===== **ARTICLES** =====

Extraction of Humic Substances from Fresh Waters on Solid-Phase Cartridges and Their Study by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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Abstract—Humic substances (**HS**) have been extracted from fresh waters on macroporous adsorbents of different types, Amberlite XAD-8 resin and Bond Elute PPL solid-phase cartridge. Comparative analysis of the extracted samples by ultra-high resolution mass spectrometry demonstrated selectivity of the selected adsorbents to the components of the molecular assembly of HS: the XAD-8 resin has affinity to the higher oxidized aromatic compounds and PPL has affinity to substances of stronger aliphatic character and nitrogen-containing compounds. Because of this fact, a comparison of the molecular composition of HS isolated from various sources must be performed taking into account selective adsorption; therefore, it is necessary to compare the products extracted by the same adsorbent for correct interpretation.

Humic substances represent the organic matrix of natural waters and soils and consist of 50 to 90% of dissolved organic matter (**DOM**) [1]. The molecular composition of HS determines the quality of water and the character of chemical and biological processes occurring in the water bodies: formation of complexes with microelements and heavy metals, solubilization of pesticides and hydrocarbons, etc. [2–5]. The extraction and determination of the structural characteristics of HS is an important problem in the prediction of the ecological state of aquatic medium.

Because of the polyelectrolyte properties of HS, which are aromatic polyoxyacids in nature, the most efficient method of their extraction from natural waters is solid-phase extraction on ion-exchange and/or macroporous adsorbents. The advantages of the method are that extraction is performed without using organic solvents and large volumes of water can be used. Ion-exchange resins are actively used in Russia for the indicated purposes. A large series of works on the adsorption of HS on synthetic anion-exchangers was published by Slavinskaya et al. from Voronezh State University; the preference of use of macroporous ion-exchangers has been demonstrated [6–8]. Macroporous anion-exchangers based on cellulose were intensively used by Varshal et al. for the extraction

and fractionation of dissolved organic matter and HS [9–11]. Works [12, 13] made a substantial contribution to the application of the method of fractionation on cellulose adsorbents for the study of the composition of DOM components. Methodological works on the extraction of fulvic acids from fresh waters using diethylaminoethylcellulose (**DEAE**) and their subsequent characterization were performed [14, 15]. It was shown that the recovery of HS attains a maximum when macroporous anion-exchangers are used, which, along with such an important advantage as high efficiency, also possess a great disadvantage of the substantial irreversibility of sorption, minimal for cellulose adsorbents. One more disadvantage of the ion-exchangers is a sharp decrease of the efficiency of extraction of HS and DOM from sea waters because of high competition with inorganic anions, chloride and sulfate.

At present nonionic macrosieve adsorbents are most widely used for the preparative isolation of HS. Amberlite XAD-8 recommended for use by the International Humic Substances Society (IHSS) and representing a hydrophilic cross-linked polymer based on polymethacrylate belongs to this type of adsorbents [16]. The decisive contribution by G.R. Aiken (United States Geological Survey, United States), who per-

formed a wide variety of studies on the efficiency of DOM extraction using a line of macrosieve Amberlite adsorbents with different polarity and demonstrated the highest recovery of DOM (up to 65%) on Amberlite XAD-8 [17−19] should be noted. As a result, this resin was recommended by IHSS as an alternative to XAD-2, which was widely used previously [20, 21]. At present this sorbent is discontinued by Rohm and Haas; therefore, the task of searching for the optimum adsorbent for HS extraction has again become important. In addition, the invention of methods of high-resolution magnetic resonance substantially increased the requirements to the purity of the isolated substance, as the presence of a mineral residue has a significant negative effect on the results of structural studies.

The approach [22] to DOM extraction based on use of Bond Elut cartridges for solid phase extraction (**SPE**), previously manufactured by Varian and at present by Agilent Technologies, has got great attention. Experiments with a series of different SPE cartridges allowed the authors [22] to recommend the PPL cartridge, representing a surface-active modified co-polymer of polystyrene and divinylbenzene, for the isolation of DOM. Such cartridge makes ensure a 75% efficiency of DOM extraction from sea waters. A substantial distinction of the extraction of DOM on the cartridge compared to XAD-8 is the use of methanol for the elution of the adsorbed substance instead of a 0.1 M NaOH solution. This procedure allows the analyst to directly obtain the acidic rather than the salt form of DOM as in the XAD case, which substantially simplifies the isolation (the stage of desalting on cation-exchangers is eliminated) and eliminates the contamination of samples by alkali metals. The indicated advantages gained wide recognition for this approach [23, 24].

It should be noted that there are virtually no systematic studies on the effect of the selection of adsorbent on the molecular composition of the isolated DOM and HS. The indicated problem is quite serious, because DOM is characterized by substantial molecular heterogeneity. Consequently, adsorbents unavoidably must manifest preferential adsorption for different classes of compounds, components of the molecular assembly of these complex natural substances. We demonstrated the dependence of the molecular composition of DOM on the type of adsorbent in a work dealing with the study of DOM of arctic rivers [25]. It was found in the comparison of the extracts obtained on DEAE cellulose, Amberlite XAD-8 resin, and PPL and C18 SPE cartridges that, as a result of the application of the PPL cartridge for DOM extraction from the Kolyma and Panteleikha rivers, products enriched with aliphatic components were obtained compared to the XAD-8 resin.

The present work, in which HS were isolated from a highly colored lake, located in the Oka-Terrace State Reserve, using a PPL SPE cartridge and an Amberlite XAD-8 resin is a continuation of our experiments. To study the adsorption selectivity, we performed a comparative study of the molecular composition of the obtained products by Fourier transform ion-cyclotron resonance mass spectrometry (**FT ICR MS**). This method possesses ultra-high resolution capability, making it irreplaceable for the study of DOM and HS at the molecular level of resolution [26, 27].

EXPERIMENTAL

Reagent and materials. Water samples for DOM extraction were taken from the Sion Lake located in the Oka-Terrace State Nature Reserve. Five liters of water were taken. NaOH and HCl of cp grade and methanol for HPLC (Lab-Scan) were used for elution and acidification. An Amberlite XAD-8 resin (Room&Haas), a modified methylmethacrylate polymer with 250 Å pore diameter and 140 m^2/g pore area, and 60 mL Bond Elut PPL cartridges (Agilent Technologies), which represent a modified surface-active copolymer of polystyrene and divibylbenzene, were used for HS extraction. Amberlite IR 120 (H^+) (Fluka) with ion capacity >1.8 eq/L was used as a cation-exchange resin.

Equipment. High-purity distilled water was prepared using a Millipore Simplicity 185 water purifying system. The elemental analysis of samples was performed on a Vario EL CHNS-analyzer (Germany). Electronic absorption spectra were recorded on a Cary 50 Probe spectrophotometer (Varian). Mass-spectrometric studies were performed on a LTQ-FT Fourier transform ion cyclotron resonance mass spectrometer (Thermo Electron Corp., Bremen, Germany) equipped with a superconducting magnet with magnetic field strength 7 T and an Ion Max electrospray source (Thermo Electron Corp., Bremen, Germany) (Prof. Nikolaev Laboratory of the Institute of Biochemical Physics, Russian Academy of Sciences). The FT ICR MS data were processed using the Transhumus software developed by A. Grigor'ev.

Method of HS extraction on an Amberlite XAD-8 resin. The extraction of HS was performed using the IHSS standard method [16]. For this purpose, 3 L of sampled colored water was passed through a 0.45 μm membrane filter for the elimination of suspended organic matter. The filtered water was acidified with 1 M HCl to pH 2; 200 g of an Amberlite XAD 8 resin was washed with 200 mL of ethanol and placed in a glass column 30 × 3 cm^2. Then the resin was washed with two volumes of water, three volumes of a 0.1 M NaOH solution, and 0.1 M HCl; 3 L of water acidified to pH 2 was passed at a flow rate of 10 mL/min. The efficiency of extraction was monitored by measuring the absorbance of the eluate at 254 nm. Afterwards, one volume of 0.01 M HCl and distilled water for the elimination of salts were passed through the column.

Elemental composition of the extracted HS samples

HS sample	Concentration of elements, wt %				Atomic ratio		
	C	H	O	N	H/C	O/C	C/N
AHF-SSX-13	39.30	4.54	55.43	0.73	1.39	1.06	62.8
AHF-SSP-13	48.70	5.67	44.27	1.36	1.40	0.68	41.8

Then, HS were desorbed by elution with a 0.1 M NaOH solution at a flow rate of 2 mL/min. As a result, 200 mL of a dark-brown eluate was obtained. For the desalting of HS, the solution was passed through a cation-exchange resin in the H-form. Then the desalinated eluate was evaporated on a rotary evaporator at 40°C and adjusted to a constant weight in a vacuum oven at the same temperature. The yield of the final product was 200 mg. The sample was labeled as AHF-SSX-13.

Method of HS extraction on a Bond Elut PPL SPE cartridge. Extraction was performed using an adapted method for sea water DOM [22]. A cartridge was activated by passing 30 mL of methanol immediately prior to extraction. Two liters of water filtered through a 0.45 μm filter and acidified to pH 2 was passed through the prepared cartridge at a flow rate of 20 mL/min. Then the cartridge was washed with 60 mL of 0.01 M HCl for the complete elimination of salts and dried in an air flow. The HS were eluted immediately after drying by passing 80 mL of methanol with 2 mL/min flow rate. The collected dark-brown eluate was evaporated on a rotary evaporator. The solid residue was dried in a vacuum oven at 40°C. The yield of the final product was 160 mg. The sample was labeled as AHF-SSP-13.

RESULTS AND DISCUSSION

Isolation and primary study of samples. The efficiency of HS extraction in both cases was monitored by spectrophotometry. Spectra were measured after 8-fold dilution, as the coloration of the initial water was too high. The ratio of water absorbances at 254 nm (characteristic wavelength for the HS aromatic fragments [28])measured before and after passing it through the column and cartridge was 20 : 7 : 1, respectively. The conclusion about more efficient extraction in using the PPL cartridge was made on this basis.

The extracted compounds were characterized by elemental analysis; the results are presented in table. As is seen, both samples are characterized by similar values of the atomic ratio H/C, which indicates that there are no substantial differences in the concentrations of aliphatic and aromatic fragments (in contrast to the regularities observed for the water of arctic rivers [25]).

At the same time, for the sample isolated on XAD-8, a substantially higher concentration of oxygen was observed, which points to a larger contribution of oxidized molecules to its composition. For the AHF-SSP-13 sample, on the contrary, a higher concentration of nitrogen was noticed, which is indicative of the contribution of amino acids and peptide residues. A similar trend was observed for samples of water from arctic rivers isolated on XAD-8 and PPL cartridges [25]. Thus, the elemental analysis of the samples, on one hand, points to the presence of common structural features of the carbon skeleton (similar H/C ratios) of the isolated samples, and, on the other hand, demonstrates substantial differences in peripheral functional groups (concentrations of oxygen and nitrogen). For the more profound characterization of the composition of isolated HS substances, one should use a high-resolution method capable of giving information on their molecular composition.

Fourier transform ion cyclotron resonance mass spectrometry is characterized by ultra-high resolution, which ensures the identification of individual molecules in complex multicomponent systems [27, 29, 30]. At the same time, the mild method of electrospray ionization allows the analyst to avoid fragmentation, because of which the obtained spectra contain only molecular ions. Figure 1 presents mass spectra of AHF-SSP-13 and AHF-SSX-13 samples. The distribution of molecular ions is similar for both samples and characterized by the presence of a maximum in the m/z 360 region. The mass spectrum of the AHF-SSP-13 sample consists of 5540 peaks, mainly corresponding to singly charged ions. For the AHF-SSX-13 sample, 11923 peaks were recorded; the amount of doubly charged ions was high, which affected the calculation of molecular formulas. The presence of singly and doubly charged ions is typical for DOM [31].

The obtained FT ICR mass spectra were used for the identification of molecular formulas using the Transhumus software based on the algorithm of the calculation of the total statistics of mass differences [32]. As a result, 1677 and 2845 formulas were identified for the isolated products AHF-SSP-13 and AHF-SSX-13, respectively. From the obtained empirical formulas, we calculated the atomic H/C and O/C ratios for each molecular component and plotted them on the Van Krevelen diagram, representing a two-dimensional correlation field with the H/C (axis of ordinates) and O/C (axis of abscissa) coordinates. The indicated diagram was first proposed for the graphical interpretation of the data on coal genesis [33]. In 2003, this diagram was proposed for the visualization of FT-
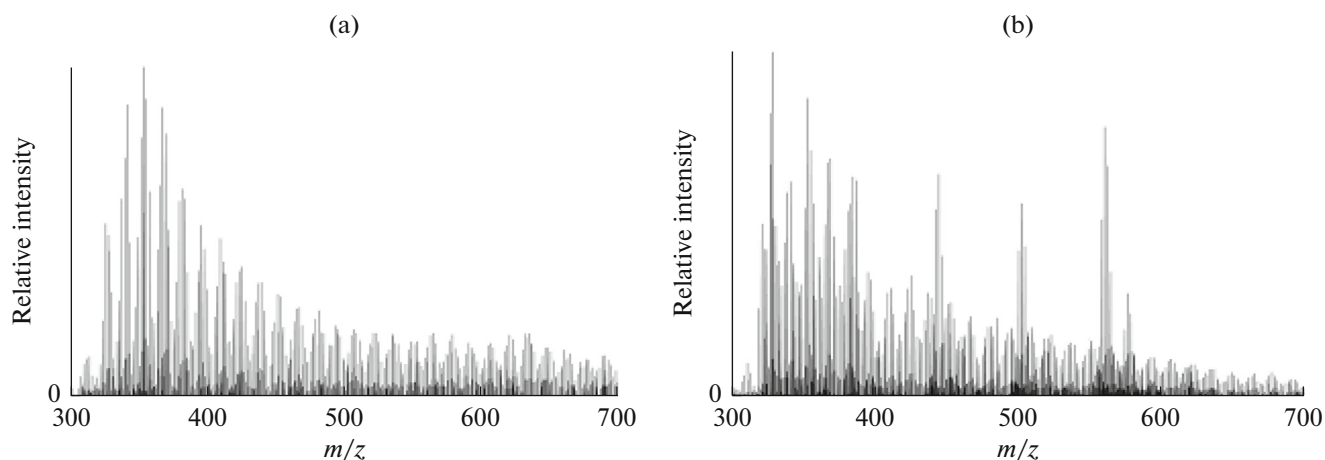
Fig. 1. FT ICR mass spectra of samples isolated on: (a) PPL solid-phase cartridge (AHF-SSP-13) and (b) Amberlite XAD-8 resin (AHF-SSX-13).

ICR MS data [34], and since that time it became a common method of the graphical representation of FT-ICR MS data. An advantage of Van Krevelen diagrams is the possibility of the subdivision of the whole field into regions corresponding to the main HS precursors, such as lignin, tannins, terpenoids, peptides, carbohydrates, etc. [35, 36]. This diagram makes possible the comparison of products by their composition. Recently, a three-dimensional image of the Van Krevelen diagrams has become widespread, in which the intensity of the third parameter is illustrated by color intensity. Such three-dimensional Van Krevelen diagrams are presented in Fig. 2.

The Van Krevelen diagrams can be described as molecular space occupied by components of complex mixtures. For the molecular space of the two studied HS samples extracted on different sorbents, we observed a series of differences. Thus, for the AHF-SSX-13 sample extracted on a XAD-8 resin, a cluster of molecules belonging to the class of condensed weakly-oxidized aromatic compounds (flavonoids or other condensed tannins) in the region of H/C values from 0.2 to 0.8 and O/C values from 0 to 0.3 can be clearly seen on the Van Krevelen diagram (the indicated cluster is virtually absent on the diagram for AHF-SSP-13). This sample is also characterized by the high occupation density of the region of hydrolyzed tannins (H/C from 0.3 to 1.3 and O/C > 0.5). At the same time, high occupation density in the region of proteins and amino acids was observed for the AHF-SSP-13 sample [35]. A similar trend was observed for the HS sample from the Kolyma River [25]. The FT-ICR MS data also confirm another trend, such as the prevalence of aromatic fragments in the composition of substances isolated with XAD-8, which was not found from the data of elemental analysis. It should be noted that the samples were characterized by 1400 common molecular formulas. Consequently, the qualitative composition of the samples is

characterized by a high degree of similarity; however, the number of components was quite different.

A deeper comparative analysis of the peculiarities of isolated HS at the molecular level allowed us to analyze the distribution of molecular weights and aromaticity index (AI) of the samples. The aromaticity index reflects the number of double C=C-bonds in a molecule and takes into account the contribution of heteroatoms to the formation of π-bonds [37]. AI is calculated based on molecular formulas containing C, H, O, N, S, and P using the following equation:

$$AI = \frac{1 + C - O - S - 0.5H}{C - O - S - N - P}.$$

Aromaticity index varies in the range from 0 to 1. If the number of heteroatoms exceeds the number of π-bonds in a molecule, the calculated AI can become negative. For example, the substance with the chemical formula $C_{23}H_{36}O_{12}$ must have AI = −0.54 because of a large number of oxygen atoms. However, since AI characterizes the density of double C=C bonds, the value of AI in this case is taken equal to zero [37].

The distribution of molecular masses by components of the HS assembly (Figs. 2a, 2b) indicates the presence of low- and high-molecular aromatic and condensed compounds in the AHF-SSX-13 sample isolated on XAD-8. The distribution of the aromaticity index AI also points to the high concentration of such compounds in this sample. This allowed us to unambiguously demonstrate the presence of aromatic and condensed structures and also to compare the aromaticity of the samples [37]. Thus, from Figs. 2c and 2d we can conclude that an insignificant amount of condensed compounds (AI $\geqslant$ 0.67) was present in both samples. However, aromatic structures with AI > 0.5 were determined in both products, and their amount was significant in the AHF-SSX-13 sample, while the AHF-SSP-13 sample consisted mainly of
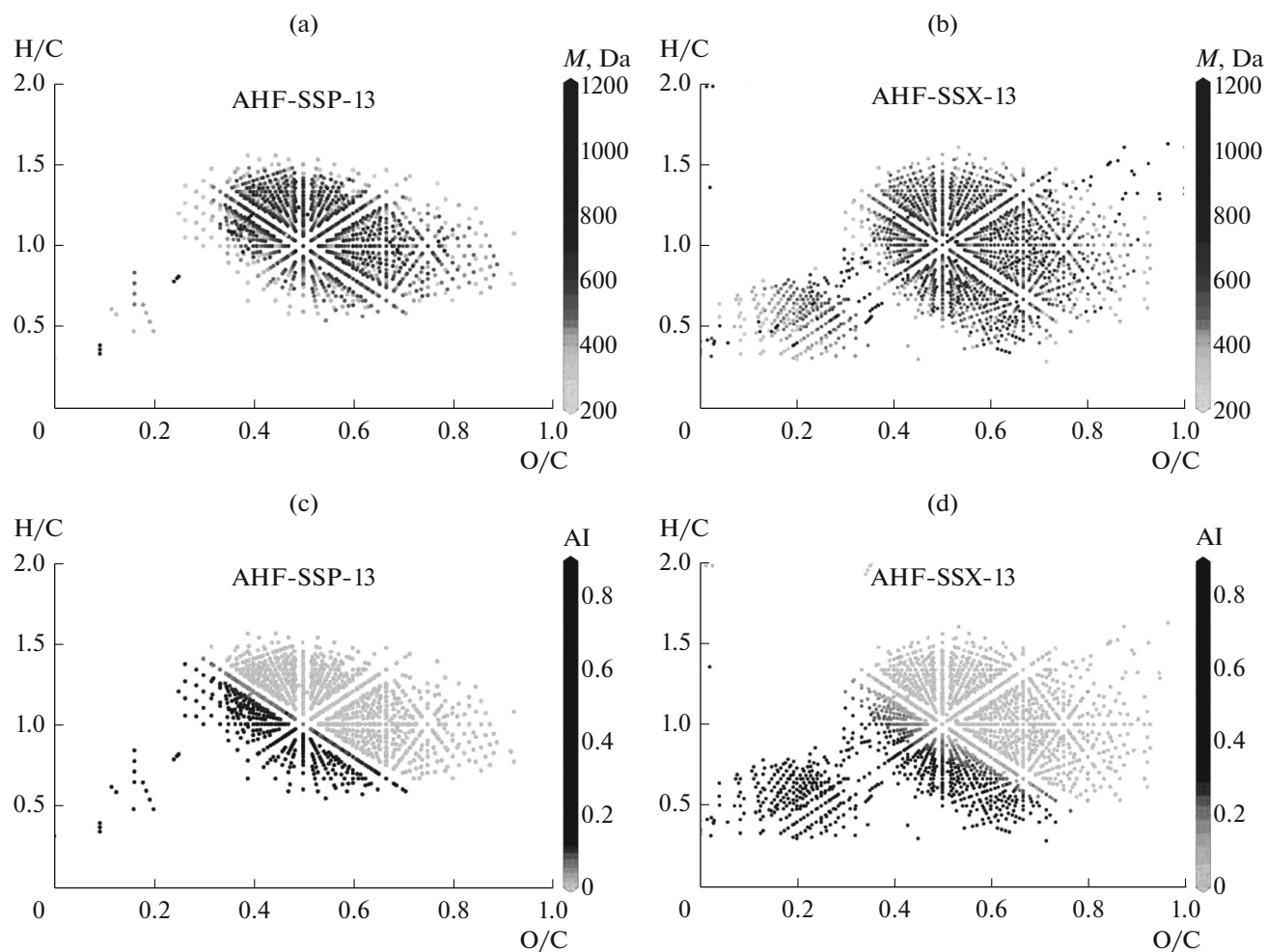
Fig. 2. 3D Van Krevelen diagrams for HS products isolated on a PPL solid-phase cartridge (AHF-SSP-13) and on Amberlite XAD-8 resin (AHF-SSX-13); the third dimension is demonstrated (a, b) by the color intensity of the point, proportional to molecular mass M; and (c, d) by aromaticity index AI.

compounds with AI < 0.5. The indicated shift in AI can be explained by the molecular selectivity of adsorbents used in the work: XAD-8 preferentially adsorbs aromatic components, while the PPL cartridge extracts compounds with larger content of aliphatic fragments rich in nitrogen.

To compare the functional composition of isolated HS samples, we calculated the distribution of oxygen-containing compounds (Fig. 3). This distribution demonstrates the ratio of peak intensities of molecules with different amounts of oxygen (CHO_x-class) to the total intensity of CHO molecules [38, 39].

Thus, most of molecules in the AHF-SSX-13 sample contained 12 oxygen atoms (CHO_{12}-class), while the CHO_{10}-class was predominant in the AHF-SSP-13 sample. Moreover, much higher intensity for molecules containing more than 12 oxygen atoms was observed for the sample isolated on XAD-8 compared to the sample obtained on PPL. The obtained data

agree with the results of elemental analysis, pointing to the higher level of oxidation of the sample isolated on the Amberlite XAD-8 resin.

CONCLUSIONS

The analysis of the obtained HS samples by FT ICR MS shows that, from one hand, both types of adsorbents ensure the isolation of a large pool of similar components present in the composition of the molecular complex of freshwater HS; however, from the other hand, it evidences a substantial selectivity of the adsorbents. In this connection, the product isolated on a XAD-8 resin (AHF-SSX-13) is of more aromatic and oxidized character, while the product obtained using a PPL cartridge (AHF-SSP-13) is characterized by a higher concentration of aliphatic and nitrogen-containing components. The indicated adsorbents could not be considered completely interchangeable: the change of the sorbent will lead to the
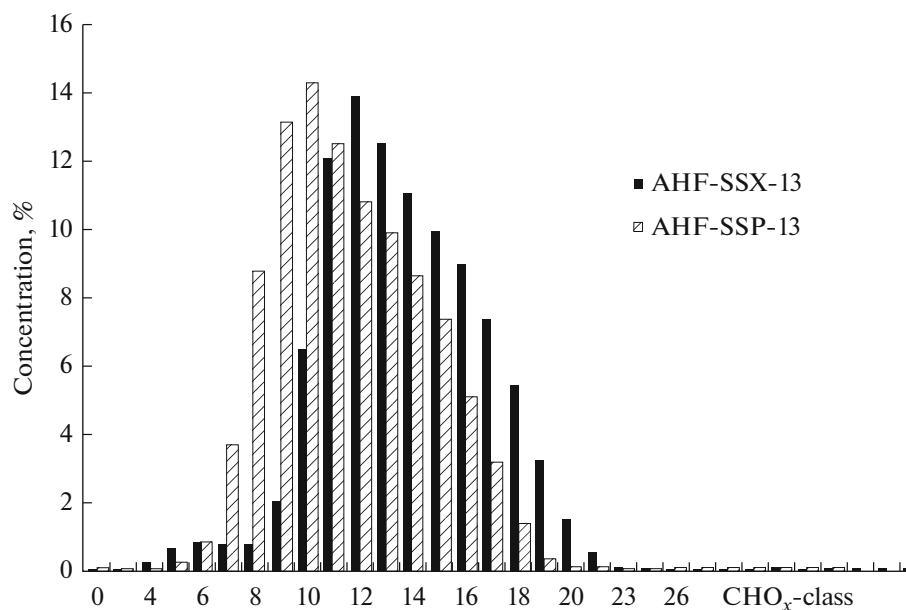
Fig. 3. Distribution of oxygen-containing compounds (CHO_x-index) in the studied HS substances isolated on various adsorbents. The product isolated on an Amberlite XAD-8 resin (AHF-SSX-13) is shown in black; the product isolated on a SPE PPL cartridge (AHF-SSP-13) is shown by hatching.

extraction of HS samples with different molecular compositions and, consequently, properties. As a consequence, when a new adsorbent (SPE PPL cartridge) is used, one should compare the samples isolated using the same sorbent, otherwise erroneous conclusions could be made. Obviously, systematic studies on the comparison of molecular space of HS isolated not only from different sources, but on various adsorbents are necessary. These studies will make possible the creation of databases necessary for the comparative analysis and classification of isolated HS samples.

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