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Characterization of Humic Acid and Fulvic Acid Extracted from Soil Samples of Cultivated Areas of North Bengal and Sikkim States, India

M. CHANDA[1], S. JHA[1,*], D. MUKHOPADHYAY[2] and M. PANDEY[1]

[1]Department of Chemistry, Sikkim Manipal Institute of Technology, Sikkim Manipal University, Majitar, Rangpo-737136, India
[2]Department of Soil Science & Agricultural Chemistry, Uttar Banga Krishi Viswa Vidyalaya, Pundibari-736165, India

*Corresponding author: E-mail: sangeeta.j@smit.smu.edu.in

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Humic acid and fulvic acid extracted from soils of cultivated areas, collected from Majitar, Darjeeling and Pundibari cities were first isolated, purified and then characterized for the physico-chemical properties (oxidizable organic carbon, electrical conductivity, pH, phosphorus, nitrogen, potassium, CEC, *etc*.). The physico-chemical studies showed that soils were neutral to acidic but are independent of altitude. Organic carbon, available phosphorus, nitrogen were higher with soil of Majitar city. The total acidity, carboxylic group, phenolic -OH group were higher in fulvic acid than in humic acid. Further, both acids were characterized by potentiometric titration, viscometric measurement, surface tension and spectral analysis. The humification index (E_4/E_6) of fulvic acid was relatively higher than humic acid. The highest molecular weight of humic acid and fulvic acid were recorded 13645.83 and 1465.54, respectively.

Keywords: Soil, Humic acid, Fulvic acid, Surface tension, Potentiometric analysis, Viscometric measurements.

INTRODUCTION

Humic acids are the combination of aromatic and weakly aliphatic organic acids. They are water insoluble and soluble under acidic and alkaline conditions, respectively. Humic acids comprise the humic substances precipitated from an aqueous solution when pH decreases to < 2. These compounds are considered flexible linear polymers existing as random coils having cross-linked bonds. An average of 35% humic acid molecules are aromatic, and the remaining molecules are aliphatic [1].

Fulvic acids are the combination of aromatic and weakly aliphatic organic acids, which are water soluble at all pH conditions. Fulvic acids are smaller molecule than humic acids and the theoretical structure of fulvic acids molecules comprises both aliphatic and aromatic groups. The oxygen content of fulvic acids is twice of that of humic acids. Fulvic acids are highly chemically reactive because they contains several hydroxyl and carboxylic groups [2].

The molecular weight of fulvic acids is a considerably low, which facilitates a substantial influence on metabolism and provides a high potential for mobility within plants. Humic substances are considerably crucial soil constituents. According to the climatic condition, soil humus contents often stabilizes at fairly definite concentrations. In soil solutions, organic acids can bind with the excessive amounts of metal ions present and can release into plants in small amounts when and as required [3]. Organic acids prevent the accumulation of large quantities of micronutrients in soil solutions, and thereby control micronutrient toxicity. Many organometallic chelates produced are water insoluble and prevent metals from entering the ground water [4].

The present study investigated the physico-chemical properties (EC, CEC, pH, organic carbon, phosphorus, nitrogen and potassium) and conducted the viscometric measurement, potentiometric titration, spectral analysis and surface tension determination of natural humic acids and fulvic acids extracted from soil in the vegetation areas of Darjeeling, Pundibari (North Bengal) and Majitar (Sikkim), India. The functional groups of the samples were also characterized to determine carboxyl groups, total acidity, phenolic groups and the spectral properties (E_4/E_6 ratio).

EXPERIMENTAL

Soil sampling: The surface (0-0.15 m) soil samples were obtained from Darjeeling (North Bengal), Pundibari (North Bengal) and Majitar (Sikkim). First, these samples were air dried and then crushed and sieved using a roller and sieve (2 mm diameter), respectively, Finally, the samples were stored in plastic bottles.

Extraction, isolation and purification of humic acids and fulvic acids: Humic acids and fulvic acids were isolated, extracted and purified by following standard procedures [5]. Both studied acids were extracted with 0.5 N NaOH and then separated. The principle employed for separation was the differential solubility of the acids in acid and alkali at different pH. The soil samples were air dried and then meshed through a 2 mm sieve. Subsequently, in a Winchester bottle, the soil samples were shaken with 0.5 N Na_2CO_3 and the final samples were stored for 14 h. The upper layer of the liquid was separated by siphoning. To precipitate out humic acids, this layer was acidified to obtain a pH of 2.0-3.4 by using HCl. To ensure total precipitation, this solution was allowed to stand overnight. This complete process was repeated to dissolve and reprecipitate humic acids. To obtained a chloride-free supernatant, it was siphoned off and dialyzed. Subsequently, the pH of supernatant was changed to 8, and then a $BaCl_2$ solution was employed to precipitate fulvic acid as Ba-fulvate. To obtain chloride-free Ba-fulvate, it was dialyzed. To acquire fulvic acid, the Ba-fulvate was passed through the cation (H^+ ion) exchange resin [6].

Physico-chemical properties: The pH of the soil sample was determined using a pH meter as soil suspension in water. The ratio followed was 1.0:2.5. The electrical conductivity of this soil suspension (soil:water::1.0:2.5) was estimated at room temperature by using a conductivity meter [7]. The oxidizable organic carbon of the soil samples was measured using a technique reported by Bray and Kurtz [8]. Ammonium saturation was employed to determine the cation exchange capacity. The Bray-P content of soils was reached by extracting 2.5 g of all the soil samples by using a solution of 0.025 M HCl and 0.03 M NH_4F (25 mL) for 5 min. Subsequently, phosphorus was estimated using a visible spectrophotometer. The status of available nitrogen in the soil was determined using a standard method [9]. The K^+ available was estimated using the flame photometer [10].

Potentiometric titration and determination of total acidity of humic acid and fulvic acid: Equal quantities of humic acid and fulvic acid suspension were placed in a series of plastic bottles. Phenolic groups and carboxylic acid were determined according to the acidity equivalents of the initial and final inflexion points as illustrated in titration curves [11].

A decrease in the pH of all the alkali-treated humic acid and fulvic acid samples was observed after titration with alkali when the samples were allowed to stand for some hours. The decreased pH was rectified and the original high pH was achieved by adding an adequate amount of the same alkaline solution. With the repeated alkali addition, the required pH were stabilized for alkali-treated humic acid and fulvic acid solutions. For each humic acid and fulvic acid sample, two values were recorded: (a) time required for stabilization and (b) total amount of fresh alkali required for pH stabilization.

Viscometric measurements: To determine viscosity, each carboxyl group of the extracted humic acid and fulvic acid samples was transformed into carboxylic form through the addition of an adequate amount of NaOH (alkali). This was related to the initial inflection point of potentiometric titration curves. Ostwald′s viscometer was employed to determine humic acid/fulvic acid (HA/FA) viscosities. In the standard procedure, the ρ_o (density of water) and η_o (viscosity of water) at the experimental temperature must be known. Viscosities were determined for these samples at five different concentrations. For each instance, ambient laboratory temperature was recorded. The mean flow time was recorded at various concentrations. To measure suspension density, a specific gravity bottle was employed.

From these experiments, specific viscosity ($\eta_{sp} = \eta_{real} - 1 = \eta/\eta_o - 1$) for all the concentrations of the HA/FA samples. A corresponding graph of $\eta_{sp}/\sqrt{C}$ *versus* $\sqrt{C}$ was plotted. From the slope and intercept of the best-fit linear plot, the A and B coefficients of the Jones-Dole's equation [12] were achieved, according to the following relation:

$$\frac{(\eta/\eta_0 - 1)}{\sqrt{C}} = \frac{\eta_{sp}}{\sqrt{C}} = A + B\sqrt{C}$$

where η_o and η represent viscosities at an identical temperature of water and HA/FA suspensions, having a percentage concentration 'C', respectively; and A and B denote the empirical constants that are sensitive to interactions among disperse phase particles and of the disperse phase with dispersion medium, respectively [13]. The B_{expt} values were similar to intrinsic viscosities [η] [12], which were employed to calculate the viscosity-average molecular weight (M) of the humic/fulvic substances, by setting $\alpha = 0.65$ and $K = 7.3 \times 10^{-4}$ according to Relan *et al.* [14] in the following modified Staudinger′s equation:

$$[\eta] = KM^{\alpha}$$

The surface tension of aqua-humic acid and fulvic acid at various concentrations was determined using a stalagmometer by maintaining a constant experimental temperature of 26 ºC within ±0.1 ºC. Each solution pH was 7. The surface tension of water was 71.47 dynes cm^{-1} at 26 ºC.

Visible spectrophotometric measurement: At 465 and 665 nm (E_4/E_6), the optical densities for the dilute solutions of HA/FA (Na-form) were estimated using a UV-visible spectrophotometer at two pH values of 7 and 9.

RESULTS AND DISCUSSION

Table-1 presents the physico-chemical properties of soils collected from three cities *viz.* Darjeeling (North Bengal), Pundibari (North Bengal) and Majitar (Sikkim). The range of oxidizable organic carbon of soils was 1.1-1.7 g/kg and the pH of soils varied from neutral to acidic. For soils, the CEC was 1.4-3.4 cmol/kg. The amount of Bray extractable phosphorous was higher in the Majitar soil than that in the Darjeeling and Pundibari soils, and the amount of exchangeable K^+ was 134.4-190.4 cmol (P^+) kg^{-1}.

The pH considerably varied but remained independent of altitude (Table-1). The types of soils and vegetation may be an underlining reason for the same. EC values were the highest and lowest for the Darjeeling and Majitar soils, respectively. In Majitar soil, the oxidizable organic C content was the highest. The available N and P amounts were the highest in Majitar

TABLE-1
IMPORTANT PHYSIOCHEMICAL PROPERTIES OF SOILS FROM CULTIVATED LAND

Location	Soil depth (cm)	pH (1:2.5)	EC (1:2.5) (dS m^{-1})	Organic C (g k^{-1})	CEC (cmol (p+) (kg^{-1})	Available		
						P (mg kg^{-1})	N (kg ha^{-1})	K^+ (cmol (p^+) kg^{-1})
Pundibari	0-15	4.7	0.09	1.1	3.4	12.21	140.50	190.4
Sikkim, Majitar	0-15	6.6	0.07	1.7	2.4	22.57	181.72	134.4
Darjeeling	0-15	4.3	0.11	1.5	1.4	9.00	126.00	156.0

TABLE-2
AMOUNT OF CARBOXYLIC GROUP, PHENOLIC GROUP & TOTAL ACIDITY OF HA/ FA SAMPLES

HA/FA sample	Amount of carboxylic (COOH) group (meq/g)	Amount of phenolic (–OH) group (meq/g)	Amount of other acids (meq/g)	Total acidity (meq/g)
1% HA Pundibari	0.825×10^{-3} (N)	2.20×10^{-3} (N)	1.65×10^{-3} (N)	4.60×10^{-3} (N)
1% HA Sikkim, Majitar	0.825×10^{-3} (N)	1.10×10^{-3} (N)	2.75×10^{-3} (N)	4.67×10^{-3}(N)
1% HA Darjeeling	1.300×10^{-3} (N)	0.55×10^{-3} (N)	2.20×10^{-3} (N)	4.10×10^{-3} (N)
1% FA Pundibari	0.825×10^{-3} (N)	3.30×10^{-3} (N)	1.10×10^{-3} (N)	5.20×10^{-3} (N)
1% FA Sikkim, Majitar	1.300×10^{-3} (N)	1.65×10^{-3} (N)	2.20×10^{-3} (N)	5.20×10^{-3} (N)
1% FA Darjeeling	0.825×10^{-3} (N)	2.20×10^{-3} (N)	1.10×10^{-3}(N)	4.60×10^{-3} (N)

HA = Humic acid; FA = Fulvic acid

soil sample, while in Pundibari soil, exchangeable K^+ content was the highest. The differences observed can be attributed to varying vegetation and soil characteristics.

Potentiometric analysis: The potentiometric results of the soil samples are shown in Table-2. After the titration of standard alkali with aqueous humic acid solutions, the pH potentiometric titration curves were acquired, which represent the characteristics nature of a weak polybasic acid. The obtained titration curves were characterized using numerous inflexion points, which corresponded to the neutralization of an acidic group. First, carboxylic groups, which were stronger than the phenolic groups, were neutralized [15-17]. According to the amounts of the standard alkali titre that corresponded to the inflexion points, carboxylic acidity, overall acidity, the other acids and phenolic acidity of the humic acid solution were calculated.

On the basis of the overall acidity, the sequential arrangement of the soil samples was as follows: fulvic acid (Majitar) ~ fulvic acid (Pundibari) > humic acid (Majitar) > humic acid (Pundibari) ~ fulvic acid (Darjeeling) > humic acid (Darjeeling). For most cases of fulvic acid, the phenolic, carboxyl and overall acidity was higher than that for humic acid. These observations are in agreement with the findings of Srilatha *et al.* [18].

The total acidity of fulvic acids is higher than that of humic acids [19]. An increase in the overall acidity with a decrease the molecular weight was observed because of the growing extent of oxidation accompanied with the low molecular weight fraction [20]. The higher acidity of fulvic acid may cause by its lower particle weight [18].

Humic acid and fulvic acid exhibit a coiling-decoiling nature, depending on basicity and acidity of a medium in which HA/FA are present. In the acidic medium, HAs/FAs exhibited the coiling nature because of intramolecular hydrogen bonding. At that stage, HAs/FAs are highly bioactive because they experience large amounts of steric hindrances. However, in the basic medium, a folded structure is obtained. At that stage, HAs/FAs are highly open and steric hindrance and intramolecular H-bonding are less. Under this condition, HAs/FAs are less stable and bioactive. The humic acid samples required longer time to achieve stable, higher pH than the fulvic acid samples did because humic acid has highly polymerized and coiled polycondensed (with phenolic -OH & -COOH groups) structure and fulvic acids exhibit a strong effect of hydrophobic hydration [21].

Viscometric measurement: The behaviour of humic substances provides an explanation of the extent of intrinsic and reduced viscosity [12]. Table-3 presents the experimental B values ($B_{expt.}$) of the HA/FA samples. The B_{expt} values were obtained from the slope of the best-fit linear graph of $\eta_{sp}/\sqrt{C}$ *versus* $\sqrt{C}$ by using the list square technique. The coiled characteristic of HA/FA molecules may be attributed to the decrease or increase in B_{expt} values [21]. The B_{expt} values of the humic acids obtained from the soil samples were higher than those of fulvic acids. These values may correspond to a higher stable coiling degree and thus to the molecular weight. The intrinsic viscosities $[\eta]$ calculated using B_{expt} values [12] were employed to estimate molecular weights (M) by applying the modified Staudinger′s equation (eqn. 2). Higher B_{expt} values result in the higher molecular weights (Table-3) [21].

TABLE-3
$B_{experiment}$ AND MOLECULAR WEIGHTS OF HUMIC AND FULVIC ACIDS

HA/FA sample	$B_{experiment}$	Molecular weight (m.w.) of humic/fulvic acid
HA Pundibari	0.3260	13645.83
HA Sikkim, Majitar	0.2950	10230.57
HA Darjeeling	0.2930	10115.79
FA Pundibari	0.0732	1196.74
FA Sikkim, Majiatr	0.0835	1465.54
FA Darjeeling	0.0690	1093.95

HA = Humic acid; FA = Fulvic acid

The results are consistent with the reported findings [16]. Under alkaline pH conditions, humic acids assume an elongated structure may be due to the negative charges present. This stimulates repulsive interactions within molecules. Humic acids can coil at a decreasing pH [16].

The molecular weight of the humic acid samples was the highest for Pundibari soil, followed by Darjeeling and Majitar soils (Table-3), which indicated that with altitudes, molecular weights decrease. The molecular weight of fulvic acid was highest in Majitar soil, followed by Pundibari and Darjeeling soils. Higher molecular weight of humic acids in comparison to fulvic acid may be due to the higher aromatic-ring condensation in humic acids [22].

This result can be caused by cooler altitudes, which may reduce the lignin degradation intensity, the formation of condensed aromatic structures and high molecular weight. Low molecular weights can be obtained because at a high altitude, humic acids with increased alcoholic OH-groups, such as polysaccharides, dominate [23].

Surface tension analysis: At the same temperature, the surface tension values of the HA/FA samples was lower than that of pure water (Table-4). The order of surface tension is as follows: Darjeeling < Majitar < Pundibari. With an increase in the concentration, surface tension decreases [24,25]. Similarly, for fulvic acids, lower surface tension was observed in Darjeeling soil followed by Majitar and Pundibari soils.

TABLE-4
SURFACE TENSION OF HA/FA SAMPLES
AT VARIOUS CONCENTRATIONS OF HA/FAs
[Surface tension of water at 26 °C = 71.47 dynes/cm]

Sample of HA/FA	Concentration (%)	Surface tension in water (dynes/cm)
HA Pundibari	0.125	70.7926
	0.250	69.7812
	0.500	68.8122
	1.000	66.9397
HA Sikkim, Majitar	0.125	70.7926
	0.250	68.7980
	0.500	66.9260
	1.000	65.1540
HA Darjeeling	0.125	69.7812
	0.250	67.8429
	0.500	66.0225
	1.000	64.2973
FA Pundibari	0.125	70.7900
	0.250	68.7900
	0.500	67.8500
	1.000	66.0300
FA Sikkim, Majitar	0.125	70.7900
	0.250	69.7800
	0.500	67.8500
	1.000	66.0300
FA Darjeeling	0.125	69.7800
	0.250	68.7900
	0.500	66.9200
	1.000	65.1500

HA = Humic acid; FA = Fulvic acid

Spectrophotometric analysis: The balance between aromatic and aliphatic compounds of HA/FA was determined using the E_4/E_6 ratio. Because the aromatic portions of HA/FA have a hydrophobic nature, they reach the innermost core of coils; subsequently, the aromatic part reaches to the outer periphery of coils [21]. Therefore, the absorbance of aliphatic groups is higher (at 465 nm) than that of aromatic groups (at 665nm) because of their conformation [26].

The E_4/E_6 ratio is consistently higher at pH 5 than that at pH 9 (Table-5). The E_4/E_6 ratio of humic acids is lower than that of fulvic acids because of the higher condensation degree of aromatic rings in humic acids [22]. These findings coincide with the results of Eshwar *et al.* [27]. The E_4/E_6 ratio array of the humic acid 1% (2.758-3.577) and fulvic acid 1 % (1.288-8.500) samples differed from those reported by other researchers may be due the differences in the climate, soil and vegetation conditions.

TABLE-5
SPECTROPHOTOMETRIC (E_4/E_6) ANALYSIS
OF HUMIC/FULVIC ACID SAMPLES

Sample of HA/FA	E_4/E_6 at pH		Δ (E_4/E_6) = (E_4/E_6 at pH 5.0- E_4/E_6 at pH 9.0)
	5.0	9.0	
HA (Stock, Pundibari)	2.265	2.150	0.115
HA (Stock, Sikkim, Majitar)	1.100	1.000	0.100
HA (Stock, Darjeeling)	2.146	1.928	0.218
HA (1%, Pundibari)	2.827	2.758	0.069
HA (1%, Sikkim, Majitar)	3.265	3.577	-0.312
HA (1%, Darjeeling)	3.243	2.963	0.280
FA (Stock, Pundibari)	4.509	4.363	0.146
FA (Stock, Sikkim, Majitar)	1.513	1.5426	-0.029
FA (Stock, Darjeeling)	8.148	8.755	-0.607
FA (1%, Pundibari)	4.800	4.650	0.150
FA (1%, Sikkim, Majitar)	1.570	1.288	0.282
FA (1%, Darjeeling)	8.500	6.800	1.700

HA = Humic acid; FA = Fulvic acid

The E_4/E_6 ratio (absorbance ratio at 465 and 665 nm), aromaticity and the condensation degree of aromatic carbons chains of humic acids were interrelated. Therefore, humic acids can be employed as humification indices [5,28]. The E_4/E_6 ratio was not affected by humic acid and fulvic acid concentration but did depend on the humic materials extracted from different soil types and manures [29].

Conclusion

The physio-chemical characteristics of the soils obtained from three locations [Darjeeling (North Bengal), Pundibari (North Bengal) and Majitar (Sikkim)] indicated that these soils had a neutral to acidic nature. Natural humic acids and corresponding fulvic acids were analyzed through viscometric analysis, potentiometric titration, surface tension and visible spectrophotometry.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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