

Spectroscopic and Potentiometric Studies on Derivatized Natural Humic Acid

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Isolated soil humic acid (HA) and commercial Aldrich HA were derivatized by esterification with methanol-thionyl and acetylation with acetic anhydride, in order to obtain derivatives with selectively blocked carboxyl and phenol groups, respectively. Results obtained by FT-IR spectroscopy and potentiometry show that the methanol-thionyl procedure is a selective, specific and efficient route for blocking carboxyl groups. The good correlation between results obtained by direct potentiometry after HA esterification and by classical calcium-acetate and baryta exchange methods suggests that esterification followed by direct acid-base potentiometric titration can be used as a method for the estimation of carboxyl and phenol group contents. Phenol groups can not be specifically identified by the acetylation method, due to the low selectivity of the acetylation method. The average values of apparent and intrinsic pK of underivatized and derivatized HAs confirm decrease in ionizable groups content due to derivatization and their values are related to the different chemical structures of the acids.

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Introduction

An understanding of the structure of humic substances (HS) is necessary in order to describe their reactivity in chemical, biological and environmental processes. Although an accurate structural formula of HS is not attainable, reasonably accurate estimates of the various functional groups in a mixture of HS can be made.¹ It is accepted that humic matters, including humic acids (HA), are heterogeneous polyelectrolyte possessing a variety of different types of functional groups which act as binding sites for proton/metal ions. The major binding sites are usually attributed to the oxygen-containing functional groups, although other less abundant functional groups, *e.g.* nitrogen and sulfur-containing groups, may also be important for cation binding.^{2,3} Carboxylic and phenolic groups are dominant oxygen-containing ionizable groups present in HA, which dissociation gives rise to a net negative charge. Thus, determination of concentration and pK_a 's of carboxylic and phenolic groups can provide a guide to modeling HA acidity.

There are various methods for determination of HA oxygen-containing functional groups,^{4,6} but they exhibit some difficulties and uncertainties due to HS complexity. Beside those methods, some authors studied the differentiation between various hydroxyl groups of HA by derivatization⁷⁻¹¹ providing HA with selectively blocked functional groups. This approach could be used for estimation of the contribution of each blocked functional group on the total humic acidity. In the present

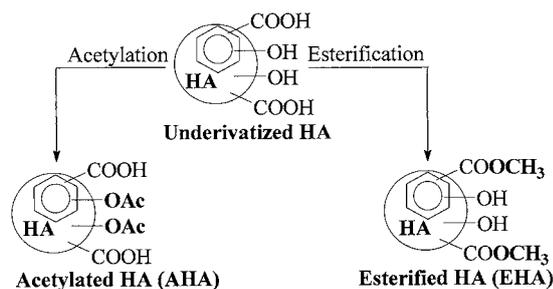
article, we show that HA derivatives with selectively blocked functional groups could be used, in comparison to untreated HA, for investigation of the influence of blocked functional groups on acidic properties of HA.

There are two basic acidity determination approaches, indirect and direct titrations, and each has some advantages and disadvantages. Direct titrations have the disadvantage of the lack of distinct inflection points in the titration curves and the overlap of pK_a values of acidic groups. Therefore, indirect titration is still in use, even though it itself shows some limitations, as well.^{12,13} Indirect titration, proposed by Schnitzer and Gupta,¹⁴ includes titration of a filtered reaction mixture to a fixed pH end point, after a 24 h equilibration with either $Ba(OH)_2$ for determination of total acidity (TA), the baryta method, or with $Ca(OAc)_2$ for determination of carboxylic acidity (CA), the Ca-acetate method. The difference between TA and CA gives the value of phenolic acidity (PA).

However, the disadvantages of direct titrations can be exceeded by performing a direct titration on a derivative with selectively blocked functional groups. Therefore, the objective of this work was to estimate the content of carboxyl and phenol groups in HA by (1) using chemical modifications specific to those individual groupings and (2) potentiometric titration data. In this context, the selective blocking of carboxyl groups was achieved by esterification, while phenolic and alcoholic hydroxyls were blocked by acetylation. The derivatizations were performed on both natural and commercial HAs.

Among many esterification methods described in the literature,¹⁵ probably one of the most attractive and efficient is the methanol-thionyl chloride procedure,¹⁵⁻¹⁸ shown in Scheme 1, which was modified and is thus described later in detail.

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Scheme 1 Reaction scheme for the synthesis of derivatized HAs.

Most of the reported esterification methods¹⁵ require long reaction times, give low yields and show low selectivity when substituents are present in aromatic carboxylic acids. The applied method was chosen because it provides derivatives with unaffected phenolic and alcoholic hydroxy groups and works equally well with aliphatic carboxylic acids.¹⁸ Also, all the reaction by-products are volatile and can be easily removed by a vacuum rotary evaporator. Due to its high selectivity, efficiency and simplicity, it is hoped that the method would be suitable for functionally very heterogeneous humic macromolecules.

Acetylation of humic substances by refluxing with acetic anhydride and sulfuric acid (Scheme 1) was chosen because it is a commonly accepted method for obtaining HS with blocked phenol alcohol groups,¹⁹ although there are no reports on systematic investigation of its selectivity.

Acid-base properties of HA, the amount of total acid titrable groups (ATG) and the average values of apparent and intrinsic pK , were evaluated by potentiometric titration with the modified Henderson-Hasselbalch interpretation.²⁰

Experimental

Reagents and chemicals

Aldrich HA (catalog H1, 675-2 lot No. S15539-264) was purified by the same procedure as soil HA, in order to remove ash content, residual fulvic acids and heavy metals.

All other chemicals were of analytical grade purity; they were supplied by Merck and used as received.

The water used for potentiometric titration experiments was double deionized (conductivity less than $0.1 \mu\text{S cm}^{-1}$).

Apparatus

UV/VIS spectra of underivatized soil HA (4 mg) dissolved in 100 ml 0.05 M NaHCO_3 solution was recorded on a Secomam Anthelie 306 spectrophotometer, using wavelengths ranging from 200 to 1000 nm.

FT-IR spectra of soil HA and Aldrich HA and their derivatives were recorded on KBr pellets (1.0 mg HAs and 150.0 mg KBr) using a Bomem Hartman & Braun MB-Series FT-IR spectrophotometer.

Potentiometric measurements were made with a Hach sension 3 pH-meter (precision of 0.1 mV or 0.001 units of pH) using a Hach gel-filled combination glass electrode (51935-00).

Elemental composition (C, H and N) of soil HA and Aldrich HA were determined directly with Vario El 3, while oxygen was obtained by the difference.

The humic acid isolation and purification

HA isolation and purification were based on the method

proposed by the International Humic Substances Society (IHSS).²¹ The soil sample was obtained from a well-humified organic horizon of old beech-forest soil (10 cm depth) in autumn 2003 using standard grinding equipment. The soil was air-dried and sieved to pass a 2.0 mm sieve. Briefly, soil HA was extracted from the soil with 0.1 M NaOH at 1:10 solid to solution (mass/volume) ratio under N_2 for 4 h. The suspension was then centrifuged at 4500 rpm for 30 min, the supernatant was acidified with 6 M HCl to pH 1.0 and the suspension was allowed to stand overnight. Precipitated HA was separated from fulvic acid by centrifugation at 4500 rpm for 30 min. The obtained HA precipitate was redissolved in 0.1 M KOH, under N_2 . Solid KCl was added to attain 0.3 M (K^+) and then the suspension was centrifuged at high speed to remove suspended solids. The soil HA was reprecipitated with 6 M HCl to pH 1.0. The soil HA precipitate was suspended in a solution of 0.1 M HCl/0.3 M HF. In order to minimize the ash content, this procedure was repeated three times. After HA purification from silicates by HCl-HF treatment, the HA fraction was dialyzed in a Spectra/Por 7 membrane (molar mass cut-off = 1000 D), until no significant change in conductivity of dialysis bag external water was observed. Bound metal ions were removed by putting the HA solution (at pH 8 adjusted with 0.1 M NaOH) into a 300×10 mm column with ion-exchange resin Dowex 50W-X8 (H^+ -form) at a flow rate of 0.5 ml min^{-1} . The soil HA suspension was diluted in a volumetric flask and stored at 4°C . The concentration of the stock was determined as 2.51 g l^{-1} , by the dry weight of a measured volume of a well-homogenized HA suspension. The concentration of the stock suspensions of Aldrich HA was determined to be 2.47 g l^{-1} .

Esterification of HA

Thionyl chloride (5 ml) was added drop wise from a dropping funnel, to a stirred solution of 500 mg of HA in 20 ml of methanol, under ice-cooling (approximately -5°C), over 2 h. The reaction mixture was then heated on a water bath for 5 min to decompose excess thionyl chloride. The suspension was then centrifuged at 2000 rpm and the separated ester was washed with distilled water until free of chlorides. The ester was dried in a rotary evaporator and finally in a vacuum desiccator over P_2O_5 . The esterification procedure was repeated and the obtained twice-esterified product is marked as EHA. The obtained ester in the second esterification procedure was not dried, but was diluted and the concentration of the stock EHA suspension was determined as 1.25 and 1.45 g l^{-1} , for soil and Aldrich HAs, respectively.

Acetylation of HA

Acetylation was performed by addition of 15 ml of acetic anhydride and 3 drops of conc. H_2SO_4 to 600 mg of dried HA in 100-ml round-bottomed flask. A Liebig reflux condenser was attached and the mixture was heated on a boiling water bath for 4 h. The content was poured into 150 ml of ice-water and vigorously stirred to assist the hydrolysis of unreacted acetic anhydride. The crystalline solid was filtered off and washed thoroughly with cold, distilled water until free of acid and then dried over P_2O_5 in a vacuum desiccator. The dry yield is 75% of the starting material. The acetylation procedure was repeated on the dry product from the first acetylation. The twice-acetylated product is marked as AHA. The product obtained from the second acetylation procedure was not dried, but was diluted in a volumetric flask and stored at 4°C . The concentration of the stock was determined as 1.05 g l^{-1} and 1.32 g l^{-1} for soil and Aldrich HAs, respectively, by dry weight of a measured volume of the homogenized suspension.

Potentiometric titrations

Suspensions of soil and Aldrich HA, and their derivatives were prepared in 0.1 M NaCl by diluting an appropriate volume of the stock suspensions with 25.00 ml 0.2 M NaCl into a 50 ml volumetric flask. The mixtures were diluted up to 50 ml with decarbonated water. The whole volume was transferred in the titration vessel. The resulting suspension concentrations were 500 mg l⁻¹. Throughout the titrations, a N₂ atmosphere was maintained over the solution, which was thermostated in a water bath at 25.0 ± 0.1°C. After introduction of the electrode, the burette tip and the N₂ conducting tube, the titration vessel was closed and maintained at a slight over-pressure of nitrogen in order to exclude atmospheric CO₂. Titrations were performed from pH 3 to 11 with standardized CO₂-free solution of NaOH (0.0970 mol l⁻¹) with the same NaCl concentration as the titrated HA suspensions. The potential was read only when its variation was smaller than 0.555 mV min⁻¹, as monitored by the drift control of the pH meter. Titrations were performed in triplicate.

Treatment of potentiometric data

The acid-base properties of HA, ATG and the average values of apparent pK (pK_{app}), were evaluated by potentiometric titration with the modified Henderson-Hasselbalch interpretation, usually used for analyzing potentiometric titration curves for weak polyacids:

$$pK_{app} = pH + \log((1 - \alpha)/\alpha) \quad (1)$$

where pK_{app} and α are the negative logarithm of the apparent acid dissociation constant and the degree of neutralization, respectively. The degree of neutralization, α , of the HA at each point of alkali addition is defined by the equation:

$$\alpha = ([base] + [H^+] - [OH^-])/ATG \quad (2)$$

where [base], [H⁺] and [OH⁻] are the molarities of added base, free hydrogen ion and hydroxide ion, respectively, and ATG is the total concentration of acid titrable groups, in mmol g⁻¹, determined by a modified Gran relationship.²² The [H⁺] and [OH⁻] ions were obtained from the pH values assuming that the activity coefficient is unity. Therefore, $\alpha = 1$ at complete neutralization. Plots of pK_{app} versus α for a monomeric acid yield a unique line with a slope of zero. However, if the molecule exists as a polymeric assembly, such as a humic macromolecule, a plot of pK_{app} versus α can give a quantitative measure of nonideality behavior by measuring any deviation from a straight line of zero slope.²⁰ The intrinsic dissociation constant (pK_{int}) was obtained by polynomial fitting to the data of the pK_{app} versus α curve, extrapolating to $\alpha = 0$ and reading off the pK_{app} value as the negative logarithm of the intrinsic dissociation constant, while the average pK_{app} value corresponds to the pK_{app} value at $\alpha = 0.5$.

Results and Discussion

The elemental compositions of underivatized and derivatized soil HA samples are summarized in Table 1. The molar elemental ratios were calculated from these values. All the obtained data for HA were consistent with typical values for soil HA.²³ The carbon content of EHA and AHA was increased by esterification and acetylation, while hydrogen and nitrogen contents remained almost the same. The changes of carbon contents of EHA and AHA indicate the preparation of methyl esters and ethers. The increase of sulfur content by acetylation

Table 1 Elemental analysis^a and molar elemental ratios of underivatized and derivatized soil HA samples

	Soil HA	Soil AHA	Soil EHA
C, %	55.74	60.02	58.63
H, %	5.45	5.23	5.47
N, %	0.86	0.62	0.72
O, %	36.55	32.93	34.65
S, %	1.40	2.85	1.57
H/C	0.10	0.09	0.09
O/C	0.66	0.55	0.59
ϵ_{280} , l mol ⁻¹ cm ⁻¹	263	—	—
Ar., %	20	—	—
M _w	1540	—	—

a. All data are on ash- and moisture-free basis.

may be attributed to the addition of H₂SO₄ as catalyst during derivatization. The estimation of the molecular weight (M_w) and aromaticity (Ar.) for the underivatized soil HA, obtained from the measurement of the molar absorption coefficient at 280 nm (ϵ_{280}), is also shown in Table 1. Since phenolic substances, aniline derivatives, benzoic acids, polyenes and polycyclic aromatic hydrocarbons, which are the main precursors or components of terrestrial HS, show absorption in the region of 270–280 nm, molar absorptivity at this wavelength yields estimation of the degree of aromaticity, extent of humification and molecular weight.²⁴ Chin *et al.*²⁴ further reported that there is strong correlation among ϵ_{280} , Ar. and M_w of HS expressed by the following equations for quick estimation of the size of HS and their aromatic contents:

$$Ar. (\%) = 0.05 \cdot \epsilon_{280} + 6.74 \quad (3)$$

$$M_w = 3.99 \cdot \epsilon_{280} + 490 \quad (4)$$

The values obtained by applying the mentioned correlations to the underivatized HA sample are shown in Table 1.

The derivatization of carboxyl and phenol groups was investigated by FTIR spectroscopy. The FTIR spectra of underivatized Aldrich and soil HA samples (Fig. 1) exhibit the typical major peaks for HA: 3400 cm⁻¹ absorption due to H-bonded OH stretching of carboxyl, phenol and alcohol; 2920 and 2850 cm⁻¹ bands for aliphatic CH stretching; 1720 cm⁻¹ for C=O stretching from COOH; 1600–1650 cm⁻¹ for C=O stretching of COO⁻, ketonic C=O and aromatic C=C conjugated with COO⁻; 1400 cm⁻¹ for aliphatic CH bending and COO⁻ asymmetric stretching.⁶

The FTIR spectra of derivatized Aldrich and soil HA samples (AHA and EHA) confirmed that esterification of the carboxyl groups and acetylation of hydroxyl groups occurred, due to the differences in the IR absorption before and after derivatization. There are three important IR regions related to these derivatization studies: (1) 3400 cm⁻¹ absorption decrease in the spectra of derivatized HA samples, comparing to a broad and strong peak of underivatized HA, indicates that practically all hydroxyl groups have been derivatized. Reduction in O-H stretching vibrations intensity is more significant in EHA than in AHA spectra, indicating effective esterification and partially achieved acetylation. Probably tertiary hydroxyls, which are acetylated under stronger conditions, were not acetylated;²⁵ (2) 1720 cm⁻¹ absorption due to stretching vibration of -C=O ester groups was increased by esterification and acetylation; and (3) 1100–1450 cm⁻¹ absorption due to C-O stretching vibrations was increased, which results from the incorporation of methyl

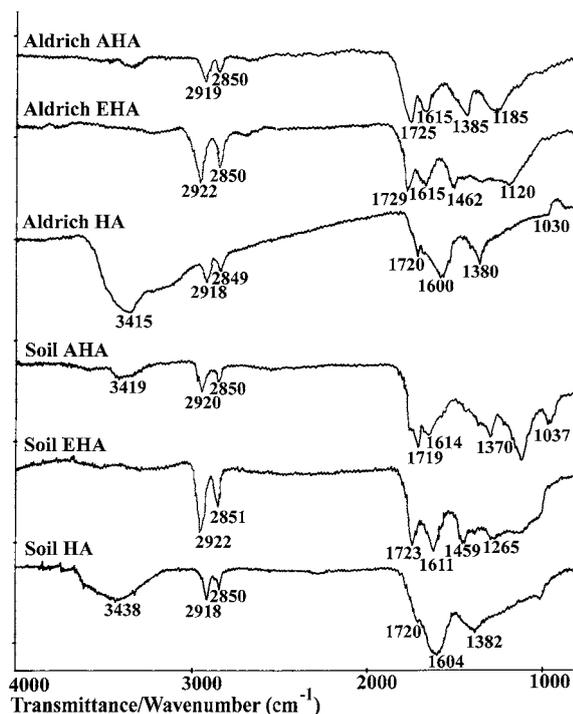


Fig. 1 FT-IR spectra of Aldrich HA and soil HA: underivatized (HA), esterified (EHA) and acetylated (AHA) humic acids.

groups into the molecule due to formation of ethers and esters. Additionally, higher OCH₃ contents of EHA derivatives are also indicated by the bands for C–H stretching at 2920 and 2850 cm⁻¹ that were stronger than in underivatized Aldrich and soil HA.

CA, PA and TA for underivatized and derivatized Aldrich HA and soil HA were obtained by classical indirect titration methods, while the average ATG values were obtained by direct titrations (Table 2). Esterification and acetylation decreased ATG value due to transformation of ionizable groups to methyl esters and methyl ethers, respectively. Obtained ATG values for HA and EHA, ATG_{HA} and ATG_{EHA}, respectively, could provide estimations of carboxyl and phenol groups contents of humic acid. Thus, after blocking of carboxyl groups in EHA derivative, the ATG_{EHA} value could be assigned to the remaining, unblocked phenolic group content, [Ph-OH]_{HA}:

$$\text{ATG}_{\text{EHA}} = [\text{Ph-OH}]_{\text{HA}} \quad (5)$$

while the difference between ATG_{HA} and ATG_{EHA} could be assigned to the carboxyl group content, [COOH]_{HA}:

$$\text{ATG}_{\text{HA}} - \text{ATG}_{\text{EHA}} = [\text{COOH}]_{\text{HA}} \quad (6)$$

The estimation of [Ph-OH]_{HA} of Aldrich HA by Eq. (5) was 2.50 mmol g⁻¹, which is in good correlation with the results obtained by classical indirect titration method: PA_{HA} = 2.54 mmol g⁻¹ and PA_{EHA} = 2.49 mmol g⁻¹ (Table 2). The estimation of [COOH]_{HA} of Aldrich HA by Eq. (6) was 3.82 mmol g⁻¹, which is similar to the result obtained by indirect titration method: CA_{HA} = 3.94 mmol g⁻¹. The results of indirect titration of Aldrich EHA derivative show that carboxyl groups are almost completely esterified, since only 7.9% of carboxyl groups (CA_{EHA} = 0.31 mmol g⁻¹) were determined in EHA derivative, while 92.1% of carboxyls were blocked, in the form of methyl esters. Likewise, the phenolic group content in

Table 2 Acidities and pK values of underivatized and derivatized Aldrich HA and soil HA (n = 3)

	ATG ^a	PA ^b	CA ^b	TA ^b	pK _{app}	pK _{int}
Aldrich HA	6.32 ± 0.15	2.54	3.94	6.48	—	—
Soil HA	4.63 ± 0.25	1.88	2.80	4.68	4.28 ± 0.05	3.98 ± 0.10
Aldrich EHA	2.50 ± 0.10	2.49	0.31	2.80	—	—
Soil EHA	1.76 ± 0.03	1.76	0.2	1.96	5.80 ± 0.04	5.50 ± 0.05
Aldrich AHA	2.05 ± 0.08	0.76	1.56	2.32	—	—
Soil AHA	1.42 ± 0.06	0.60	1.12	1.72	5.17 ± 0.02	5.15 ± 0.08

a. Obtained by direct titrations. Standard deviations for three replicates.

b. Obtained by indirect titrations (the baryta and Ca-acetate methods).

Aldrich EHA derivative was decreased by only 2.0%, confirming the high selectivity of the esterification method.

The trends observed in acidity change have been checked on the isolated soil HA. The estimation of [Ph-OH]_{HA} of soil HA by Eq. (5) gave 1.76 mmol g⁻¹, which corresponds to the results obtained by classical indirect titration method: PA_{HA} = 1.88 mmol g⁻¹ and PA_{EHA} = 1.76 mmol g⁻¹. The estimation of [COOH]_{HA} of soil HA by Eq. (6) was 2.87 mmol g⁻¹, which is similar to the value of 2.80 mmol g⁻¹ obtained by indirect titration of soil HA. In the case of soil HA, carboxyl groups are also almost completely esterified, since only 7.1% of carboxyl groups (CA_{EHA} = 0.20 mmol g⁻¹) were determined in EHA derivative, while the phenolic group content in EHA derivative was decreased by only 2.0%.

These observations agree with IR data and lead to the conclusion that the applied methanol-thionyl procedure is a selective, specific and efficient route for blocking carboxyl groups. Also, esterification followed by direct potentiometric titration can be used as a method for the estimation of carboxyl and phenol group contents.

However, similar relations are not found for acetylated HA. After the blocking of phenol groups in Aldrich AHA derivative, the ATG_{AHA} value, which is assigned to the content of the remaining unblocked carboxyl groups, was 2.05 mmol g⁻¹. However, this is only half of the value obtained by indirect titration of Aldrich HA (CA_{HA} = 3.94 mmol g⁻¹) (Table 2). The result obtained is probably a consequence of the low selectivity of the acetylation method. Acetic anhydride, beside blocking phenol groups, blocked carboxyl groups as well, which is confirmed by the decrease in COOH content in AHA derivative for even 60.4% (only 1.56 mmol g⁻¹ carboxyl groups were not in the ester form) (Table 2). The estimation of [Ph-OH]_{HA} determined by the difference between ATG_{HA} and ATG_{AHA} was 4.27 mmol g⁻¹, which is surprisingly high comparing to the PA_{HA} value of 2.54 mmol g⁻¹ obtained by the indirect titration of underivatized Aldrich HA. The reason for this is probably the fact that phenolic groups cannot be completely blocked by acetylation, as 29.9% of phenolic groups were not blocked (0.76 mmol g⁻¹) and were still determined after derivatization (Table 2). Similarly, after blocking of phenol groups in soil AHA derivative, a significant discrepancy was found between results obtained by direct titration after acetylation and by indirect titration.

The Henderson-Hasselbalch plots for soil HA and its derivatives are not linear, confirming that HA possesses different types of ionic groups (Fig. 2). The pK_{app} value depends on α, which also confirms the polyacidity of soil HA (Fig. 3). As was expected, all three acids exhibit different acidic strengths. The pK_{int} and the average pK_{app} increased after derivatization in

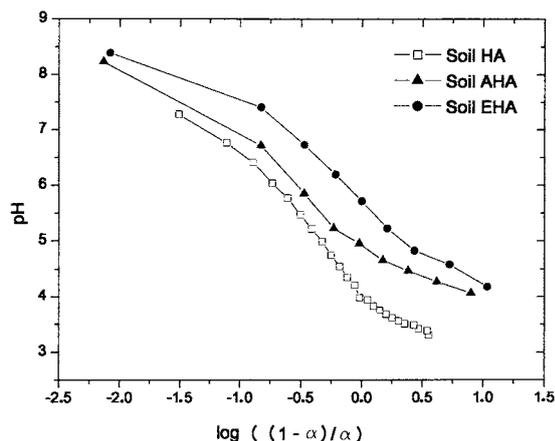


Fig. 2 Henderson-Hasselbalch plot of underivatized and derivatized soil HA samples.

accordance with the different chemical structures of the acids. The soil HA is the strongest acid (pK_{app} 4.28 and pK_{int} 3.9), as it possesses free carboxylic, phenolic and alcoholic groups. Soil AHA is a weaker acid than HA (pK_{app} 5.17 and pK_{int} 5.1), due to blocking of phenolic and alcoholic groups, but it does have carboxylic groups available for proton interaction. Soil EHA is the weakest acid (pK_{app} 5.80 and pK_{int} 5.5), because more acidic carboxylic groups are blocked, while less acidic phenolic groups are available, in this case, for proton interaction. Finally, the pK_{app} values distribute in the ranges 3.8–5.8 for HA, 4.9–6.1 for AHA and 5.2–6.6 for EHA (Fig. 3). The change in pK_{app} over the range of α values is the largest for HA ($\Delta pK_{app} = 2.0$ pK units) compared to EHA ($\Delta pK_{app} = 1.4$ pK units) or AHA ($\Delta pK_{app} = 1.2$ pK units). The distribution of pK_{app} values over the range of α values can give a quantitative measure of deviation from a straight line of zero slope which is characteristic for monomeric acid.²⁰ Thus, the highest pK_{app} distribution was found in underivatized soil HA-polyprotic acid with the greatest number of free carboxylic, phenolic and alcoholic groups.

Conclusions

The results indicate that valuable information on carboxyl and phenol group contents in HA may be achieved by a combination of derivatization and direct titration approaches. Results obtained for soil HA and Aldrich HA samples by direct titration after esterification are in agreement with results obtained by classical baryta and Ca-acetate exchange methods. Obtained esterified derivatives of soil HA and Aldrich HA include over 92% of blocked carboxyl groups and only 2% of blocked phenolic groups. Along with data from IR spectra, this result confirms the high selectivity of the esterification method. Thus, due to high efficiency, selectivity and simplicity of the esterification/direct titration method, it is suitable for carboxyl and phenol group content estimations of humic macromolecules. However, the acetylation derivatization method is neither effective enough nor selective enough, because it provides acetylated derivatives with only 70% of blocked phenol groups and even 60% of blocked carboxyl groups. Therefore, it is not suitable for phenol group content estimation. The values of pK_{int} , pK_{app} and pK_{app} distribution decrease by derivatization methods, confirming the reduction in

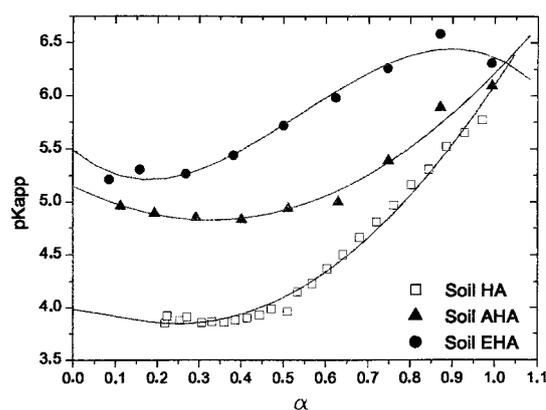


Fig. 3 α vs. pK_{app} plot for underivatized and derivatized soil HA samples.

the number of free carboxylic, phenolic and alcoholic groups.

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