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The Carbon Distribution among the Functional Groups of Humic Acids Isolated by Sequential Alkaline Extraction from Gray Forest Soil

V. A. Kholodov[a], A. I. Konstantinov[b], and I. V. Perminova[b]

[a] Dokuchaev Soil Science Institute, Russian Academy of Agricultural Sciences, per. Pyzhevskii 7, Moscow, 119017 Russia
[b] Faculty of Chemistry, Moscow State University, Moscow, 119991 Russia
E-mail: vkholod@mail.ru

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Abstract—Preparations of humic acids (HAs) were isolated from a gray forest soil by sequential alkaline extraction. From a sample of 500 g, HA preparations of 2.24, 0.23, and 0.20 g were obtained from the first, second, and third alkaline extracts, respectively. The structure of the preparations was determined by ^{13}C NMR spectroscopy. At each next extraction step, the portion of aliphatic fragments in the HA preparations increased and the content of aromatic structures decreased. 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) [15]. Both methods are based on the general principle of the alkaline extraction of humic substances from soils; however, they have a number of significant methodological differences.

One of them is the number of extractions used for the isolation of the HA preparation: according to the method used in Russia, the soil is extracted by 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; the isolation of the HAs is, hence, performed from the primary alkaline extract.

Other differences include (a) the alkaline extraction under a nitrogen atmosphere according to the IHSS procedure [15]; (b) the coagulation of the mineral suspensions: it is performed in the alkaline extract with sodium chloride according to the method used in Russia [7]; in the IHSS method, it is performed with the HAs separated from the fulvic acids (FAs) using KCl as a coagulating agent [15]; and (c) the treatment of the HA precipitate with a mixture of 0.1 M HCl and 0.3 M HF for the final removal of the fine mineral suspensions as recommended by the IHSS method [15].

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 expenses and the duration of the HA isolation procedure and, second, affects the degree of alteration of the 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 provide more representative HA preparations and with a higher yield, but this procedure is significantly more laborious. In addition, the holding of the soil suspension in the 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 its 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 preparations isolated in accordance with the protocol used in Russia and that recommended by the IHSS.

We previously showed with a typical chernozem as an example that a single extraction is sufficient for obtaining a representative HA sample [10]. In this work, a similar problem was considered for gray forest

Table 1. Some physicochemical parameters of the gray forest soil

Parameter	$\bar{x} \pm t_{\alpha} s_{\bar{x}}^{*}$
pH_{water}	6.7 ± 0.1
C_{org}, %	1.59 ± 0.1
C_{HA}/C_{FA}	1.6 ± 0.1
Exchangeable bases, meq/100 g	
Ca^{2+}	10.7 ± 0.2
Mg^{2+}	8.6 ± 0.2
K^{+}	0.50 ± 0.05
Na^{+}	–
Content of particles, %	
<0.01 mm	35.8 ± 0.2
<0.001 mm	15.5 ± 0.2

* ($\pm t_{\alpha} s_{\bar{x}}$) confidence interval calculated for $\alpha = 0.05$ and $n = 3$.

soils: either the HA preparation obtained by the single extraction is representative or a multiple alkaline extraction is necessary for obtaining a representative HA sample.

Liquid-state ^{13}C NMR spectroscopy is one of the most informative methods for the structural study of humic substances, including HAs. It allows describing the distribution of carbon in HA molecules among the structural fragments with different chemical shifts of their carbon nuclei. Thus, liquid-state ^{13}C NMR spectroscopy can quantitatively assess the content of structural units in the carbon skeleton of HAs [5, 12, 14], which allows describing the changes occurring in the HA structure at each next extraction stage.

The aim of this work was to study the changes occurring in the structure of HAs sequentially extracted from a decalcified gray forest soil.

EXPERIMENTAL

HA preparations were isolated from the humus-accumulative horizon of a virgin gray forest soil according to the 1977 classification [3] (or a gray soil according to the 2004 classification [4]). The sampling site was located at the research station of the Dokuchaev Soil Science Institute not far from the town of Ozery in Moscow oblast.

The soil sample was composed of five individual samples of about 2 kg that were taken from the humus-accumulative horizon at a depth of 5–15 cm in a plot about 5 m^2 in size.

An average sample (1.5 kg) was formed by quartering the obtained 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, the exchangeable bases [1], the physical clay and clay (by the pyrophosphate method [2]), the organic carbon, and the C_{HA}/C_{FA} ratio [7]. The data obtained are given in Table 1.

The determined parameters corresponded to the ranges reported for gray forest soils [8, 9]. Thus, the soil was representative of the type of gray forest soils, which completely answered the purposes of the study.

From the soil sample prepared and characterized, HA preparations were obtained according to the pattern combining both methods considered [7, 15]. 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 structure of the preparations obtained was analyzed by ^{13}C NMR spectroscopy.

Isolation of the HA preparations. A soil sample (500 g) was decalcified by the addition of 1 M HCl to reach a suspension pH in the range 1–2; 0.1 M HCl was then added to a final soil : solution weight ratio of 1 : 10. The obtained suspension was periodically shaken for 6 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 a final 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 reach 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 [15]. The treated HA suspension was purified by dialysis, dried on a rotor evaporator at 65°C, and stored in a desiccator over P_2O_5 for at least 21 days. The HA-GF1, HA-GF2, and HA-GF3 preparations were thus obtained from extracts 1, 2, and 3, respectively. The yield of the HAs during each extraction was assessed by weighing the preparations obtained.

Structural characterization of the obtained HA preparations. The structure of the preparations was determined by liquid-state ^{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 then centrifuged at 18 g for 15 min; the solution was separated from the precipitate and transferred into an NMR ampoule. The ^{13}C NMR spectra were recorded on a Bruker Avance-400 spectrometer operating at 100 MHz for carbon 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 wide-band decoupling of protons was on during the signal recording and off during the relaxation delay to exclude the Overhauser nuclear effect [11]. The duration of one NMR determination was about 12 h.

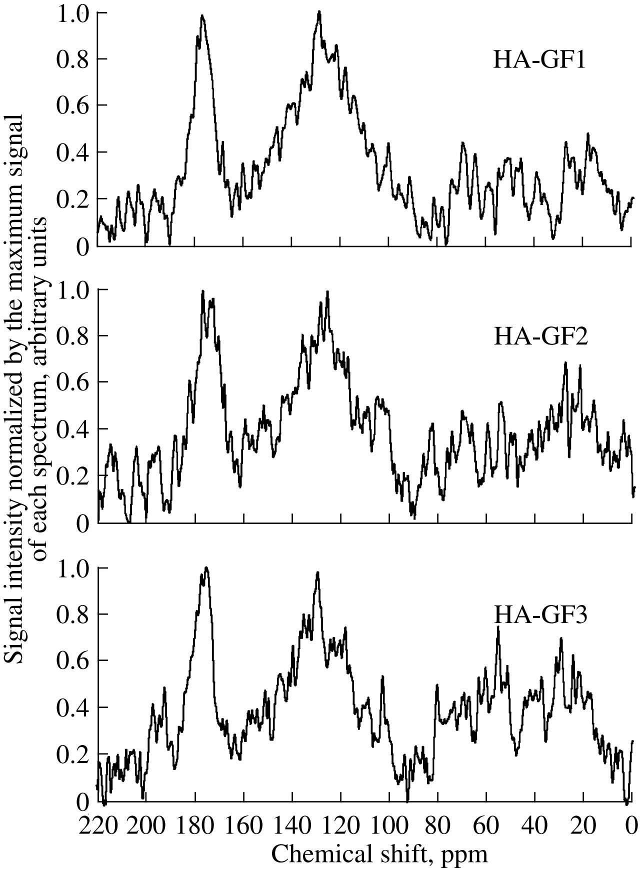
To improve the signal-to-noise ratio, the recorded signals of free induction decay were multiplied by the tapered exponent with a spectral line broadening parameter (lb) of 100 Hz and subjected to Fourier transformation for converting the time-sweep signal into a frequency-sweep spectrum with the frequency being measured in ppm of the spectrometer's operating frequency (100 MHz for ^{13}C nuclei). The processing and transformation of the spectra were performed using the MestReC software package. The integration of the spectra over the intervals was performed using GelTreat software (licence of A.V. Kudryavtsev).

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

The obtained data on the structure of the HAs were used for comparing the preparations isolated.

RESULTS AND DISCUSSION

Using 500 g of the gray forest soil sample, 2.24 g of HA-GF1, 0.23 g of HA-GF2, and 0.20 g of HA-GF3 were obtained from the first, second, and third alkaline extract, respectively. Thus, the preparative yield of HAs after the first extraction was higher than those after the second and third extractions by about 10 times. The preparative yields of HAs from the second and third extracts were similar. Thus, it was shown that most of the HAs were extracted with 0.1 M NaOH from the decalcified gray forest soil during the first extraction step. We previously showed in a similar experiment with a typical chernozem from an annually mown steppe of Kursk oblast that the preparative yield of HAs decreased by 3–4 times with each next extraction step [10]. The higher yield of HAs in the first extract from the gray forest soil could be attributed to the fact that this soil contained less hardly extractable HAs (e.g., those bound to calcium or clay minerals) compared to the chernozem [6].



^{13}C NMR spectra of HAs obtained from sequential alkaline extracts.

To assess the structural changes occurring in the HAs from one extraction step to another, the distribution of carbon among the structural groups of HAs was determined by ^{13}C NMR spectroscopy. The spectra recorded are shown in the figure.

On the whole, the characteristic peaks of humic substances were present in all the spectra, including the regions of aromatic carbon (165–108 ppm), carboxyl groups (187–165 ppm), and substituted (105–48 ppm) and unsubstituted (48–5 ppm) aliphatic carbon. The signal intensity in the region of aromatic carbon was higher for the HA-FG1 preparation than for the HAs isolated from the next extracts. At the same time, the spectra of the HA-FG2 and HA-FG3 preparations contained more intensive peaks in the regions of 108–48 ppm, where the signals of carbon substituted by heteroatoms (O, N) occur, including the carbohydrate and amine fragments, and 48–5 ppm, where the aliphatic carbon signals are recorded.

Table 2. Distribution of carbon among the structural fragments of HA preparations obtained by sequential alkaline extraction from the gray forest soil (% of C_{total})

Preparation	Spectral ranges, ppm					
	5–48 ($C_{Alk-H,R}$)	48–108 ($C_{Alk-O,N}$)	108–145 ($C_{Ar-H,R}$)	145–165 ($C_{Ar-O,N}$)	165–187 ($C_{COO-H,R}$)	187–220 ($C_{C=O}$)
HA-GF1	14	18	34	10	17	7
HA-GF2	21	20	28	8	16	7
HA-GF3	20	23	27	8	15	7
Wa*	15	18	33	10	17	7
$\pm t_{\alpha} s_{\bar{x}}^{**}$	3	1	2	2	1	2

Notes: * Weight-average value for all the preparations;
** ($\pm t_{\alpha} s_{\bar{x}}$) confidence interval calculated for $\alpha = 0.05$ and $n = 3$.

Data on the quantitative assessment of the distribution of the spectral intensity in the ^{13}C NMR spectra for the HA preparations isolated from the three sequential alkaline extracts are given in Table 2.

The presented data show that the structure of the preparation isolated from the first extract significantly differed from that of the HAs isolated by the second and third extractions, which were similar to each other. It can be seen that the HA-GF2 and HA-GF3 preparations significantly differed only in the content of aliphatic carbon atoms substituted by hetero atoms with the difference in this parameter being relatively small (3% in modulus). The difference in the content of aliphatic carbon atoms substituted by hetero atoms in the HA-GF2 and HA-GF3 could be related to the hydrolysis of nonhumic compounds (cellulose and protein residues) present in the soil.

The most significant structural difference between the HA-GF1 and the two other preparations was in the content of aromatic, substituted aliphatic, and unsubstituted aliphatic carbon. The total content of aromatic carbon in the HAs from the first extract (43%) was higher than that in the HA-GF2 and HA-GF3 (36%) by 7%. At the same time, the lowest relative content of aliphatic fragments (14%) was observed in the HA-GF1 preparation compared to the two other preparations (20–21%). A regular increase in the content of aliphatic carbon bound to hetero atoms from 18 to 23% was also observed in the series of the preparations obtained from the HA-GF1 to HA-GF3. A tendency toward a decrease in the content of carboxyl carbon in the HAs with each next step was also notable.

The tendencies revealed for the gray forest soil (an increase in the content of aliphatic fragments and a decrease in the contribution of aromatic structures) well agree with the reported data on the structural and group composition of the HAs isolated from the sequential alkaline extracts of marsh peat soil [13]. They also agree with our data obtained earlier for the typical chernozem [10].

Table 2 also contains the weight-averaged values of the carbon distribution among the structural fragments. These values were calculated for the HAs obtained by combining all three extracts into one extract with account for the weight proportion of each HA preparation. This composition would be typical of the integral HA preparation isolated using the Russian protocol [7], while the composition of the preparation isolated according to the IHSS procedure [15] corresponded to the HA-GF1 preparation. It can be seen that, in spite of the relatively significant differences between the preparations obtained from the different extracts, the calculated weight-averaged values were close to the data obtained for the HAs from the first extract. Thus, the structural–group composition of the HAs of the combined three sequential alkaline extracts from the gray forest soil are very similar to that of the HAs of the first extract. Therefore, the preparations of HAs isolated from the gray forest soil by the Russian procedure [7] can be compared to the preparations obtained in accordance with the IHSS recommendations [15].

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 nor components entering into the humin complex of a specific soil. The separate study of each sequential extract can be recommended for obtaining the most complete knowledge.

CONCLUSIONS

Our studies showed that, in the case of the gray forest soil, the structure 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 of the structural parameters. The multiple alkaline extractions could favor the hydrolysis of nonhumic compounds (cellulose and protein residues) present in the soil and their simultaneous extraction with the HAs.

On the whole, a decrease in the content of carbon, an increase in the portion of aliphatic fragments, and a decrease in the content of the aromatic groups in the preparations were observed from one extraction to another. A tendency toward an increase in carbohydrates with each next extraction was also noted.

It was shown that, in terms of the preparative yield, most of the HAs from the gray forest soil were extracted during the first extraction: the yield of HAs from the first extract was higher than those from the next extracts by about 10 times. Therefore, the first extraction made the major contribution to the structure of the integral HA preparation obtained by combining all the extracts.

Taking into consideration the high labor expenses for the three-stage extraction and the possibility of 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.

REFERENCES

1. E. V. Arinushkina, *Handbook on the Chemical Analysis of Soils* (Mosk. Gos. Univ., Moscow, 1970) [in Russian].
2. A. F. Vadyunina and Z. A. Korchagina, *Methods for Studying Soil Physical Properties* (Agropromizdat, Moscow, 1986) [in Russian].
3. *Classification and Diagnostic System of Soils of the Soviet Union* (Kolos, Moscow, 1977) [in Russian].
4. *Classification and Diagnostic System of Russian Soils* (Oikumena, Smolensk, 2004) [in Russian].
5. D. V. Kovalevskii, A. B. Permin, I. V. Perminova, and V. S. Petrosyan, “The Choice of Conditions for Recording ^{13}C NMR Spectra of Humus Acids,” Vestn. Mosk. Gos. Univ., Ser. 2 (Chemistry), **41** (1), 39–42 (2000).
6. M. M. Kononova, *Soil Organic Matter* (Akad. Nauk SSSR, Moscow, 1963) [in Russian].
7. D. S. Orlov and L. A. Grishina, *Practical Manual on Humus Chemistry* (Mosk. Gos. Univ., Moscow, 1981) [in Russian].
8. *Pedology. Soil Types, Their Geography, and Their Use*, V. A. Kovda and B. G. Rozanov (Eds.) (Vysshaya Shkola, Moscow, 1988) [in Russian].
9. *Soils of Moscow Oblast and Their Management*, L. L. Shishov and N. V. Voitovich (Eds.) (V.V. Dokuchaev Soil Science Inst., Moscow, 2002), vol. 1 [in Russian].
10. V. A. Kholodov, A. I. Konstantinov, E. Yu. Belyaeva, et al., “Comparative Characterization of Humic Acids Obtained during a Sequential Extraction from a Typical Chernozem,” Accepted for publication by the journal Pochvovedenie (2009).
11. T. D. W. Claridge, *High-Resolution NMR Techniques in Organic Chemistry* (Elsevier Science Ltd., Amsterdam–Lausanne–New York–Oxford–Shannon–Singapore–Tokyo, 1999).
12. N. Hertkorn, A. B. Permin, I. V. Perminova, et al., “Comparative Analysis of Partial Structures of a Peat Humic and Fulvic Acid Using One and Two Dimensional Nuclear Magnetic Resonance Spectroscopy,” J. Environ. Qual. **31**, 375–387 (2002).
13. L. Li, W. Huang, P. Peng, et al., “Chemical and Molecular Heterogeneity of Humic Acids Repetitively Extracted from a Peat,” Soil Sci. Soc. Am. J. **67**, 740–746 (2003).
14. C. M. Preston, “Applications of NMR to Soil Organic Matter Analysis: History and Prospects,” Soil Sci. **161**, 144-166 (1996).
15. R. S. Swift, Organic Matter Characterization (Chapter 35), in *Methods of Soil Analysis* (Soil Sci. Soc. of America, Madison, WI, 1996), pp. 1018–1020.