Article

An Alternative Methodology for the Extraction of Humic Substances from Organic Soils

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Este trabalho apresenta uma metodologia alternativa para extração de Substâncias Húmicas (SH) de solos. Nesta metodologia a extração é feita com KOH 0,5 M por 3 h, na razão solo/extrator 1:20 (m/v) sob atmosfera de nitrogênio. As SH são separadas por centrifugação, com base em sua solubilidade em pH alcalino. Esta metodologia foi comparada com o procedimento usualmente utilizado em três diferentes solos e uma amostra de vermicomposto. Determinou-se rendimento, razão E_4/E_6, teor de cinzas e composição elementar (C, H, O, N) das SH extraídas. Os grupos funcionais foram identificados utilizando-se espectroscopia na região do infravermelho com transformada de Fourier. Esta metodologia acrescenta uma nova perspectiva à extração das SH devido ao menor tempo e elevado rendimento da extração em relação ao procedimento usual.

The present study reports on an alternative methodology for the extraction of Humic Substances (HS) from the soil. The extractions were carried out with 0.5 M KOH for 3 h, at a ratio of 1:20 (m/v) under a nitrogen atmosphere. The HS were separated by centrifugation based on their solubility in alkaline solution. This methodology was compared with the usual procedure in three different soil samples and in one sample from vermicompost. The yield, E_4/E_6 ratio, ash content, and the elemental composition (C, H, O, N) of the extracted HS have been determined. The functional groups were identified by Fourier-transform infrared spectroscopy. This novel procedure adds a new perspective to the extraction of humic substances due to the short time and high performance of the extraction in relation to the usual procedure.

Keywords: *humic substances, extraction, characterization*

Introduction

The most important class of naturally occurring complex agents is the humic substances (HS). They comprise a complex mixture of physically and chemically heterogeneous substances which show a relatively high molecular weight with a large number of oxygen-containing functional groups: -COOH and -OH in particular[1]. The HS are formed by secondary synthesis reactions during the decay process and by transformation of biomolecules originating from dead organisms and microbial activity[2]. These compounds are important in soil conservation, in water holding capacity[3], and in the complexation of metals in aquatic systems[4].

The extraction procedure is a very important step in relation to the characterization of humic substances (HS). Several extraction procedures using alkaline, complexing and salt solutions have been discussed by Hayes[5]. The International Humic Substances Society (IHSS) has recommended[6,7] a standard procedure, based on a treatment with 0.1 M NaOH at room temperature for 4 h, under N_2 atmosphere. Despite the numerous techniques used for this purpose[8-11], HS specific structural characteristics still remain unknown, chemical analyses of untreated samples have provided only limited information and many questions

concerning the factors that influence the extraction proce-dure need to be answered[12].

The purpose of the present work is to report on an alternative methodology for the extraction of HS which required a short experimental time period and give high yields when compared with the usual procedure[6,7].

Materials and Methods

Samples

The study has been made with three samples collected from soil surfaces of different regions of São Paulo State - Brazil. Two samples are from the peat (a recent and an old one) collected from the regions of Ribeirão Preto and Batatais, respectively. A sample of sediment from the Cananéia island and a vermicompost from a vivarium located in the city of São Carlos.

The proportion of organic material was determined by calcination at the temperature of 700 °C for 3 h[13]. The characterization of the soils in terms of fertilization (Ca, K, Mg, P, S, CEC) was done through the methodology de-scribed by Raij, *et al.*[14]. Physical and chemical charac-teristics of these soils are given in Table 1.

Extraction, separation and purification of the humic substances

Alternative methodology

The dried soil samples were ground and passed through a 2 mm sieve. Five grams of the resulting material were transferred to a round-bottom flask and 20 mL of KOH (0.5 M) were added per gram of soil. The suspension was stirred under N_2 atmosphere for 3 h. Then, the humic mixture was centrifugeted at 2012 g for 10 min. The non-dissolved fraction was separated and discarded. The supernatant (HS) was evaporated *in vacuo* and dried in a forced ventilation stove at 55 °C for further determination of extraction yields and ash content. Next, the HS were purified following the procedures 'separation of the non-humic contaminants' and 'separation of the inorganic contaminants' for its sub-sequent characterization.

Usual methodology [6,7]

The dried soil samples were ground and passed through a 2 mm sieve. Five grams of the resulting material were extracted with a volume of 0.1 M HCl equal to ten times the weight of the sample. The pH of the solution was adjusted between 1 and 2 with 1.0 M HCl. The soil/HCl mixture was shaken for 1 h and the suspension was, then, allowed to settle. The mixture was centrifuged at 1478 g for 10 min, and the supernatant separated from the sedi-ment. The sediment was neutralized with 1.0 M NaOH to pH 7.0, and a volume of 0.1 M NaOH equal to ten times the weight of the sample was added under nitrogen atmos-phere. The mixture was shaken for 4 h and then allowed to settle overnight. The supernatant was separated from the sediment by centrifugation at 1478 g for 10 min, and the sediment was discarded. The supernatant (HS) was evapo-rated *in vacuo* and dried in a forced ventilation stove at 55 °C for further determination of extraction yields and ash content. Next, HS were purified following the procedure 'separation of the non-humic contaminants' and 'separa-tion of the inorganic contaminants' for its subsequent char-acterization.

Purification of the humic substances

Separation of the non-humic contaminants

The HS extract containing non-humic substances was applied to a macroporous resin AMBERLITE XAD-8[15] column. After previous treatment of the resin with metha-nol/deionized water, the HS were diluted in deionized water and eluted in a 3 mL/min descendent flow. The elution of the HS with NaOH 0.1 M was performed in a 1 mL/min flow.

Separation of the inorganic contaminants

Dried HS were purified during 4 h shaking at room temperature with a 10 mL solution composed of 0.5 mL of HCl (conc.), 4.5 mL of HF solution (40%) (v/v) and deion-ized water up to 1 litre. Next, the samples were transferred to dialysis tubing and dialysed in order to remove the salt contents. The external water was replaced three times after 3-5 h and then every 12 h until the Cl⁻ could not be detected

Table 1. Physical and chemical characteristics of soil samples (ovendried basis).

Sample	Color	pH	OM (%)	Mg	Ca	K	S	CEC*	P (µg/g)	C/N
						meQ/cm³				
Batatais Soil	black	4.2	50.92	3	8	1.4	12.2	183.8	16	11.81
Ribeirão Preto Soil	gray	4.1	24.16	1.8	7.1	0.48	9.4	25.9	37	11.35
Cananéia Soil	yelow dark	3.1	23.32	50	55	6.3	111.7	421.1	5	7.77
Vermicompost	brown	4.8	31.46	54	120	105.3	279.2	342.3	188	5.36

*CEC - cation exchange capacity.

(this procedure decreased the ash contents of the HS below 3% m/m). After that, the HS were evaporated *in vacuo*, dried in a forced ventilation stove at 55 °C and characterized by the spectroscopic analysis in the visible and infrared region and also by elemental analysis.

Dialysis tubing purification

Dialysis tubing (molecular weight cut-off 14 000) was cleaned by boiling in 2% (m/m) sodium hydrogencarbonate - 0.01 mol/L Na_2EDTA for 10 min, followed by rinsing in boiling deionized water. Clean tubing was stored in deionized water at 6 °C[16].

Characterization

Yields

The yield of HS was calculated based on the soil mass submitted to extraction (5.00 g) and the weighed organic material (OM) extracted. The results are given in Table 2.

Ash content

The ash content of the extracted HS was obtained after ignition at 750 °C for 4 h[17].

UV/VIS spectroscopy

E_4/E_6 ratios were determined by dissolving 2.0 mg of HS in 10 mL of 0.05 M $NaHCO_3$ solution (pH between 9.1 - 9.5 in all cases) and by measuring optical densities at 465 and 665 nm[17] on a Varian GBC - 911 A spectrophotometer. The results of these measurements are given in Table 2.

Infrared spectroscopy

The functional groups of the HS were characterized by Fourier-transform IR spectroscopy (Fig. 1). The spectra were recorded by using a Nicolet 730 SX-FT spectrometer. KBr discs were used to prepare the samples[18].

Elemental analysis

The elemental analysis of the HS extracted was determined by using a Perkin-Elmer 240 - C elemental analyser. The results of these measurements are given in Table 2.

Results and Discussions

Table 2 shows the results of the elemental composition (C, H, O, N), yields of the extraction and E_4/E_6 ratio of the HS extracted by the alternative methodology and by the usual procedure in the different soil samples and vermicompost.

Yields and extraction time

The extraction of the HS from the soils using alkaline solvents comprises the ionization of the acid groups and solvation of the conjugated bases in aqueous medium. At first, any system which displaces or removes divalent and polyvalent cations and allows the negative charges on the macromolecular matrix to repel each other, and to give rise to the expansion of the molecules, should promote solvation. The consequent dissociation, molecular expansion, solvent penetration, etc., result in the transference of the humic substances from the solid to the solution phase[6,19]. Consequently, the solubility of humic substances (and the molecular size distribution of the soluble components) is dependent on pH, ionic strength and nature of the electrolyte ions[16].

The increase of the yields and the reduction of the HS extraction time using the alternative methodology is associated with the substitution of the alkali used in the extraction. The counter ions Na^+ and K^+, responsible for the interaction and solvation of the negative charges dissociated from the humic macromolecule, have different behaviours in aqueous medium (Table 3)[20,21].These different characteristics resulted in the increase of the yields and on the reduction of the extraction time, when KOH was used.

Table 2. Comparison between the elemental composition, C/H, C/N and E_4/E_6 ratios and yields of HS extracted by the usual procedure (A) and by the alternative methodology (B).

| Sample | Yields (%) | | Elemental composition (%) (ash-free) | | | | | | | | C/H | | C/N | | E_4/E_6 | |
| | | | C | | H | | O* | | N | | | | | | | |
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
HS - 1	18.25	30.19	52.60	50.51	5.21	4.85	41.59	43.93	0.60	0.72	10.10	10.41	87.67	70.15	3.87	3.54
HS - 2	13.92	18.31	45.24	49.85	5.00	4.49	47.60	44.55	2.16	1.11	9.05	11.10	20.94	44.91	4.03	4.01
HS - 3	7.23	10.28	47.92	48.41	6.16	6.15	44.48	44.12	1.44	1.32	7.78	7.87	33.28	36.67	4.62	4.22
HS - 4	8.66	9.12	40.74	42.18	9.23	9.01	47.30	46.36	2.73	2.45	4.41	4.68	14.92	17.22	4.75	4.14

* obtained by difference.
HS - 1 - HS extracted from Batatais soil.
HS - 2 - HS extracted from Ribeirão Preto soil.
HS - 3 - HS extracted from Cananéia island soil.
HS - 4 - HS extracted from vermicompost.

The smaller ionic radius of K^+ in relation to Na^+ allows a greater efficiency in the expansion of the macromolecule, in the counterbalance and in the capacity of interaction of the intra and intermolecular charges present in the HS. This efficiency contributed significantly to the solvation and to the increase of the extraction yields using KOH. The reduction of the extraction time of the HS by the alternative methodology, is justified by a better mobility, of the K^+ ions in aqueous solution. This mobility reflected directly on the increase of the speed of the interaction between the charges of the polyelectrolyte and of the counter ion.

The difference of the yields between the two methodologies was greater in the peat samples, mainly the Batatais peat, in which the humic level and the organic material contents are higher. This shows that the increase of the condensation level makes the Na^+ counter ion mobility in the process of solvation of the macromolecule difficult. These results show that the characteristics of the matrix and the properties of the HS are relevant for choosing the adequate solvent for the effective extraction of the HS.

Another factor that contributed to the increase of the yields of the alternative methodology, in relation to the usual methodology, was the omission of an acidification step of the soil because there is a loss of organic material in the form of low molecular weight fulvic acid [22].

Characterization of HS

The decomposition of the organic material present in the soil tends to lead to the formation of phenolic structures derived from the lignin. It is harder for these structures to suffer degradation in relation to sugars and proteins[23]. Therefore, the C/H and C/N ratio are important indicators of the aromaticity and of the organic material decomposition level. The highest C/H ratio found in the HS proceeding from the peat show higher aromaticity in relation to the HS extracted from the sediments and vermicompost. This occurs due to the different characteristics of the soils used in the study (Table 1). The higher C/N ratios of the samples from the Batatais and Ribeirão Preto soils indicates greater decomposition which is a characteristic of the peat. The samples of the sediments and vermicompost, instead, showed lower C/N ratios because of the low humic level of organic material. The results of the elemental analysis of the HS extract (Table 2) are compatible with the values found in the literature [13,17,24].

Table 3. Thermodynamic properties of Na^+ and K^+ in aqueous solution.

Íon	Ionic radius hidrated[20] (10^{-9} m)	Ionic mobility[21] $u/10^{-18}$ cm^{2}s^{-1}v^{-1}, 298 K
K^+	300	7.62
Na^+	450	5.19

These characteristics related to the decomposition level and aromaticity can be reassured by the values of the E_4/E_6 ratio (Table 2). The E_4/E_6 ratio depends on the humification degree and molecular size of the humic substances[25]. Thus, low E_4/E_6 ratios are indicative of relatively extensive molecular condensation, while higher ratios are associated with materials having better access to their internal functional groups[26]. The values found are in accordance to the literature[17,26,27].

Some differences are observed when the values of the elemental analysis of the extracted HS by the two methodologies are compared. In the soils studied, the extracted HS by the alternative methodology present higher values in the ratios C/H and lower in the ratio E_4/E_6 indicating higher level of condensation when compared with the usual procedure that has been utilized. Differences in the oxygen content of the extracted HS by the two methodologies are also observed. In all extractions done by the usual procedure, higher oxygen contents was observed. This fact can be associated with the oxidation of the HS during the process of extraction. In this case, the use of KOH would decrease the oxidation of the HS during the extraction and would minimize possible structural alterations in the HS (these observations are still being studied).

The infrared spectra of the purified materials (Fig. 1) show absorption bands compatible with the literature[17,18,28].

1 - bands around 3750 cm^{-1} associated with a ν O-H related to clay;

2 - broad bands around 3400 cm^{-1} assigned to ν O-H of alcohols, phenols and carboxylic acids and N-H of amines and amides;

3 - weak bands between 2920 and 2850 cm^{-1} attributed to ν C-H aliphatic;

4 - strong bands near 1700 cm^{-1} assigned to ν C=O of carboxylic acids and their derivatives: ketones, quinones, amines and amides;

5 - strong bands near 1640 cm^{-1} attributed to ν C=C of aromatic rings and to ν C=O of conjugated ketones and carboxylate ions;

6 - bands near 1530 cm^{-1} assigned to δ N-H of amides;

7 - bands between 1400 and 1380 cm^{-1} attributed to δ O-H of alcohols, phenols and carboxylic acids, ν C-O phenolyc, δ C-H aliphatic and ν COO of carboxylate ions;

8 - medium bands in the regions of 1110 and 1030 cm^{-1} are assigned to ν C-O of polysaccharides, alcohols and δ Si-O.

Differences between the nature of the functional groups present in the structure of the HS cannot be observed when compared with the spectrum of extracted HS by the two methodologies from the different soils. It indicates structural resemblance between the HS present in soils with different characteristics (Table 1). The main difference
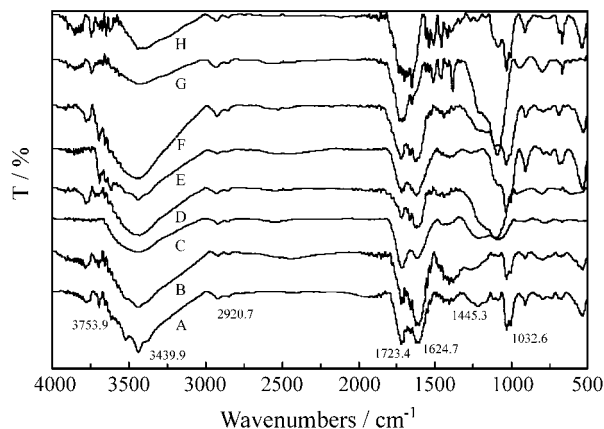
Figure 1. IR spectrum of the HS extracted by the usual procedure (1) and alternative methodology (2). (A = HS extracted from Batatais soil by procedure 1, B = HS extracted from Batatais soil by procedure 2, C = HS extracted from Ribeirão Preto soil by procedure 1, D = HS extracted from Ribeirão Preto soil by procedure 2, E = HS extracted from Cananéia soil by procedure 1, F = HS extracted from Cananéia soil by procedure 2, G = HS extracted from vermicompost by procedure 1, H = HS extracted from vermicompost by procedure 2).

between the HS seems to be associated with the content and the characteristic of the oxygenated groups, as it can be observed by the results of the elemental analysis (Table 2).

Conclusions

The extraction and purification of the HS are indispensable requisites for the studies of its characteristics and properties. These stages are considered boring, time consuming and tiring for researchers. This methodology adds a new perspective to the extraction of humic substances due to the short time and high yields of the extraction in relation to the usual, especially for peat samples which present higher levels of condensation and high contents of organic material.

Acknowledgments

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