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SOIL CHEMISTRY

Comparison of the Properties of Humic Acids Extracted from Soils by Alkali in the Presence and Absence of Oxygen

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Abstract—Humic acids (HAs) make up to 30–50% of the soil organic matter, which is the main reservoir of organic carbon in the biosphere. The common isolation protocol for HAs implies alkaline extraction from soils followed by acidification of the extract with HCl to pH 2. International Humic Substances Society (IHSS) recommends isolation of HAs in oxygen-free atmosphere (e.g., under nitrogen or inert gas purging) to prevent oxidative transformations of HAs during the extraction process. In the Russian school of soil science, extraction is usually conducted without the use of nitrogen. In the present work, we compared the physicochemical properties of HAs isolated from A1 horizons of soddy-podzolic soil (Retisol) and chernozem (Chernozem) by 0.1 M NaOH in the presence and absence of oxygen. The soils used in this study represented zonal types of southern taiga and steppe, respectively, and differed markedly with respect to humus formation conditions. The yield of humic substances (C_{org} content in the extracts), their elemental composition, functional groups content, molecular-weight distributions (gel filtration on Sephadex G-75), paramagnetic properties, and absorption spectra in the visible, UV, and IR regions were studied. For both soils, no statistically significant differences were found in the quantitative yield, molecular weight distribution, absorption spectra in the visible, UV and IR regions between HAs isolated by alkaline extraction in the presence and absence of oxygen. At the same time, for the HAs extracted from the Retisol soil in the presence of oxygen, higher O : C ratios, higher contents of quinone and carboxyl groups, and significantly higher content of free radicals were observed. This was revealed with the use of elemental analysis, potentiometric titration, ^{1}H and ^{13}C NMR spectroscopy, and electron paramagnetic resonance spectroscopy. For the Chernozem HAs, these differences were not observed. The obtained results suggest that partial oxidation of the soil organic matter components takes place during alkaline extraction from the Retisol in the presence of oxygen. In the Chernozem, humification process is apparently accompanied by significant oxidative transformation of organic residues, so the presence of molecular oxygen does not cause further oxidation of HAs under alkaline conditions. Our results indicate that, for the isolation of HAs from the mineral horizons of Chernozems, the use of oxygen-free atmosphere is optional. In the case of Retisols, the use of oxygen-free atmosphere is desirable, especially if it is intended to study the reactions of HA oxidation upon, for example, enzymatic catalysis.

Keywords: soil organic matter, alkaline extraction, humic acids, elemental composition, gel filtration, spectroscopy, NMR

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INTRODUCTION

Humic substances are widely distributed in soils, sediments and natural waters [30]. They are described as dark-colored amorphous products of transformation of organic residues and were considered to make up to 90% of soil organic matter (SOM) [6, 19], the main reservoir of C_{org} in the biosphere [14]. The classification of humic substances is based on their solubility: alkali-soluble but insoluble at pH <2 humic acids (HAs), soluble at all pH values fulvic acids (FAs), and the insoluble residue from the alkaline extraction, or humin.

Humic substances largely determine the fertility and biospheric functions of soils, though their origin, molecular structure, and stability are the matter of discussion [27, 33]. Recently, the concept of humification and the fact of the existence of humic substances as a distinct class of organic compounds have been questioned. It has been suggested that SOM can be considered a continuum of biomolecules at different stages of degradation [22]. To describe the organic matter (OM) of soils and natural waters, the term natural organic matter is applied along with the term humic substances [24]. Nevertheless, the results of quantitative solid-phase ^{13}C NMR spectroscopy indi-

cate that HAs of Chernozem and peat differ significantly from the biomolecules of organic residues (lignin, cellulose, and proteins) in a higher content of condensed and O-substituted aromatic fragments and carbon of ketones and carboxyl groups [15]. These results do not support the view that the NMR signals of humic substances can be attributed exclusively to intact or degradable biomolecules [20], and that the substances of alkaline extracts are intermediate products of the transformation of organic residues [22].

One of the main problems of studying SOM is its strong association with mineral components and the method of its isolation based on alkaline extraction. About 40–80% of C_{org} is transferred to the alkaline extract (depending on the type of soil) [32], and a significant part of SOM remains in the "humin" fraction. Under alkaline conditions, especially in the presence of molecular oxygen, oxidation of phenolic compounds—key aromatic components of soil humus—can take place [35]. As a result of oxidation, both the destruction of humus compounds and their oxidative condensation are possible [6, 32]. Therefore, it is believed that alkaline extraction creates artifacts, and the compounds of alkaline extracts have little in common with the native SOM [22]. Nondestructive methods of SOM analysis [13], as well as sequential extraction of OM components by organic solvents [17, 25], are being developed. However, alkaline extraction is the only generally accepted method for extracting HAs from soil in preparative amounts; it is also a method for obtaining commercial humic preparations for their use in agriculture [29]. The International Humic Substances Society (www.ihss.org) has recommended to isolate HAs in a nitrogen atmosphere in order to reduce the effect of oxidative transformations on the structure of recoverable compounds [34]. The method for isolating HAs adopted in the Russian school of soil science does not involve the use of nitrogen or inert gas [7]. Therefore, there is a large amount of data on the properties of HA preparations isolated from various objects without using nitrogen [1, 6, 10, 26]. Studies devoted to the comparison of the structure and physicochemical properties of HAs isolated from mineral soil horizons by alkaline extraction in the presence and absence of O_2 are few in number and are limited to the study of individual HA properties, e.g., their molecular weight distributions [3]. This make it difficult to compare the data on HAs obtained by alkaline extraction in nitrogen atmosphere and without it and to evaluate the effect of the presence of O_2 in alkaline extracts on the properties of HAs from different types of soils.

The aim of our study was to compare the structure and physicochemical properties of HAs isolated from soils by alkaline extraction under nitrogen atmosphere and without it. The humus horizons of two soil types with contrasting conditions of humus formation—soddy-podzolic soil (Retisol, RT) and chernozem (Chernozem, CH) were chosen as the objects of this study. These are zonal soil types in the southern taiga and steppes, respectively, and HA preparations from these soils are often used in studies of soil humus.

OBJECTS AND METHODS

Soils. Samples from the humus horizons (A1) of silt loamy soddy-podzolic soil (Moscow oblast, Russia, N 56.226819, E 37.951602) and leached chernozem (Lipetsk Region, Russia, N 53.496117, E 38.990066) were used. Soddy-podzolic soil (Stagnic Retisol (Loamic, Humic), according to WRB 2015) is developed under the dead-floor spruce forest and consists of the following horizons: O (0–1 cm)–A (1–6(10) cm)–AE (6–20 cm)–EB (20–36 cm)–Bg (36–70 cm). Leached chernozem (Luvic Chernozem (Siltic, Pachic), according to WRB 2015) under a 20-year-old fallow is developed under steppe vegetation and has the following horizonation: A1 (0–43 cm)–AB (43–67 cm)–Bt (67–100 cm)–BCk (100–122 cm). A mixed sample from the A1 horizon of the Retisol (10 kg) was taken at the beginning of May 2017 from a depth of 2–10 cm on an area of about 3 m^2. A mixed sample from the A1 horizon of the Chernozem (10 kg) was taken in June 2017 from a depth of 10–40 cm on an area of about 2 m^2. The soil samples were air dried, ground, and sieved through a 1-mm sieve. The pH_{H_2O} was determined in soil suspensions (solid phase : water ratio 1 : 5) by potentiometry; the contents of organic carbon and nitrogen, on a Vario LIII analyzer (Germany); and the C_{HA}/C_{FA} ratio, by the method of Ponomareva and Plotnikova with slight modification [7]. For this, the OM was extracted from the samples with 0.1 M NaOH (24 h) after washing with 0.1 M H_2SO_4; then, the extraction with 0.02 M NaOH (6 h at 80°C) was performed. The HAs in the extracts were separated from the FAs by acidifying the solution to pH 2. The concentration of total dissolved C_{org} and C_{FA} in the extracts was determined on a TOC analyzer (Shimadzu, Japan) after filtering the solutions through filter paper washed with distilled water. The C_{HA} content was calculated as the difference between the total dissolved C_{org} and C_{FA}. The $C_{HA} : C_{FA}$ ratio was found taking into account the contents of HAs and FAs in two consecutive extractions. Some properties of the soils are given in Table 1. The humus horizon of the Retisol was characterized by acid reaction (pH 3.8 in the layer of 0–6 cm, pH 5.0 in the layer of 6–10 cm; average pH 4.49), the carbon content of 5.12%, the nitrogen content of 0.32%, and by the humate–fulvate type of humus (C_{HA}/C_{FA} 0.6). The A1 horizon of the Chernozem had the pH 5.31, the carbon content 4.35%, the nitrogen content 0.33%, and the humate type of humus (C_{HA}/C_{FA} 2.6). In general, the properties of sampled soils were characteristic of their types, except for the carbon content. A rather high carbon content in the Retisol can be associated with an admix-

Table 1. Some properties of the studied soils

Soil	pH_{H_2O}	C, %	N, %	C_{HA}/C_{FA}
Soddy-podzolic	4.41	5.12	0.32	0.60
Chernozem	5.28	4.68	0.33	2.61

ture of fine fragments of plant detritus. A relatively low carbon content in the Chernozem may be due to carbon losses during the period of agricultural use of this soil.

Extraction and purification of HAs. The extraction of HAs was performed by adding 0.1 M NaOH to the soil samples (3 kg) with the soil : extractant ratio of 1 : 5. Three successive extractions (24 h each) were carried out under nitrogen atmosphere (HA_{N2}) and without it (HA). The Chernozem was preliminarily decalcified with 0.1 M HCl (48 h, soil : solution ratio 1: 5); then, the soil was washed two times with distilled water (15 L each time, 24 h). The alkaline extracts were acidified to pH 2 (HCl), and HAs were separated by centrifugation (10 min, 7000 *g*). The ash content of untreated HAs determined by combustion at 800°C (4 h) was 30–60%. The isolated HAs were purified from ash components by salting out with 0.4 M NaCl [7]. The precipitate (ash content > 70%) was separated by centrifugation (16000 *g*, 10 min). The purified HAs in the supernatant solution were transformed to the H-form by triple reprecipitation with concentrated HCl. The precipitate of HAs was washed by distilled water to remove excessive Cl^- ions, centrifuged, and dried on a water bath at 50°C. All procedures with HA_{N2} under alkaline conditions were performed in nitrogen atmosphere.

The yield of HAs in the alkaline extracts obtained in the presence and absence of O_2. In order to estimate the amount of HAs in the alkaline extracts obtained in the presence and absence of O_2, three consecutive extractions with 0.1 M NaOH under nitrogen atmosphere and without nitrogen were performed. Extractions were carried out in Eppendorf microcentrifuge tubes (2 mL) in triplicate; 1.5 mL of 0.1 M NaOH was added to 300 mg of soil, the extracts were shaken for 24 h on a thermoshaker (Biosan, Latvia) at 22°C. In the case of extraction under nitrogen atmosphere, the alkali was first purged with nitrogen gas for 15 min, then the alkaline solution was blown with nitrogen gas for 2 min immediately after adding alkali to the soil. The supernatants were separated by centrifugation (18000 *g*, 15 min), and the soil between the extractions was washed with H_2O (2 times, 1 mL). Total dissolved carbon in the extracts and C_{FA} after precipitation of HAs were determined on a TOC analyzer (Shimadzu, Japan). The solutions were diluted with distilled water before the analysis. The C_{HA} content was found by the difference between C_{tot} and C_{FA}.

Molecular weight distribution of humic substances upon successive alkaline extractions. Molecular weight distributions of humic substances in alkaline extracts were obtained by gel filtration using Sephadex G-75 gel. The Amicon column (1.2 × 60 cm, Amicon Corporation, Japan) and 0.025 M Tris-HCl (pH 8.2) buffer with addition of 0.05 M NaCl and 0.1% SDS to suppress ionic and hydrophobic interactions, respectively. The elution rate was 7 mL/h. Elution profiles were recorded at 280 nm using a 2238 UVICORD SII detector (LKB, Sweden). The void volume of the column and the total volume of mobile phase were determined using Blue Dextran 2000 and $(NH_4)_2Cr_2O_7$, respectively. Probes (2 mL) of alkali extracts from soils (humic substances) were passed through a desalting column (1.0 × 8 cm) filled with Sephadex G-10 gel to transfer them into elution buffer. The relative contents of high- and low-molecular-weight fractions in the humic substances were estimated by the areas under chromatographic peaks as described in [36].

Physicochemical properties of HAs preparations.

Elemental composition. The elemental composition of HAs was determined on a CNH analyzer (Vario LIII, Germany). The oxygen content was found by difference. The ash content of the preparations was investigated after ashing at 800°C for 4 h.

The content of functional groups. The content of functional groups and the pK_a values were determined by potentiometric titration. Samples of HAs (50 mg) were dissolved in 25 mL of 0.05 M NaOH. Then, 5 mL of 1 M KCl, and 8.5 mL of 0.1 M HCl were added. The volume of the solution was adjusted to 50 mL with distilled water. The final concentration of HAs before titration was 1 mg/mL; the pH of the solution was about 11.2, and the ionic strength was 0.1. The solution of HAs was titrated with 0.01 M HCl (via adding 0.1-mL HCl each time) on an automatic titrator Mettler Toledo DG58 (USA) under nitrogen atmosphere. The functional groups content and pK_a values were determined as described elsewhere [1].

Absorption spectra in the visible and UV regions and the infrared spectra. The spectra of HAs in the visible region were recorded on a Specord M40 spectrophotometer (Germany). HAs were dissolved in 0.05 M NaOH purged by N_2. Infrared spectra were recorded using KBr technique on a Bruker Tensor 27 spectrophotometer (Germany).

^1H NMR and ^{13}C NMR spectroscopy of HA preparations. For ^1H NMR and ^{13}C NMR spectroscopy in the liquid phase, deuterium water (D_2O, 99.95% D) and 40% NaOD in D_2O (99+% D) (Aldrich, Milwaukee, WI) were used.

The ^1H and ^{13}C NMR spectra were recorded in ampoules for NMR spectroscopy with a diameter of 5 mm on a Bruker Avance 400 spectrophotometer (Germany) with a carrier frequency for protons of 400 MHz. Fourier transformation, phase correction, and integration of the transformed spectrum were performed using the MestReC software (Mestrelab Research, USA). Integration of the obtained NMR spectra was performed using the GelTreat software

developed by A.V. Kudryavtsev (Chemical Faculty, Lomonosov Moscow State University).

For ^{1}H NMR spectroscopy, 10 mg of HAs was dissolved in 0.5 mL of 0.3 M NaOD/D_2O. The spectra were recorded with a relaxation delay of 2 s, the number of accumulations of the spectrum was from 30 to 40 scans. The distribution of skeletal protons by structural fragments (% of the total area under the spectrum) was determined by integrating the spectral regions using the following assignments (in ppm): 0.0–1.8 for H alkyl units (CH_n protons); 1.8–2.9 for H alkyl units in α-position with respect to COOH-groups or aromatic ring (α-CH_n); 2.9–4.5 for H alkoxygroup (CH_nO); 6.0–12.0 for aromatic protons (H_{Ar}) [21].

To record ^{13}C NMR spectra, 40 mg of HAs was dissolved in 0.5 mL of 0.3 M NaOD/D_2O. The mixture was homogenized on a Vortex shaker for 10 min and centrifuged for 5 min at 10000 rpm. When recording the ^{13}C NMR spectra, the INVGATE pulse technique was used (to eliminate the Overhauser nuclear effect and to obtain a quantitative spectrum) and the CPMG pulse sequence as described in [10]. The signal acquisition time was 0.2 s, the relaxation delay time was 7.8 s, and the duration of one NMR experiment was 10 h. The distribution of carbon atoms among the main structural fragments of HAs was determined by integrating the spectral regions using the following assignments [4] (ppm): 220–185 for carbonyl carbon of ketone and quinone groups (C=O); 185–165 for carbon of carboxyl, ester, and amide groups ((C=O)–O, N); 165–145 for aromatic carbon substituted by heteroatoms (C_{Ar}–O, N); 145–108 for unsubstituted or C-substituted aromatic carbon (C_{Ar}–H, C); 108–90 for carbon bonded by single bonds to two heteroatoms (in HAs, this is mainly acetal carbon in O–O, N cyclic saccharides); 90–48 for carbon with a single bond to a heteroatom and entering the composition of aliphatic fragments; 48–0 for carbon of alkyl units not bound to heteroatoms (CH_n). Spectral range from 90 to 48 ppm was divided into three parts: 90–64, carbon in the groups (CH–O, N); 64–58, carbon in the groups (CH_2–O, N); and 58–48, carbon of methoxyl groups (CH_3O).

The content of free radicals in HAs according to electron paramagnetic resonance (EPR) data. The EPR spectra were recorded on a Radiopan SE/X-2547 spectrometer (Poland) in the X-range at room temperature, with a high-frequency power of 1 mW and an amplitude of high-frequency modulation of 0.06 mT. The concentration of free radicals in soils and in the HA preparations was determined by comparing the areas under the relative integral intensities of the EPR signals of the standard and the sample [5, 12]. Diphenylpycrylhydrazyl was used as a standard. The coefficient of variation in determining the concentration of free radicals in this case does not exceed 5% [5, 12, 28]. The linewidth (in Gauss) was calculated from the distance between the extreme points of the absorption line.

Molecular weight distribution of HA preparations. The molecular weight distributions of HAs were obtained by gel filtration on Sephadex G-75 gel as described above. The probes of HAs (1 mg/mL in 0.05 M NaOH) were preliminarily passed through a Sephadex G-10 column (1.0 × 8 cm) for transferring them to elution buffer. The molecular weights corresponding to the center of the chromatographic peaks were calculated using the Determan formula for globular proteins, and the relative contents of fractions were estimated from the areas under chromatographic peaks as described in [36].

RESULTS AND DISCUSSION

Quantitative yield and molecular weight distributions of humic substances upon their extraction in nitrogen atmosphere and without it. The extraction of humic substances in an alkaline medium is accompanied by oxygen consumption [32]. The alkaline medium and O_2 can affect not only humic substances (oxidation, condensation reactions) [34] but also the mineral components of the soil (dissolution of oxides, hydroxides, and silicates) [16]. We assumed that if the presence of oxygen during alkaline extraction has a destructive effect on the mineral matrix of soils, this should affect the quantitative yield of humic substances (it should increase). Oxidative polymerization of humic substances in an alkaline medium in the presence of oxygen may lead to an increase in the yield of HA fraction in the extracts. Therefore, we have studied the yield of humic substances (HAs and FAs), as well as the amount of HA fractions in three consecutive extractions under nitrogen atmosphere and without it. The yields of humic substances (HAs + FAs) and of HA fractions turned out to be similar in each of three consecutive alkaline extractions for the extractions with and without nitrogen purging (Fig. 1). The total yield of $C_{HA + FA}$ in three extractions with nitrogen and without it reached 38.9 ± 0.8% and 40.4 ± 1.2% of C_{org}, respectively, for the Retisol and 69.2 ± 3.5% and 67.6 ± 4.3% of C_{org}, respectively, for the Chernozem. The total yield of C_{HA} in the extraction with nitrogen (without it) was 27.8 ± 0.8% (28.5 ± 0.8%) of C_{org} for the Retisol and 64.9 ± 3.4% (62.8 ± 4.3%) of C_{org} for the Chernozem. Thus, the presence of oxygen in alkaline extracts did not affect the amount of humic substances extracted by alkali from the humus horizons of Retisol and Chernozem. The method of gel filtration also attested to the absence of the polymerizing effect of oxygen on OM in alkaline extracts. The organic matter of alkaline extracts of the studied soils consisted of high- (>75 kDa) and low-molecular-weight fractions (10–30 kDa), the ratio of which did not differ in the extraction variants with and without nitrogen both in the Retisol and in the Cher-

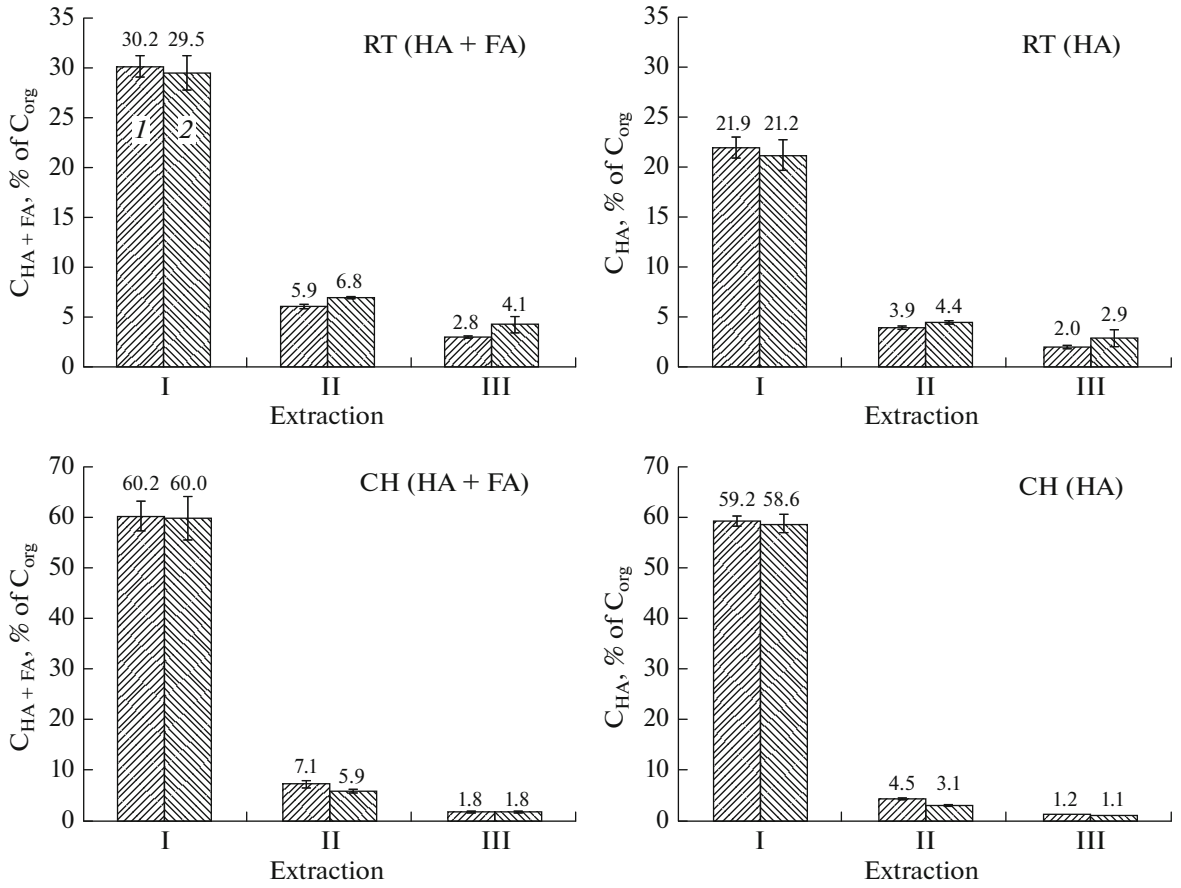

Fig. 1. The carbon contents of humic substances (HA + FA) and humic acids (HA) in alkaline extracts from the Retisol (RT) and Chernozem (CH) isolated under (*1*) nitrogen atmosphere and (*2*) without it.

nozem (Fig. 2). Indeed, we have shown recently that for the polymerization of HAs in the presence of oxygen, even under alkaline conditions, the presence of a biocatalyst (laccase) is necessary [23].

The yield of humus acids ($C_{HA + FA}$) sharply decreased from the first to the second and third extractions (Fig. 1). In the Retisol, about 75% of C_{org} of three extractions passed into the first extract, and about 20 and 5% of C_{org} passed into the second and third extracts, respectively. The same tendency was found for the Chernozem. About 86% of C_{org} from three extractions passed into the first alkaline extract; about 10 and 3%, passed into the second and third extracts, respectively (Fig. 2). The study of the molecular weight distributions of HAs in alkaline extracts showed an increase in the relative content of high-molecular-weight fraction from the first to the second and third extractions for both soils. The high-molecular-weight components present in alkaline extracts from the Chernozem are most likely represented by organomineral complexes with the high ash content, because they are removed at the stage of purification by salting out. The increase in the content of these components from the first to the second extraction may be due to the dissolution of the mineral particles and mobilization of high-molecular-weight organic-mineral compounds. It should be noted that the absence of differences in the molecular weight distributions of HAs from extraction to extraction shown in [11],

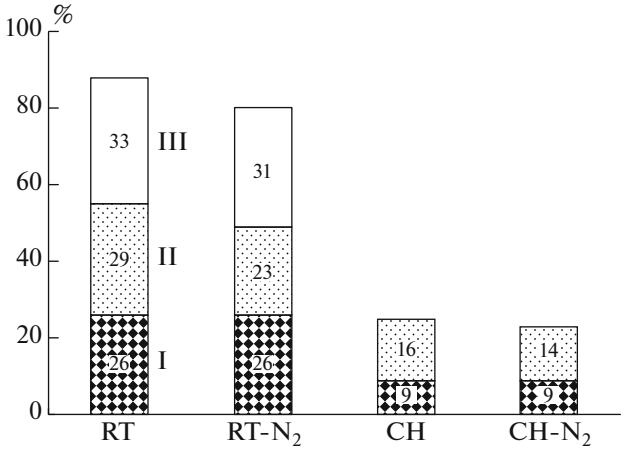

Fig. 2. The contents of high-molecular-weight fraction (>75 kDa) in the organic matter of three consecutive alkaline extractions (number of extraction is indicated by Roman numerals); N_2 denotes the extraction in nitrogen atmosphere.

Table 2. Elemental composition of humic acids and their optical properties

HA	Ash, %	Contents, wt % / at %				Atomic ratios			$E_{465}^{0.001\%\,HA}$	E_{465}/E_{650}
		C	H	N	O	H : C	O : C	C : N		
HART	2.6	52.9/35.3	5.2/42.1	4.5/2.6	40.0/20.0	1.2	0.6	13.7	0.03	5.0
$HART_{N_2}$	4.8	59.6/38.6	5.4/41.6	5.3/2.9	34.7/16.9	1.1	0.4	13.1	0.03	5.0
HACH	2.0	57.4/45.3	3.2/29.9	3.7/2.5	37.7/22.3	0.7	0.5	18.0	0.12	3.1
$HACH_{N_2}$	3.3	55.7/45.3	2.8/27.0	3.5/2.4	41.5/25.3	0.6	0.6	18.6	0.11	3.1

The coefficient of variation in determinations of the elemental composition of humic acids did not exceed 2%.

Table 3. The contents of functional groups and pK_a values of humic acids according to the results of potentiometric titration

Preparation	Functional groups, mmol/g				pK_a		
	pH 3.9–5.6	pH 5.6–8.7	pH 8.7–10.3	Σ	pK_a-1	pK_a-2	pK_a-3
HART	1.64	2.05	1.23	4.92	4.7	6.7	9.7
$HART_{N_2}$	1.45	1.87	1.24	4.56	4.8	6.6	9.5
HACH	1.84	2.45	1.02	5.31	4.5	6.4	9.6
$HACH_{N_2}$	1.86	2.48	1.03	5.37	4.6	6.4	9.3

The coefficient of variation in determinations of the contents of functional groups did not exceed 10%.

may be due to the use of Toyopearl gel, which has affinity for hydrophobic compounds. The relative hydrophobicity of the components of HAs increases with an increase in their molecular weight [2]. The hydrophobic/high-molecular-weight components of HAs are sorbed on the gel (data not shown), which may be the reason for their apparently low content on the chromatograms presented in [11]. In general, the results of this work are consistent with published data on gray forest soil [9] and soddy-podzolic soil and chernozem [11]; one alkaline extraction can be used to obtain representative humic sample in preparative amounts significantly reduces labor costs. This is especially true for the Chernozem, in which the second and third extractions are accompanied by pronounced peptization of colloids. The latter significantly increases the time of precipitation of mineral particles and complicates further purification of the HA preparation.

Physicochemical properties of HAs. The elemental composition of HA preparations is in the range of values typical of the studied soil types. The nitrogen content in the HAs from the soddy-podzolic soil (Retisol, RT) (HART) (2.6–3.0 at %, Table 2) is slightly higher than the average values for such soils (2.4 at %, [6]), but generally agrees with the data of other authors [10]. The HART preparation obtained without nitrogen has a slightly higher O : C ratio compared with the HART preparation isolated under nitrogen atmosphere. This ratio is one of the indicators of the degree of HA oxidation [6]. No other differences were found in the elemental composition of preparations isolated under nitrogen atmosphere or without it.

The spectra of HA and HA_{N_2} preparations in the visible and UV regions were identical (data not shown). The optical properties of HAs in the visible region (E values) are consistent with the literature data [6, 32]. The significantly higher optical density of HAs from the Chernozem (HACH) compared with HAs from the Retisol (HART) is attributed to a more developed chain of conjugated double bonds and to a higher degree of oxidation [6].

The HART and HACH preparations isolated with and without nitrogen do not differ in terms of the content of functional groups, except for HAs from the Retisol. In the HART preparation, compared with the $HART_{N_2}$ preparation, the content of groups titrated in the pH ranges of 3.9–5.6 and 5.6–8.7 is somewhat higher (Table 3). In the first pH range, carboxyl groups are supposedly titrated; in the second pH range, carboxyl, nitrogen-containing and, partially, phenolic groups are titrated [1]. The increased content of acidic functional groups in $HART_{N2}$ compared with HART is consistent with the results of elemental analysis (O : C ratio, Table 2) and may indicate a slightly higher degree of oxidation of the preparation isolated without using nitrogen.

The IR spectra of the HART and HACH preparations were identical for the extractions with and without nitrogen (Fig. 3). The spectra contain a set of absorption bands typical of humic acids from soils [6, 31, 32]. Significant differences were found between the spectra of the HART and HACH preparations (Table 4). HAs from both soils contain a wide band

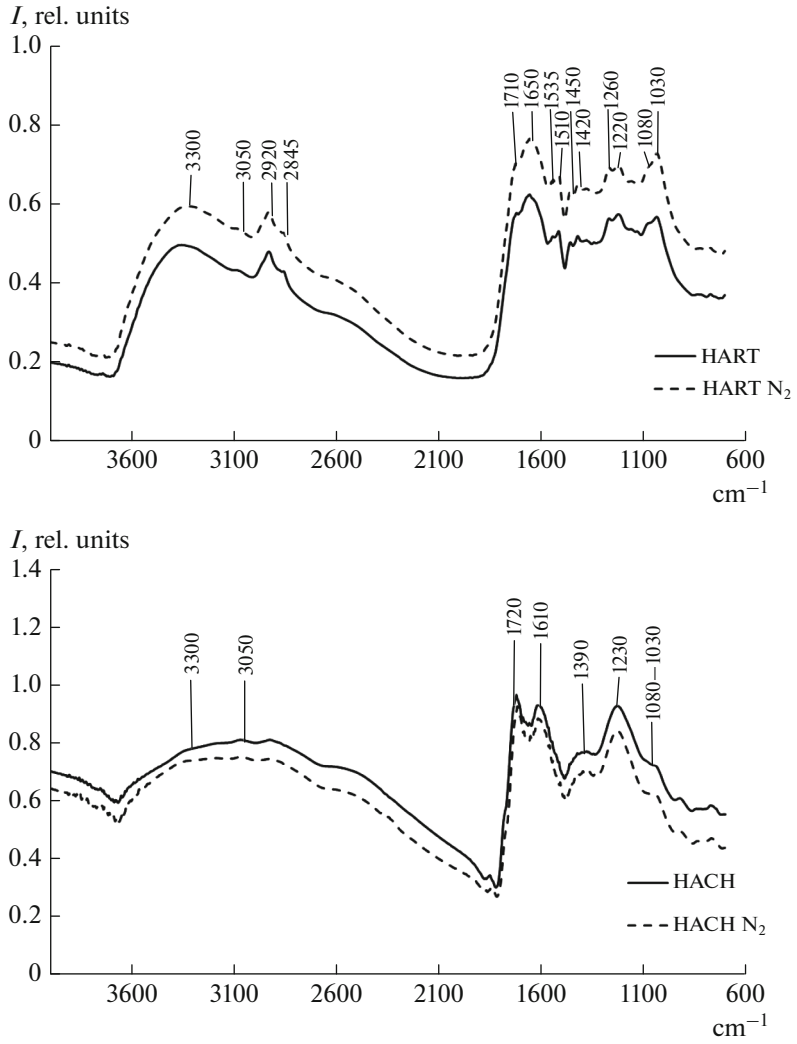

Fig. 3. Infrared spectra of humic acids: HART—HAs from Retisol; HACH—HAs from Chernozem; N_2 denotes the extraction in nitrogen atmosphere.

attributed to the stretching of OH- and NH-groups linked by intermolecular hydrogen bonds (3300 cm^{-1}), but this band has a much higher intensity in HART. The HAs of both soils contain a shoulder in the region of 3050 cm^{-1} attributed to aromatic C-H groups stretching [31]. The weak intensity of this band may be due to the presence of substituents in the aromatic rings of HAs or band overlapping from the broad band of the OH stretching [31]. The HART preparation contains pronounced peaks of aliphatic CH_2 and CH_3 groups stretching (2930 and 2845 cm^{-1}), and weak peak of the deformation vibrations of these groups at 1450 cm^{-1}. These bands are absent in the HACH preparation, but latter, in contrast to HART, contains an intense band of the aromatic C=C groups stretching (1610 cm^{-1}). Thus, the data of IR spectroscopy indicate a higher degree of aromaticity of HACH compared to HART, which is consistent with the data of elemental analysis (H : C ratio) and spectroscopy in the visible region and is characteristic of HAs of these soil types [6]. In the region of 1710–1030 cm^{-1}, the IR spectrum of the HART preparation contains a range of absorption bands of varying intensity (Fig. 3), while the HACH spectrum contains three strong absorption bands at 1720, 1610 and 1230 cm^{-1} and two shoulder-like bands at 1390 and 1080–1030 cm^{-1} (Fig. 3). In the region of 1710–1720 cm^{-1}, the band of C–O stretching of COOH group is observed which is characteristic of the H-form of the preparation [6, 31]. This band is well expressed in the HACH preparation; this HA has also a strong band at 1610 cm^{-1} and a band at 1390 cm^{-1}, which are partly due to symmetric and asymmetric stretching of ionized COO groups, respectively. In the HART preparation, the band of COOH groups (1710 cm^{-1}) is expressed in the form of a shoulder, due to almost complete overlapping from the band of amide groups (amide I, 1650 cm^{-1}). Nar-

Table 4. Absorption bands in the IR spectra of humic acids *

Maximum of the absorption band, cm^{-1}		Group and oscillations
HART and $HART_{N_2}$	HACH and $HACH_{N_2}$	
3300 s	3300 s	O–H stretching, N–H stretching (trace), intermolecular hydrogen bonds
3050 w	3050 w	Aromatic C–H stretching
2920, 2845 m	–	C–H stretching in aliphatic CH_2, CH_3 groups
1710 shoulder	1720 s	C=O stretching of COOH, aldehydes and ketones
–	1610 s	Aromatic C=C stretching, COO^- symmetric stretching
1650 s	–	C=O stretching of amide groups (amide I), C=O of quinones and/or H–bonded conjugated ketones
1535 w	–	N–H deformation and C=N stretching (amide II)
1510	–	aromatic C=C stretching
1450 w	–	C–H in CH_2 (or CH_3) deformation
1420 w	–	C=N stretching of primary amides (amide III)
–	1390 m	COO^- antisymmetric stretching O–H deformation and C–O stretching of phenolic OH groups
1260, 1220 m	1230 s	C–O stretching of COOH O–H deformation of COOH C–O stretching of aryl ethers and phenols
1030 s	1080–1030 shoulder	C–O stretching of polysaccharides

* Identification of bands according to [6, 23]: s—strong, m—medium, and w—weak.

row bands of weak intensity at 1535 (amide II) and 1420 cm^{-1} (amide III) also indicate the presence of N-containing components in the HART preparation. Similar spectra of HAs, in which the carboxyl group band is overlapped with the amide I band, are described in the literature, for example, for HAs of brown and podzolic forest soils from the reference base EUROSOILS (Rendzinas, Cambisols, and Luvisols) [31]. Such spectra indicate a high content of peptides or proteins in the HART, and this may serve as an explanation of an increased nitrogen content in this preparation. The stretching C–O and O–H stretching in COOH (1260–1220 cm^{-1}) are much more pronounced in the HACH compared to the HART, which is consistent with a higher content of functional groups titrated in the pH range <8 in the HACH preparation. In the HART, in the region of 1080–1030 cm^{-1}, an intense band appears which can be attributed to C–O stretching of polysaccharides; in the HACH, this band is expressed as a shoulder. Thus, the data of IR spectroscopy indicate a higher content of aliphatic, nitrogen-containing, and polysaccharide components in the HART compared with the HACH, which is consistent with the conditions of humus formation in these soils and a lower degree of humification of OM in the soddy-podzolic soils (Retisols) [6, 31].

Table 5. Distribution of skeletal protons by structural fragments in HA preparations according to 1H NMR spectroscopy, % of the total area of NMR spectrum

Preparation	Structural group (spectral region, ppm)			
	CH_n (0–2.05)	α-CH_n (2.05–3.2)	CH_nO (3.0–4.4)	H_{AR} (6.2–12.0)
HART	26.2	19.4	35.2	19.3
$HART_{N_2}$	26.8	16.0	33.0	24.2
HACH	16.6	16.1	35.4	31.9
$HACH_{N_2}$	15.8	16.4	34.8	33.1

1H NMR spectra are characteristic of HAs (Fig. 4). The content of protons of aromatic fragments in the HACH is slightly higher than in the HART (Table 5), which is consistent with the data of visible and IR spectroscopy and indicates a greater aromaticity of the HACH. There were no differences between the spectra of HA and HA_{N_2} preparations from Chernozem, whereas in the HART the contents of alkoxy groups (3.0–4.4 ppm) and α-CH_n groups (2.05–3.2 ppm) were somewhat higher, and the content of aromatic protons was somewhat lower in the preparation obtained without nitrogen. However, this trend cannot be seen (with the exception of CH_nO groups) in the ^{13}C NMR spectra (Table 6). The ^{13}C NMR spectra contain peaks of quinone (187–220 ppm) and carboxyl (165–187 ppm) groups, aromatic carbon (108–165 ppm),

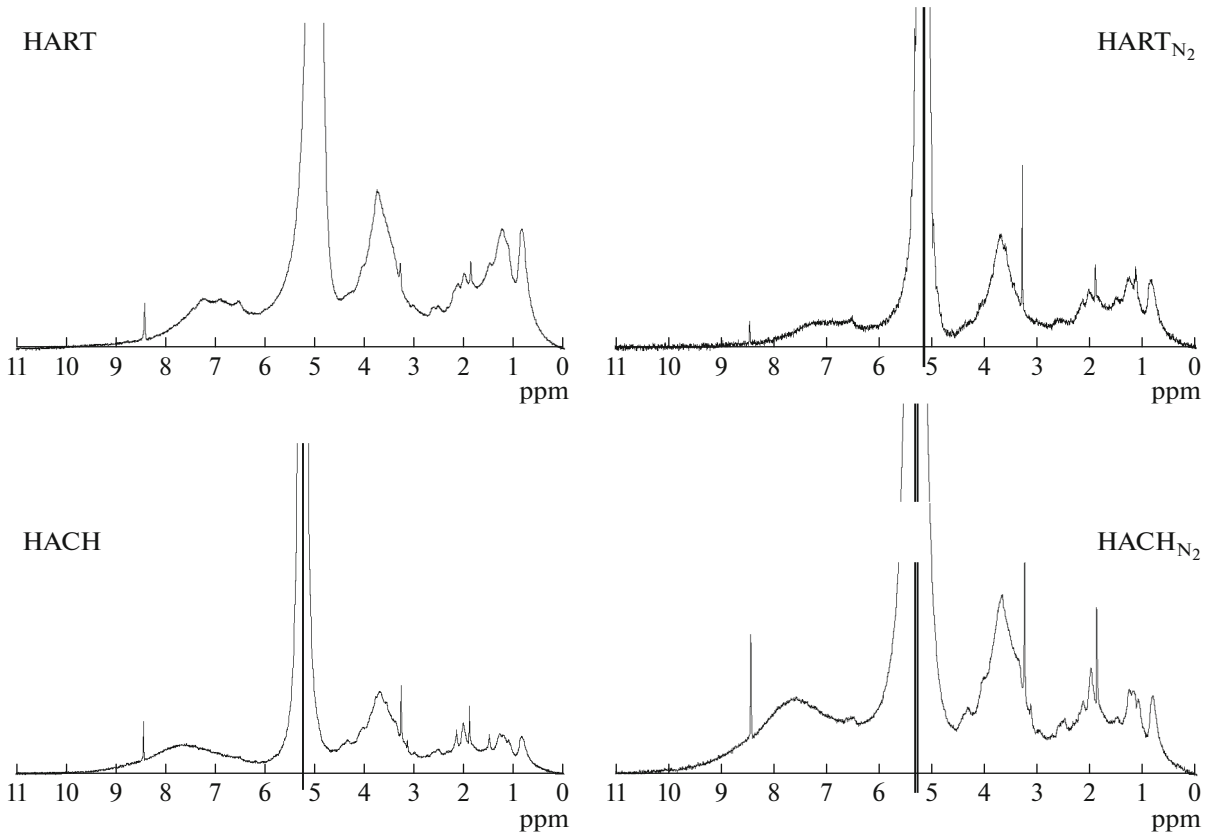

Fig. 4. NMR spectra of HA preparations.

substituted (48–108 ppm), and unsubstituted (5–48 ppm) aliphatic carbon, which is characteristic of HAs. The content of carboxyl groups in the HACH is significantly lower than in the HART, which is not entirely consistent with the data of potentiometric titration (the content of the groups titrated in the acidic range is somewhat higher in the HACH). The total content of aromatic carbon (108–165 ppm) is slightly higher in the HACH, and the content of aliphatic groups (108–5 ppm) is slightly lower in the HACH as compared to HART (Table 7), which is consistent with a higher degree of aromaticity of the HACH according to the elemental analysis, visible and IR spectroscopy, and ^{1}H NMR spectroscopy. It was not possible to acquire well-resolved spectrum of the HACH preparation isolated under nitrogen atmosphere, which made it impossible to compare data from different extraction options. The HART, obtained by the extraction without nitrogen was characterized by the higher relative content of quinone and

Table 6. Distribution of carbon atoms by structural fragments in HA preparations according to ^{13}C NMR spectroscopy data, % of the total area of NMR spectrum

Preparation	Structural group (spectral region, ppm)								
	aliphatic fragments				polysaccharides	aromatic fragments		carboxyls, ketones, and quinones	
	CH_n (5–48)	CH_3–O,N (48–58)	CH_2–O,N (58–64)	CH–O (64–90)	OC–O,N (90–108)	C_{AR} (108–145)	$C_{AR}O$ (145–165)	COO (165–187)	C=O (187–220)
HART	13	7	3	13	3	21	13	20	8
HART$_{N_2}$	20	5	4	12	9	24	9	12	4
HACH	15	5	7	11	7	31	8	8	7

For the HACH$_{N_2}$ preparation, the spectrum of appropriate quality could not be obtained.

Table 7. The contents of free radicals in soils and HA preparations according to EPR data

Sample	Spin/g	Linewidth, G
Chernozem	1.30×10^{16}	5.01
HACH	1.51×10^{18}	3.56
$HACH_{N_2}$	1.30×10^{18}	3.83
Retisol	3.49×10^{15}	5.20
HART	8.06×10^{16}	5.58
$HART_{N_2}$	6.89×10^{16}	4.26

The coefficient of variation in determinations of the concentration of free radicals did not exceed 5%.

carboxyl groups and aromatic fragments substituted by oxygen, which is consistent with the elemental analysis data (O : C ratio is higher in the HART, Table 2) and may indicate the oxidation of the HAs from this soil in the course of the extraction.

The EPR method established a slightly higher content of free radicals of the semichinoid type in all HA preparations compared with the initial soil (Table 7), which can be explained by the action of alkaline hydrolysis [12]. Similar data were obtained earlier by other authors [18, 28]. The scatter of the energy characteristics of free radicals (corresponding to the width of the EPR spectral line) in HA preparations is noticeably lower than in the EPR spectra of soil samples. except for the HART preparation obtained without nitrogen. The content of free radicals in the Chernozem and in the HAs isolated from this soil is higher than that in the Retisol and in the HART by one and two orders of magnitude, respectively. At the same time, the HACH content of free radicals is only 15% higher than that of the $HACH_{N_2}$, whereas in the Retisol the effect of the presence of oxygen during the isolation of HA preparations turned out to be more significant: the HART contains two times more free radicals than the $HART_{N_2}$. In addition, in the HACH, the width of the EPR spectral line increased significantly. This indicates a greater variation in the energy characteristics of free radicals in the HART, which exceeded the scatter even for the EPR spectra of the soil samples. The EPR data confirm the results of NMR spectroscopy, potentiometric titration, and elemental analysis and indicate the initially lower degree of oxidation of the organic matter in the Retisol, which is also confirmed by literature data [12, 28]. As a result, alkaline extraction without nitrogen leads to a significant increase in the concentration of oxygen-induced free radicals in the HAs from this soil.

In addition to the optical properties, structural groups content, and elemental composition, molecular weight distributions of HAs were studied. The HART preparation consisted of two fractions: a high-molecular-weight fraction eluted at the void volume of the column (molecular weight >75 kDa, relative content 23%) and a low-molecular-weight fraction with peak molecular weight of about 29 kDa, the relative content of which is 77–78% (Table 8). The HACH preparation contains much higher amounts of low-molecular-weight components: the content of the high-molecular-weight fraction is only 2%, and the low-molecular-weight fraction constitutes 98% of the preparation; its molecular weight is about 10 kDa. Differences between preparations isolated with and without nitrogen have been identified. The results obtained for the HACH are consistent with literature data. It was shown that O_2 does not affect the molecular-weight distributions of humic substances isolated by alkaline extraction from chernozem [3]. Thus, the presence of oxygen in alkaline extracts does not lead to the oxidative polymerization or depolymerization of HAs in the studied soils. The relatively low-molecular nature of the HACH is consistent with the conditions of humus formation in the Chernozem. In these soils, a period of biological activity is rather long, which contributes to the deep transformation of plant residues and humic substances, so that the latter become more oxidized and have a lower molecular weight [6]. In the Retisols, which are zonal types for the middle and southern taiga regions characterized by cold humid climate, the decomposition of plant residues and humification rate are slowed down. Therefore, conditions are

Table 8. Molecular masses of HAs and the contents of their fractions according to gel-filtration method

Preparation	High-molecular-weight fraction		Low-molecular-weight fraction	
	MM, kDa	share, %	MM, kDa	share, %
HART	>75	23	27	77
$HART_{N_2}$	>75	24	27	76
HACH	>75	2	13	98
$HACH_{N_2}$	>75	2	12	98

The coefficient of variation in determinations of molecular masses of humic acids and the contents of their fractions did not exceed 5%.

created in these soils for the preservation and accumulation of high-molecular-weight humus compounds.

CONCLUSIONS

The study of the structure and physicochemical properties of HAs from the Retisol and Chernozem showed no differences in the yield of preparations; molecular weight distributions and absorption spectra in the UV, visible, and IR regions between the preparations isolated by alkaline extraction in the presence of nitrogen or without it. Elemental analysis, potentiometric titration, and ^{1}H and ^{13}C NMR spectroscopy revealed somewhat higher O : C ratios, slightly higher content of acidic groups titrated in the pH range 3.9–8.7, and increased content of quinone (187–220 ppm) and carboxyl (165–187 ppm) groups in the HART preparation in comparison with the $HART_{N_2}$, which indicates progressive oxidation processes of the components of the alkaline extract from the Retisol during the extraction in the presence of molecular oxygen. Oxidation is also indicated by an increase in the content of free radicals in the HART in comparison with the $HART_{N_2}$. At the same time, there were no differences found for HA and HA_{N_2} samples from Chernozem, which may indicate a deep oxidative transformation of OM during humification in this soil, so that no further oxidation during the extraction in an alkaline medium in the presence of oxygen takes place. The use of nitrogen for the isolation of HAs from the mineral horizons of Chernozem can be considered inexpedient. In the case of Retisols, the extraction under nitrogen atmosphere is desirable, especially, if it is intended to study the reactions of HA oxidation, e.g., during enzymatic catalysis.

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