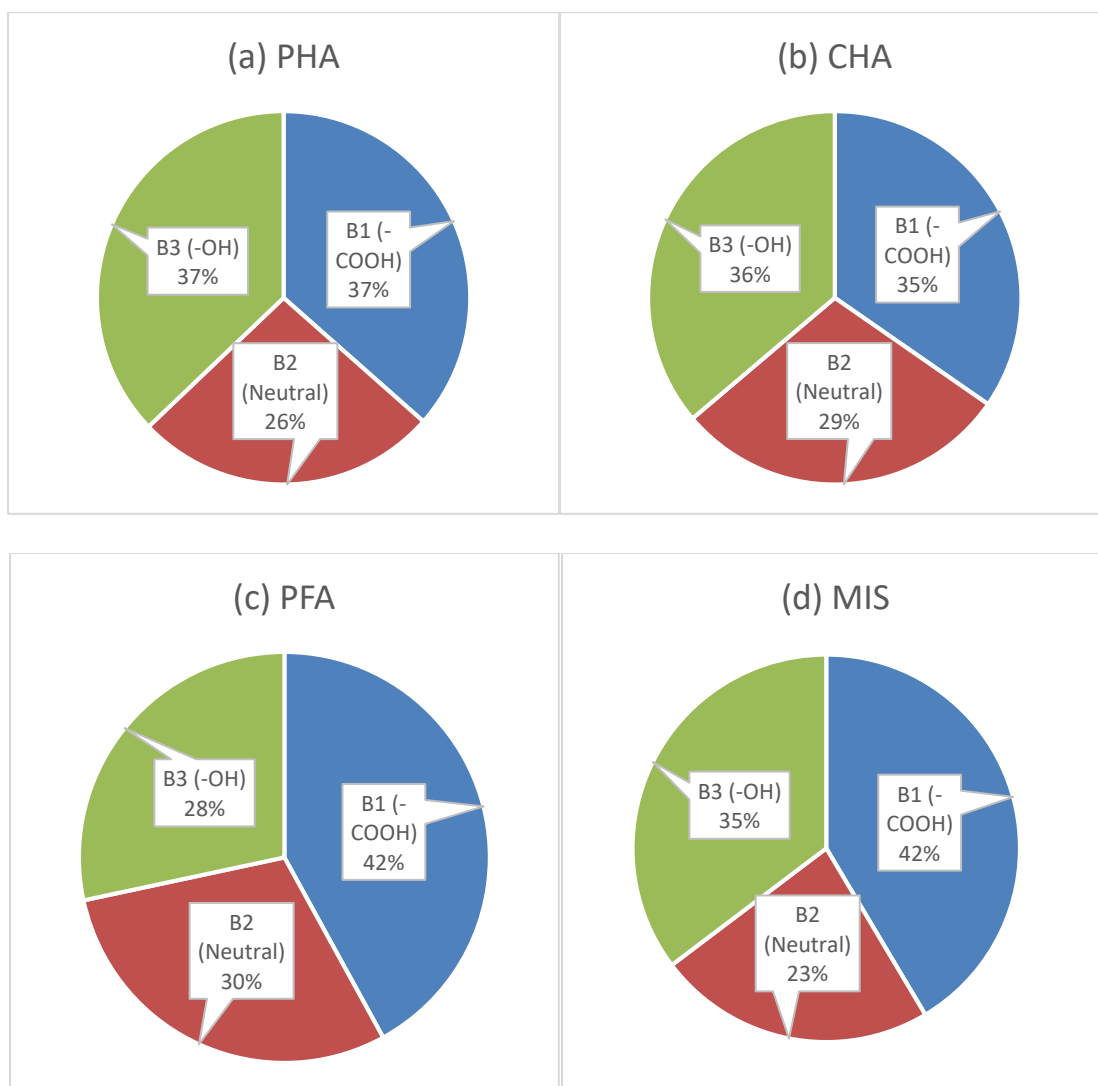


Figure 16. Pie charts (a), (b), and (c) correspond to the percent binding site density contribution from HS samples B₁ - COOH ($\text{pK}_a < 6$), B₂ - neutral ($6 < \text{pK}_a < 8$), and B₃ - OH ($\text{pK}_a > 8$) functional groups, as per Table 7.

Figure 16 shows the percent site density contributions in the form of pie charts. Subplots a, b, c and d correspond to each HS sample as labeled on the top of each chart. Binding site B₂, arbitrarily labeled as neutral, was added to the table to show the mathematical contribution to the HS besides carboxylic group and phenolic group for percentage calculation purpose even though functional group for the site was not identified in this study. Subplot a. PHA, indicates a carboxylic group site, neutral group site, and phenolic group site contribution of 37, 26, and 37% respectively. Subplot b. CHA shows the proportion of the site contribution of 35, 29, and 36%. Each site contribution clearly shows that the percentage of both humic acids, PHA and CHA, are similar to each other even though the pK_a values and the site density values are different. This suggests that contribution of carboxylic group and phenolic group to the humic acid are in approximately same percentage and the contribution of both functional groups are almost the same, 35~36%, as shown in the pie chart a. and b. of Fig 16. Each site density contribution of PFA and MIS (subplots c and d) show different results from HAs in percentage as well as pK_a values. As already discussed earlier, both PFA and MIS acids, showed fulvic acid like property in physical observation and pK_a values. PFA and MIS also showed the same percentage of carboxylic group and the approximately same pK_a values on carboxylic group. However, it is interesting that pK_a values of both PFA and MIS were very close, 4.17 ± 0.02 and 4.14 ± 0.02 , respectively. The percentage of carboxylic group higher in MIS and PFA by 7% than two HS samples, PHA and CHA which represents that a PFA and MIS compound contains more oxygen than carboxylic and phenolic groups. That PFA and MIS have higher percentages in carboxylic group

than the two HA samples is good agreement with that fulvic acid contains higher oxygen content compared to HA and humin (Hedges and Oades, 1997) because Soil humic acids typically show low H/C ratios near 1 (Schnitzer, 1978), consistent with a higher aromatic component than in corresponding fulvic acids (Hedges and Oades, 1997). This is also good agreement that PFA and MIS contains higher oxygen than PHA and CHA shown in the Table 1. Humic Substances Properties. Based on the observation in this work, it suggests that aquatic HS, MIS shows characteristic more like fulvic acid and soil HS, CHA, is more like humic acid. The lower value of the total site density of PHA compared to the other 3 samples (CHA, PFA and MIS) can be explained with that the numbers of carboxylic and phenolic groups might have been washed off during purification process in the lab along with other impurities as suggested earlier.

Temperature effects

The Influence of temperature on the acid dissociation properties of carboxylic and phenolic functional groups in selected HS samples was studied to obtain thermodynamic properties, including ΔG , ΔH and ΔS for proton dissociation.

Temperature experiments with potentiometric titrations at 6 °C, 25 °C and 35 °C were carried out and the results and the combined graph are shown in Table 8 and Fig. 17. As shown in Fig. 17, pK_a values of carboxylic group on both PHA and PFA seemed to stay about the same or went up very slightly along with temperature increase.

Table 8. pK_a values of selected HSs titrated at at 6 °C, 25 °C and 35 °C (SD = standard deviation)

HS samples	Temp (°C)	Carboxylic group (-COOH)		Phenolic group (-OH)	
		pK_a values	K_a values	pK_a values	K_a values
PFA	6 °C	4.14 ± 0.03	7.21E-05	9.12 ± 0.03	7.64E-10
	25 °C	4.17 ± 0.02	6.75E-05	9.59 ± 0.02	2.57E-10
	35 °C	4.14 ± 0.06	7.23E-05	9.42 ± 0.08	3.79E-10
CHA	6 °C	4.81 ± 0.04	1.54E-05	9.55 ± 0.04	2.81E-10
	25 °C	4.31 ± 0.08	4.88E-05	9.55 ± 0.06	2.81E-10
	35 °C	4.16 ± 0.06	6.89E-05	9.47 ± 0.00	3.43E-10
PHA	6 °C	4.84 ± 0.10	1.45E-05	9.43 ± 0.07	3.75E-10
	25 °C	4.88 ± 0.04	1.32E-05	9.70 ± 0.04	2.02E-10
	35 °C	4.85 ± 0.08	1.42E-05	9.74 ± 0.07	1.83E-10

However, CHA showed significant decrease on pK_a values of carboxylic group and slight decrease on pK_a value of phenolic group along with temperature increase. This shows that deprotonation of both carboxylic and phenolic group on CHA is more active at higher temperature which results higher acid dissociation constant.

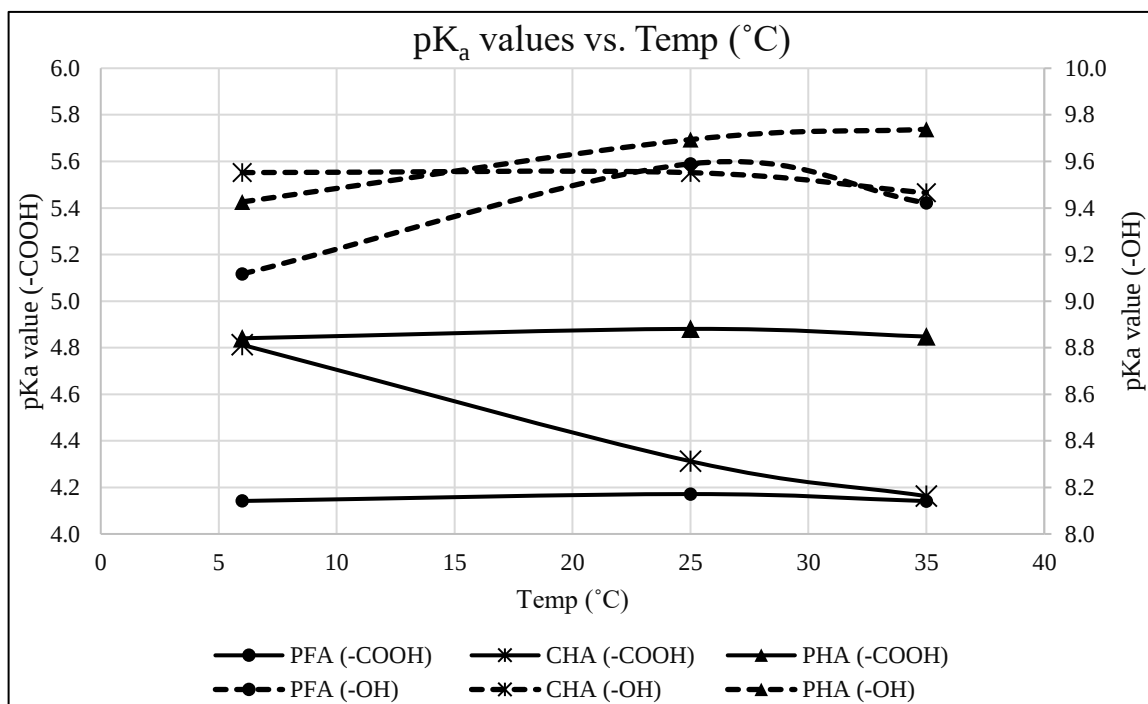


Figure 17. pK_a value changes depending on the temperature

The charge excess graph of CHA in Fig. 18 visualizes the charge excess dependence on temperature. At the temperature 6 °C, the net surface charge is the least and charge (mol/L) increased as temperature increased. This is the evidence that the dissociation of H^+ increases at higher temperature.

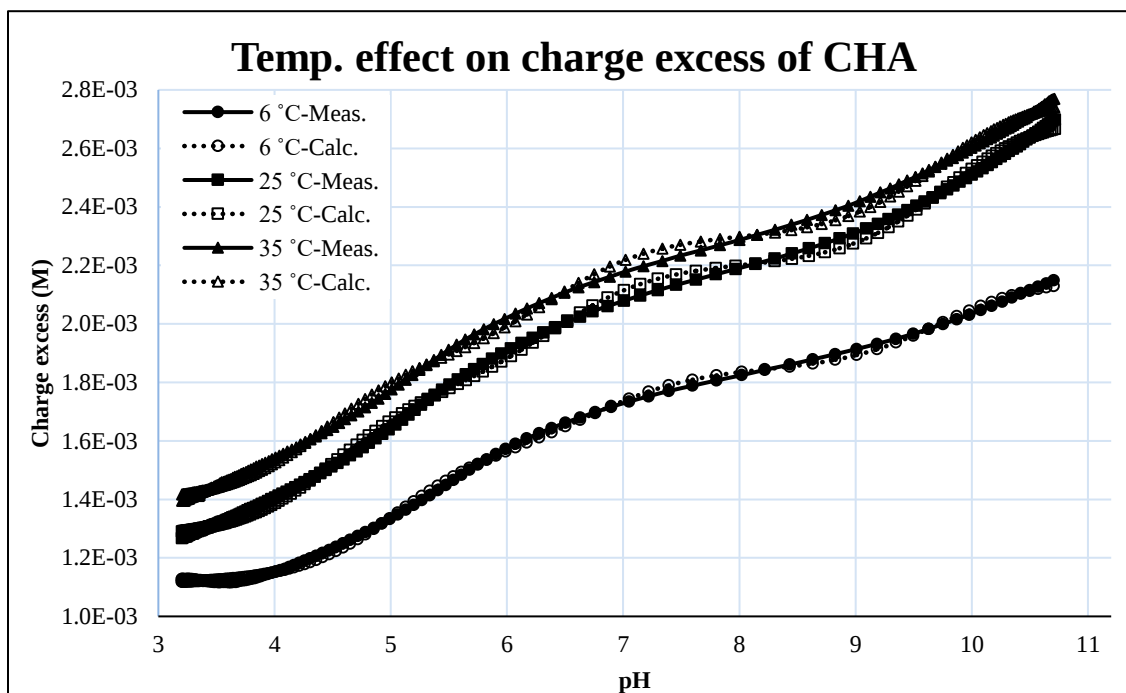


Figure 18. Charge excess of CHA dependence on temperature

Thermodynamic properties were evaluated along with determination of pK_a value. Gibbs free energy (ΔG), enthalpy (ΔH) and entropy (ΔS) were obtained using the titration data conducted at three different temperatures, 6 °C, 25 °C and 35 °C. According to the classic definition of thermodynamic terms, the reaction is endothermic when $\Delta H > 0$, and the reaction is exothermic when $\Delta H < 0$. When $\Delta S > 0$, the products are more disordered than the reactants and when $\Delta S < 0$, the products are less disordered than the reactants. For Gibbs free energy, when $\Delta G > 0$, the reaction is disfavored, and the reaction is favored when $\Delta G < 0$ in their standard states.

To obtain ΔG , ΔH and ΔS for proton dissociation, each value was calculated using the K_a values in the table 8. K_a values at different temperature were converted into $\ln K_a$ value to obtain ΔH values by running linear regression in Excel. ΔH value was calculated

using Eq. 6. The Gibbs free energy, ΔG , of deprotonation of the functional group at selected HS sample were determined by Eq. 7 using acid dissociation constant, K_a , which was determined by potentiometric titration with FOCUS model. Each ΔS value was then, calculated by applying the ΔH and ΔG to Eq. 6.

In thermodynamic terms, it is generally known that ΔG is temperature dependent and assumed that the ΔH and ΔS values are independent of temperature as long as the reaction does not involve a phase change. In this work, it is observed that ΔG was temperature dependent whereas ΔH and ΔS are not related to temperature changes. In Table 9. that ΔG value increased as the temperature increases except ΔG value of the carbolic group on CHA slightly decreased along with temperature increase. Even though ΔH was observed as independent on temperature in this research, it was observed increasing tendency of ΔH along with the ionic strength increase because rich counter ions such as K^+ and NO_3^- provided by the higher ionic strength condition is considered to reduce the de-hydration energies from (COO^-) and (H_3O^+) (Kimuro et al., 2015).

Table 9. ΔG , ΔH and ΔS values at 6 °C, 25 °C and 35 °C

HS Sample	Func. group	ΔH_{H^+} (kJ/mol)	ΔG_{H^+} (kJ/mol)			ΔS_{H^+} (kJ/mol-K)		
			6 °C	25 °C	35 °C	6 °C	25 °C	35 °C
PFA	-COOH	-0.36	22.12	23.80	24.42	-0.08	-0.08	-0.08
	-OH	-20.71	48.70	54.71	55.55	-0.25	-0.25	-0.25
CHA	-COOH	37.65	25.71	24.60	24.54	0.04	0.04	0.04
	-OH	4.16	51.02	54.49	55.81	-0.17	-0.17	-0.17
PHA	-COOH	-0.78	25.86	27.84	28.59	-0.10	-0.10	-0.10
	-OH	-18.42	50.35	55.31	57.42	-0.25	-0.25	-0.25

As shown in Table 10, ΔG values of acid dissociation (deprotonation) for -COOH group lie within close range which is average 25.41 ± 2.14 kJ/mol and for -OH group the average is 54.84 ± 0.42 kJ/mol which is in good agreement with the protonation ΔG value for acetic acid and phenol, -26.0 kJ/mol and -55.9 kJ/mol respectively (Martell et al., 2004). It suggests that ΔG value may be specific for functional group within certain range. In this work, all the ΔG values (Table 9 and Table 10) were positive which is normal when the dissociation constants are small. A reaction is thermodynamically spontaneous under standard conditions if ΔG° is negative or, equivalently, if $K > 1$ and the reaction is thermodynamically not spontaneous if ΔG° is positive ($K < 1$) (Harris, 2010).

Table 10. Thermodynamic Values for Dissociation of Humic Substances at 25 °C and I=0.01M

Humic Substance	Func. Group	pK _a	K _a	ΔG_{H^+} (kJ/mol)	=	ΔH_{H^+} (kJ/mol)	-	T ΔS_{H^+} (kJ/mol)	ΔS_{H^+} (kJ/mol-K)
PFA	-COOH	4.17	6.74E-05	23.80		-0.36		-24.15	-0.08
CHA	-COOH	4.31	4.87E-05	24.60		37.65		13.04	0.04
PHA	-COOH	4.88	1.32E-05	27.84		-0.78		-28.62	-0.10
PFA	-OH	9.59	2.57E-10	54.71		-20.71		-75.42	-0.25
CHA	-OH	9.55	2.81E-10	54.49		4.16		-50.33	-0.17
PHA	-OH	9.69	2.02E-10	55.31		-18.42		-73.73	-0.25

However, the temperature can be the deciding factor in spontaneity when ΔS and ΔH have opposite signs. In a chemical reaction, the reaction is clearly favored when ΔH is negative and ΔS is positive. When ΔH is positive and ΔS is negative, the reaction is clearly disfavored (Harris, 2010). If ΔH is negative, and -T ΔS positive, the reaction will be spontaneous at low temperatures (decreasing the magnitude of the entropy term) or if

ΔH is positive, and $-T\Delta S$ negative, the reaction will be spontaneous at high temperatures (increasing the magnitude of the entropy term). Among the HS samples used for this work, phenolic group of CHA can be determined based on the previous statement while all other data show that both ΔS and ΔH have same signs. Phenolic group of CHA has positive ΔH value and negative ΔS value, therefore it is more spontaneous at higher temperature as the K_a value at 35 °C is higher than that of at 6 °C and 25 °C.

The degree of disorder, Entropy (ΔS), were also determined with using Eq. 6 as shown in Table. 9 and Table. 10. In HS titration, the carboxyl or phenol is breaking into two parts upon dissociation, which is more disordered. However, only carboxylic group on CHA showed positive ΔS while none of other data showed negative ΔH and positive ΔS which represents thermodynamically spontaneous reactions.

Five of the ΔS values are negative which suggest the reactions have tendency toward less disordered state.

After running the data analysis on regression, the square of the correlation coefficient, R^2 values were obtained along with thermodynamic properties (Fig. 19). R^2 is a measure of goodness of fit of data points to a straight line. The closer R^2 is to 1, the better the fit is. For a major component of an unknown, a value of R^2 above 0.995 or, perhaps, 0.999, is deemed a good fit for many purposes (Harris, 2010). Fig. 19 shows the $\ln K_a$ graph plotted with the titration data along with $1/T$ based on Eq. 8 and displays each R^2 values on the graph. The R^2 values in this work distributed in the range of 0.034 ~ 0.990, as shown in Fig. 19. Only a few values fitted to a straight line in this work as shown in Fig 19. The most successful fit, $R^2=0.990$, was obtained with carboxylic group

on CHA, which demonstrated pK_a value dependency on temperature in Fig. 18. The least of R^2 was obtained from carboxylic group of PFA which measured data may be the least reliable compared to other data.

Even though the R^2 value is not scientifically reliable because many of the R^2 is a lot smaller than 1, it is still useful to determine if the reaction is whether it is endothermic or exothermic by looking at the linear regression graph. In Fig 19., negative slope means ΔH is positive therefore the reaction is endothermic (subplot c and d) and positive slope means ΔH is negative therefore the reaction is exothermic (subplot a, b, e and f).

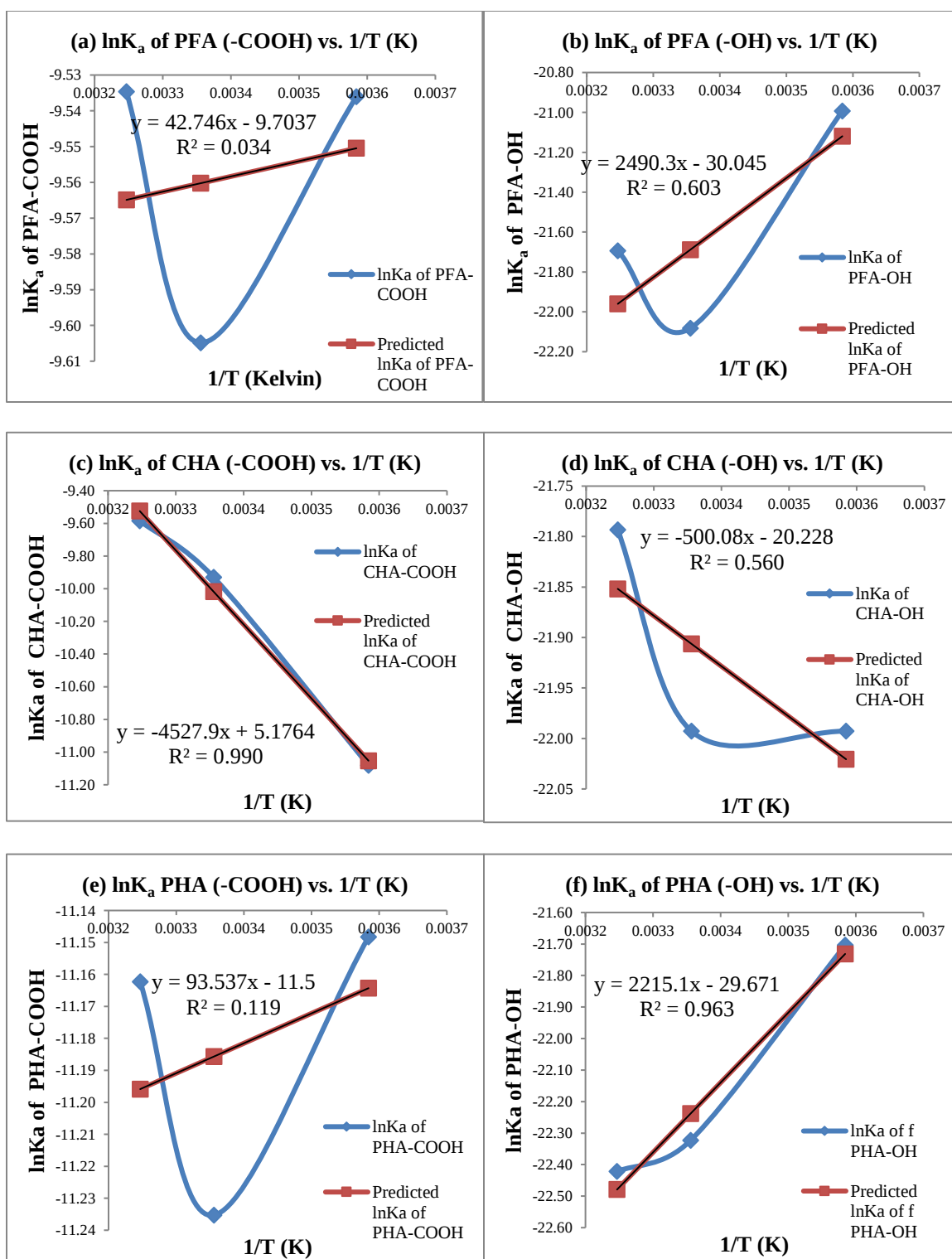


Figure 19. $\ln K_a$ vs $1/T$ (K) graph of -COOH and -OH on each HS sample

Model compounds

Small molecules of model compound that have common structural motifs in natural humic substances were evaluated to understand how the electronic environment immediately surrounding the carboxyl and phenol groups effects the magnitude of dissociation constants in humic substances. It is not clear that the small molecules reflect the dissociation process in the macromolecular nature of HS, however, this experiment is useful to determine structural variations in dissociation of proton. The pK_a values of the model compounds were evaluated via potentiometric titration using G10S. As mentioned earlier in this paper, benzofuran derivatives were selected as model motif for HS to evaluate influence on dissociation of the carboxyl and phenol group's structural variation on benzofuran rings.

The raw titration graph, G10S generated, shows nice and clean one reflection point for equilibrium that is a typical feature of monoprotic acid (Fig. 20). The titration results in Fig. 21 show that K_a value increases (i.e., pK_a decreases) as the carboxylic group is closer to the oxygen on furan ring. In other word, a carboxylic group close to the oxygen on furan ring dissociates better than a carboxylic group positioned further away. As a result, larger acid dissociation constant, K_a value is observed which means smaller pK_a value. The further the carboxylic group is positioned from the oxygen on the furan ring the larger the pK_a (i.e., smaller K_a) value is observed.

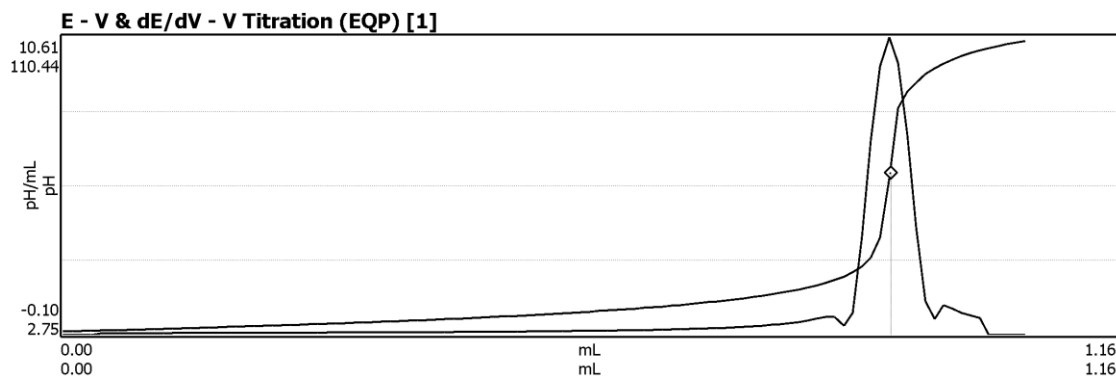


Figure 20. Titration curve and 1st derivative curve for a model compound, Benzofuran-2-carboxylic acid (B2CA), titrated with 0.1M KOH at 25 °C. One significant equilibrium point at pH 7.019 was observed.

Due to the limited availability of the model compounds in the market to purchase, it wasn't available to evaluate the influence of phenyl versus furan ring on carboxyl dissociation even though the pK_a on phenyl ring showed the largest value. This result suggests that pK_a value can change dramatically (from 3.21 to 4.63) depending on the position of carboxylic functional group on the same compound (Fig 21). Demonstration of different pK_a values on the same benzofuran ring is explained with inductive effect, more precisely negative inductive effect in this case. Oxygen is much more electronegative than hydrogen, thus, this cause electron-withdrawing effect on the carboxyl group. The closer the electron-withdrawing oxygen is to the carboxylic group, the stronger its effect in decreasing the pK_a because more protons are dissociated that increases K_a . The electron-withdrawing effect on the carboxylic group induces negative charge and increase the stability of the ionized form (Schwarzenbach et al., 2017).

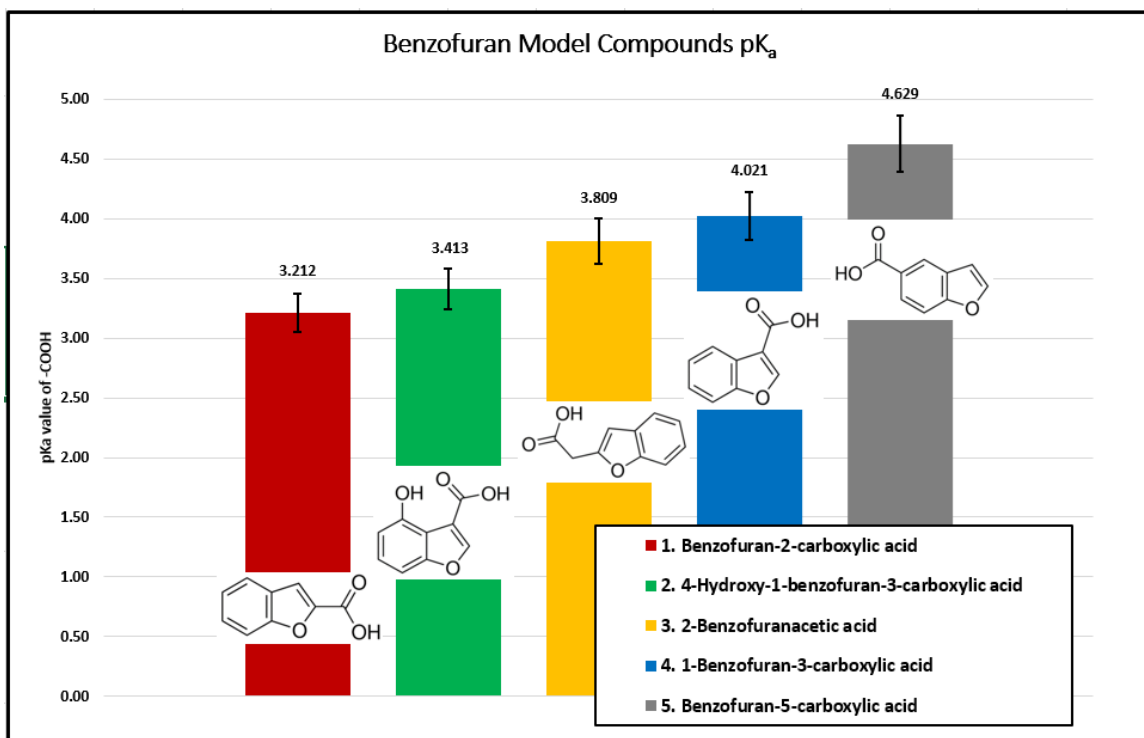


Figure 21. Each Benzofuran Model Compound and its pK_a value

The same result is also observed on furan carboxylic acids. 2-furoic acid has larger pK_a value (pK_a = 3.6) than 3-furoic acid (pK_a = 4.5) due to electron withdrawing effect (Bansal, 2008). Depending on the position of carboxylic group on acid, the dissociation rate can be as big as 10 folds or larger. As for the pK_a value, another interesting result was observed in that 4-Hydroxy-1-benzofuran-3-carboxylic acid (2nd bar in green) and 1-benzofuran-3-carboxylic acid (4th bar in blue) are the identical benzofuran compounds except for a phenolic group on 4-hydroxy-1-benzofuran-3-carboxylic acid. The chemical with the phenolic group showed smaller pK_a (pK_a=3.413) value than the one without phenolic group (pK_a=4.021). It is possible that negative inductive effect resulted the big difference between 4-hydroxy-1-benzofuran-3-carboxylic acid (2nd bar in green) and 1-

benzofuran-3-carboxylic acid (4th bar in blue) because OH is also known as negative inductive substituents (Schwarzenbach et al., 2017) and the OH group is positioned near the carboxylic group on the compound possibly induced electron withdrawing that lower the pK_a value on 4-hydroxy-1-benzofuran-3-carboxylic acid. Another possibility is proximity effect. As illustrated in Fig. 22, the effect of intramolecular hydrogen bonding on hydroxy-benzoic acid leads to a big difference in pK_a value in the two isomers. The stabilization of the carboxylate anion formed by the hydroxyl hydrogen in ortho-hydroxy-benzoic acid leads to a much lower pK_a value (pK_a = 2.97) compared with para-hydroxy-benzoic acid (pK_a = 4.48), in which no intramolecular hydrogen bonding is possible (Schwarzenbach et al., 2017). The big difference in pK_a between 4-hydroxy-1-benzofuran-3-carboxylic acid (2nd bar in green) and 1-benzofuran-3-carboxylic acid (4th bar in blue) can be explained with the same proximity. The intramolecular hydrogen bonding between carboxylic group on furan ring and phenolic group on benzene ring stabilize the carboxylate anion and this results much smaller pK_a. Even though those two substituents were positioned on two different rings one each, their physical distance was probably close enough to form a hydrogen bond.

In thermodynamic perspective, larger K_a (i.e. smaller pK_a) value means that ionized form is more favorable than natural form of the compound in aqueous solution. In this case, the Gibbs free energy, ΔG^0 decreases when ionized form increases.

For the ΔS and ΔH analysis of acid dissociation on carboxylic group, Benzofuran-2-carboxylic acid (B2CA) titration data may be a good index for thermodynamic property

since B2CA contains only one carboxylic group on it. Any changes made during titration in values along with temperature changes means mostly changes on carboxylic group.

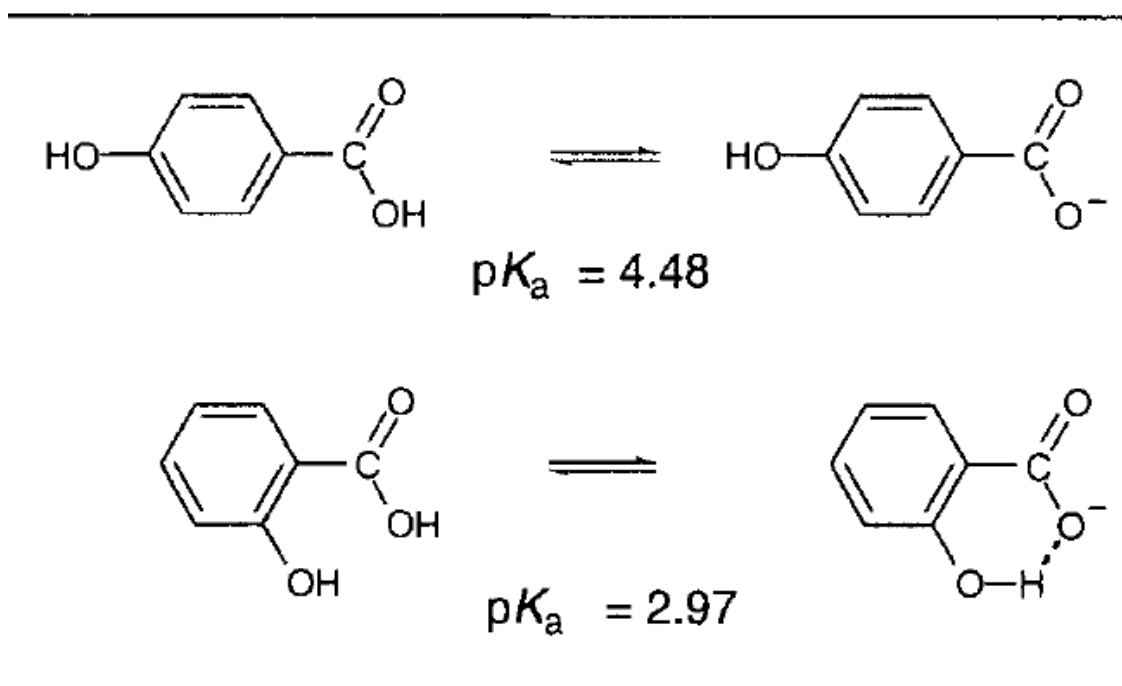


Figure 22. Examples of proximity effect on the acidity constant: hydrogen bonding (Schwarzenbach, 2017)

However, R² value (=0.670) is quite lower however, the linear regression is good enough to determine whether ΔH is positive or negative and the B2CA thermodynamic data may be used as a reference to evaluate temperature effect on a carboxylic group on the compound.

In this work, it was observed that pK_a value went up as the temperature increased which suggests deprotonation on carboxylic group increases at higher temperature. The

ΔG value also increased as the temperature increased. Thermodynamic values in this work showed that both negative values of ΔS and ΔH which doesn't give enough data to determine if this reaction is favored or not. With the positive ΔG , it can be terminated that the reaction is not spontaneous and disfavored.

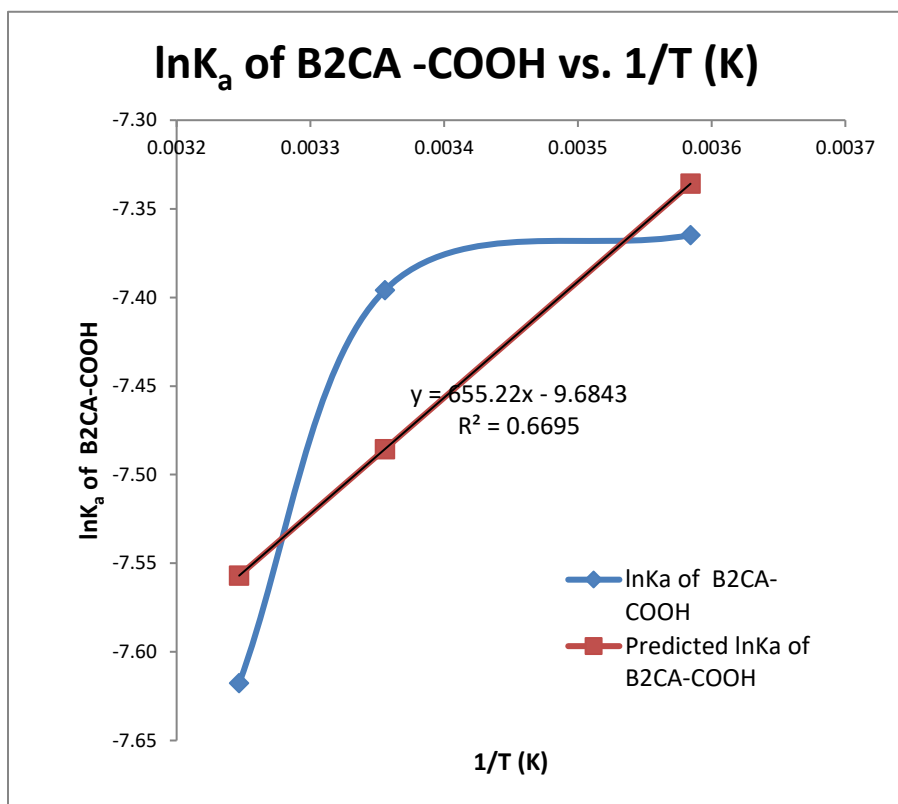


Figure 23. B2CA Linear regression graph of lnK_a

Table 11. Thermodynamic data of Benzofuran-2-carboxylic acid (B2CA)

Benzofuran-2-carboxylic acid (B2CA)				
Temp.	279 K	298 K	308 K	Note
K_a	6.331E-04	6.138E-04	4.917E-04	
pK_a	3.20	3.21	3.31	
ΔG_{H^+} (kJ/mol)	17.08	18.32	19.51	Reaction is disfavored
ΔS_{H^+} (kJ/mol-K)	-0.081	-0.081	-0.081	Products less disordered than reactants
ΔH_{H^+} (kJ/mol)	-5.45			Heat is absorbed (Endothermic)

CONCLUSION

To characterize the acid-base properties of selected humic substances, potentiometric titration was performed on the HSs and the model compounds. Potentiometric titration is the most common method for determining acid groups in HSs and it was used to determine the surface charge excess, K_a and pK_a values of carboxylic and phenolic group on each selected HS. With fitting data to FOCUS model, it was available to estimate pK_a values of carboxylic and phenolic group of each HS with the assumption that each HS is a mixture of monoprotic acids. In this study, the pK_a values found to be in the range of 3.890 ± 0.018 to 4.881 ± 0.036 for carboxylic group and 8.712 ± 0.017 to 9.738 ± 0.071 for phenolic group. These values are in good agreement with literature. FOCUS model was usefully used to determine the surface acidity on a bacterial cell by Martinez et al. and it was found that FOCUS model can be used for HS analysis through this study. Thermodynamic properties, ΔH , ΔS and ΔG , were determined with titration carried out at 6 °C, 25 °C and 35 °C. However, every functional group on every HS had different property so it wasn't available to find out common properties within those HSs.

Model compound experiments suggested that pK_a value changes depending on the position of carboxylic group on the same compound. Temperature effect on carboxylic group on a model compound was also observed that pK_a value went up as the temperature

increased which means that acidity decreased as temperature increased. To evaluate the effect of electro and stereochemical properties on the model compound, additional studies need to be followed.

APPENDIX A: FOCUS MODEL AND SOLVER OPTIMIZATION PROCEDURE

To estimate the unknown parameters K_a , B_j and S_o in Eq. 5, a nonlinear fitting-optimization (FOCUS model, fully optimized continuous pK_a spectral) was performed by Solver and was available in Excel. FOCUS optimization provided pK_a spectra for the humic substances upon minimization of the error between the measured and calculated charge excess quantities. Solver can extract these parameters by comparing the experimental values of $Q_{\text{mas},i}$ (Eq. 2) with the calculated values of $Q_{\text{calc},i}$ (Eq. 5) through a residual minimization routine. Solver extracts four K_{a_i} and $[B_j]$ values corresponding to unique dissociation sites.

Equation 2

$$Q_{\text{mas},i} = C_{bi} - C_{ai} + [H^+]_{\text{bulk},i} - [OH]_{\text{bulk},i}$$

Equation 5

$$Q_{\text{calc},i} = \sum_{j=1}^m \left(\frac{K_{aj}^{\text{app}}}{K_{aj}^{\text{app}} + [H]_{\text{bulk},i}} \right) \times [B_j] + S_o$$

Once a predicted K_a value is entered for each site, Solver displayed the values in the example below in Table A for purified Aldrich humic acid.

The Solver result shown in Table A was derived using a FOCUS model for four dissociation sites. Solver extracted three legitimate values, pK_{a1} through pK_{a3} ; the fourth

site had a site density (B_{j4}) that was too low to be meaningful as shown in Table B. For that reason, Solver yielded only meaningful pK_a values. FOCUS yielded pK_a values for carboxylic group (pK_a 4.92~4.93) and phenolic groups (pK_a 9.73~9.75) based on the literature. However, it was not possible to assign a particular acidic functional group to the third binding site pK_{a2} (BS 2 is Table A).

Table A. Tetraprotic model Solver result of 0.3 mg/ml purified Aldrich humic acid (PHA) titrated with 0.1M KOH at 25 °C.

Binding Site	K_a	pK_a	mg/mL	0.30	Humic sample concentration
BS 1	1.41E-05	4.85	C_a	0.0001	Initial acid concentration
BS 2	1.10E-07	6.96	C_b	0.0954	Initial base concentration
BS 3	2.31E-10	9.64	V_a	50.50	Volume of HS analyte to titrate
BS 4	1.00E-11	11.00			
MW	(Eqiv wt)	2002.71			
$pK_{a1}B_{j1}$	5.41E-09	8.27	B_{j1}	3.84E-04	M
$pK_{a2}B_{j2}$	3.17E-11	10.50	B_{j2}	2.88E-04	M
$pK_{a3}B_{j3}$	9.05E-14	13.04	B_{j3}	3.93E-04	M
$pK_{a4}B_{j4}$	7.87E-23	22.10	B_{j4}	7.87E-12	M
S_o	1.01E-03	2.99	B_j -Total	1.06E-03	M (mols/L)
SSQR		1.93E-08			

The model was considered more reliable if entering different initial guesses of pK_a yielded similar optimized values of pK_a , which occurred with the tetraprotic FOCUS model (Table B). The average values in Table B were used for the data analysis and standard deviation calculation.

Table B. Repeated solver results for tetraprotic model in Table A

pK _{ax}	It1	It2	It3	It4	It5	It6
pK _{a1}	4.85	4.85	4.85	4.84	4.86	4.85
pK _{a2}	6.96	6.97	6.96	6.95	6.99	6.97
pK _{a3}	9.64	9.65	9.64	9.64	9.65	9.64
pK _{a4}	11.00	11.00	11.00	11.00	10.00	10.00
MW	2003.77	1003.65	1985.11	1064.34	1961.16	1043.74
pK _{a1} B _{j1}	8.27	8.26	8.27	8.26	8.27	8.26
pK _{a2} B _{j2}	10.50	10.51	10.50	10.49	10.53	10.51
pK _{a3} B _{j3}	13.04	13.05	13.04	13.04	13.06	13.05
pK _{a4} B _{j4}	22.10	22.10	22.10	22.10	22.10	22.10
So	2.99	3.06	3.00	3.05	3.00	3.06
SSQR	1.93E-08	1.98E-08	1.93E-08	1.97E-08	1.93E-08	1.97E-08

Diprotic and triprotic Solver models were also tested before selecting the tetraprotic model as the preferred estimation method; however, those two models did not extract the pK_a values with the lowest SSQR values or provide values that were consistent with literature pK_a values for humic substances. The diprotic model (Table C) yield two Ka values, but with a large SSQR. The SSQR values were the lowest in the tetraprotic model, so, thus, it was considered the most reliable.

Table C. Diprotic model Solver result of 0.3 mg/ml purified Aldrich humic acid (PHA) titrated with 0.1M KOH at 25 °C.

Binding Site	K _a	pK _a	mg/mL	0.32	Humic sample concentration
BS 1	6.86E-04	3.16	C _a	0.0006	Initial acid concentration
BS 2	1.24E-11	10.91	C _b	0.0860	Initial base concentration
			V _a	50.00	Volume of HS analyte to titrate
EqWt		1266.75			
SSQR=		0.2223			

To resolve the issue present in the diprotic model, the triprotic model was used (Table D), expecting well spread pK_a values throughout three binding sites. However, it yielded three dissociation sites with pK_a values inconsistent with literature reports. One of the pK_a values was close to 1, which is an unknown dissociation site. The SSQR value in this case was too large to consider adoption of it as a final model. The same issue present in the diprotic model was present as well. With the tetraprotic model, meaningful pK_a values for three major binding sites were successfully extracted with very low SSQR values.

Table D. Triprotic model Solver result of 0.3 mg/ml purified Aldrich humic acid (PHA) titrated with 0.1M KOH at 25 °C.

Binding Site	K_a	pK_a	mg/mL	0.30	Humic sample concentration
BS 1	1.00E-01	1.00	C_a	0.0007	Initial acid concentration
BS 2	8.24E-04	3.08	C_b	0.0954	Initial base concentration
BS 3	6.02E-08	7.22	V_a	50.50	Volume of HS analyte to titrate
EqWt		932.09			
SSQR=		0.2452			

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