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Extraction of humic acids and their fractions in poly (ethylene glycol)-based aqueous biphasic systems

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Abstract

The possibility of extraction and fractionation of humic acid (HA) in the aqueous biphasic system (ABS) was shown for the first time. The 10% PEG–10% $(NH_4)_2SO_4$–H_2O and 5% PEG–7.5% dextran (or dextran sulphate, sodium salt)–H_2O systems were used. HA originated from peat, soddy-podzolic soil and chernozem were studied. The distribution coefficients were measured for HA of different origin, size of the molecules, molecular weight (MW) of the polymers and pH of the system. The PEG–$(NH_4)_2SO_4$–H_2O system was found to be better for preconcentration and isolation of HA, whereas the PEG–dextran–H_2O system is preferable for HA fractionation. The extractability of HA in ABS increases with increasing the MW of HA molecules. Peat HA are extracted in ABS with higher distribution coefficients compared with chernozem and soddy-podzolic soil HA. It is consistent with higher hydrophobicity of peat HA revealed by hydrophobic interaction chromatography. ABS are promising for HA separation into fractions that differ in their hydrophobic/hydrophilic properties as well as for comparing HA of different origin by their relative hydrophobicity. © 2001 Published by Elsevier Science B.V.

Keywords: Humic acids; Aqueous biphasic systems; Fractionation; Membrane filtration; Gel permeation chromatography

1. Introduction

Humic substance (HS) are natural organic compounds ubiquitous in soils, sediments and waters. They are formed in the process of humification from different structural precursors of biotic origin and have irregular structure and varying composition [1,2]. Generally HS can be described as a complex mixture of related polyelectrolyte-like macromolecules which differ in chemical composition and size. Because of the heterogeneity and polydispersity of HS their molecular structure is not well understood yet, however, it is generally accepted that they consist of aromatic rings and aliphatic chains which carry O-, N- and S-containing functional groups [1,3].

Due to a broad spectrum of polar groups and non-polar fragments HS interact with metal ions, minerals and organic compounds [4–6], thus playing an important role in soil formation processes and binding of environmental pollutants. For better understanding of the mechanisms of these reactions and predicting the HS behaviour in natural processes, more detailed information is needed about molecules, structural constituents and groupings involved, as well as about their complexing capabilities. For such

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complex mixtures as HS their better characterisation can hardly be achieved without application of fractionation techniques based on different properties of the macromolecules.

A traditional fractionation method applied to HS is based on their separation into humic acid (HA) and fulvic acid (FA) according to different solubilities of HS components in acidic medium. HA are soluble in alkali but precipitated at pH 1–2, while FA are soluble in both alkaline and acidic aqueous solutions.

Various other separation procedures have been employed for HS: gel permeation chromatography (GPC), membrane filtration (MF) to fractionate HS by their size (molecular weight (MW)) [7–9]; electrophoresis to separate HS on the basis of their size and charge [10,11]; hydrophobic interaction chromatography to obtain distribution patterns of hydrophobic and hydrophilic fragments of HS [12]. Combinations of various fractionation and extraction methods provide more complete information about HS and their fractions which can have different properties, e.g. different complexing abilities [13].

Liquid–liquid extraction is one of the most useful techniques for the recovery, separation and investigation of organic and inorganic substances. However, hydrophilic HS are poorly extracted to conventional organic solvents in traditional oil/water systems. We have suggested that HS should be better extracted in aqueous polymer-based two-phase systems. Such aqueous biphasic system (ABS) have been successfully used in biotechnology for the fractionation of cell particles and macromolecules [14,15], for analytical and radiochemical separations of metal ions as their complexes with inorganic ligands and water-soluble organic reagents [15–19], but have never been applied to extracting HS.

Biphasic systems can be obtained by mixing aqueous solutions of two dissimilar polymers or of a polymer and an inorganic salt. Usually two-phase aqueous systems are prepared on the basis of poly(ethylene glycol) (PEG), which is salted out from a solution of inorganic salt or another polymer and forms a separate top phase. Both PEG-rich and bottom phases contain significant amounts of water (80–90%), that reduces the effect of hydration on the transfer of the compound to be extracted from one phase to another. This allows one to separate strongly hydrated species whose extraction in traditional solvent extraction systems is difficult or impossible.

Separation of macromolecules in ABS is based mainly on their different hydrophilic/hydrophobic properties. Due to water structuring near the polymer chains, hydrophobic interactions between $–CH_2–CH_2–$ groups of PEG and non-polar groups of the extracted organic macromolecules are entropy favourable. The presence of aromatic structures, accessible to hydrophobic interactions, is important because purely aliphatic organic molecules are poorly extracted to the PEG-rich phase.

The distribution of a biomolecule between two phases in ABS is characterised by distribution coefficient (D). It is dependent on different parameters of extraction system as well as on the size of macromolecules, their charge and conformation, which determine the type and number of groups accessible to the solvent molecules [14].

In our investigations, we used aqueous systems of PEG−ammonium sulphate, PEG−dextran sulphate, sodium salt (NaSD), and PEG−dextran which provide rather high rates of phase separation. The distribution coefficients were measured for HA of different origin, size of the molecules, MW of the polymers and pH of the system.

2. Experimental

2.1. Reagents

The following chemicals were used in the experiments: NaOH, HCl, NaCl, KCl, $(NH_4)_2SO_4$, $(NH_4)_2Cr_2O_7$ (Reakhim, Russia); Tris (Reanal, Hungary); sodium dodecyl sulphate (SDS) (Serva, Germany); Blue dextran 2000 (Pharmacia, Sweden); PEG with MW of 4, 6, 12 and 20 kDa (Loba Chemie, Germany); dextran T70 and T500 with MW of 70 and 500 kDa, respectively (Loba Chemie, Germany); NaSD with a MW of 500 kDa (Pharmacia Fine Chemicals, Sweden). Aqueous solutions of all reagents were prepared using Milli-Q (Millipore) distilled water.

2.2. Extraction and preparation of humic acid

HA from peat (HA_p, "Merck" preparation), soddy-podzolic soil (HA_s) and chernozem (HA_{ch}) were stud-

ied. HS were leached by aqueous 0.1 M NaOH solution from A1 horizons of respective soils using the conventional procedure [20]. HA were precipitated from the obtained extract by its acidification to pH 1–2 by concentrated HCl. Then HA were purified by salting-out with 0.4 M NaCl solution in water and gel filtration on a 1.0 cm × 26 cm column (Pharmacia, Sweden) filled with Sephadex G-10 gel as described elsewhere [21].

2.3. Size fractionation of humic acid by GPC and MF

High- and low-MW fractions of HA_p were obtained by GPC on a K 2.6 cm × 70 cm column (Pharmacia, Sweden), filled with Sephadex G-75 gel (Pharmacia Fine Chemicals, Sweden) using 0.05 M NaOH as the eluent at a flow rate of 20 ml h^{-1}. Then the HA were precipitated from the obtained solutions by HCl to adjust pH <2 and air dried.

Samples of HA_p (0.5 mg ml^{-1} in 0.05 M Tris–HCl buffer, pH 8.2) were also fractionated using an on-line multi-stage MF device (OMFD-2) [22]. The total inner volume of the device is 15 ml, it contains five polymethacrylate discs having non-spiral channels in the upper part and bearing 47 mm Millipore polysulphone membranes with pore sizes of 0.45, 0.1, 0.05, 0.03 and 0.005 μm; 15 ml of 0.05 M Tris–HCl buffer were pumped through the device (at a pressure of 2.5 bar) and after that the sample was passed through. Portions of liquid fractions from every filtration stage were taken after obtaining 30 ml of the filtrate.

2.4. Characterisation of humic acid

The elemental composition was determined on a CHNS analyser "Karlo-Erba" 1106 (t = 1000 °C). UV–VIS spectra of HA solutions (pH 12) were recorded on a Shimadzu UV-2501 PC spectrophotometer.

The distribution of hydrophobic and hydrophilic fragments in HA was estimated on a 1.0 cm × 10 cm column (Amicon) filled with octyl-Sepharose CL-4B gel (Pharmacia, Sweden). The elution was carried out at a rate of 10 ml h^{-1}, firstly in 0.05 M Tris–HCl buffer and after that in 0.05 M Tris–HCl + 0.3% SDS buffer with a step buffer change gradient.

The molecular weight distributions of unfractionated and MF-fractionated HA were obtained on a K 0.9 cm × 60 cm column (Pharmacia, Sweden) filled with Sephadex G-100 gel (eluent: 0.025 M Tris–HCl buffer + 0.05 M NaCl + 0.1% SDS, elution rate 3 ml h^{-1}). The elution profiles were recorded at 280 nm using a 2238 UVICORD SII detector (LKB, Sweden). The MW distributions of HA fractionated by GPC were obtained on a K 1.6 cm × 70 cm column (Pharmacia, Sweden) filled with Sephadex G-75 gel (eluent: 0.025 M Tris–HCl buffer + 0.05 M NaCl + 0.1% SDS, elution rate 3 ml h^{-1}). Each column was calibrated by Blue dextran 2000 and $(NH_4)_2Cr_2O_7$ solutions in the eluent buffer to determine a column void volume and a total gel bed volume, respectively.

There are no proper of HA-like substances with defined MW that could be used as standards for accurate MW determination. We had to calculate the average MW of HA using the Determan's empirical formula for globular proteins [23]

$$\text{Sephadex G} - 75\,\text{gel}: \quad \log \text{MW} = 5.624 - 0.752\frac{V_e}{V_o} \tag{204}$$

$$\text{Sephadex G} - 100\,\text{gel}: \quad \log \text{MW} = 5.941 - 0.847\frac{V_e}{V_o} \tag{205}$$

where MW is molecular weight, V_o the column void volume and V_e is the elution volume, each measured from the start of the sample application to the centre of elution peak.

2.5. Preparation of aqueous biphasic system

The 10% PEG–10% $(NH_4)_2SO_4$–HA–H_2O systems were prepared by mixing 1 ml of 30% PEG solution (pH 3.5) with 1 ml of 30% solution of ammonium sulphate (pH 5.6) and 1 ml of HA (2 mg ml^{-1} in 0.05 M NaOH). The systems of 5% PEG–7.5% dextran (or NaSD)–HA–H_2O were prepared by mixing 0.5 ml of 30% PEG with 1.5 ml of 15% solution of the second polymer (pH 7.0) and 1 ml of 2 mg ml^{-1} solution of HA or its fraction, obtained by GPC. Solutions of HA fractionated by MF were taken just from the OMFD-2 reservoirs. NaCl was added to the PEG–NaSD–H_2O system to adjust an ionic strength of 0.3. The mixtures were shaken for 1 min. After complete phase separation for an hour aliquots were taken from each phase

and diluted by 10 times in KCl–NaOH buffer (pH 12). Then the absorbances at 465 nm were measured on a SF-46 spectrophotometer (LOMO, Russia). The distribution coefficients were calculated as following:

$$D = \frac{\text{absorbance at 465 nm of the top (PEG} - \text{rich) phase}}{\text{absorbance at 465 nm of the bottom phase}}$$

The pH-dependence of HA distribution was studied using a PEG 12000−dextran 70000 aqueous system. A set of two-phase systems was prepared where pH at equilibrium varied from 4 to 12.1 (pH was adjusted by 0.3 M HCl). The pH values were measured using a pH meter Horiba F-16 with a combined electrode of Horiba 6367-10D.

3. Results and discussion

3.1. Characterisation of humic acid

The HA elemental composition and E values (absorbance of 0.001% HA solution at 465 or 650 nm, pH 12) are shown in Table 1. With a certain approximation, H:C ratios can be used to assess a contribution of aliphatic chains bearing ≡CH, =CH_2, –CH_3 groups to the HA structure. The H:C ratio is highest for HA_p which may contain the largest number of aliphatic fragments among the studied HA, while HA_{ch} is most aromatic. The UV–VIS spectra obtained were typical for HA and had no maxima except for HA_s spectra where a shoulder in the region of 440–480 nm and small maxima at 575 and 620 nm were observed. These maxima are usually attributed to the presence of green P_g pigment, commonly found in preparations of HA originated from soddy-podzolic soils [1]. The E values increased in the order $HA_s < HA_p < HA_{ch}$. Highest E values were found for HA_{ch} that is in agreement with elemental analysis data, suggesting that this HA is most aromatic.

The reversed-phase hydrophobic interaction chromatography (RPHIC) profiles are shown in Fig. 1.

RPHIC is based on the distribution of organic molecules between a liquid polar mobile phase and a non-polar stationary phase (hydrophobic gel matrix). The larger the content of hydrophobic fragments of the molecules, the stronger is their affinity to the hydrophobic matrix. Molecules are eluted from the column in the order of their hydrophobicity. Detergents are often added to the mobile phase to elute most hydrophobic molecules. Thus, RPHIC permits to separate organic molecules according to their relative hydrophobicity. It should be noted that the distribution profiles in RPHIC are highly dependent on the matrix and eluent used. Therefore, the distribution patterns obtained are valid for operational experimental conditions.

HA macromolecules are amphiphilic, i.e. they contain both hydrophobic and hydrophilic fragments. Hydrophilic fragments are represented by O- and N-containing polar groups. The hydrophobicity of HA may be due to the presence of aromatic structures (for example, aromatic rings) and non-polar aliphatic chains [1]. The molecules containing a larger number of hydrophilic fragments were eluted first from the column (Fig. 1). More hydrophobic HA molecules were stronger retained by the hydrophobic matrix and eluted after addition of an ionic detergent (SDS) to the eluent. Assuming that the absorbances of hydrophobic and hydrophilic fractions were the same for all HA studied, the relative hydrophobicity of HA was estimated taking into consideration the corre-

Table 1
Elemental composition of humic acids and their UV–VIS data

HA	Content (at.%)				Atomic ratios			UV–VIS data	
	C	H	O[a]	N	H:C	O:C	N:C	E_{450}	E_{450}:E_{650}
HA_p	35.9	40.7	21.7	1.7	1.13	0.60	0.05	0.051	3.9
HA_s	37.3	41.3	18.7	2.7	1.12	0.50	0.07	0.043	4.3
HA_{ch}	41.8	35.3	19.7	3.2	0.84	0.47	0.08	0.070	3.8

[a] O content is found from the difference.

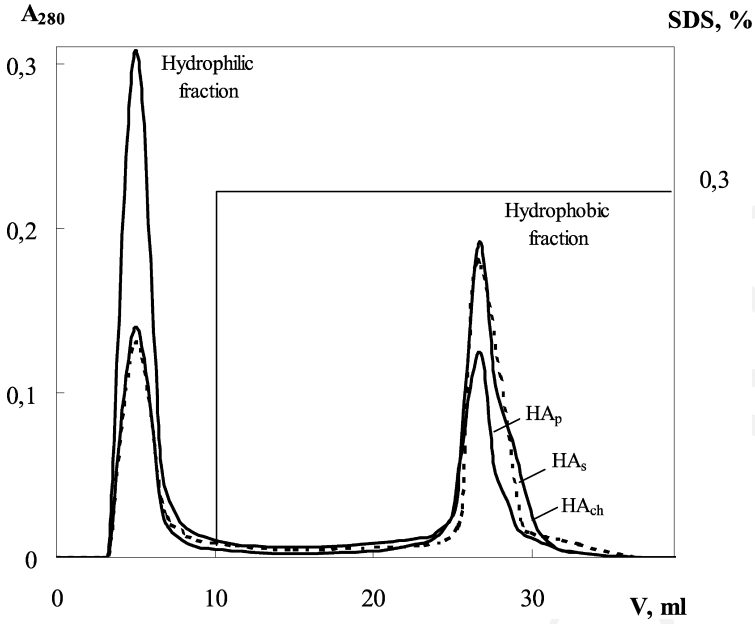

Fig. 1. Reversed-phase hydrophobic interaction chromatography profiles of humic acids. Column 1.0 cm × 10 cm, octyl-Sepharose CL-4B gel, elution by 0.05 M Tris–HCl buffer and after that by 0.05 M Tris–HCl + 0.3% SDS buffer with a step buffer change gradient, flow rate 10 ml h^{-1}.

sponding peak areas in the chromatograms. The ratios between the areas of hydrophobic and hydrophilic peaks for peat, soddy-podzolic soil and chernozem HA were 1.5, 1.0 and 0.7, respectively, thus HA_p is most "hydrophobic" among the studied HA.

Gel-chromatographic profiles of HA consist of three fractions (Fig. 2): a high molecular weight (HMW) fraction eluted in a void volume (MW > 150 kDa), a middle molecular weight (MMW) fraction (MW 60–72 kDa) and a low molecular weight (LMW) fraction (MW 6–10 kDa).

The percentage of each fraction in HA (percentage of total chromatogram area) was calculated taking into consideration the corresponding peak areas in gel-chromatograms. The peak areas of HMW and LMW fractions were obtained by multiplying the squares of segments A and B by a factor of two, respectively (Fig. 3). The peak area of MMW fraction was found by difference between the total chromatogram area and the areas for HMW and LMW fractions. The results are presented in Table 2. The LMW fraction is of the largest content in all HA studied. The mean MW of this fraction is highest for HA_p.

3.2. Size fractionation of humic acid

GPC was used to obtain HMW and LMW fractions of HA_p. The MW of HMW fraction eluted in a void volume was >75 kDa. The average MW of LMW fraction was about 5 kDa.

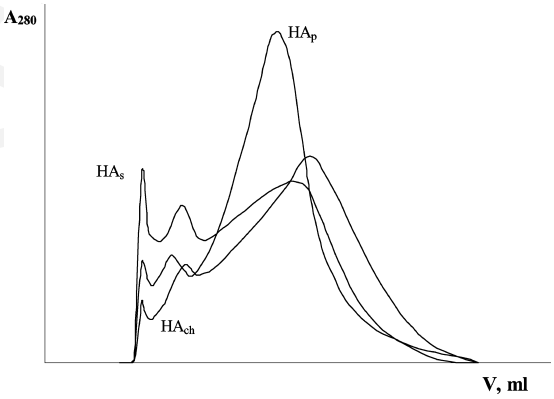

Fig. 2. Gel-chromatographic profiles of humic acids. Column K 0.9 cm × 60 cm, Sephadex G-100 gel, elution by 0.025 M Tris–HCl (pH 8.2) + 0.05 M NaCl + 0.1% SDS buffer, flow rate 3 ml h^{-1}.

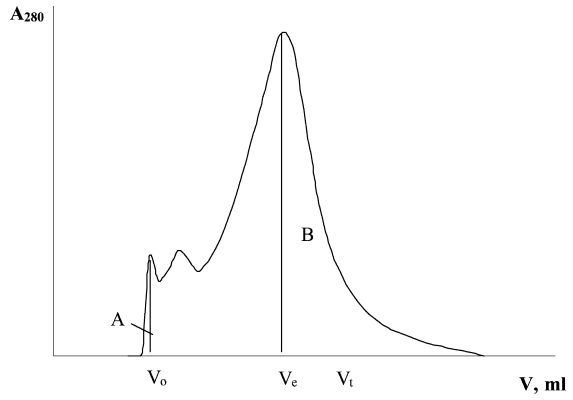

Fig. 3. Gel-chromatografic profile of peat HA. The elution peak areas of high and low molecular weight fractions were obtained by multiplying by a factor of 2 the squares of segments A and B, respectively. The peak area of middle molecular weight fraction is found by difference.

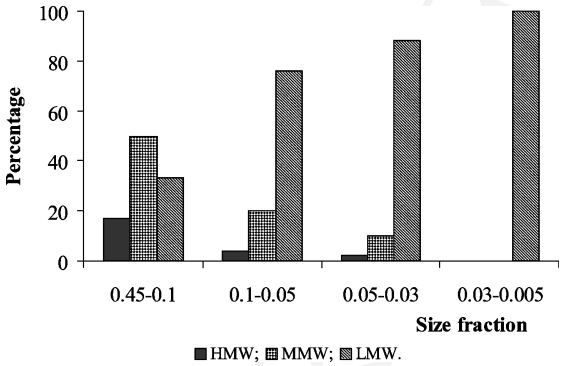

Fig. 4. Percentage of high, middle and low molecular weigh fractions in MF-fractionated HA samples.

Peat HA was also fractionated by MF. A filtrate obtained with a starting membrane (0.45 μm) was further fractionated with membranes of decreasing pore sizes down to 0.005 μm. The HA molecules, which are able to penetrate all the membranes used, are found in the filtrate of the last membrane. Each HA fraction obtained by the MF procedure contain not only molecules passed through a certain membrane but also molecules from further filtration steps.

The molecular sizes between 0.005 and 0.45 μm are roughly within an HS MW from $<10^3$ to $>10^6$ kDa [9]. More detailed information about the molecular size distribution of HA in MF filtrates can be provided by GPC. The gel-chromatographic profiles of 0.45–0.1 and 0.1–0.05 μm HA samples consisted of three fractions: HMW eluted in a void volume (MW > 150 kDa), shoulder-like MMW (62 kDa) and LMW (5 kDa). The percentage of each fraction observed in the gel-chromatograms was calculated according to the procedure described above (Fig. 3). The results are shown in Fig. 4. It can be seen from the figure that the 0.1–0.45 μm size fraction contains about 17% of HMW molecules. The HA fractions with molecular sizes of 0.03–0.05 and 0.005–0.03 μm contain 88 and 100% of LMW molecules, respectively. Thus, with decreasing the membrane pore size the percentage of LMW molecules increases in the respective filtrates (size fractions).

Table 2
Average molecular weights (kDa) and relative contents (%) of fractions in HA

HA	Fraction 1		Fraction 2		Fraction 3	
	MW	Content	MW	Content	MW	Content
HA_p	>150	4	72	14	10	82
HA_s	>150	7	72	31	8	62
HA_{ch}	>150	2	60	16	6	82

3.3. Fractionation of humic acid in ABS

Concentrations of the polymers necessary for the formation of two phases were chosen on the basis of phase diagrams [14]. A diagram for the PEG 6000–dextran 500000 system is shown in Fig. 5.

The bold curve represents a binodial below which the system is homogeneous. The straight line connecting points B and C is tie line. Point A characterises the initial composition of the whole system chosen for our studies, points B and C characterise the individual compositions of top and bottom phases after phase separation. The approximate phase volume ratio can be estimated by segments AB and AC [14]. It can be seen from Fig. 3 that although each phase contains certain amounts of both polymers, the bottom phase is enriched by dextran and the top phase is enriched by PEG. The binodial position depends on the type of polymer, its concentration and MW, temperature, pH and other variables.

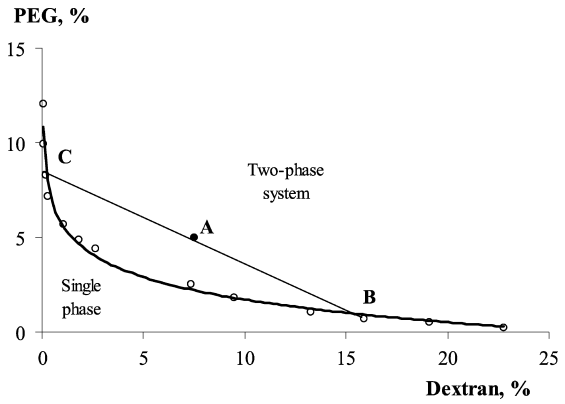

Fig. 5. Phase diagram of the PEG 6000−dextran 500000−H_2O system. Point A characterises the initial composition of the whole system, points B and C characterise the individual compositions of top and bottom phases after phase separation. The approximate phase volume ratio is estimated by segments AB and AC.

The results presented in Table 3 show that HA can be extracted in ABS. At pH about 12 the distribution coefficients are higher than unity and much higher than those in HA extraction in traditional oil/water systems. This is most probably due to hydrophobic interactions between non-polar fragments of HA molecules and $–CH_2–CH_2–$ groups of the linear polyether molecules. It is important that the presence of polar and dissociated groups in HA molecules does not impair the extraction that is the principal difference of ABS from traditional water-organic solvent systems.

The D values are maximum for the PEG–$(NH_4)_2SO_4$–H_2O system and the distribution coefficients for the PEG–NaSD–H_2O system are somewhat higher than for the PEG–dextran–H_2O system. The relative hydrophobicity of phases in polymeric systems studied decreases in the order [14]

$$\text{PEG} > \text{dextran} > \text{NaSD} > (NH_4)_2SO_4$$

Thus, top and bottom phases of the PEG–$(NH_4)_2SO_4$–H_2O system are most contrast by their hydrophobic properties, comparing with other systems under study. This can serve as an explanation why HA are extracted with highest D values in this system, while the distribution of HA in more "soft" two-polymer-based systems is more uniform.

Thus, one can operate either with hard or soft systems depending on the problem to be solved. The PEG–$(NH_4)_2SO_4$–H_2O system best suits for preconcentration and isolation of HA, whereas the PEG–dextran–H_2O system is better for HA fractionation.

Relationships between the MW of the polymers and the distribution coefficients for HA have also been studied. No effect of MW has been found for the PEG–$(NH_4)_2SO_4$–H_2O system within the studied range of PEG MW (from 4 to 20 kDa). The systems based on two polymers exhibit more narrow heterogeneity regions than the PEG–salt–H_2O system [18]. With decreasing the PEG MW, the binodial for a two-polymer system shifts upward and the equilibrium composition of the two phases become closer to

Table 3
Distribution coefficients of HA in ABS

HA	PEG, MW (kDa)	PEG–$(NH_4)_2SO_4$	PEG–dextran T70	PEG–dextran T500	PEG–NaSD
HA_s	20	4.4	–	–	9.5
	12	5.5	2.7	4.0	3.6
	6	3.5	1.4	2.3	–
	4	4.3		No phase separation	
HA_{ch}	20	5.0	–	–	16.4
	12	4.4	2.6	4.2	4.8
	6	5.5	1.2	1.9	–
	4	3.8		No phase separation	
HA_p	20	18.8	–	–	16.9
	12	17.0	3.5	5.2	4.3
	6	21.0	2.0	2.8	–
	4	11.1		No phase separation	

Table 4
Distribution coefficients for GPC-fractionated HA_p in PEG–dextran 70000–H_2O system

Sample	PEG 12000	PEG 20000
LMW	2.1	2.8
HMW	5.7	10.6

each other at a given initial composition of the system. This might be a reason for more uniform distribution of the solute between the two phases and consequently for lower *D* values (Table 3). At as low PEG MW as 4 kDa, the two-polymer systems are homogeneous at the chosen concentrations of the components.

The data on the extraction of GPC-fractionated HA in PEG–dextran 70000–H_2O systems show that the *D* values are higher for the HMW fraction (Table 4). This can be due to higher relative hydrophobicity of the HMW fraction. The results of extraction of MF-fractionated HA in the same ABS are presented in Table 5. It can be seen from this table that the overall tendency remains the same: the distribution coefficients are higher for HMW fractions.

Of three HA studied, the highest distribution coefficients were obtained for HA_p. The other two HA had similar *D* values. It was interesting to compare these results with those obtained by GPC and RPHIC. It was shown that the larger the average MW of the compound under study the higher is its distribution coefficient in ABS (Tables 4 and 5). The LMW fraction was of the largest content in all HA studied, and its average MW was highest for HA_p (Table 2). This may explain a better extractability of HA_p in the ABS. According to the RPHIC data (Fig. 1), HA_p are most hydrophobic that is a reason for better transfer to the more hydrophobic PEG-rich phase.

Table 5
Distribution coefficients for MF-fractionated HA_p in PEG–dextran 70000–H_2O system

MW of PEG (kDa)	Size of the fractions (μm)			
	0.45–0.1	0.1–0.05	0.05–0.03	0.03–0.005
12	2.5	1.5	1.7	1.1
20	7.9	3.6	4.1	1.9

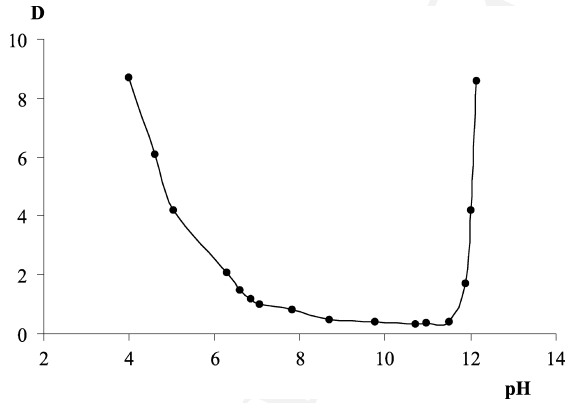

Fig. 6. Dependence of HA_p distribution on equilibrium pH for 5% PEG–7.5% dextran 70000–H_2O system.

Finally, pH-dependence of HA distribution in ABS was studied. It can be seen from Fig. 6 that the relationship is rather complicated. The *D* values decrease from 8.7 to 0.35 with pH increasing from 4 to 11. Further increase in pH leads to a drop in the *D* values (to 8.6 at pH 12.1). The preferential transfer of HA to the PEG-rich phase at lower pH values could be explained by suppressed ionisation of functional groups in acidic medium and conformational changes of HA molecules. However, it is difficult to interpret a sharp increase in *D* values on going from pH 11.5 to 12. Further studies are needed to understand such a complicated pH-dependence.

4. Conclusions

The principal difference of ABS from traditional water–organic solvent extraction systems, is that they enable to achieve the extraction and separation of strongly hydrated molecules of HA, containing polar and dissociated groups. The extraction of HA to any organic solvents in conventional liquid–liquid systems is difficult or impossible. Compared to other HA fractionation techniques the new approach proposed is rather simple, time saving, and cheap. Extracted HA can be easily separated from the polymers by either precipitation with concentrated HCl or filtration through Sephadex G-10 gel. It is recommended to use the extraction in ABS in combination with other techniques for fractionation and characterisation of HA.

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References

[1] D.S. Orlov, Gumusovie kisloty pochv i obshchaya teoriya gumifikatsii (Humic Acids of Soils and the General Theory of Humification), MSU, Moscow, 1990 (in Russian).
[2] B. Allard, H. Boren, A. Crimvel, Humic Substances in the Aquatic and Terrestrial Environment, Springer, Berlin, 1991.
[3] F.J. Stevenson, Humus Chemistry: Genesis, Composition, Reactions, 2nd Edition, Wiley, New York, 1994.
[4] J.H. Weber, in: F.H. Frimmel, R.F. Christman (Eds.), Humic Substances and their Role in the Environment, Wiley, New York, 1988, pp. 165–178.
[5] B.K.G. Theng, Formation and Properties of Clay–Polymer Complexes, Elsevier, Amsterdam, 1979.
[6] E.M. Murphy, J.M. Zachara, S.C. Smith, J.L. Phillips, Sci. Total Environ. 117/118 (1992) 413.
[7] R.S. Swift, in: M.H.B. Hayes, P. MacCarthy, R.N. Malcolm, R.S. Swift (Eds.), Humic Substances. II. In Search of Structure, Wiley, Chichester, 1989, p. 450.
[8] P. Burba, V.M. Shkinev, B.Y. Spivakov, Fresenius' J. Anal. Chem. 351 (1995) 74.
[9] P. Burba, B. Aster, T.I. Nifant'eva, V.M. Shkinev, B.Y. Spivakov, Talanta 45 (1998) 977.
[10] J.M. Duxbury, in: M.H.B. Hayes, P. MacCarthy, R.N. Malcolm, R.S. Swift (Eds.), Humic Substances. II. In Search of Structure, Wiley, Chichester, 1989, p. 257.
[11] O.A. Trubetskoj, O.E. Trubetskaya, G.V. Afanas'eva, O.I. Reznikova, C. Saiz-Jimenez, J. Chromatogr. A 767 (1997) 285.
[12] E.Y. Milanovskij, Eurasian Soil Sci. 6 (2000) 706.
[13] T.I. Nifant'eva, V.M. Shkinev, B.Y. Spivakov, in: D.L. Pyle (Ed.), Separations for Biotechnology. 3. SCI, Cambridge, 1994, p. 358.
[14] P.-O. Albertsson, Partition of Cell Particles and Macromolecules, 3rd Edition, Wiley, New York, 1986.
[15] R.G. Rogers, M.A. Eiteman, Aqueous Biphasic Separations: Biomolecules to Metal Ions, Plenum Press, New York, 1995.
[16] R.D. Rogers, C.B. Bauer, J. Chromatogr. B 680 (1996) 237.
[17] V.M. Shkinev, N.P. Molochnikova, T.I. Nifant'eva, B.Y. Spivakov, B.F. Myasoedov, Y.A. Zolotov, J. Radioanal. Nuclear Chem. 88/1 (1985) 115.
[18] B.Y. Spivakov, T.I. Nifant'eva, V.M. Shkinev, in: R.D. Rogers, M.A. Eiteman (Eds.), Aqueous Biphasic Separations: Biomolecules to Metal Ions, ACS, 1994.
[19] H. Walter, D.E. Brooks, D. Fisher, Partitioning in Aqueous Two-Phase Systems, Theory, Methods, Uses, and Application to Biotechnology, Academic Press, Orlando, FL, 1985.
[20] D.S. Orlov, L.A. Grishina, Praktikum po khimii gumusa (Laboratory Manual for Humus Chemistry), MSU, Moscow, 1981 (in Russian).
[21] A.G. Zavarzina, V.V. Demin, Eurasian Soil Sci. 32 (10) (1999) 1246.
[22] K.E. Geckeler, E. Bayer, V.M. Shkinev, N.N. Gomolitskii, B.Y. Spivakov, Intern. Labmate 47 (1992) 17.
[23] G. Determan, Gel Khromatographiya (Gel Chromatography), Mir, Moscow, 1970 (in Russian).