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Article

Potentiometric Acidity Determination in Humic Substances Influenced by Different Analytical Procedures

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Os valores de acidez carboxílica (CA), fenólica (PhA) e total (TA) de um conjunto de cinco amostras padrão da Sociedade Internacional de Substâncias Húmicas (IHSS) e de quatro compostos modelo foram determinados pelo método de titulação potenciométrica. Os dados das curvas de titulação foram tratados pelo programa BEST7 assim como pela equação de Henderson-Hasselbalch modificada (MHHM) e os parâmetros obtidos mostraram-se extremamente dependentes do modelo matemático aplicado. Para as amostras de substâncias húmicas (SH), a aplicação da MHHM resultou em uma alta CA e uma baixa PhA, sendo o resultado praticamente oposto quando o programa BEST7 foi empregado. No caso dos compostos modelo entretanto, os valores de acidez convergiram aos valores esperados (teóricos) independente do modelo aplicado. Para investigar as razões das discrepâncias, variações na condução das titulações (p.e., titulação rápida ou lenta) também foram consideradas. O conjunto de informações mostrou que a determinação da acidez de SH por titulação potenciométrica é extremamente dependente do modelo matemático, assim como das condições experimentais aplicadas.

Carboxylic (CA), phenolic (PhA) and total (TA) acidity contents of five International Humic Substances Society (IHSS) standards and four model compounds were determined via the potentiometric titration method. Titration curves were scrutinized both by the BEST7 algorithm and the modified Henderson-Hasselbalch model (MHHM). In the case of IHSS samples, the fitting data depended on the analytical procedure undertaken. Whilst high CA and low PhA were usually recorded using the MHHM, the opposite trend was observed employing the BEST7 algorithm. In contrast, in the case of model compounds the acidity values matched well with theoretical data regardless of the procedure. In order to better understand the reasons for such discrepancies changes in the titrations procedure (e.g.: fast or slow) were also considered. General data strongly suggest that acidity determination of humic substances (HS) by potentiometric methods is extremely dependent on both the choice of mathematical model to fit experimental data points as well as the experimental conditions employed.

Keywords: humic substances, potentiometry, carboxylic acidity, phenolic acidity, total acidity

Introduction

Interaction between trace metals and natural organic matter (OM) is of great importance for nutritional, transport and sedimentary processes of the latter. However, the understanding of physical-chemical processes associated with chelation reactions still remains a challenge, fundamentally due to the dynamic nature and complexity of OM systems. Modeling such interactions requires, among other parameters, accurate determination of acid-base properties (concentrations and equilibrium constants).[1-9] A major fraction of dissolved OM in natural ecosystems is in fact formed by humic substances (HS), which are characterized by the presence of several functional groups with labile protons such as carboxylic acids, phenols and amines. These moieties are able to bind protons and metal cations that not only affect the geochemistry of natural systems, but also regulate the buffer capacity of waters, soils and sediments, and metal speciation and transport.[1-5] The

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assessment of concentrations and equilibrium constants of carboxylic and phenolic groups is, therefore, a key step in the characterization of acidity, ion exchange capacity, and charge development properties of HS systems.[3,6-9]

Within this context, two main approaches have been employed in the determination of acid contents of fulvic acid (FA) and humic acid (HA) systems: indirect and direct titrations. In the first case, the analyses consist in the titration of filtered reaction mixtures to a fixed pH end-point after a 24 h equilibrium time with either barium hydroxide or calcium acetate, and have the advantage of simplicity.[10,11] Direct potentiometric titrations, on the other hand, provide more details on the thermodynamics of proton binding processes since the solution pH is monitored continuously as increments of a given titrant are added.[5] However, the lack of well-defined inflection points in the titration curves and the overlap of wide ranges of pK_a-values associated with acid groups in FA and HA structures, makes difficult the task of characterizing unequivocally the contribution from each chemical function. Other possible sources of inaccuracy are the downward pH drifts that suggest the occurrence of acid-generating side reactions and assumptions implied in the mathematical model used to fit experimental data points.[4-6]

In this work, direct and indirect titrations were carried out in order to determine acid-base properties of a set of International Humic Substances Society (IHSS) standard samples. The potentiometric data collected during the titration experiments were then studied by two distinct approaches: the modified Henderson-Hasselbalch model (MHHM) formerly employed by Ritchie and Perdue[5] and the BEST7 algorithm.[6,12] The main goal in such an endeavor is to evaluate the influence of analytical methods on FA and HA acidity quantifications. In order to get insight into these aspects, experiments using different model compounds were also performed.

Experimental

Materials

Five IHSS standard samples were selected for this investigation: two FA (Suwannee River FA (1S101F), Pahokee Peat FA (1S103F)) and three HA (Pahokee Peat HA (1S103H), Elliot Soil HA (1S102H) and Leonardite HA (1S104H)). The model systems were comprised of two benzoic acid derivatives (phtalic acid and 3,5-dihydroxybenzoic acid), an aldehyde (4-hydroxybenzaldehyde) and a dipeptide (DL-alanyl-DL-alanine).

All solutions were prepared using twice-distilled CO_2-free water and chemicals of the highest purity available from Sigma-Aldrich without any further purification, unless otherwise specified. The pH measurements were done using a Micronal B 375 pH-meter equipped with a glass electrode and an Ag/AgCl reference electrode.

Direct potentiometric titration

Titration experiments were carried out using standardized 0.10 mol L^{-1} KOH solutions under stirring in electrochemical cells thermostated at 25 ºC. An inert atmosphere was maintained throughout the experiments using argon gas which was previously washed with 5% pyrogallol in 0.10 mol L^{-1} KOH in order to eliminate O_2 and CO_2. In all measurements, the ionic strength (I = 0.10 mol L^{-1}) was controlled with KCl. Before each experiment, the system was standardized with dilute HCl solutions (pH *ca.* 2.0 and I = 0.10 mol L^{-1}). Solutions containing 140 mg L^{-1} HA and 250 mg L^{-1} FA were prepared directly in the electrochemical cell as follows. Typically, in a 10.0 mL aliquot of 0.02 mol L^{-1} KOH were dissolved 10.0 mg of HA. Subsequently, 35.0 mL of water and 25.0 mL of 0.01 mol L^{-1} HCl were added to neutralize the excess of KOH and reduce the pH. In the case of FA, in a 40.0 mL aliquot of twice-distilled CO_2-free water were dissolved 10.0 mg of sample. Finally, the titrant was added in 0.02 mL aliquots, allowing the solutions to reach equilibrium (*i.e.*: constant pH readings) after each increment. Solutions from model compounds were prepared similarly by dissolving 10.0 mg of each compound in 40.0 mL of water. All measurements were done starting pH to *ca.* 3.0 and in triplicate.

The modified Henderson-Hasselbalch model (MHHM)

Titration curves were firstly analyzed following strictly the MHHM and procedures described in detail in literature by Ritchie and Perdue.[5] The reader is referred to the cited article and references therein for a comprehensive MHHM description. Here, we provided a brief outline of the method, which consists in fitting the titration data for two classes of proton-binding sites (carboxylic groups and phenolic groups) in order to extract the parameters that satisfactorily describe the curves. To this end, the total charge (Q_{TOT}) in solution originated from organic compounds is calculated at each titration point on the basis of the equation (equation 1) electroneutrality and taking into account the dilution-corrected concentrations of all species in solution, assuming that IHSS samples consist in a mixture of monoprotic acids. Q_{TOT} is obtained by normalizing equation 1 to the dilution-corrected concentration of dissolved OM and plotted against the solution pH. The resulting curves are fitted according to the MHHM equation (equation 2), whose fitting parameters are Q_1 and Q_2 (concentrations of

carboxylic acid and phenolic groups, respectively), K_1 and K_2 (respective aqueous dissociation constants), and n_1 and n_2 (constants reflecting the range of pK_a-values within each distribution of proton-binding sites). A nonlinear iterative fitting routine was applied in this work using Origin software. The process was completed (convergence was reached) when the difference between reduced chi-square values of two successive iterations is less than certain tolerance value (confidence level for curves = 95%).[5]

$$\sum_i [Org_i^-] = [Na^+] + [H^+] - [Cl^-] - [OH^-] \quad (1)$$

$$Q_{TOT} = \left(\frac{Q_1}{1 + (K_1[H^+])^{1/n1}} \right) + \left(\frac{Q_2}{1 + (K_2[H^+])^{1/n2}} \right) \quad (2)$$

The BEST7 algorithm

The BEST7 algorithm has been successfully employed in our laboratory[6] and elsewhere[5,12] to resolve equilibrium data of multiple ligands, both in presence and absence of metal ions. The algorithm is capable of determining equilibrium constants of ligands and complexes, as well as their accurate concentration. In general, the input is the variation of pH as a function of added base or acid, the number of mmol of all reagents (ligands, metals, acids or bases), the initial volume and the set of constants relative to all known equilibria existing in the system. Unknown equilibrium constants can be estimated by comparison with values reported for systems with similar characteristics. The approximate amount of carboxylic acid and phenolic groups, in mmol, was previously determined by the Gran's function.[13] Finally, the mass balance for each species present in solution is established and the equations for hydrogen ion concentrations are resolved by least squares fitting method.

Indirect titration

The acidity of the IHSS standards was also evaluated by the indirect titration method formerly devised by Schnitzer and Gupta.[10] Briefly, ion exchange reactions involving HS and either calcium acetate or barium hydroxide are performed in order to determine the carboxylic acidity (CA) and the total acidity (TA), respectively. The difference between these two values is then ascribed to phenolic acidity (PhA). In a typical experiment, 10.0 mg of HS were dissolved in 10.0 mL of 0.20 mol L^{-1} $Ca(CH_3COO)_2$. Subsequently, the mixture was diluted to 50.0 mL with water and maintained under inert atmosphere for 24 h. Then, the supernatant was separated by filtration and the precipitate was further washed with water. The mixture was filtrated and liquid phase added to the previous supernatant. CA in the resulting solution was finally determined by potentiometric titration using 0.02 mol L^{-1} NaOH as titrant under inert atmosphere. The TA was determined following a similar procedure, however using 0.05 mol L^{-1} $Ba(OH)_2$ in the dissolution step and 0.10 mol L^{-1} HCl as titrant. Solutions from model compounds were prepared similarly by dissolving 10.0 mg of each compound in 50.0 mL of water. All measurements were done in triplicate.

Results and Discussion

HS titrations

CA, PhA and TA values of the IHSS samples as determined from titration curves resolved by the MHHM and BEST7 approaches are given in Table 1. A careful analysis of these results straightforwardly reveals that potentiometric acidity quantification in HS can be strongly influenced by data treatment procedure. Furthermore, the behavior might vary from one series of samples to another. Indeed, according to titration data summarized in Table 1 for both FA- and HA-containing systems, the CA values estimated using the BEST7 algorithm were in general much lower than those recorded employing the MHHM. In the case of PhA values, fairly consistent results were observed among the FAs (Table 1, entries 1 and 2), whereas for HAs (Table 1, entries 3-5) the values generated by the BEST7 program differed deeply from those obtained by the MHHM. In this latter case, however, it is very interesting to note the TA was approximately constant regardless of the analytical method. This remark suggests that the CA and the PhA might have been computed differently in each case (*i.e.*: CA < PhA using the BEST7; CA > PhA using the MHHM). At this point, it is worth noting that Ritchie and Perdue[5] had investigated the acidity of a series of 14 standard and reference materials from the IHSS using MHHM approach. Those authors found practically the same results as discussed above for the same samples listed in the Table 1 (*i.e.*: CA > PhA).

Given such highly important observations and their potentially profound implications for correct understanding of HS systems with complex chemical structures and dynamics, the raw titration curves and procedures were investigated in detail as given. The experimental titration data and best fit curves for two selected IHSS samples (Table 1, entries 2 and 4) as obtained using the BEST7 program and the modified Henderson-Hasselbalch model (MHHM) are shown in Figure 1A and Figure 1B, respectively. At a glimpse, both methods (BEST7 and MHHM) described

Table 1. Acidity of IHSS samples as determined using the MHHM and BEST7 methods

Entry	Sample	Method	CA[a] / (mequiv. g^{-1})	PhA[a] / (mequiv. g^{-1})	TA[a] / (mequiv. g^{-1})
Fulvic Acids (FA)					
1	Suwannee river (1S101F)	BEST7	2.54 ± 0.30	1.24 ± 0.10	3.78 ± 0.31
		MHHM	6.06 ± 0.26	1.89 ± 0.70	7.95 ± 0.74
2	Pahokee peat (1S103F)	BEST7	2.69 ± 0.30	1.22 ± 0.32	3.91 ± 0.44
		MHHM	5.89 ± 0.50	1.65 ± 0.37	7.54 ± 0.62
Humic Acids (HA)					
3	Elliot soil (1S102H)	BEST7	4.59 ± 0.32	10.24 ± 0.51	14.83 ± 0.60
		MHHM	11.08 ± 0.29	1.92 ± 0.44	13.00 ± 0.53
4	Pahokee peat (1S103H)	BEST7	4.88 ± 0.25	11.15 ± 0.56	16.90 ± 0.50
		MHHM	13.50 ± 0.32	2.50 ± 0.50	16.00 ± 0.59
5	Leonardite (1S104H)	BEST7	4.18 ± 0.39	11.51 ± 0.52	15.69 ± 0.35
		MHHM	11.19 ± 0.45	1.61 ± 0.62	12.80 ± 0.75

[a] Abbreviations: CA = carboxylic acidity, PhA = phenolic acidity and TA = total acidity. Standard deviations obtained from three replicates.

satisfactorily the proton-binding properties of the samples, as long as the fitting curves are in excellent agreement with the experimental data points. In such HS systems, two distinct proton-binding sites are known to exist, which are attributed to CA and PhA groups, as mentioned earlier in the text. Depending on their respective pK_a range, titration of these moieties results, presumably, in two inflection points in the titration curve due to the consumption of base in the acid pH range for neutralization of carboxylic groups, and in the moderately basic pH range for neutralization of phenolic groups. As is evident in Figure 1 for the Pahokee Peat HA sample, two well-defined pH buffer regions do exist in such a case (Figure 1A, see the arrows). When the same experimental data set was handled in order to apply MHHM, however, poorly defined or no discrete inflection points were detected (Figure 1B). In fact, such a behavior is frequently revealed in studies dealing with the potentiometric properties of HS,[4,14,15] being attributed to the overlap of a wide range of pK_a-values associated with a diversity of acid groups.

The observation that both data treatment approaches describe in detail the raw experimental data (curve fittings), but give different information on the relative carboxylic and phenolic acidities is, at least, an intriguing issue.

Following, we address the different aspects related to the aqueous dissociation behavior of HS and how they are taken into consideration during the analysis. As one can anticipate, the choice of any proposed mathematical model to fit experimental data points implies the assumption of a number of statements and boundary conditions, which can obviously have different impacts on the outcomes. For example, the influence of pK_a- and *n*-values on the overall profile of Q_{TOT} *vs.* pH curves derived from the modified Henderson-Hasselbalch equation (equation 1) is shown in

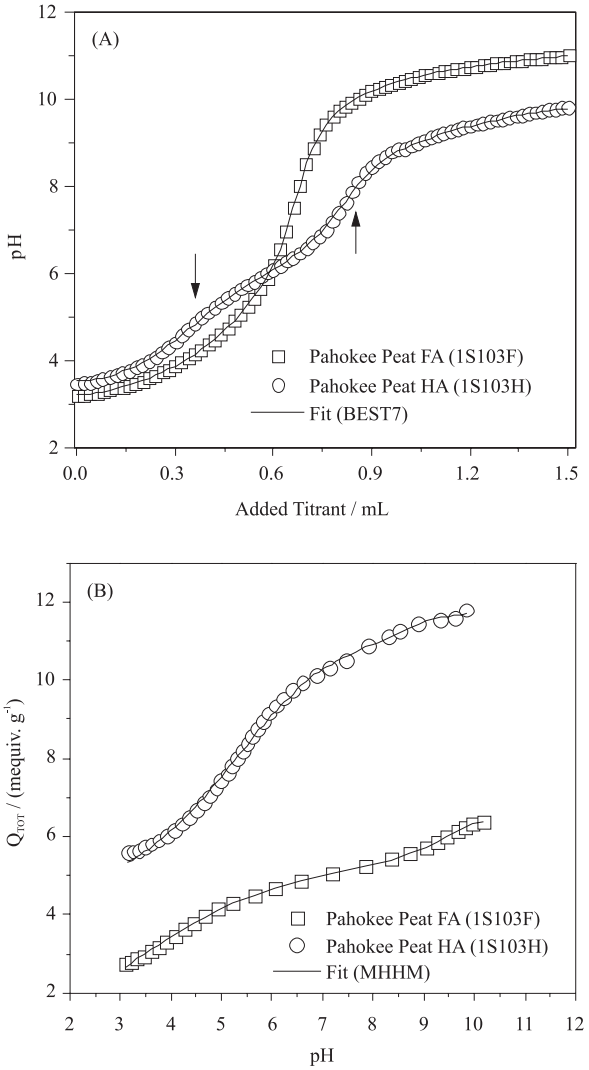


Figure 1. Titration data and fitting curves as obtained using the BEST7 (A) and MHHM (B) methods for selected IHSS samples.

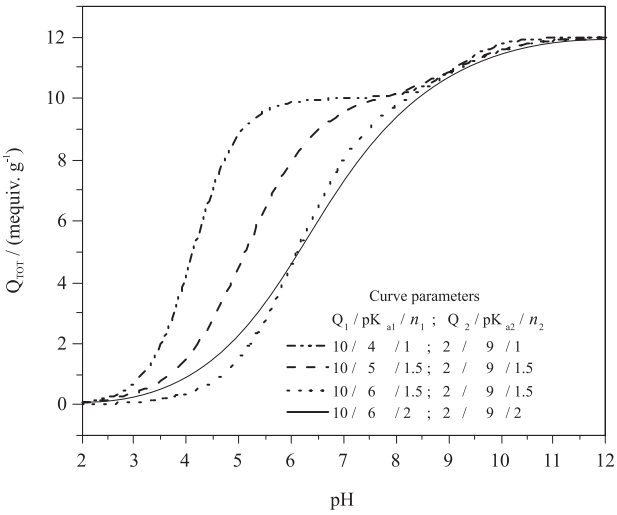


Figure 2. Influence of pK_a- and *n*-values on the overall profile of Q_{TOT} *vs.* pH curves derived from the MHHM.

Figure 2 for a hypothetical HS system. These plots clearly illustrate the difference between systems with well-defined proton-binding properties (*i.e.*: small pK_a variations (small n_1 and n_2) within adequately separated ranges corresponding to carboxylic and phenolic groups; pK_{a1} well-apart from pK_{a2}) and those formed by substances exhibiting a complex and diverse mixture of proton-binding functions (*i.e.*: large pK_a variations (large n_1 and n_2) sometimes with overlapping within each distribution of functional groups; pK_{a1} close to pK_{a2}). In this latter case, the inflection points almost disappeared, as indicated in Figure 2 (solid line). Therefore, it is absolutely not surprising that in HS titrations a fraction of phenolic groups may in fact be computed as carboxylic acid content,[16] hence leading, some times, to overestimated CA values. For this reason, carboxylic equivalence (or CA) is often taken at pH-values lower than equivalence point. Likewise, other chemical groups with intermediate pK_a-values such as amines might contribute to eventual inconsistencies in the pH-based determinations.[17,18]

Model compound titrations

In order to gain insight into these questions, solutions with well-known acid-base properties (model compounds and mixtures) were studied following exactly the same procedure as above. Phtalic acid, 3,5-dihydroxybenzoic acid, 4-hydroxybenzaldehyde, DL-alanyl-DL-alanine can be regarded as model compounds since they reasonably simulate the equilibrium properties of HA and FA solutions. For instance, the 3,5-dihydroxybenzoic acid presents carboxylic acid and phenolic groups within a wide range of pK_a-values (pK_{a1}(R-COOH) = 3.90, pK_{a2}(Ph–OH) = 9.03, pK_{a3}(Ph–OH) = 10.30).[19] Meanwhile, phtalic acid does not have phenolic moieties, but instead it has two carboxylic acid groups with distinct dissociation constants (pK_{a1}(R-COOH) = 2.78, pK_{a2}(R-COOH) = 5.08).[19] The 4-hydroxybenzaldehyde exhibits a very low pK_a for the phenolic group (pK_{a1}(Ph–OH) = 7.04).[17] The dipeptide DL-alanyl-DL-alanine (pK_{a1}(R-COOH) = 3.05, pK_{a2}(R-NH_2) = 8.16)[17] was chosen to evaluate both the possible hydrolysis of peptide linkages during the titrations[18,20] and, most importantly, the influence of functional groups such as R-NH_2, which have aqueous dissociation behavior similar to R-COOH but contribute differently to the total organic charge in solution (see hereinafter), on the potentiometric titration process.

Table 2 shows the CA and PhA results obtained by applying the BEST7 and MHHM data treatment procedures to raw titration points recorded for solutions of model systems. Also included in this table are the acidity values calculated by indirect titration as well as the theoretical values. The corresponding aqueous dissociation constants (pK_a) are given separately in Table 3. Considering the acidity results from direct titrations (MHHM and BEST7), very good agreement between experimental and theoretical values was observed independently of the mathematical model (Table 2, entries 1-4), except for the dipeptide (Table 2, entry 5) due to reasons discussed below. Therefore, both MHHM and BEST7 approaches described satisfactorily the proton-binding properties of model compounds. Indirect titrations, on the other side, were found to produce consistent results for CA but failed in assessing the actual PhA, which was remarkably lower than the theoretical amount (see Table 2, entries 1, 3 and 5). The lack of reliability in PhA determinations by such a method has been largely discussed in literature, being most often reported for systems containing phenolic moieties with high pK_a-values.[6,10,11,18,21] This is in part due to incomplete reactions between Ph-OH groups and $Ba(OH)_2$ that lead to underestimated TA. Since PhA = TA – CA in this case (see Experimental section), lower-than-expected PhA-values are consequently obtained.

DL-alanyl-DL-alanine dipetide exhibited a very distinctive behavior among the model compounds investigated in this work, highlighting potential effects of such a kind of chemical structures on potentiometric HS acidity determinations. Not surprisingly, however, the results could be interpreted on the basis of characteristic aqueous dissociation processes, which are depicted in Scheme 1. In aqueous medium, peptide properties are consistent with a dipolar ion structure, where the acidic group is, in fact, the ammonium ion (Zwitterionic species). The experimental CA (*ca.* 2.3 mequiv. g^{-1}, in average considering all results) was markedly lower than the theoretical value (6.24 mequiv. g^{-1}) (see Table 2, entry 5). The underestimate CA-values originate from the incomplete titration of carboxylic acid functions in

Table 2. Acidity of model compounds and mixtures as determined using the MHHM and BEST7 methods

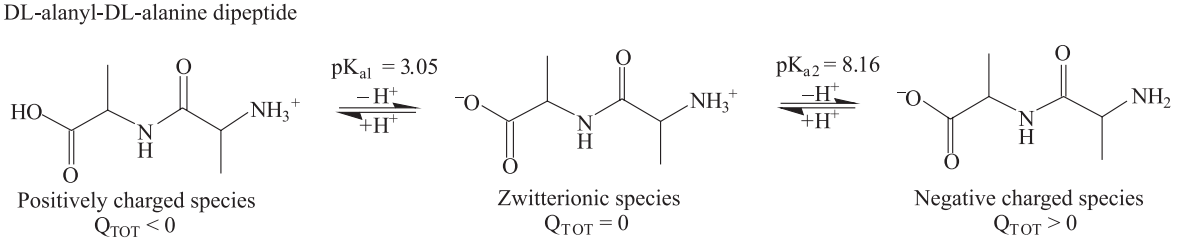
Entry	Sample	Method	CA[a] / (mequiv. g^{-1})	PhA[a] / (mequiv. g^{-1})	TA[a] / (mequiv. g^{-1})
Mixture of Acids					
1	Phtalic acid and 3,5-dihydroxybenzoic acid	BEST7	9.42 ± 0.35	6.35 ± 0.43	15.77 ± 0.55
		MHHM	9.16 ± 0.29	5.72 ± 0.80	14.88 ± 0.85
		Indirect Titration[b]	9.05 ± 0.26	3.25 ± 0.43	12.30 ± 0.50
		Theoretical value	9.38	6.25	15.63
Individual Compounds					
2	Phtalic acid	BEST7	12.55 ± 0.46	-	12.55 ± 0.46
		MHHM	12.19 ± 0.86	-	12.19 ± 0.86
		Indirect Titration[b]	10.92 ± 0.46	-	10.92 ± 0.46
		Theoretical value	12.04	-	12.04
3	3,5-Dihydroxybenzoic acid	BEST7	6.32 ± 0.08	12.66 ± 0.22	18.98 ± 0.23
		MHHM	5.75 ± 0.24	12.19 ± 0.89	17.94 ± 0.92
		Indirect Titration[b]	6.37 ± 0.03	5.93 ± 0.50	12.30 ± 0.50
		Theoretical value	6.49	12.98	19.48
4	4-Hydroxybenzaldehyde	BEST7	-	8.94 ± 0.28	8.94 ± 0.28
		MHHM	-	9.21 ± 0.49	9.21 ± 0.49
		Indirect Titration[b]	-	8.35 ± 0.40	8.35 ± 0.40
		Theoretical value	-	8.19	8.19
5	DL-Alanyl-DL-alanine	BEST7	2.04 ± 0.25	6.54 ± 0.16	8.58 ± 0.30
		MHHM	2.62 ± 0.20	4.14 ± 0.22	6.76 ± 0.10
		Indirect Titration[b]	3.24 ± 0.25	11.76 ± 0.35	14.98 ± 0.29
		Theoretical value	6.24	6.24[c]	12.48

[a] Abbreviations have the same significance as in Table 1; [b] considering total deprotonation; [c] acidity related to amine groups. Standard deviations obtained from three replicates.

Table 3. Summary of pK_a-values for model compounds and mixtures as determined using the MHHM and BEST7 methods

Entry	Sample	Method	pK_{a1}	pK_{a2}	pK_{a3}
1	Phtalic acid	BEST7	2.84 ± 0.15	4.99 ± 0.43	-
		MHHM	2.15 ± 0.27	4.77 ± 0.32	-
		Reported values	2.78	5.08	-
2	3,5-Dihydroxybenzoic acid	BEST7	3.78 ± 0.26	9.76 ± 0.21	10.35 ± 0.28
		MHHM	3.49 ± 0.07	9.64 ± 0.06	ND
		Reported values	3.90	9.03	10.30
3	4-Hydroxybenzaldehyde	BEST7	-	7.59 ± 0.28	-
		MHHM	-	7.51± 0.09	-
		Reported values	-	7.04	-
4	DL-Alanyl-DL-alanine	BEST7	2.96 ± 0.19	8.47 ± 0.20	-
		MHHM	3.10 ± 0.14	8.34 ± 0.06	-
		Reported values	3.05	8.16	

ND = Not detected; the MHHM describes HS systems by two proton-binding constants (see equation 2) associated with CA and PhA. Therefore, additional dissociation processes cannot be determined using this model. Standard deviations obtained from three replicates.



Scheme 1. Aqueous dissociation behavior of DL-alanyl-DL-alanine.

the DL-alanyl-DL-alanine structure. At the solution pH from which potentiometric data were collected (pH *ca.* 3.6) an important fraction of these moieties was already deprotonated (*ca.* 50%, as judged from chemical equilibrium calculations using pK_{a1} ((R-COOH) = 3.05) and the titrant was then not basic enough for this proton removal, hence not contributing to pH-based estimates of CAs. Consequently, the carboxylic acidity value was detected due to the much higher excess of titrant relatively to peptides, in solution. On the other hand, amine groups constitute an additional source of acidity that was computed as PhA, in this case. They behave as weak bases in aqueous medium due to protonation/deprotonation of nitrogen atoms (Scheme 1, right), exhibiting equilibrium constants close to phenols (see, for instance, the pK_{a2} of 3,5-dihydroxybenzoic acid and DL-alanyl-DL-alanine in Table 3, entries 2 and 4, respectively). Therefore, these findings demonstrate that amines cannot be distinguished from phenols in typical potentiometric measurements, thus unavoidably contributing to overall PhA of HS systems. The same comments also apply for secondary and tertiary amino groups.

As defined by the eletroneutrality or charge balance equation (equation 1), anionic (e.g.: R–COO^-) and cationic (e.g.: R–NH_3^+) species originate, respectively, positive and negative Q_{TOT}-values. It is worth noting that negative Q_{TOT}-values were observed in Q_{TOT} *vs*. pH plots. Hence, the overall profile in Figure 3 adequately reflects the different dissociation processes illustrated in Scheme 1. There exists an isoelectric point (pI = 5.60; see pK_a data in Table 3) at which the overall organic charge is theoretically zero because only zwitterionic species exist in solution. Nevertheless, such an observation (negative Q_{TOT}-values) should not have implications for HS acidity determination given that the amount of base titrant used to neutralize the system is taken into account during the data analysis.

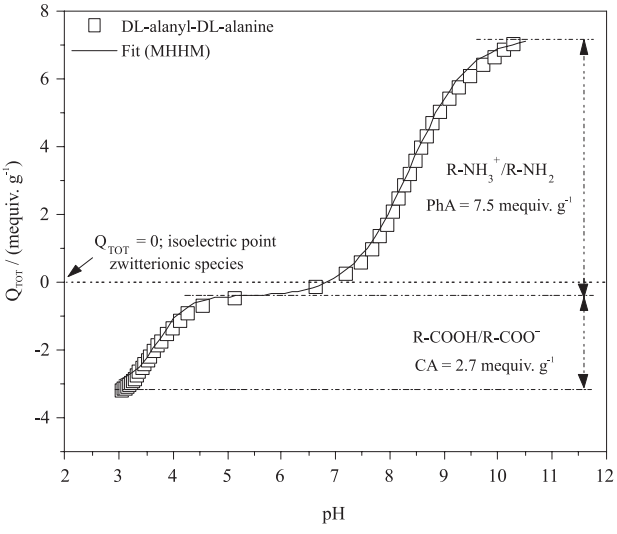


Figure 3. Titration data and fitting curve using the MHHM for a solution of DL-alanyl-DL-alanine dipeptide.

In general, it has been observed that both analytical protocols (MHHM and BEST7) describe satisfactorily the acid contents in solutions containing model compounds, with a few exceptions related to specific molecular properties already discussed above. Thus, differences in CA and PhA seen in HS systems are apparently a consequence of distinct manners of approaching the complex chemical structure of HS in each model, with their abundant diversity of proton-binding groups (carboxylic acids, phenols, amines, etc) and dissociation behaviors (low or high pK_as, overlapping pK_a ranges, etc).

Besides the aforementioned sources of uncertainty concerning HS potentiometric studies, there are additional aspects that may influence the results.[16,22-24] Among others, side reactions (e.g.: hydrolysis of peptides and esters[18]) that generate additional acidity during experiments have been reported for direct titrations of HS systems.[4,16,18,24] In contrast to characteristically fast proton-transfers between acids and bases in aqueous media, these secondary reactions are slow and occur mainly in the alkaline pH range, ultimately leading downward drifts in pH measurements during titrant additions.[4,5] On this basis, fast titrations would minimize secondary reactions. On the other hand, slow titrations would allow acid-base exchanges to reach the equilibrium state, hence improving accuracy and leading to more representative acidity quantifications. Thus, in addition to the above-cited sources of uncertainty concerning HS potentiometric studies, there are also those associated with the different experimental conditions used to perform the titrations.[16,22-24] In order to analyze these aspects we compare the data obtained in this study (slow titration) with those (fast titration) published by Ritchie and Perdue[5] for the same set of FA and HA. The data, presented in Table 4, together with those generated by the indirect titration method, were handled with the MHHM approach and are discussed in the following section.

Procedure implications

From Table 4 it can be seen that for HA (entries 3-5), carboxylic, phenolic and, consequently, total acidities, are clearly higher when slow titration is adopted. For FA, differences are less in evidence, with a slight decrease in CA and an increase in PhA for slow titration data. Such a reverse trend produced practically identical slow and fast titration TA values. Because of this, and also because even though in the present study the FA titrations (Table 4, entries 1 and 2) were relatively fast (the pH was rapidly stabilized after each titrant addition), only the HA data

Table 4. Acidity, in mequiv. g^{-1}, of the IHSS samples calculated using distinct procedures. The slow and fast titration data were handled by the MHHM. The indirect titration data were measured using the Schnitzer and Gupta[10] method

Entry	Sample	Method	CA[a] / (mequiv. g^{-1})	PhA[a] / (mequiv. g^{-1})	TA[a] / (mequiv. g^{-1})
Fulvic Acids (FA)					
1	Suwannee River (1S101F)	Slow titration	6.06 ± 0.26	1.89 ± 0.70	7.95 ± 0.74
		Fast titration[b]	6.29	0.77	7.06
		Indirect titration	5.15 ± 0.05	14.99 ± 0.06	20.14 ± 0.04
2	Pahokee Peat (1S103F)	Slow titration	5.89 ± 0.50	1.65 ± 0.37	7.54 ± 0.62
		Fast titration[b]	7.17	0.38	7.55
		Indirect titration	5.93 ± 0.08	21.07 ± 0.51	27.00 ± 0.50
Humic Acids (HA)					
3	Elliot Soil (1S102H)	Slow titration	11.08 ± 0.29	1.92 ± 0.44	13.00 ± 0.53
		Fast titration[b]	5.17	0.49	5.66
		Indirect titration	3.90 ± 0.05	11.35 ± 0.25	15.25 ± 0.25
4	Pahokee Peat (1S103H)	Slow titration	13.50 ± 0.32	2.50 ± 0.50	16.00 ± 0.59
		Fast titration[b]	5.43	0.53	5.93
		Indirect titration	4.46 ± 0.01	16.79 ± 0.27	21.25 ± 0.25
5	Leonardite (1S104H)	Slow titration	11.19 ± 0.45	1.61 ± 0.62	12.80 ± 0.75
		Fast titration[b]	5.22	0.77	5.99
		Indirect titration	3.32 ± 0.02	19.93 ± 0.29	23.25 ± 0.25

[a] Abbreviations have the same significance as in Table 2; [b] these data, which are presented here in mequiv. g^{-1}, come from Ritchie and Perdue.[5] Standard deviations obtained from three replicates.

will be discussed here. For these, due to the recurrent drift of pH values (see Experimental section), titrations consumed longer periods of time. These drifts seem to be a consequence of secondary reactions such as hydrolysis of peptides and esters that consume hydroxyls, accounting for the total acidic content.[16,18] This effect is not noticeable in FA titrations because these compounds have less diversified chemical compositions. The results given in Table 4 clearly show the influence of these reactions on the final results: data from slow titrations (*i.e.*, up to equilibration) are always higher than those obtained with fast titrations. None of the data are comparable to those obtained *via* indirect titration, which presented the highest values of TA and PhA. As has previously been shown, under extreme conditions of the reaction with $Ba(OH)_2$, hydrolysis of the peptide linkages occurs, consuming extra hydroxyl groups and increasing artificially the phenolic content.[18]

Finally, if the acidities measured with the BEST7 (slow titration, Table 1) are included in this table, we have four different sets of values for each sample, with the highest discrepancies found in the PhA data.

Conclusions

Potentiometric acidity quantification in HS systems may be significantly influenced by analytical methods. In particular, the procedure undertaken to fit experimental titration data points is of great importance, as judged from the results obtained using the modified Henderson-Hasselbalch model (MHHM) and the BEST7 routine. While high carboxylic and low phenolic contents were usually recorded using the MHHM, the opposite was observed employing the BEST7-based routine. In contrast, titration curves for model solutions containing compounds that mimic some of the HS properties were described satisfactorily by both approaches, with a few exceptions due to specific molecular properties.

The dependence of CA and PhA on the method applied to fit titration curves of HS systems are essentially a consequence of distinct ways by which the models (MHHM and BEST7) resolve the aqueous equilibrium processes of HS with their complex chemical structures and diversity of functional groups. Experimental handling (e.g.: low or fast titration) is also a critical issue on HS potentiometric acidity determination.

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