ISSN 0361-5219, Solid Fuel Chemistry, 2022, Vol. 56, No. 4, pp. 253–258. © Allerton Press, Inc., 2022.
Russian Text © The Author(s), 2022, published in Khimiya Tverdogo Topliva, 2022, No. 4, pp. 20–25.

Antioxidants in Humic Acids of Various Origins

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Received February 14, 2022; revised February 17, 2022; accepted March 30, 2022

Abstract—With the use of a voltammetric method, we found that humic acids (HAs) of different origin extracted from soils, sapropels, peats, and brown coals exhibit pronounced antioxidant properties. According to the results of a correlation analysis, it was shown that the antioxidant activity of HAs depends on the concentration of phenolic hydroxyls and carboxyl groups in a range of pH 10–5.5. Peat HAs were characterized by a maximum antioxidant activity.

Keywords: soil, sapropels, peat, brown coal, humic acids, composition, antioxidant properties

DOI: 10.3103/S0361521922040097

INTRODUCTION

Natural antioxidants are widely distributed in the biosphere, and they affect the processes of biochemical transformation of organic matter to provide oxidation resistance. The most effective oxidation inhibitors include compounds with functional groups containing mobile hydrogen atoms (phenols, aromatic amines, aminophenols, pigments, etc.) capable of converting free radicals into an inactive form [1–4]. Compounds with relatively weak –OH and –NH bonds interact with peroxide radicals to terminate the main chain of oxidation [5].

Humic acids (HAs), which are heterogeneous redox-active organic macromolecules, are able to regulate redox processes. A special characteristic of HAs is their antioxidant activity, which affects the concentration and lifespan of reactive oxygen species in soils and water systems [6]. Humic acids can also perform protective functions in ecosystems by interrupting radical reactions and preventing damage to cell membranes and biological macromolecules [7, 8].

It was found that, at given values of pH and Eh, the electron-donating ability of HAs under oxidizing conditions correlates well with the concentration of phenolic fragments [6]. Phenolic fragments, which are formed from more complex precursor molecules such as lignin and tannins, have been proposed as the main electron-donor fragments of HAs responsible for their antioxidant capacity.

Based on the results of a comparative analysis of the reactivity indices of the model structure of HAs, criteria for evaluating the antioxidant capacity based on the energy of adsorption of molecular oxygen by the hydroxyl group were developed [9].

The values of pH have an important influence on the reducing ability of antioxidants. Under acidic conditions, the reducing ability can be limited due to the protonation of antioxidant compounds, while the proton dissociation of phenolic compounds under basic conditions would increase the reducing ability of the sample [10]. The antioxidant activity of HAs in an acidic range of pH is mainly associated with the presence of nitrogen-containing groups, such as peptides and amino acid residues [11–13].

Various methods are widely used in the determination of antioxidant activity: chemiluminescence [14], spectrophotometry [15], electrochemical [16, 17] and gasometric methods [18], etc. Voltammetry, which is very sensitive to the presence of oxygen and its active radicals in the medium, is a convenient method for the determination of antioxidants and their activity.

The purpose of this work was to study the antioxidant activity of HAs of various origins depending on the molecular structure and the pH of medium.

EXPERIMENTAL

Humic acids isolated from chernozem (ChP), soddy-podzolic (DP), and podzolic (PZ) soils; sapropels of the organic (OR), organoferrous (OZh), and carbonate (CR) types; high-moor fuscum (TVF), high-moor woody herbal (TDT), and lowland woody (TD) peats from the area of the Vasyugan natural province were used in this study. Oxidized coals from

Table 1. Physicochemical characteristics of humic acids

HA sample	Atomic ratio			Concentration of functional groups, mg-equiv/g		
	H/C	O/C	C/N	$C_{alk}COOH$	$C_{Ar}COOH$	$C_{Ar}OH$
Chernozem ChP	0.86	0.51	14.8	Not determined	Not determined	Not determined
DP	1.01	0.50	16.4	–	–	–
Podzolic	1.08	0.56	11.9	–	–	–
Sapropel OR	1.36	0.62	21.5	–	–	–
Sapropel OZh	1.61	0.57	13.6	–	–	–
Sapropel KB	1.89	0.77	17.0	–	–	–
Peat TVF	1.24	0.66	29.7	3.2 ± 0.1	7.5 ± 0.1	10.2 ± 0.3
Peat TDT	1.22	0.48	35.4	3.1 ± 0.2	6.5 ± 0.1	9.2 ± 0.2
Peat TD	1.21	0.50	28.1	3.1 ± 0.1	6.8 ± 0.1	8.5 ± 0.2
Chui-Kenul OU	1.20	0.46	37.3	1.6 ± 0.1	6.1 ± 0.2	8.4 ± 0.1
Baganur OU1	1.19	0.45	41.3	2.0 ± 0.1	5.8 ± 0.2	8.3 ± 0.1
Ching-Chai BU	1.10	0.39	72.3	2.4 ± 0.1	5.1 ± 0.2	8.1 ± 0.1
Barandat BU1	1.13	0.38	71.9	0.8 ± 0.1	4.7 ± 0.2	8.0 ± 0.2
Aldrich HFA	1.15	0.71	39.7	2.3 ± 0.1	6.1 ± 0.3	8.7 ± 0.2

Humic acids isolated from chernozem (ChP), soddy podzolic (DP), and podzolic (PZ) soils; sapropels of the organic (OR), organoferrous (OZh), and carbonate (CR) types; high-moor fuscum (TVF), high-moor woody herbal (TDT), and lowland woody (TD) peats from the area of the Vasyugan natural province were used in this study. Oxidized coals from the Chui-Kenul (OU, China) amd (OU1, Mongolia) deposits and brown coals from the Ching-Chai deposit (BU, China) and the Barandat deposit of the Kansk-Achinsk coal basin (BU1) served as the source of HAs of more transformed caustobioliths. HAs from Merk (Germany) and an unfractionated HFA mixture of humic and fulvic acids from Sigma-Aldrich (the United States) were used as reference standards.

the Chui-Kenul (OU, China) and Baganur (OU1, Mongolia) deposits and brown coals from the Ching-Chai deposit (BU, China) and the Barandat deposit of the Kansk-Achinsk coal basin (BU1) served as the source of HAs of more transformed caustobioliths. HAs from Merk (Germany) and an unfractionated HFA mixture of humic and fulvic acids from Sigma-Aldrich (the United States) were used as reference standards.

The caustobioliths were preliminarily crushed in a Nossen 8255 disintegrator to a particle size of 1–3 mm.

Humic acids were isolated from the caustobioliths using a 0.1 N solution of NaOH at a temperature of 20°C with stirring for 1 h. Alkaline extraction was repeated three times. Humic acids in an alkaline solution were precipitated with 4% HCl to pH 2. A brown amorphous precipitate of HAs was separated by centrifugation; then, it was washed with distilled water to pH 7 and dried in a Petri dish in a vacuum oven to constant weight.

The concentration of acidic ionogenic groups was determined by potentiometric titration. In the course of the titration of HAs, the ionic strength of the solution was maintained at a constant level with a saturated solution of sodium chloride. Three distinct inflection points were distinguished in the obtained titration curves in the ranges of pH 10–11 (phenolic hydroxyls of $C_{Ar}OH$), pH 5.5–8.5 (carboxyl groups at aromatic rings $C_{Ar}COOH$), and pH 2.5–5.0 (carboxyl groups at hydrocarbon chains $C_{Alk}COOH$). The equivalence point was calculated using numerical interpolation.

The elemental composition of HAs was determined on a Vario El Cube elemental analyzer (Germany).

The IR spectra of HAs were recorded on a Nikolet 5700 Fourier transform IR spectrometer with a Raman module (Thermo Electron Corporation, the United States) in pellets with KBr at a ratio of 1 : 300, respectively, in a frequency range from 400 to 4000 cm^{-1} with the compensation of adsorbed water signals. To quantify the intensity of absorption bands, the method of baselines and relative optical densities (*D*) was applied using a computer program. The concentration of structural groups was calculated as a ratio between optical densities at the maximum of a characteristic absorption band and a reference absorption band at 1465 cm^{-1}.

The fragment composition of HAs was analyzed by ^{13}C NMR spectroscopy on a Bruker 300 radiospectrometer (Germany) with an operating carbon frequency of 125 MHz using an accumulation Fourier transform procedure. The accumulation time varied from several hours to days. The spectra were processed using the Bruker WINNMR software. In addition to recording the NMR spectra, the integral was recorded to determine the relative concentrations of magnetic carbon nuclei belonging to one or another group of atoms.

Table 2. Structural group composition of HAs according to Fourier transform IR-spectroscopic data

HA sample	Concentration of structural groups, arb. units			
	D_{3350}/D_{1465}	D_{2910}/D_{1465}	D_{1720}/D_{1465}	D_{1270}/D_{1465}
Sapropel OR	0.88	0.91	0.86	0.83
Sapropel OZh	0.87	0.89	0.89	0.85
Sapropel KR	0.86	0.90	0.82	0.82
Peat TVF	0.89	0.82	1.02	0.85
Peat TDT	1.05	0.88	0.93	0.82
Peat TD	0.85	0.86	0.89	0.82
Chui-Kenul OU	0.83	0.75	0.88	0.78
Baganur OU1	0.80	0.71	0.82	0.72
Ching-Chai BU	0.76	0.69	0.84	0.78
Barandat BU1	0.72	0.63	0.81	0.75

The antioxidant properties of HAs were determined by the voltammetric method of cathodic oxygen reduction with the use of a mercury film electrode (Antioxidant AOA) [17]. The measurement error did not exceed 10%. The supporting electrolytes were a phosphate buffer solution (pH 6.8) and a borate buffer solution (pH 9.18). The HA concentration in the cell was varied from 0.5×10^{-5} to 2.0×10^{-5} g/mL. Based on the results of the determinations, a graph of the dependence of $(1 - I/I_0)$ on time (t) was constructed. This graph was processed by the least squares method. The slope $d(1 - I/I_0)/dt$ was determined from the linear part of the curve, and the kinetic criterion K (μmol L^{-1} min^{-1}) was calculated according to the formula

$$K = C^0_{O_2}(1 - I/I_0)/t,$$

where I is the electroreduction (O_2 ER) current in the presence of HAs in solution, μA; I_0 is the O_2 ER current in the absence of HAs in the solution, μA; $C^0_{O_2}$ is the initial concentration of oxygen in the solution, μmol/L; and t is the process time, min.

The following materials were used: NaOH (chemically pure, Reakhimpribor), HCl (chemically pure, OOO Khimtorg), NaCl (chemically pure, OOO Khimtorg), phosphate buffer solution (a 100-fold concentrate, pH 6.8, Abris+), and borate buffer solution (pH 9.18, LenReaktiv).

RESULTS AND DISCUSSION

Humic acids are aromatic hydroxypolycarboxylic acids, the peripheral part of their macromolecules is enriched in oxygen-containing groups. As found by Khil'ko et al. [8], the numbers of functional groups per HA macromolecule are 50 for –COOH and 25 for –OH.

Tables 1 and 2 summarize the physicochemical characteristics of HAs isolated from soils, sapropels, peats, and coals. The test HAs differed significantly in their molecular structure characteristics. The highest values of H/C atomic ratios were typical for HAs from sapropels and peats; they indicated a greater saturation of the molecular structure with hydrogen and the predominance of aliphatic and acyclic fragments. HAs from soils and coals were characterized by low H/C ratios. The latter were characterized by more condensed structures with a smaller fraction of hydrogen in them. The transformation of organic matter (OM) in the course of humification in the peat–coal system was accompanied by the condensation of molecules and led to a decrease in the H/C atomic ratio in HAs.

In the process of OM transformation, not only the fraction of hydrogen but also the fraction of nitrogen in the structure of HAs decreased. The nitrogen content of the test HA molecules decreased in the following order: soil > sapropel > peat > oxidized coal > brown coal. The second largest element in HA molecules after carbon is oxygen, which determines the concentration of acidic functional groups. The maximum values of O/C atomic ratios were typical for HAs from sapropels, soils, and peat, and they decreased in HAs from oxidized and brown coals. In HFA from Aldrich, the O/C ratio was 0.71.

According to the results of potentiometric titration, the concentrations of phenolic $C_{Ar}OH$ and carboxyl $C_{Ar}COOH$ groups in peat HAs and HFA from Aldrich were higher than those in coal HAs (Table 1).

The IR-spectroscopic data indicate higher fractions of alkyl substituents D_{2910}/D_{1465}, hydroxyl groups D_{3350}/D_{1465}, and carbonyl groups D_{1720}/D_{1465} and D_{1270}/D_{1465} in HA macromolecules from peat and sapropels than those in HAs from coals (Table 2).

The molecular-structure characteristics of HAs are based on the values calculated from the results of ^{13}C NMR spectrometry (Table 3) [19]. The ratio $C_{alk}O/C_{alk}$, which characterizes the number of heteroalkyl carbon with respect to alkyl carbon atoms, in soil HAs decreased in the following order: chernozem >

Table 3. Molecular-structure characteristics of humic acids

HA sample	$C_{Alk}O/C_{Alk}$	$\Sigma C_{Ar}O + C_{Ar}CH$	$C_{Ar}O/C_{Ar}O + C_{Ar}CH$	K, μmol L^{-1} min^{-1}
Soil ChP	0.67	43.9	0.27	0.32
Soil DP	0.56	39.6	0.17	0.24
Soil PZ	0.38	35.5	0.35	0.25
Sapropel OR	1.0	33.5	0.32	0.77
Sapropel OZh	0.95	32.4	0.22	0.59
Sapropel KR	0.91	28.4	0.20	0.89
Peat TVF	0.91	20.8	0.11	0.91
Peat TDT	0.89	32.7	0.17	0.73
Peat TD	0.88	28.4	0.56	0.62
Chui-Kenul OU	0.57	44.5	0.29	0.41
Baganur OU1	0.67	50.0	0.25	0.39
Ching-Chai BU	0.47	50.0	0.35	0.32
Barandat BU1	0.38	45.6	0.28	0.27
Merk HA	0.67	35.8	0.11	0.43
Aldrich HFA	0.68	43.8	0.24	0.49

soddy-podzolic soil > podzolic soil. The highest degree of oxidation of alkyl fragments was observed in HA molecules from sapropels and peats. The degree of oxidation of alkyl fragments noticeably decreased in HAs during the transformation of organic matter in a transition from peats to oxidized coals and brown coals.

Significant differences in HAs were observed in terms of the number of aromatic fragments $\Sigma C_{Ar}O + C_{Ar}CH$ (Table 3). In HA macromolecules isolated from chernozem and coals, the aromatic fragments contained from 43.9 to 50 rel % of carbon. Peat, sapropel, and Merk HAs were characterized by close values of the degree of aromaticity. The HAs of TVF and TDT peats and soddy-podzolic soil showed a low degree of oxidation of aromatic fragments, calculated as $C_{Ar}O/(C_{Ar}H,C + C_{Ar}O)$, which was comparable to the degree of oxidation of these fragments in the Merk HAs.

Table 3 shows the values of the kinetic criterion K of the antioxidant activity of humic acids, which reflects the amount of oxygen and active oxygen radicals that reacted with the antioxidant for 1 min at pH 10. It is believed [5–8] that phenolic fragments formed from more complex plant precursor molecules such as lignin and tannins are responsible for the antioxidant activity of HAs. Maximum values of K were noted for HAs from peats and sapropels, and minimum values were observed in HAs from DP and PZ soils and HFA from Aldrich.

Due to the presence of a wide range of functional groups of different nature in HAs, their antioxidant properties can manifest themselves in the entire range of pH. Figure 1 shows the effect of pH in a range of 10–5.5 on the value of K for humic acids isolated from peats and coals. Under basic conditions, the proton dissociation of phenolic compounds increases the reducing ability of HAs, while the reducing ability can be limited under acidic conditions due to the protonation of antioxidant fragments [10]. At pH 10, HAs from high-moor TVF peat were characterized by a maximum antioxidant capacity. For HAs from brown coals, the lowest value of K was noted. A decrease in pH to 6.5 led to a decrease in the value of K for all HA samples. In this range of pH, carboxyl groups dissociate.

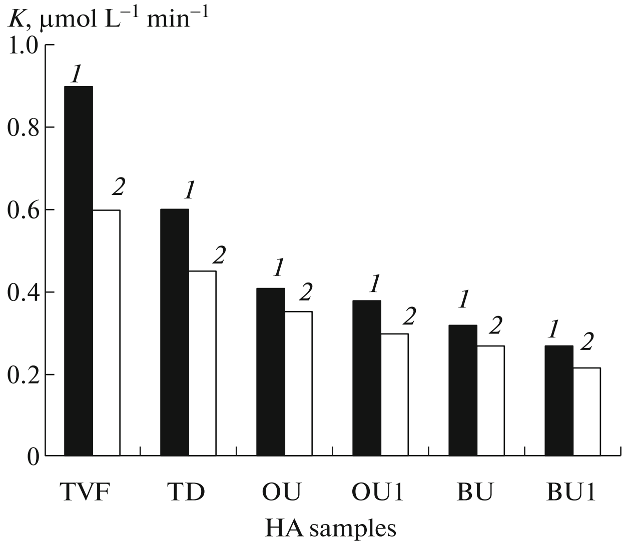

Fig. 1. Influence of the pH of medium on the antioxidant activity of peat and coal HAs.

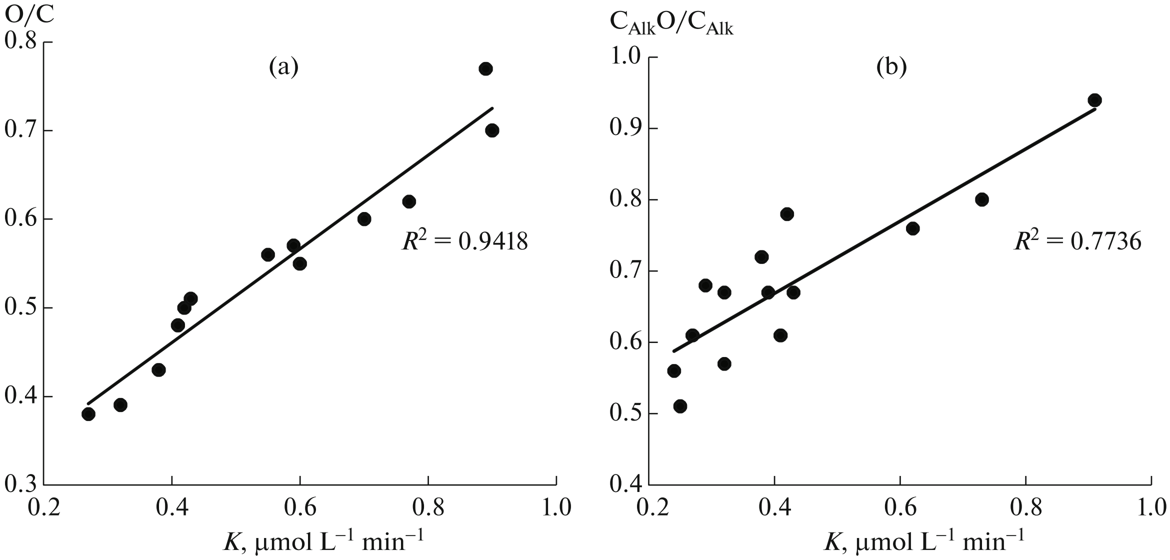

Fig. 2. Dependences of the value of K on the atomic ratios (a) O/C and (b) $C_{Al}O/C_{Al}$ in humic acids.

To understand the relationship between the antioxidant properties and structural features of HAs, we performed a correlation analysis of the elemental composition and data obtained by ^{13}C NMR spectroscopy and potentiometric titration with the concentrations of antioxidants. Figure 2a shows the dependence of K on the O/C atomic ratio in HA macromolecules, which is characterized by a positive linear correlation with a high degree of approximation due to the presence of acidic functional groups in the structure of HAs. Based on an analysis of ^{13}C NMR-spectroscopic data for HAs, a correlation was established between the degree of oxidation of alkyl fragments $C_{Al}O/C_{Al}$ in HAs and the value of K (Fig. 2b).

Figure 3a shows the relationship between the concentration of phenolic groups $C_{Ar}OH$ in HAs determined by potentiometric titration and the kinetic criterion K at pH 10. The highest concentration of phenolic groups was found in TVF peat HAs and the lowest, in brown coal HAs, which affected the values of their antioxidant activity. The reliability of the approximation was satisfactory.

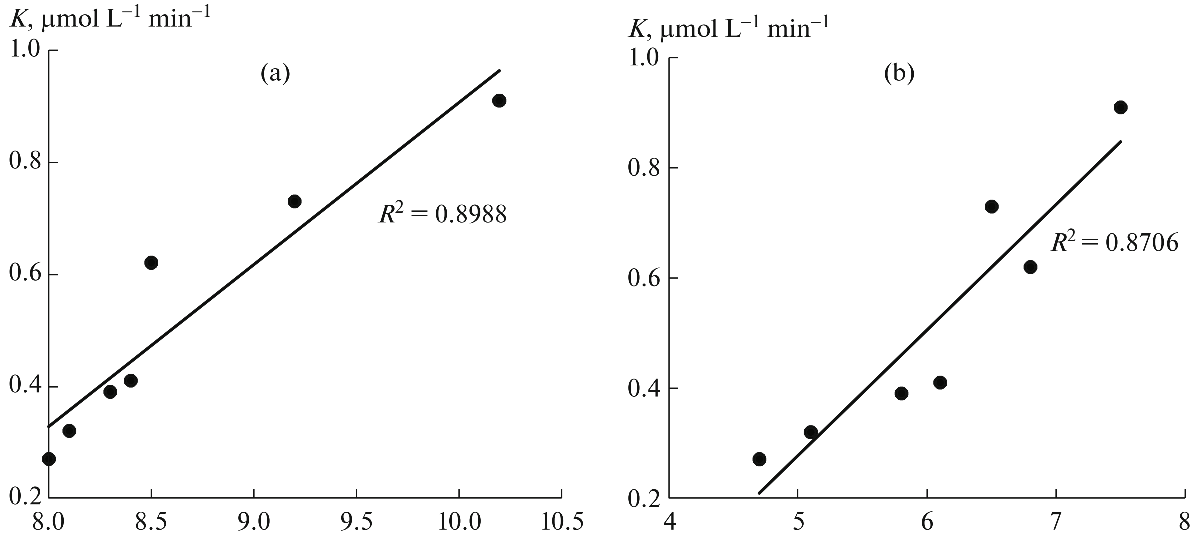

Fig. 3. Dependences of the value of K in the peat and coal HAs on (a) the $C_{Ar}OH$ content at pH 10 and (b) the $C_{Ar}COOH$ content at pH 5.0–7.5.

Figure 3b shows the dependence of K at pH 6.5 on the concentration of $C_{Ar}COOH$ groups in the HAs of peats and coals. With a decrease in the concentration of carboxyl groups in the order peat HAs > oxidized coal HAs > brown coal HAs, their antioxidant activity decreased with a fairly high degree of reliability.

The reactivity of similar groups in a HA macromolecule depends on their spatial arrangement. Depending on the location of carboxyl groups, the antioxidant activity of phenolic hydroxyls can decrease due to the removal of electron density from the phenolic ring, and the antioxidant activity of phenolic hydroxyls increases upon their deprotonation at pH 6.5 [20].

CONCLUSIONS

The antioxidant activity of HAs depends on their molecular structure, which is determined by their origin. The HA macromolecules isolated from soils are characterized by low hydrogen saturation and high nitrogen content. Aliphatic and acyclic fragments and acidic functional groups predominate in the composition of peat and sapropel HAs enriched in hydrogen and oxygen.

The antioxidant activity of HAs, depending on the pH of the medium, is determined by compounds that include –OH and –NH bonds. The values of antioxidant activity K for peat and coal HAs decreased as the basicity decreased from pH 10 to pH 5.5. A positive linear dependence of the antioxidant activity values of HAs on the concentration of phenolic hydroxyls at pH 10 and on the concentration of carboxyl groups at pH 6.5 was established.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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Translated by V. Makhlyarchuk