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SOIL CHEMISTRY

Structure of Humic Acids Isolated by Sequential Alkaline Extraction from a Typical Chernozem

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Abstract—It was shown with the isolation of a humic acid (HA) preparation from a typical chernozem by sequential alkaline extraction as an example that the preparative yield of HAs decreased at each sequential extraction stage by 3–4 times. On the basis of studying the obtained preparations using elemental analysis, gel-penetration chromatography, and ^{13}C NMR spectroscopy, the tendencies of the changes in the structural-group and molecular-weight compositions of the HAs from one extraction stage to the next one were revealed. The conclusion was drawn that a single extraction is sufficient for obtaining a representative HA sample.

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INTRODUCTION

Two main methods are presently used for the isolation of humic acid (HA) preparations from soils: the method accepted in the Russian school of soil science [7] and that recommended by the International Humic Substances Society (IHSS) [17]. Both methods are based on the following general principle: the alkaline extraction of humic substances from soil. However, they have a number of significant methodological differences. One of them is the number of extractions used for the isolation of an HA preparation. According to the method used in Russia, the soil is treated with alkali at least three times, and the extracts are combined for obtaining an integral sample, from which the HA preparation is then isolated. The procedure recommended by the IHSS involves a single extraction with an alkali; hence, the isolation of the HAs is performed from the primary alkaline extract.

Other differences include the following:

(a) The alkaline extraction is performed under a nitrogen atmosphere according to the IHSS procedure [17].

(b) The coagulation of the mineral suspensions is performed in the alkaline extract by sodium chloride according to the method used in Russia [7]; in the IHSS method, it is performed with the HAs previously separated from the fulvic acids (FAs) using KCl as a coagulating agent [17].

(c) The treatment of the HA precipitate with a mixture of 0.1 M HCl and 0.3 M HF for the ultimate removal of the fine mineral suspensions as recommended by the IHSS method [17].

The assessment of the effect of each difference on the composition of the HA preparations is necessary for the correct intercalibration of the above methods.

First of all, the effect of the number of extractions on the structure and properties of the preparations obtained should be elucidated. The number of alkaline extractions determines, first, the labor content and the duration of the HA isolation procedure and, second, affects the degree of alteration of soil organic matter during the isolation procedure.

As was noted above, the IHSS method includes a single extraction of humic substances from the soil after decalcification, while the Russian version recommends at least three extractions, because it is believed that the humic substances in the first extract portions can differ in their properties and composition from those of the substances extracted later [7]. As a result, the Russian method should give more representative HA preparations and their higher yield, but this is a significantly more laborious procedure. In addition, the occurrence of a soil suspension in alkali for a long time (no less than three days) and the attendant processes of deep hydrolysis can result in the extraction of a preparation significantly differing in structure and properties from the humic substances present in the soil.

Thus, the comparison of the structures and properties of the HA preparations obtained by the single and repeated alkaline extractions from soils is an essential condition for the comparison of the preparations isolated in accordance with the protocol used in Russia and that recommended by the IHSS.

Table 1. Some physicochemical parameters of the deep typical chernozem under study

Parameter	$\bar{x} \pm t_\alpha s_{\bar{x}}^*$
pH water	6.8 ± 0.1
C_{org}	5.52 ± 0.1
C_{ha}/C_{fa}	1.7 ± 0.1
Exchangeable bases, meq/100 g	
Ca^{2+}	30.8 ± 0.2
Mg^{2+}	6.9 ± 0.2
K^+	0.5 ± 0.05
Na^+	1.1 ± 0.05
Content of particles, %	
<0.01 mm	40.0 ± 0.2
<0.001 mm	21.2 ± 0.2

* Here and below, $\pm t_\alpha s_{\bar{x}}$ the confidence interval calculated for $\alpha = 0.05$.

The aim of this work was to study the elemental, structural-group, and molecular-weight compositions of the HAs isolated from the sequential alkaline extracts of a decalcified typical chernozem.

EXPERIMENTAL

The HA preparations were isolated from the humus-accumulative horizon of a deep typical chernozem on the annually mown steppe of the Alekhin Central Chernozemic State Biosphere Nature Reserve in Kursk oblast. Soil samples (200 g) were taken from the 5- to 15-cm layer at randomly selected sampling points in a plot 10 × 10 m in size. An average sample (1.5 kg) was formed by quartering the combined sample (about 10 kg). The large roots were removed from the average sample, which was then triturated and passed through a 1-mm sieve.

For the soil sample thus prepared, the main physicochemical parameters were determined: the pH, exchangeable bases [1], physical clay and clay (by the pyrophosphate method [4]), organic carbon, and the C_{ha}/C_{fa} ratio [7]. The results are given in Table 1. The determined parameters correspond to the ranges reported for typical chernozems [9]. In addition, the values of the pH and C_{ha}/C_{fa} ratio and the contents of organic carbon, exchangeable calcium and magnesium, physical clay, and clay particles were close to those previously obtained for typical chernozems of the Central Chernozemic Zone [10, 11]. Thus, the soil was representative of the typical chernozem subtype, which completely satisfied the purposes of the study.

From the soil sample prepared, the HA preparations were obtained according to a pattern combining both considered methods [7, 17]. Several alkaline extractions of humic substances were performed according to the method used in Russia; however, the extracts obtained were not combined, and the HA preparations were separately isolated from each extract. The elemental, structural-group, and molecular-weight compositions were determined for these HA preparations.

Isolation of the HA preparations. A soil sample (500 g) was decalcified by the addition of 1 M HCl until reaching a suspension pH in the range of 1–2; then, 0.1 M HCl was added to reach a soil : solution weight ratio of 1 : 10. The obtained suspension was shaken for 1 h and left to stand for 24 h; the supernatant was separated from the solid phase by decantation. The decalcified soil was neutralized by the addition of 1 M NaOH to pH 7, and 0.1 M NaOH was added to reach a soil : solution weight ratio of 1 : 10. The suspension was periodically stirred for 6 h and left to stand overnight; 24 h after the beginning of the extraction, the alkaline extract (extract 1) was drawn off through a siphon and stored. Then, a new portion of 0.1 M NaOH was added to the soil at a ratio of 1 : 10, the mixture was periodically stirred for 6 h and left to stand overnight, and the alkaline extract (extract 2) was drawn off after 24 h and stored. The procedure was repeated to obtain extract 3. Each of the extracts obtained was used to isolate the HA preparations by the separation of the FAs and the purification of the HAs from inorganic impurities. The HAs were separated from the FAs by precipitation under acidification with 6 M HCl to pH 1–2 and centrifugation of the precipitate. The HAs were then dissolved in a minimum volume of 0.1 M KOH, and KCl was added to a K^+ concentration of 0.3 M for coagulating the fine mineral particles. The coagulated solid impurities were separated by centrifugation. Next, the HAs were reprecipitated and treated with a mixture of 0.1 M HCl and 0.3 M HF to remove the silicon-containing impurities according to the IHSS procedure [17]. The treated HA suspension was purified by dialysis, dried on a rotor evaporator, and stored in a desiccator over P_2O_5 for at least one week. The HA1, HA2, and HA3 preparations were thus obtained from extracts 1, 2, and 3, respectively. The yield of HAs during each extraction was assessed by weighing the preparations obtained.

Characterization of the preparations isolated. The elemental composition was determined using a Carlo Erba elemental analyzer (model 1106; Carlo Erba Strumentazione, Italy). The content of hygroscopic water in all the preparations was taken equal to 8% [8]. The content of ash in the isolated HAs was determined by ignition in quartz tubes under an oxygen atmosphere at 750°C for 40 min. The content of oxygen was calculated as the difference between the weight of the dry ashless sample and the total content of C, H, and N.

The molecular-weight distribution of the HAs was determined by gel-penetration chromatography [15]. The gel-chromatography system (Gilson Abimed, France) included a column, a pump, an automated sampler, and a flow-through ultraviolet detector. The gel-chromatography column was 25 mm in diameter and

Table 2. Elemental composition of the HAs obtained by three sequential alkaline extractions from a typical chernozem

HA preparation	Elemental composition, wt %*				Atomic ratios			Ash, %
	C	H	N	O	H/C	O/C	C/N	
HA 1	56.3	3.3	3.9	36.6	0.70	0.49	16.9	2.1
HA 2	54.8	3.8	4.5	36.9	0.83	0.51	14.0	2.7
HA 3	54.5	3.7	4.7	37.1	0.81	0.51	13.5	2.0
$\pm t_\alpha s_{\bar{x}}$	0.5	0.3	0.3	1.0	0.06	0.02	0.8	0.2

* Calculated for a dry ashless sample. The content of hygroscopic water in all the preparations was taken equal to 8% [8].

20 cm in length. Gel Toyopearl TSK HW-55S (Toso Haas, Japan) was used as the stationary phase; a 0.028 M phosphate buffer solution with pH 6.8 was used as the mobile phase. The column void volume was determined with blue dextran (20000 kDa); its total volume was determined with acetone (48 Da). They were 12 and 34 ml, respectively. Sodium salts of polystyrene sulfonic acids with peak molecular weights of 14.00, 20.70, 45.10, and 80.84 kDa (Polymer Standard Service, Germany) were used for the calibration. From the obtained chromatograms and calibration curves, the peak molecular weight (M_p) was calculated, and the weight-average and number-average molecular weights (M_w and M_n, respectively) were determined, as well as the polydispersity (M_w/M_n) [3, 13]. To assess the possible sorption of substances on the gel, the substance yield from the column was determined. The experiments showed that 85% of the HAs on average were recovered from the column.

The distribution of the HA carbon among the structural fragments was determined by ^{13}C NMR spectroscopy [5]. The HA samples for the NMR study were prepared by dissolving a sample (80 mg) in 0.6 ml of 0.3 M $NaOD/D_2O$. The mixture was placed in an ultrasonic bath for 30 min and centrifuged at 18 g for 5 min; then, the solution was separated from the precipitate and transferred into a 5-ml NMR ampoule. The ^{13}C NMR spectra were recorded on a Bruker Avance-400 spectrometer operating at 400 Hz for hydrogen nuclei using a CPMG sequence with an initial 90° pulse; the registration time of the free induction decay was 0.2 s, and the relaxation delay between the pulses was 7.8 s. The duration of one NMR determination was about 12 h.

The redistribution of carbon atoms among the different structural fragments was determined by the integration of the corresponding spectral regions. The following assignments were made in the spectrum according to [5] (ppm): 220–187, carbon of ketone and quinone groups ($C_{C=O}$); 187–165, carbon of carboxyl and ester groups ($C_{COO\text{-}H,\,R}$); 165–145, carbon of O, N-substituted aromatic fragments ($C_{Ar\text{-}O,\,N}$); 145–108, carbon of unsaturated and C-substituted aromatic fragments ($C_{Ar\text{-}H,\,R}$); 108–48, carbon of O, N -substituted aliphatic fragments ($C_{Alk\text{-}O,\,N}$); 48–5, carbon of aliphatic fragments unbound to heteroatoms ($C_{Alk\text{-}H,\,R}$).

The data obtained for the structural-group and molecular-weight composition of the HAs were used for comparing the isolated preparations.

RESULTS AND DISCUSSION

The yield of the HA preparations obtained by the three sequential extractions from 500 g of typical chernozem was 6.4, 1.6, and 0.5 g for the HA1, HA2, and HA3, respectively. Thus, the amount of the preparation obtained after each extraction decreased by 3–4 times compared to the previous one.

The elemental compositions of the obtained HA preparations are given in Table 2. On the whole, they were within the ranges reported for HAs [2, 6, 8]. Within these ranges, HA1 significantly differed from HA2 and HA3. The elemental compositions of HA2 and HA3 differed statistically insignificantly. The content of carbon was 56.3% in HA1, 54.8% in HA2, and 54.5% in HA3 at a confidence level of 0.5%. A similar situation was observed for hydrogen and nitrogen: HA2 and HA3 differed from each other by a value lower than the confidence level, and the parameter of HA1 reliably differed from those of HA2 and HA3. It should be noted that all three preparations insignificantly differed in the content of oxygen.

The HA1 preparation obtained by the first extraction had a higher content of carbon and lower contents of hydrogen and nitrogen. The H/C ratio was 0.70 for HA1; its values for HA2 and HA3 were significantly higher (0.81 and 0.83, respectively). This fact could be indicative of an increase in the share of aliphatic fragments in the HAs obtained by the second and third alkaline extractions. Similar O/C values were observed for all the preparations; this suggested that the observed changes in the elemental composition of the HAs were caused by the extraction of organic substances differing in their properties from HA1 during the following extractions rather than by the oxidative destruction of organic matter.

The molecular-weight distributions of the obtained HAs were studied by gel-penetration chromatography. The gel chromatograms of all three HA preparations are shown in Fig. 1.

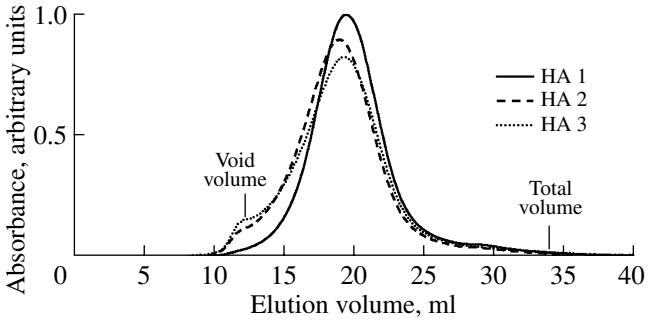

Fig. 1. Gel-chromatograms of HA samples obtained by sequential alkaline extractions.

It can be seen that the chromatograms of all the obtained preparations had unimodal distributions, which indicated the significant compensation for non-exclusion effects [13]. In distinction from HA1, the chromatograms of HA2 and HA3 contained a small peak in the region of the void volume, which most probably indicated the appearance of a high-molecular-weight fraction.

From the chromatograms obtained, the main parameters of the molecular-weight distributions were calculated (Table 3): the peak (M_p), weight-average (M_w), and number-average (M_n) molecular weights, as well as the preparation polydispersity (M_w/M_n).

The minimum peak value of the molecular weight (16.8) was obtained for HA1; the maximum value was observed for HA2 (Table 3).

The weight-average molecular weights increased in the following series: HA1 < HA2 < HA3. The molecular weights of HA2 and HA3 were close to each other and significantly differed from that of HA1. It is noteworthy that the observed tendencies agreed with the results of other authors obtained for HAs in sequential extracts from marsh peat soil [14]. This confirmed the above supposition about the significantly different HAs in the first extract and similar HAs in the subsequent extracts, which was based on the data of the elemental analysis.

Table 3. Molecular-weight distribution of the parameters of the HAs obtained by sequential alkaline extractions from a typical chernozem

HA preparation	M_p	M_w	M_n	M_w/M_n
	kDa			
HA 1	16.8	23.1	2.9	8.0
HA 2	19.6	35.7	6.1	5.9
HA 3	18.3	37.2	2.0	18.6
$\pm t_\alpha s_{\bar{x}}$	0.2	0.5	0.5	0.8

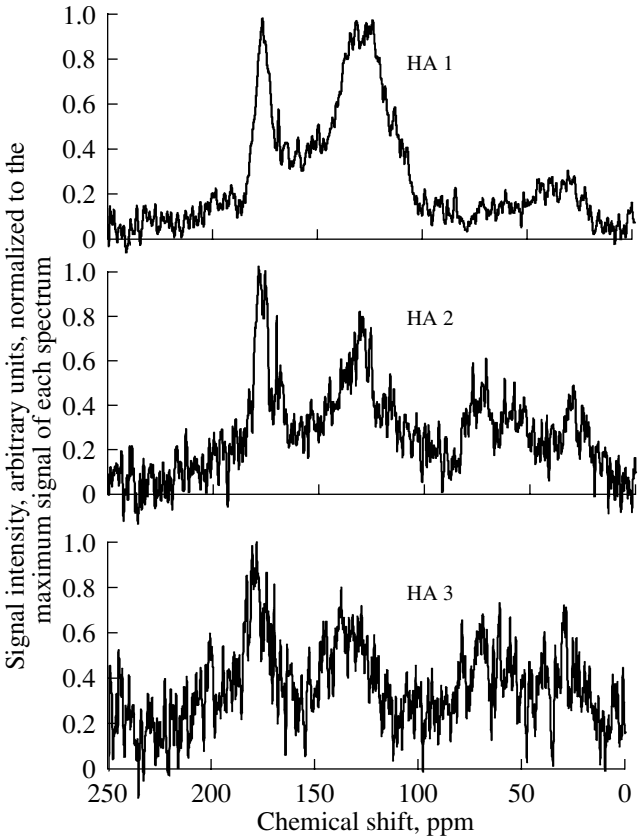

Fig. 2. ^{13}C NMR spectra of HAs obtained by sequential alkaline extractions.

The maximum number-average molecular weight was observed for HA2, and the minimum one was found for HA3. From the data obtained, the M_w/M_n ratio characterizing the polydispersity of the HA preparations was calculated [3]. The values obtained for HA1 and HA2 were close to those reported for the soil HAs [8, 12, 13, 16]; however, the values of HA3 were significantly higher. This could be related to the fact that various substances, probably of nonhumin nature, were also extracted in the third extraction.

This conclusion was confirmed by the ^{13}C NMR spectroscopic data for the preparations studied. It can be seen (Fig. 2) that the spectrum of HA1 was characterized by the presence of intensive signals in the region of aromatic carbon (165–108 ppm), carboxyl groups (187–165 ppm), and unsaturated aliphatic carbon (48–5 ppm). An almost complete absence of spectral intensity was observed in the region of 108–48 ppm, where the signals of heteroatoms (O, N), including carbohydrate and amine fragments, are located. At the same time, relatively intensive peaks appeared in the carbohydrate carbon region of the HA2 and HA3 spectra. Data on the quantitative assessment of the spectral intensity distribution in the ^{13}C NMR spectra for the HA preparations isolated from three sequential alkaline extracts are given in Table 4.

Table 4. Carbon distribution among the structural fragments in the HA preparations obtained by sequential alkaline extractions from a typical chernozem (% of C total)

Preparation	Spectral regions, ppm					
	5–48	48–108	108–145	145–165	165–187	187–220
	$C_{Alk-H,R}$	$C_{Alk-O, N}$	$C_{Ar-H,R}$	$C_{Ar-O, N}$	$C_{COO-H,R}$	$C_{C=O}$
HA 1	9	10	44	13	19	5
HA 2	15	26	25	9	17	8
HA 3	18	26	20	8	17	11
Weight-average value for all the preparations	11	14	39	12	19	6
$\pm t_\alpha s_{\bar{x}}$	4	1	3	2	2	3

The presented data show that the preparations most significantly differ in the content of aromatic and heteroatom-substituted aliphatic carbon. The total content of aromatic carbon in HA1 (57%) was almost double those in HA2 and HA3 (34 and 28%, respectively). At the same time, the lowest relative content of oxygen-substituted aliphatic fragments (10%) was found in HA1 compared to the other preparations (26%). The content of organic carbon regularly decreased with each next extraction. At the same time, the content of carbon in the aliphatic groups (both the nonsubstituted and oxygen-substituted ones) increased from HA1 to HA3. It should also be noted that the content of carbon in the ketone and quinone groups of the HA preparations significantly increased with each next extraction.

The above changes in the spectra of the preparations isolated from the second and third alkaline fractions could be related to the hydrolysis of nonhumin compounds: cellulose and protein residues present in the soil. Therefore, the HA2 and HA3 preparations should rather be classified as humin-like compounds. On the whole, it should be noted that the NMR data well agreed with the results of the elemental analysis, which indicated an increase in the share of aliphatic fragments in the structure of the HAs sequentially extracted by alkali from the soil.

These tendencies (the increase in the content of carbohydrate fragments and the decrease in the contribution of aromatic structures) agreed with the reported data on the structural-group composition of the HAs isolated in sequential alkaline extracts from marsh peat soil [14].

The weight-average carbon distribution among the structural fragments is given in Table 4. The tabulated values were calculated for the HAs that would be obtained by combining all three extracts with account for the weight proportion of each HA preparation. This composition would be typical for the integral HA preparation isolated by the Russian method, while the composition of the preparation isolated by the IHSS procedure would be similar to that of the HA1. It can be seen that, in spite of the significant differences between the preparations from the different extracts, the calculated weight-average values were close to those obtained for the HA1. Thus, the structural-group composition of the combined HA preparation obtained from a typical chernozem by the three-step alkaline extraction would be similar to that of the HAs isolated by the first extraction. Therefore, the HA preparations isolated by the method used in Russia [17] can be compared to the preparations obtained according to the IHSS recommendations [7], at least for typical chernozems.

Our results suggest that a single extraction is sufficient for obtaining an HA preparation with the properties most typical for this soil type, which agrees with the procedure recommended by the IHSS. At the same time, the multistage extraction according to the protocol used in Russia can be recommended for acquiring more profound comprehension of the specific minor components entering into the humin complex of a specific soil.

CONCLUSIONS

Our studies showed that, in the case of a typical chernozem, the properties of the HA preparations isolated by the multiple extractions regularly changed from one extraction step to another.

The HAs isolated from the first extract significantly differed from the HAs isolated by the second and third extractions, which were similar to each other in most parameters of the structural-group and molecular-weight compositions. On the whole, a decrease in the content of carbon, an increase in the H/C ratio, an increase in the portion of aliphatic fragments and the content of aromatic and carboxyl groups, and an increase in the weight-average molecular weights of the HA preparations were observed from one extraction to another.

It was shown that the preparative yield of HAs at each extraction stage decreased by 3–4 times compared to the previous one. Therefore, the first extraction made the major contribution to the structural-group and molecular-weight compositions of the average HA

preparation obtained by combining all the extracts. Taking into consideration the high labor expenses of the three-stage extraction and the possibility of the partial degradation of organic substances during the isolation, the single-step alkaline extraction can be recommended for the soil type studied. However, multiple extractions should be used in specific cases, e.g., for studying the minor components of the humin complex.

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