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Comparative study for separation of aquatic humic-type organic constituents by DAX-8, PVP and DEAE sorbing solids and tangential ultrafiltration: elemental composition, size-exclusion chromatography, UV–vis and FT-IR

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Abstract

Aquatic humic-type solutes were separated in parallel from the same fresh water source by four different procedures: non-ionic polymethyl methacrylate (DAX-8) and functional cross-linked polyvinylpyrrolidone (PVP) resins, functional diethylaminoethyl cellulose (DEAE) and tangential ultrafiltration completed with a weakly basic anion exchange resin (IRA-67). The similarity–dissimilarity between the quantities and qualities of the different humic samples is discussed, especially in the light of the original dissolved organic matter (DOM). During the past two decades, a significant progress has occurred in the aquatic humic research due to the so-called hydrophobic–hydrophilic properties possessed by certain non-ionic sorbing solids. As a result of many coincidences, it may be justifiable to examine critically the prevailing isolation techniques of aquatic humic solutes and to try to update their complicated definitions. For that reason, it is reasonable to summarize the leading principles of different isolation techniques in Section 1 of this article. The results of the present study strongly support the applicability of the PVP resin, alone or completed in sequence with a suitable non-ionic sorbing solid, for isolation of aquatic humic-type solutes from both quantitative and qualitative points of view. In certain cases, the DEAE cellulose gives a useful alternative for conventional sorbing solids in the isolation of the bulk of aquatic humic solutes. The base-catalyzed ester hydrolysis of the HM during the chromatographic isolation of the DOM seems to be relative minor.

Keywords: Humic substances; DOM; DAX-8; PVP; DEAE; SEC; FT-IR

1. Introduction

The importance of the macromolecular, unidentifiable and very complicated heterogenous mixture of natural organic matter (NOM) in all environmental systems can be considered as a consensus of opinion. These scattered mosaic-like organic constituents are most frequently extracted from the solid NOM with aqueous bases (e.g. [1]). These extracts of so-called humic substances are further partitioned into functional humic (HA) and fulvic (FA) acids based on their solubilities in aqueous acids and bases. This humic–fulvic acid fractionation at strongly acidic conditions (pH ≈ 1) is believed [2] to be useful and meaningful for characterization and separation of humic substances. The nomenclature and definition of these humic substances are not easy-to-understand and they have features of scientifically hair-splitting [3]. The common denominator in the humus chemistry is that all classifications and definitions of humic substances, regardless of the nature of the sample (dissolved or solid), are only operational based on the procedure used for their isolation and no ideal system is available that would satisfy each scientist.

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The carbon cycling in aquatic ecosystems is extremely complicated and the origin of aquatic humic solutes can be dated back to many complex interacting sources (e.g. [4]). The formation of aquatic humic solutes occurs as the result of several processes in the aquatic environment and it is a dynamic process with no unidirectional vector, as has been pointed out in the literature (e.g. [5]). In general, the dissolved organic matter (DOM) in natural water is classified [6] roughly into two groups: (i) non-humic solutes, consisting of compounds belonging to the well-known classes of organic substances such as amino acids, hydrocarbons, carbohydrates, fats, waxes, resins, low-molecular acids, etc. and (ii) very complicated heterogenous humic solutes. These two groups are not completely, neither physically nor chemically, distinguishable from each other, because some natural non-humic solutes, such as carbohydrates, can be an integral part in the structural composition constructing humic solutes.

Nevertheless, the phrase of aquatic humus is very popular in water chemistry, this term is as indefinite as that of the humic substances, and it remains open what is dealt with: DOM, humic solutes in full without partition or something else. The use of specific terms of fulvic and humic acids is based on an assumption that they represent real entities of organic constituents. On the contrary, the abbreviation of humic matter (HM) refers to the generic term of all humic solutes regardless of certain specific isolation–fractionation procedure. The HM, which may, at best, account for as much as 90% of the DOM, has an essential role in the carbon cycle of the dissolved organic carbon (DOC). The ability of the HM, e.g. to inactivate various pesticides and other organic pollutants via complexation–copolymerization [7], to influence transport processes of organic and inorganic pollutants [8], to lower bio-availability of harmful heavy metals via complexation [9], to act as precursors for the formation of several mutagenic organic chemicals during chlorine treatment of natural waters [10] in addition to the structural chemistry, especially in the light of its environmental impact (e.g. [11]), have led to world-wide interest in research of this natural organic material.

The modern humus chemistry has progressed strongly recently, thanks to modern analytical techniques. However, the major problem in the aquatic humus chemistry is still how to separate the HM selectively from other organic and inorganic solutes for obtaining a representative sample. Because of the dilute solutions of the aquatic NOMs, they must be concentrated for further studies. Several techniques, with their advantages and disadvantages, for concentrating and also simultaneously for isolating the aquatic HM from the DOM are available [12], including freeze-drying, chemical precipitation, solvent extraction, reverse osmosis, ultrafiltration and adsorption to solids.

The most frequently applied procedures for simultaneous concentration and fractionation of aquatic humic solutes from most other dissolved constituents are at present the column chromatographic methods by non-ionic sorbing solids (such as XAD resins or analogues). Unfortunately, the manufacture of the reliable XAD-8 resin (Amberlite®) was ceased some years ago. It has been reported [13,14] that XAD-8 resin possibly can be substituted by Supelite™ DAX-8 resin. The potential of the DAX-8 resin as a research tool in the humic sciences has been also tested using several analyses of structural fine-chemistry [15–17]. Despite some promising attempts, it seems, however, to be troublesome and uncertain to substitute the workable XAD-8 resin for a comparable one. In the light of this problem, it may be justifiable to try to adopt a more physical definition for humic-type constituents, not solely based on some fictional hydrophobic–hydrophilic interactions at certain acidity but more real and distinctive functionalities.

The leading principle in using non-ionic sorbing solids is that the method classifies organic solutes in a water sample at preadjusted acidity (pH $\approx$ 2) into fictional hydrophobic and hydrophilic fractions (in fact, to be dissolved, the original DOM at natural acidities must in reality be quite hydrophilic anyhow). According to certain preadjusted hydrophobic–hydrophilic interactions between organic solutes and the non-ionic sorbing solid, the relatively most hydrophobic macromolecular organic acids (humic substances [18]) are retained onto the adsorbent. This primary organic fraction is most frequently partitioned at strongly acidic conditions (pH $\approx$ 1) into humic- and fulvic-type acids. The background of the multi-stage non-ionic sorbing solid technique is thoroughly reported and discussed in the literature [18,19]. It has been underlined [20,21] that the utilization of this technique alone may include certain risks for uncontrolled fractionation, reactions and conclusions.

The most peculiar characteristic for different kinds of humic-type constituents is the occurrence of acidic functional (mainly carboxylic) groups which render them into polyelectrolytes. This quality permits the isolation of practically all humic-type solutes in one step from water by certain anion exchange resins. The most popular material for this purpose has been the DEAE cellulose (Sigma, [25249-54-1]) which is a weak anion exchanger with tertiary amine functional groups bound to a hydrophilic matrix ($-OC_2H_4N(C_2H_5)_2$). The quantities of ionized organic humic solutes isolated with this procedure have generally been relatively high (about 80% to nearly 100% of the DOM) in fresh water as compared to the so-called XAD technique [22–27]. The optimum recovery of organic acids of the DOC occurs between pH 4 and 6, and it is possible to isolate almost all organic acids from a fresh water sample without any pH adjustments. On the other hand, it has been reported [28] by studying aquatic marine HM that even the relatively low salinity of, e.g. brackish water will decrease the retention of acidic solutes, especially those with lower molecular sizes, onto the DEAE cellulose. The utilization of anion exchangers in the humus chemistry has been discussed more closely in the literature (e.g. [19]).

Other peculiar characteristics of humic-type organic constituents are their relatively high content of phenolic functional groups, in addition to those of acidic groups, and the

abundance of aromatic C=C moieties. These properties permit the utilization of the cross-linked PVP for the fractionation of the DOM into humic-type constituents and other organic residues (primarily comprising carbohydrates, proteins, amino acids and uronic acids) under acidic conditions (pH ≈ 2). Although PVP in its insoluble form has been used in several fields of research, including also the HM beginning in 1968 [29], its utilization in the humus chemistry is lesser-known. However, this does not prove the unsuitability of PVP resins for the isolation–fractionation of the HM [30–35]. The PVP procedure is similar to that of non-ionic sorbing solids, concerning the acidity of the original water, which must first be acidified to about pH 2. The PVP resin forms strong hydrogen bonds with phenolic, hydroxyl and carboxyl groups of the DOM (e.g. [33]), while non-ionic macroporous copolymers classify at a given preadjusted acidity organic humic solutes in a water sample according to their relative hydrophobic–hydrophilic interactions between the surface of the sorbent bed.

Tangential-flow membrane-ultrafiltration provides a method for concentrating the original DOM according to its molecular size. In addition, this procedure (continuous operation) will minimize (e.g. [36,37]), as recently verified [38–40], a number of problems connected (e.g. [41–43]) with the bath operation (dead-ended) even though a slight fouling of the membranes cannot be avoided (e.g. [44]), and large volumes of water can be easily processed for obtaining gram quantities of DOM concentrates with different molecular sizes, e.g. for freeze-drying.

Molecular weight-size distributions are essential properties for estimating physical and chemical characteristics of dissolved humic-type organic constituents. The most extensively used technique for this purpose is apparently the high-performance size-exclusion chromatography (HPSEC) by UV-detection. In all, the UV–vis spectroscopy has its own function in the study of aquatic organic solutes, e.g. UV 254 nm is the most commonly utilized wavelength for monitoring water quality changes, but the absorptivity of smaller molecular size fractions at this wavelength is somewhat reduced. In summary, determining molecular sizes of DOM by different HPSEC applications is not a simple task as reported and reviewed previously (e.g. [39,45]). Worth of considering in this context, is the view stressed in literature that a chromatographic column of the TSK G3000SW-type and 10 mmol sodium acetate buffer at pH 7 as the eluent is the only system being able to separate efficiently different molecular size fractions of the aquatic very heterogenous NOM [46–48].

In the present study, three different sorbing solids (namely DAX, PVP and DEAE) and tangential ultrafiltration (UF, 1 kDa of nominal molecular size, NMW, cutoff), followed with XAD-8/2 (65/35 (w/w)), weakly basic anion (IRA-67) and strongly acidic cation (Dowex 50W x-8) exchange resins, were applied for isolation–fractionation of humic-type organic solutes from the same lake water sample. Several humic isolates separated previously by XAD-8 and DEAE resins from different fresh water sources were utilized as reference samples. Principal basic analyses (molecular size distribution, elemental organic analyses, UV–vis and Fourier-transform infrared (FT-IR) characteristics) were performed to illustrate the general ability of different sorbing solids in isolating humic constituents in comparison with the original DOM.

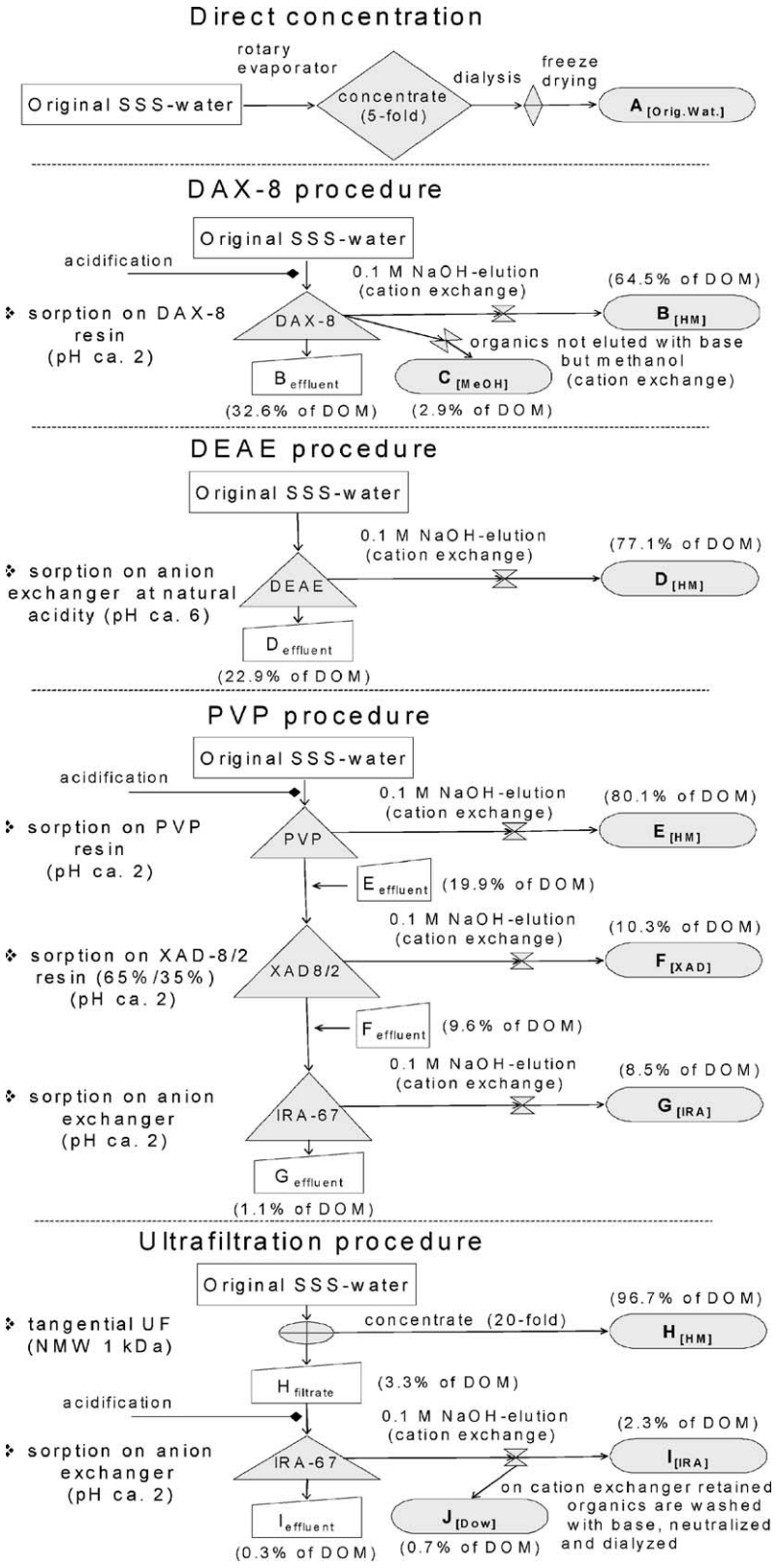
2. Experimental

2.1. Origin and isolation of samples

A natural fresh water sample was collected from Lake Savojärvi (SSS) situated in a marshy region in the southwestern part of Finland, in autumn 2002. Lake Savojärvi has very brown water (colour as cobalt–platinum units about 150 mg Pt l^{-1}; DOC 20 mg C l^{-1}; conductivity at 25 °C, about 6 mS m^{-1} and pH 5.8) [39]. The SSS-water sample (about 150 l) was collected 1 m below the surface into glass containers. The original water sample was at first prefiltrated (0.2 μm, Nuclepore polycarbonate filter cartridge, no. 611101) directly after sampling and, thereafter, stored in hermetic containers in the dark at 4 °C during the analysis and isolation procedures. The International Humic Substances Society reference samples of Nordic aquatic fulvic acid (No. FA, code IR105F) and humic acid (No. HA, code IR105H) were isolated by the conventional XAD-8 technique in summer 1986 from the runoff water (colour about 200 mg Pt l^{-1} and DOC 20 mg C l^{-1}) of a Norwegian mire (symbols R8 and R9 in Table 1, respectively). The reference samples R1–R7 were isolated in 1994 from Lake Savojärvi (SS, [26]) by an enlarged XAD technique, XAD-8 → cation exchanger → weakly basic anion exchanger, and in 1989, by the conventional XAD-8 technique from Lake Mekkojärvi (M3, [38]), also a highly coloured lake (colour about 200 mg Pt l^{-1} and DOC about 22 mg C l^{-1}, [39]).

The different analytical procedures applied in this study for isolation and fractionation of the DOM are shown in Scheme 1. A part of the prefiltrated original SSS-water (5 l) was first directly concentrated with a rotary evaporator to 1 l and then dialysed (from about 1.3 mS cm^{-1} to 70 μS cm^{-1}, 20 °C) with Spectra/Por 6 dialyse tube (1 kDa of NMW cut-off) against distilled water and finally freeze-dried (fraction $A_{[Orig.Wat.]}$), in order to obtain a point of reference for comparisons.

The chromatographic isolation methods of HMs by DAX-8 resin and DEAE cellulose in Scheme 1 have been thoroughly reported previously [15–19,26,27]. The organic constituents not eluted from the DAX-8 resin with base (so-called [18] hydrophobic neutrals being relatively too hydrophobic to be eluted with base) were eluted with methanol and labelled as [MeOH] (fraction $C_{[MeOH]}$). It is notable that the alkaline extracts of the primary HM solutes (hydrophobic humic substances) eluted from the DAX-8 (fraction $B_{[HM]}$) and DEAE (fraction $D_{[HM]}$) sorbents were not, in the present study, further divided at strongly acidic



Scheme 1. Analytical procedures for classification of the DOM into different humic-type fractions. For symbols of samples, see Table 1.

Table 1
Major elemental organic analyses and some compositional characteristics for the different humic-type isolates obtained by several isolation procedures[a]

Method[b]	Symbol[b]	Sample[b]	Ash (%)	$\bar{M}_n$	$\bar{M}_w/\bar{M}_n$	$\in$[c]	E_2/E_3[d]	Elemental analysis (%)					Atomic ratios				Average molecular formula: $C_vH_wN_xO_yS_z$						
								C	H	N	S	O	H/C	O/C	N/C	S/C	C_v	H_w	N_x[e]	O_y	S_z[e]	ϕ_{total}[f]	Ar. (%)[g]
F.D.	$A_{[Orig.Wat.]}$	$SSS_{concentrate}$	2.6	2750	4.20	331	4.75	51.1	6.4	2.3	1.7	38.4	1.494	0.564	0.038	0.013	117	175	4	66	1	12	22
DAX-8	$B_{[HM]}$	$SSS_{isolate}$	3.1	2630	3.70	325	4.77	53.1	4.7	1.7	0.8	39.7	1.064	0.561	0.028	0.005	116	124	3	65	*1.6*	22	22
DAX-8	$C_{[MeOH]}$	$SSS_{isolate}$	3.4	760	8.23	145	6.20	56.2	6.9	1.6	0.7	34.7	1.455	0.463	0.025	0.004	36	52	*1.1*	16	*6.5*	15	11
DAX-8	$B_{effluent}$	$SSS_{effluent}$		950	4.01	182	5.85																13
DEAE	$D_{[HM]}$	$SSS_{isolate}$	1.9	2740	4.43	329	4.74	53.1	4.3	1.0	0.9	40.8	0.954	0.577	0.017	0.006	121	116	2	70	*1.4*	24	22
DEAE	$D_{effluent}$	$SSS_{effluent}$		370	3.81	35	6.88																5
PVP	$E_{[HM]}$	$SSS_{isolate}$	3.1	2570	3.36	320	4.83	52.1	4.7	1.2	1.3	40.6	1.076	0.585	0.020	0.009	112	120	2	65	1	21	21
PVP	$E_{effluent}$	$SSS_{effluent}$		1260	2.62	220	5.56																16
PVP	$F_{[XAD]}$	$SSS_{isolate(XAD8/2)}$	2.3	1350	4.52	227	5.51	55.4	5.4	1.7	1.1	36.4	1.171	0.494	0.026	0.007	62	73	2	31	*2.2*	20	16
PVP	$F_{effluent}$	$SSS_{effluent(XAD8/2)}$		930	3.22	175	5.88																13
PVP	$G_{[IRA]}$	$SSS_{isolate(IRA-67)}$	3.8	560	3.71	105	6.44	37.4	2.9	1.7	1.4	56.7	0.915	1.138	0.039	0.014	17	16	*1.5*	20	*4.1*	19	9
PVP	$G_{effluent}$	$SSS_{effluent(IRA-67)}$		320	3.42	30	6.86																5
UF	$H_{[HM]}$	$SSS_{concentrate}$	2.2	2860	4.04	336	4.69	51.7	5.8	2.4	1.6	38.5	1.337	0.559	0.040	0.012	123	165	5	69	1	15	22
UF	$H_{filtrate}$	$SSS_{filtrate}$		970	2.30	182	5.85																13
UF	$I_{[IRA]}$	$SSS_{isolate(IRA-67)}$	3.7	660	3.05	126	6.26	36.0	2.7	1.8	1.2	58.4	0.888	1.219	0.042	0.012	20	18	*1.2*	24	*4.2*	19	10
UF	$J_{[Dow]}$	$SSS_{isolate(Dowex)}$	3.6	770	8.04	152	6.15	44.5	6.4	4.6	2.7	41.9	1.703	0.706	0.088	0.023	29	49	3	20	*1.5*	8	12
UF	$I_{effluent}$	$SSS_{effluent(IRA-67)}$		320	3.71	21	6.90																4
DEAE	$R1_{[HM]}$	SS.[DEAE]	2.2	2840	2.43	354	4.79	53.9	4.2	1.0	0.7	40.2	0.920	0.560	0.016	0.005	127	117	2	71	*1.6*	25	23
XAD-8	$R2_{[HM]}$	SS.FA	2.4	2232	2.61	332	4.58	54.9	4.4	0.7	0.7	39.5	0.944	0.540	0.010	0.004	102	96	1	55	*2.2*	25	22
XAD-8	$R3_{[HM]}$	SS.HA	3.1	4451	4.98	407	3.51	55.9	4.2	1.7	0.9	37.3	0.890	0.501	0.026	0.006	207	185	5	104	1	27	26
XAD-8	$R4_{[MeOH]}$	SS.[MeOH]	3.9	654	4.72	152	6.18	56.3	6.7	1.4	1.1	34.6	1.413	0.462	0.021	0.007	31	43	*1.5*	14	*4.5*	16	12
XAD-8	$R5_{[IRA]}$	SS.[IRA]	2.1	660	4.99	246	6.41	45.0	1.9	0.4	0.5	52.2	0.505	0.870	0.008	0.004	25	13	*5.1*	22	*10.8*	30	11
XAD-8	$R6_{[HM]}$	M3.FA	3.8	4475	2.37	356	4.33	56.9	4.3	0.6	1.0	37.2	0.901	0.490	0.009	0.007	212	191	2	104	1	26	23
XAD-8	$R7_{[HM]}$	M3.HA	5.8	6508	5.28	452	3.70	59.0	4.6	1.2	1.1	34.1	0.925	0.435	0.018	0.007	320	296	6	139	2	27	29
XAD-8	$R8_{[HM]}$	No.FA	1.3	4750	1.56	410	4.08	53.1	4.6	0.8	0.8	40.7	1.032	0.575	0.013	0.005	210	217	3	121	1	22	26
XAD-8	$R9_{[HM]}$	No.HA	2.2	6204	3.77	466	3.47	54.6	4.5	1.0	0.9	39.0	0.988	0.536	0.016	0.006	282	279	5	151	2	24	30

[a] Elemental composition is given on ash- and moisture-free basis.

[b] Different [-HM] fractions obtained from the SSS-water were not divided into so-called humic- (HA) and fulvic- (FA) acid subfractions, thus representing their combined mixtures; F.D., freeze-drying; R1–9, reference samples; for other symbols, see Scheme 1.

[c] $\in$, molar absorptivity at 280 nm (1 mol^{-1} cm^{-1} of OC).

[d] E_2/E_3, quotient of absorbances at 250 and 365 nm.

[e] The decimal numbers in italics indicate frequency of occurrence, i.e. one per stated number of molecules with a given molecular weight.

[f] ϕ_{total}, estimated total amount of unsaturation in mmol g^{-1}.

[g] Ar. (%), estimated aromaticity.

conditions into functional HA- and FA-type fractions. The amounts of the original SSS-water used for the DAX-8 and DEAE procedures were 10.0 and 44.9 l, respectively. All cation exchanged concentrates $B_{[HM]}$ (1.00 l), $C_{[MeOH]}$ (0.25 l) and $D_{[HM]}$ (1.50 l) were finally freeze-dried.

As shown in Scheme 1, the PVP (Sigma [25249-54-1]) resin was first washed with distilled water, dilute NaOH, 10% HCl, distilled water until free of Cl^-, acetone, distilled water, and thereafter, suspended in 0.01 M HCl (pH 2). Pretreated slurry of PVP resin (160 g dry weight) was placed in a glass bottle (10 l) and the original SSS-water samples (7–9 l per an isolation cycle, in all, 45.0 l), acidified (HCl) to pH 2, were added and the mixture was stirred for 16 h (batch method). The suspension was filtered and the resin slurry washed with distilled water before eluting (0.1 M NaOH) the retained humic-type organics (fraction $E_{[HM]}$). The combined acidic (pH 2) filtrates ($E_{effluent}$, in all, 40.0 l) were further treated by the bath method, first with XAD-8/2 resin (fraction $F_{[XAD]}$) and next with IRA-67 anion exchanger (fraction $G_{[IRA]}$) for obtaining the rest of humic-type macromolecular organic acids. All cation exchanged concentrates $E_{[HM]}$ (4.43 l), $F_{[XAD]}$ (0.21 l) and $G_{[IRA]}$ (0.60 l) were finally freeze-dried.

The UF procedure in Scheme 1 was carried out using a tangential-flow membrane-filtration system (4 GPM Pellicon Cassete System by Millipore). The original SSS-water sample (51.16 l) was concentrated (concentration degree, 20; the consistency of the molecular size distribution with that of original water was verified by HPSEC during the concentration process) by a membrane of 1 kDa NMW (filter code: PCAC, cellulosic material by Millipore). The dialysed (from about 290 to 85 $\mu S\,cm^{-1}$) UF concentrate $H_{[HM]}$ (1.00 l) was freeze-dried for obtaining solid HM/DOM for analyses. The UF filtrate ($H_{filtrate}$, 46.65 l) was further acidified (HCl) to pH 2 and treated with weakly basic anion exchanger (IRA-67) followed with cation exchanger for characterizing the nature of remained humic-type solutes with NMW of $\leq$1 kDa. The basic extract from the IRA-67 resin was cation exchanged for obtaining the actual macromolecular organic acids/humic-type constituents of smaller molecular sizes (fraction $I_{[IRA]}$). Onto the cation exchanger retained various organic constituents (fraction $J_{[Dow]}$) were eluted with NaOH solution. The basic extract of the fraction $J_{[Dow]}$ was neutralized to pH 7 with HCl and the salt (NaCl) formed was removed by dialysis. The purified concentrates $I_{[IRA]}$ (0.76 l) and $J_{[Dow]}$ (0.87 l) were finally freeze-dried.

2.2. Chemical, chromatographic and spectroscopic analyses

The determination of the moisture and ash contents of the freeze-dried HMs as well as their elemental compositions (carbon, hydrogen, nitrogen and sulphur; the content of oxygen was taken as a difference from 100%) has been discussed previously [15]. Table 1 shows the results of the elemental organic analyses on ash- and moisture-free basis for the different humic isolates.

The number- ($\bar{M}_n$) and weight-averaged ($\bar{M}_w$) molecular weights before freeze-drying the different HM-type concentrates (diluted about 10-fold with the sodium acetate eluent solution) were determined by HPSEC using a silica-based TSK G3000SW column (7.5 mm × 300 mm with a 7.5 mm × 75 mm precolumn) and 10 mmol sodium acetate solution (pH was adjusted to 7.0 with acetic acid) as an eluent at 20 °C [39,46–48]. The flow rate of the eluent was 0.80 ml min^{-1} and the injection volume, 80 μl. The pumping system was an L-6200A Intelligent Pump (Merck Hitachi) and the eluted solute was detected at 254 nm (L-4250 UV–vis Detector, Merck Hitachi). The void (V_o, 6.67 ml) and total permeation (V_o + V_i, 15.98 ml) volumes were determined using Blue Dextran 2000 and acetone, respectively; the total volume (V_t) of the gel bed was 16.57 ml. Elution parameters were calculated as a distribution coefficient [39]: $k'_D = (V_e - V_o)/((V_o + V_i) - V_o)$, where V_e, elution volume of the solute. Averaged $\bar{M}_n$ and $\bar{M}_w$ values for the heterogenous humic mixtures were calculated by the equations: $\bar{M}_n = \Sigma n_i/\Sigma n_i/MW_i$ and $\bar{M}_w = \Sigma n_i MW_i/\Sigma n_i$, where n_i is the number/weight (in this case, relative response at 254 nm) of a solute i with the molecular weight MW_i. Distribution of MW_i values for sample solutes, at some eluted volume *i*, were estimated by the calibration equation presented previously [39] for this HPSEC column system. The maximum absorbance-scale for each SEC chromatogram in Fig. 1 is the same, thus making the results more comparable with each other. Relative UV_{254}-absorbances {(UV abs at 254 nm/OC in the original solution, mg l^{-1}) × 100} were calculated for the chromatograms, irrespective of the fact that the real OC concentration of the eluted sample was not exactly known but the injection volumes were constant, in order to bring the samples into the same line. The quotient $\bar{M}_w/\bar{M}_n$ is a coarse estimate for the degree of polydispersity of the mixture (the value 1 is given for homogenous polymers). The previous results obtained by different aromatic acids implied that the charge exclusion effect of the applied HPSEC system is quite insignificant, i.e. the charged aquatic humic solutes have a fair chance to permeate into the stationary phase pores while using a 10 mmol sodium acetate, at pH 7.0, as the eluent [39].

The UV–vis spectrophotometric analyses for the different HM-type concentrates (diluted about 10-fold with the sodium acetate eluent solution) were performed on a dual-beam Spectrometer Lamda 12 (Perkin-Elmer) for the calculation of the E_2/E_3-ratio (absorbances at 250 and 365 nm) and ∈ (molar absorptivity at 280 nm, l mol^{-1} cm^{-1} of OC). Samples were placed in a 1 cm quartz window cuvette and scanned (60 nm min^{-1}; data interval, 0.20 nm) from 670 to 200 nm. The previously isolated reference samples have been also dissolved in corresponding sodium acetate eluent solution.

Fourier-transform infrared (FT-IR) spectra for different solid samples in Fig. 2 were collected in the transmission mode using a Galaxy 6030 FT-IR spectrophotometer (Matt-

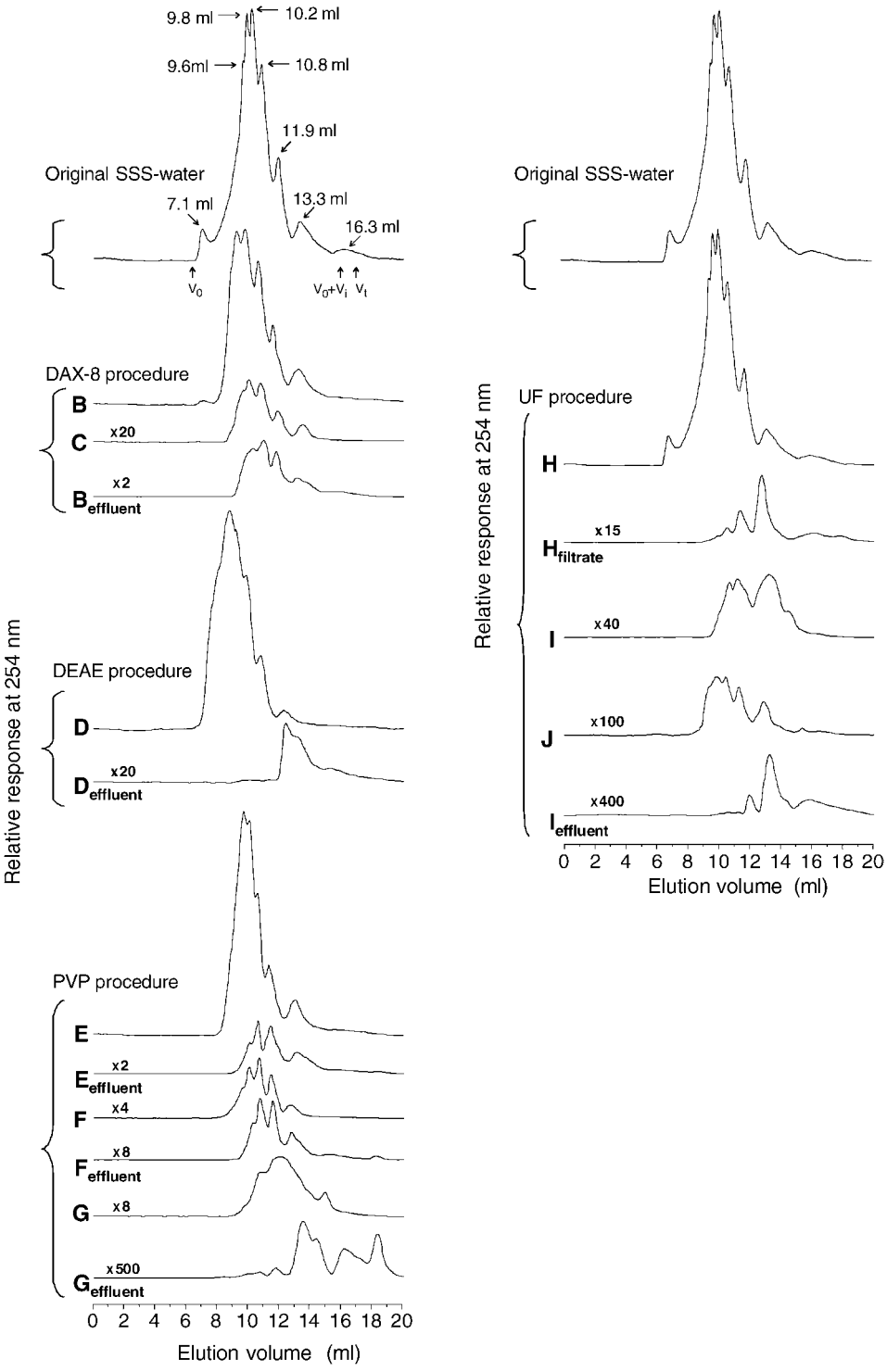


Fig. 1. Molecular size distributions for the organic solutes in the original water sample and different fractions obtained by several separation procedures. TSK G3000SW, 7.5 mm × 300 mm with a 7.5 mm × 75 mm guard column; 10 mmol sodium acetate; pH 7.0; 20 °C; sample loop, 80 μl; flow rate, 0.80 ml min^{-1}; V_o = 6.67; V_o + V_i = 15.98 and V_t = 16.57 ml. Some chromatograms are enlarged 2- to 500-fold for better visualizing. For symbols of samples, see Table 1.

son Instruments) equipped with a DTGS detector. The original spectral bandwidth was 4 cm^{-1}. About 1.5 mg of the freeze-dried sample (desiccator dried) and 200 mg of KBr powder (dried in an oven at 100 °C) were ground together and hydraulically pressed into a small pellet (−13 mm in diameter, −0.5 mm thick). The pelletized KBr samples were further dried overnight in a desiccator prior to analysis to minimize interferences from absorbed water. All samples were measured at a constant temperature (20 °C). The intensities of the recorded FT-IR spectra were standardized

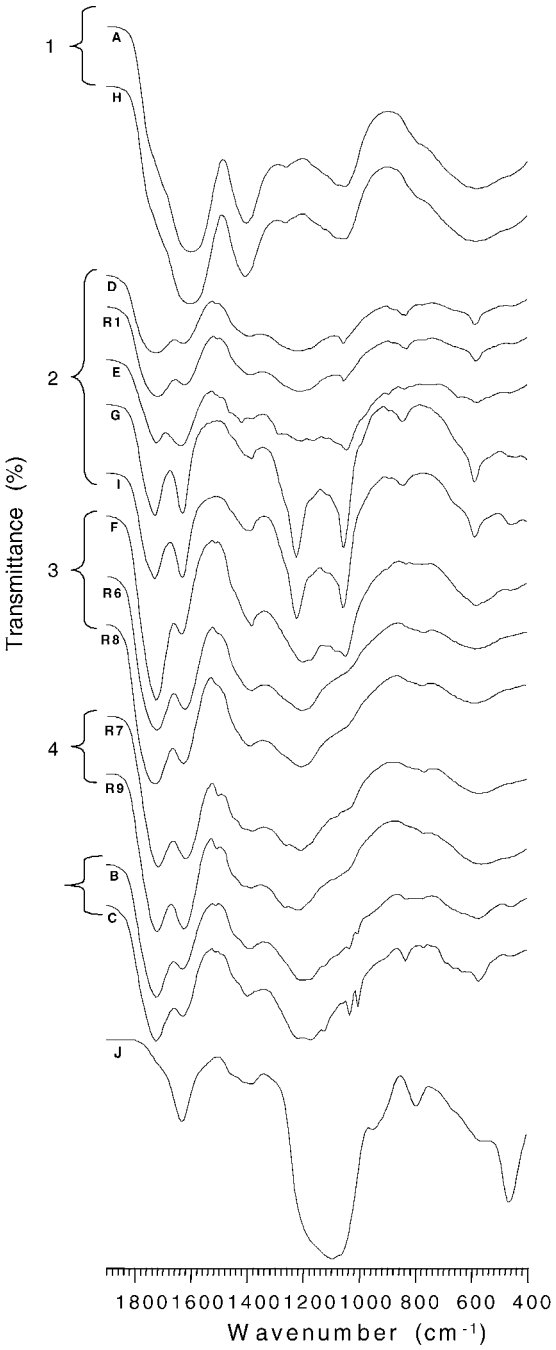


Fig. 2. FT-IR spectra in the zone 1900–400 cm^{-1} for the original water sample and different humic-type fractions obtained by several separation procedures. The numbers of the regrouped spectra point to the clusters in Fig. 4. For symbols of samples, see Table 1.

against the OM contents of the samples to reduce their weighing differences and ash contents for obtaining more semi-quantitative spectra. Although the FT-IR spectra were scanned between 4000 and 400 cm^{-1}, Fig. 2 shows only the region 1900–400 cm^{-1} where the major differences were observed. The transmittance-scale for each spectrum is the same thus making the results more comparable with each other.

3. Results and discussion

3.1. Effects of different isolation methods on the quantities of humic-type organic solutes, molecular size distributions of separated mixtures

Scheme 1 sums up the efficiency of a given isolation method (percent of the original DOM) in dividing original organic solutes into different specific subfractions. Likewise, Fig. 1 illustrates the molecular size distributions of obtained mixtures in relation to their quantities. The applied HPSEC system separated the heterogenous mixture of the original SSS-water sample into eight different molecular size groups, i.e. humps of the chromatogram at the elution volumes of about 7.1, 9.6, 9.8, 10.2, 10.8, 11.9, 13.3 and 16.3 ml corresponding to molecular sizes of about 115300, 11200, 9300, 6400, 3660, 1300, 360 and <100 Da, respectively. The exclusion volume of the last hump at 16.3 ml was clearly greater than the total permeation volume (V_o + V_i, 15.98 ml) indicating that some interactions occur between these small-molecular size constituents and the gel matrix.

3.1.1. DAX-8 procedure

The ability of the non-ionic DAX-8 resin (in Scheme 1) to divide the original DOM at pH 2 into so-called hydrophobic acids ($B_{[HM]}$, "humic substances"), hydrophobic neutrals ($C_{[MeOH]}$) and less hydrophobic effluent ($B_{effluent}$) was of the same magnitude as reported previously (e.g. [15–17,26]). The quantitatively significant and relatively more hydrophilic organic constituents remaining in the $B_{effluent}$ are occasionally further divided by weakly basic anion exchangers or certain suitable non-ionic sorbing solids into a so-called "transphilic" fraction (hydrophilic acids, about 14–25% of the fresh water DOC [19,26]) and hydrophilic neutrals. It has been recently [16] demonstrated that the substitute DAX-8 resin can, at best, isolate about 20% more hydrophobic HM-type organic constituents than the previous XAD-8 resin. However, it appears that certain failures occur in the manufacturing of the DAX-8 resin thus perverting the reliability of the isolation. This, among other thinks, speaks for arguments to test also other isolation techniques.

Fig. 1 indicates that the molecular size distribution of different humic-type constituents in fraction $B_{[HM]}$ resembles closely to that of the original SSS-water. This single HPSEC analysis verifies the previous statement, obtained [27,40] by complicated fine-structural analyses, that HM-type solutes obtained by the DAX–XAD procedure must play a role as certain definite entities in the original DOM and they cannot be merely accidental products of the isolation process. However, during the isolation procedure, some aggregation–rearrangement of certain constituents were taken place which is also visible from the decreased $\bar{M}_w/\bar{M}_n$ value in Table 1, i.e. the molecular size distribution became narrower and the fingerprint humps in the chromatogram shifted slightly towards higher molecular sizes. The shapes of the elution profiles in the chromatograms obtained for

the more hydrophobic $C_{[MeOH]}$ fraction and transphilic effluent ($B_{effluent}$) are rather similar to those of the original SSS-water and the fraction $B_{[HM]}$. Most noteworthy is the continuous existence of the relatively high-molecular size constituents at the elution volumes of 9.8 and 10.8 ml. The loss of these constituents during the conventional single isolation procedures is a notable drawback because previous studies [49,50] prove that the organic constituents belonging to this, so-called non-humic, category contain qualitatively the same principal building blocks and the most powerful discriminating factor appears to be their relative content.

3.1.2. DEAE procedure

Scheme 1 proves the high retaining capacity of the DEAE cellulose for HM-type organic macromolecular acids (about 17% more HM, fraction $D_{[HM]}$, was obtained than with the DAX-8 procedure) analogously to previous studies [15,16,26]. It has been recently verified [11,26,27], by fine-structural analyses, that the integrated whole of macromolecular organic acids isolated by the DEAE technique resembles both quantitatively and qualitatively very closely to an average combination of the four different acidic fractions obtained by the multi-stage DAX–XAD procedure, i.e. so-called: (1) hydrophobic FA- and (2) HA-type acids, (3) hydrophobic neutral solutes ([MeOH]) and (4) hydrophilic acids (a transphilic fraction). This is an essential advantage from the structural chemistry point of view and shows that the DEAE isolate represents a clear-cut average HM for fresh waters. From a practical point of view, it should be emphasized that a complete back-elution of the adsorbed humic solutes from the DEAE cellulose is somewhat more difficult than in the case of DAX-8 resin or its homologues.

Fig. 1 proves that the same fingerprint information as shown in the chromatogram of the original SSS-water is partly retained in that of the fraction $D_{[HM]}$; namely, two humps at the elution volumes of about 9.8 and 10.8 ml and one more slightly after 11.9 ml (at 12.2 ml, equal to 990 Da). In contrast, the maximum elution of organic solutes appears now, as early as, at about 8.7 ml (equal to 26,000 Da). This discrepancy shows that certain rearrangements take place among the macromolecular constituents during the isolation procedure. However, the averaged $\bar{M}_n$ and $\bar{M}_w/\bar{M}_n$ values in Table 1 speak for that these compositional changes are not, in reality, so drastic as compared to the situation predominating in the original water. On the other hand, the effluent of the DEAE column ($D_{effluent}$) was mainly composed of organic constituents with relative small molecular sizes, about 87% of organics were eluted after 12.2 ml contributing to the benefits of the DEAE technique.

3.1.3. PVP procedure

Scheme 1 demonstrates that the ability of the PVP resin to retain HM-type solutes at pH 2 (fraction $E_{[HM]}$), even by a simple batch technique, was particularly effective corresponding to the results of Chen et al. [34,35]. The content of organic solutes in the $E_{effluent}$ was of the same order of magnitude (19–23% of the original DOM) than in the case of the DEAE procedure. The DAX–XAD technique (in this case, XAD-8/2 resin) was able to isolate a significant part (ca. 52%) of organic solutes from the $E_{effluent}$ as HM-type constituents (fraction $F_{[XAD]}$). The weakly basic IRA-67 anion exchanger further retained from the $F_{effluent}$ very effectively (ca. 89%) certain kinds of macromolecular HM-type acids (fraction $G_{[IRA]}$, which corresponds, according to the XAD terminology, to a so-called transphilic fraction). The final content of organic solutes in the last $G_{effluent}$ was comparatively low (ca. 1% of the original DOM).

Fig. 1 confirms that PVP is a workable resin for retaining HM-type constituents, which are found in the original water. Three elution humps of the fraction $E_{[HM]}$ positioned at about 9.8, 10.2 and 10.8 ml and two humps at 11.3 and 13.0 ml (2300 and 470 Da, respectively). However, the distinct high-molecular size hump at the elution volume of about 7.1 ml (115,300 Da), which was visible in the chromatogram of the original SSS-water, was now missing. Likewise, the molecular size distribution was narrower for the fraction $E_{[HM]}$ than that obtained for the original SSS-water (cf. $\bar{M}_n$ and $\bar{M}_w/\bar{M}_n$ values of $A_{[Orig.Wat.]}$ in Table 1) speaking for the fact that certain compositional rearrangements take place during the isolation procedure. The chromatogram of the $E_{effluent}$ contained almost the same elution humps, yet with somewhat improved resolution power, which were also obtained for the fraction $E_{[HM]}$ (namely at 10.2, 10.8, 11.3 and 13.0 ml, only the elution hump for the largest molecular size at 9.8 ml was absent). This outcome is apparently a result of the slightly different retaining capacity of the PVP resin. The fraction $F_{[XAD]}$ contained the three elution humps at 10.2, 10.8 and 11.3 ml also characteristic for the $E_{[HM]}$ and $E_{effluent}$ fractions. However, the relative proportion of the elution hump at about 10.2 ml was significantly higher, compared with the situation in the original $E_{effluent}$ fraction, and the small elution hump at about 9.6 ml, specific for the original DOM, was now visible in the fraction $F_{[XAD]}$, in addition to the molecular size of the latest elution hump being slightly increased from about 470 to 620 Da (13.0 versus 12.7 ml). The $F_{effluent}$ contained again the same four molecular size fractions (namely those at 10.2, 10.8, 11.3 and 12.7 ml) which were specific for the preceding $F_{[XAD]}$ fraction but the molecular size distribution was now narrower (cf. Table 1). On the other hand, the fine structure of the chromatogram for the $G_{[IRA]}$ fraction was fully different, only a small shoulder was visible at about 10.8 ml indicating a possible similarity with the previous $F_{effluent}$. The dissimilarity may be caused by the aggregation degree of these small-molecular size organic macromolecular HM-type acids being too high for the complete penetration into the gel matrix. The remaining $G_{effluent}$ fraction was split into several scattered elution humps with small molecular sizes. The intensive elution humps at about 16.3 ml and especially at 18.3 ml (total volume of the gel bed (V_t) was ca. 16.6 ml) indicate strong interactions with the gel matrix. The sequence of the six HPSEC chromatograms given for the enlarged PVP application demonstrates that the isolation of HM-type or-

ganic solutes is not a straightforward and very simple task, especially since after a certain separation phase the remaining organic constituents seem to attain again a certain compositional equilibrium state resembling those of the previous fractions in the isolation flow chart. Similar re-configuration of humic solutes after separation has been postulated earlier [39,51].

3.1.4. UF procedure

Scheme 1 shows that the 20-fold concentrate using a membrane of 1 kDa cutoff includes about 97% organic solutes of the original DOM with molecular size greater than the applied NMW value (fraction $H_{[HM]}$). The cutoff range of the applied tangential UF membrane was in accordance with the HPSEC results (cf. Table 1) and previous findings [39]. The ability of the weakly basic IRA-67 resin to retain small-molecular size constituents (organic acids, etc.) from the $H_{filtrate}$ was very effective (ca. 91% of the remaining organic solutes were removed from the parent solution). Cation exchanger retained about 21% of miscellaneous organic constituents from the basic extract of the IRA-67 resin (fraction $J_{[Dow]}$; amines, amino acids, peptides, etc.), and the rest (ca. 70%) consisted of certain kinds of macromolecular HM-type acids (fraction $I_{[IRA]}$). The content of organic constituents in the final $I_{effluent}$ was negligible and thus it represents practically mere water.

Fig. 1 proves that a controlled concentration of the original water sample does not increase the relative content of small-molecular size constituents, as stated [52] previously, but the fingerprint information of the chromatogram $H_{[HM]}$ was totally identical with that obtained for the original SSS-water (cf. $\bar{M}_n$ and $\bar{M}_w/\bar{M}_n$ values of $A_{[Orig.Wat.]}$ in Table 1). The chromatogram of the $H_{filtrate}$ possessed three new elution humps at about 10.5, 11.3 and 12.8 ml (4850, 2300 and 570 Da, respectively), which significantly differed from the corresponding candidates obtained for the previous $H_{[HM]}$ sample, one minor hump at 16.3 ml (<100 Da) being common with the previous head samples and a new very marginal one at about 17.8 ml ($\lll$100 Da) indicating possibly slight interactions with the gel matrix. This outcome speaks for the tendency, occurring after removing of the DOM, that the remaining organic solutes take readily a new re-configuration, i.e. find a new equilibrium state. The molecular size distribution of organic constituents in the fraction $I_{[IRA]}$ was quite different from that obtained for the previous parent solutes in the $H_{filtrate}$. In accordance with this outcome, the molecular size distribution of the $J_{[Dow]}$ fraction, obtained during the purification procedure of the fraction $I_{[IRA]}$ by a cation exchanger, had nothing in common with those obtained in the whole isolation flow chart. On the other hand, as a peculiarity, the molecular size distribution of the final $I_{effluent}$ fraction resembled very closely to the parent $H_{filtrate}$ fraction. This irregularity illustrates that certain kinds of organic constituents of the original heterogenous DOM do not behave as definite entities but after they are removed from their original equilibrium state they form new aggregates.

3.2. Spectroscopic behaviours of organic solutes during different isolation procedure

Each isolation procedure in Scheme 1 divided the original DOM into a specific main fraction and an effluent-filtrate, which was further divided into several subfractions. The different fractions of each isolation procedure, weighted with their relative contents, were computationally combined for estimating how well the sum functions of these individual pieces correspond to the molecular size distribution of the original state of the DOM, and what was the effect of the isolation procedure on the absorbance-abilities of chromophores at 254 nm. The combined chromatograms correlated surprisingly well, in the case of DAX-8, DEAE and PVP procedures, with that obtained for the original water sample ($r = 0.943 \pm 0.001$, $P_{0.95}$), and the decrease in the total chromatogram area was only $8 \pm 1\%$ which was, considering experimental errors, almost insignificant. This recalculation indicates that the absorbance-ability of different chromophores remains reversible in the different chemical treatments and each isolation method gives, to a certain degree, a representative candidate for the HM model, especially in the light of the molecular size distribution.

3.3. Differentiation of solid isolates obtained by applied procedures

In addition to experimental results in Table 1, some values were derived by certain theoretical or experimental equations. Molar absorptivities ($\in$, l mol^{-1} cm^{-1} of OC) were measured at 280 nm. A good correlation was observed, by means of equations presented [39] previously, between $\in$, total aromaticity (Ar. (%)) and $\bar{M}_n$ values being also consistent with the results of Chin et al. [53]. It has been stated [54] that the absorbance of humic waters at 365 nm will increase relatively more with increasing molecular size than that at 250 nm thus permitting an estimate (E_2/E_3-ratio) for the relative degree of humification. The correlation obtained in the present study between the quotient E_2/E_3 and $\in$ was acceptable ($r = 0.89$, $P_{0.95}$) demonstrating that when the E_2/E_3-ratio increased, the estimated Ar. (%) and $\bar{M}_n$ values decreased. The total amount of unsaturation (ϕ_{total}) in mmol g^{-1} was calculated, for better describing the nature of different isolated fractions, by means of the method presented in [55] specially for the complex mixture of humic solutes: $\phi_{total} = C_{total} + N_{total}/2 - H_{total}/2 + 1000/\bar{M}_n$, where C_{total}, N_{total} and H_{total} are the contents of carbon, nitrogen and hydrogen in mol g^{-1} and $\bar{M}_n$ is the number-averaged molecular size. It is notable that ϕ_{total} is the sum of carboxyl (ϕ_{COOH}), carbonyl ($\phi_{C=O}$, a pi-bond), aromatic (ϕ_{Ar}) and aliphatic (ϕ_{al}, cycloalkyl groups) unsaturations and unsaturation due to alkenes, esters, amides, etc. (ϕ_{xs}). The different ϕ_{total} values obtained in the present study for HM-type isolates were in good accordance with those reported [11,27,40] previously for various HM-type mixtures. The average molecular formulas for different solid isolates in Table 1 were calculated, by means of unprocessed number-

averaged $\bar{M}_n$ values estimated by HPSEC, for better visualizing the composition of a sample. The use of more reliable $\bar{M}_n$ values, obtained by vapor–pressure osmometry (VPO), as a standard for comparison is a common practice in humus chemistry. The relationship between the $\bar{M}_n$ values obtained by the VPO and the applied HPSEC was [39]: $\bar{M}_{n\,\mathrm{VPO}} = 591.249 + 0.049 \times \bar{M}_{n\,\mathrm{HPSEC}} + 7.531\mathrm{E}^{-6} \times \bar{M}_n^2$, ($r^2 = 0.984$, $n = 19$, $P_{0.95}$), which was also previously adopted [11,27,40] for calculating structural characteristics per an average humic molecule.

Table 1 shows that although some variation occurred, e.g. between the elemental compositions of different actual HM-type isolates, the parameters were fairly similar with each other. Likewise, the discriminating power between the hydrogen-to-carbon (H/C) and oxygen-to-carbon (O/C) ratios (van Kravelens diagram [56], whose application is still under intensive studies [57]) is not very easy to realize. For better extracting the small differences among the nineteen different isolates and to find a possible similarity–dissimilarity between samples, the original data was slightly manipulated. The multi-dimensional dataset of Table 1 was examined closer with a statistical–graphical principal components analysis (PCA). The fundamental idea of the PCA is to reduce the numerous variables and to seek for linear combinations of those variables explaining most of the variability. Accordingly, PCA (eigenanalysis) and subsequent inspection of the eigenvector plots is one of the first and foremost procedures that can be done when tackling a multi-dimensional dataset. The credit of multivariate methods is that a huge amount of data can be presented in a graphical form, which would be very hard to do using tables of numbers or univariate statistics. For the PCA, from the dataset of Table 1, a 19 × 6 matrix (19 objects (samples) and 6 variables: $\bar{M}_n$, ∈, H/C, O/C, N/C and E_2/E_3) was generated for calculating variances and covariances. From this, eigenvectors and eigenvalues have been extracted.

Fig. 3 shows the biplot (scatterplot) of the six variables for the different samples (19 cases) isolated and processed with different techniques and chemical treatments (level of statement, 86% for the first two PC). The six arrowhead lines intersecting at (0,0) represent the weights of the variables. The direction and length of each vector (variable) are proportional to its contribution to the principal components, and the angle between any two is inversely proportional to the correlation between them. According to the statistical–graphical analysis of Fig. 3, the actual HM-type isolates ($B_{[HM]}$, $D_{[HM]}$, $E_{[HM]}$, $R1_{[HM]}$ and $R2_{[HM]}$) formed a compact cluster I, regardless of the applied isolation procedure. In other words, the averaged values of the studied parameters for these samples were almost similar. The original DOM ($A_{[Orig.Wat.]}$) and its UF concentrate ($H_{[HM]}$) formed, as expected, also a compact cluster II quite close to that of I (the only significant discriminating factor between clusters I and II was the H/C-ratio). The so-called hydrophobic neutral isolate $C_{[MeOH]}$ and its reference $R4_{[MeOH]}$ fell into cluster III, and the strongest discriminating parameters in respect of their HM homologues (cluster I) were $\bar{M}_n$, ∈ and E_2/E_3. The same three structural parameters discriminated also the HA-type $R3_{[HM]}$ sample from its FA-type $R2_{[HM]}$ counterpart and the HA-type $R7_{[HM]}$ and $R9_{[HM]}$ samples from their FA-type $R6_{[HM]}$ and $R8_{[HM]}$ counterparts (respectively). The environmental impact was also revealed clearest by these three parameters, i.e. the FA-type $R6_{[HM]}$ and $R8_{[HM]}$ samples became clearly separated from their $R2_{[HM]}$ homologue, and the HA-type $R7_{[HM]}$ and $R9_{[HM]}$ samples were positioned far from their $R3_{[M]}$ homologue. The discriminating effect of these three parameters prevailed also for the $F_{[XAD]}$ sample, i.e. the E_2/E_3-ratio was slightly greater and $\bar{M}_n$ as well ∈ values smaller than

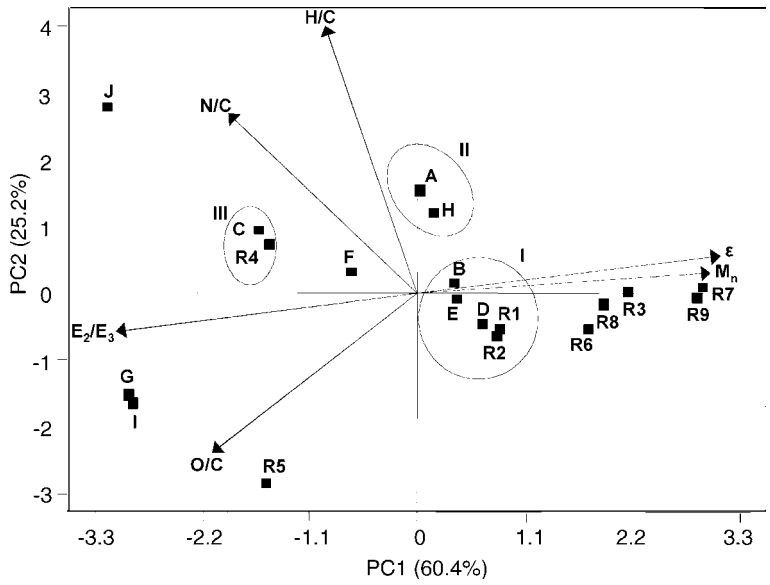


Fig. 3. Graphical two-dimensional perspective of projections (biplot) of the six basic structural variables on the first two principal components for the distribution of the different humic-type isolates obtained by several procedures. For symbols of samples and variables, see Table 1.

those obtained for the corresponding main $D_{[HM]}$ fraction. The O/C-ratio was exceptionally high for $G_{[IRA]}$, $I_{[IRA]}$ and $R5_{[IRA]}$ samples resulting, together with other parameters, in their positioning far from other samples. The divergence of $R5_{[IRA]}$ from $G_{[IRA]}$ and $I_{[IRA]}$ is due to their different isolation procedures. The special $J_{[Dow]}$ sample formed its own group, far from other samples, particularly owing to very high N/C- and H/C-ratios, in addition to other parameters. The statistical–graphical analysis of Fig. 3 confirms that the molecular size distribution and certain simple spectroscopic properties are very powerful discriminating parameters between different natural organic isolates.

3.4. FT-IR analysis

Infrared spectroscopy has been widely used for gross characterization of humic-type constituents and can provide valuable information on the structural and functional properties of NOMs, e.g. oxygen-containing functional groups, occurrence of protein and carbohydrate moieties and relative proportions of aromatic versus aliphatic moieties. However, the unambiguous assignments of different spectral bands are not possible for humic-type very heterogenous organic material.

All spectra in Fig. 2 are characterized by a number of absorption bands, exhibiting variable relative intensities, typical of humic-type materials [32,34,58–61]:

- A band around 1724 cm^{-1}, typically associated to the C=O stretching of carbonyl functions, particularly aldehydes, ketones and carboxyl groups (COOH);
- The range of 1650–1600 cm^{-1}, C=O stretch (amide), aromatic C=C, hydrogen bonded C=O, double bond conjugated with carbonyl and COO^- vibrations, COO^- symmetrical stretch;
- A discrete small band at about 1510 cm^{-1}, possible ascribed to C–C stretching of aromatic rings (lignin indicator), to conjugated C=N systems and amino functionalities;
- A broad band around 1400 cm^{-1} describing several functionalities, aliphatic C–H, CC–H_3, C–H stretching of methyl groups, C–H bending, O–H deformation and C–O stretching of phenolic groups, COO^- antisymmetrical stretch, salts of COOH;
- Near 1224 cm^{-1}, C–O stretching and O–H deformation of COOH groups, aromatic and ester linkage C–O, phenolic C–OH;
- Around 1054 cm^{-1}, C–C, C–OH, C–O–C typical of glucosidic linkages, Si–O impurities, especially C–O stretches of carbohydrates and peptides;
- A small band at 840 cm^{-1}, aromatic C–H vibrations;
- For low-energy vibrations a broad band at about 600 cm^{-1}, specific for inorganic and organometallic compounds.

3.4.1. Differentiation of samples according to FT-IR analysis

Fig. 2 verifies that it is practically impossible to draw definite conclusions about the influence of the isolation procedure on the structural compositions of separated fractions directly from their multidimensional functionalities. The only IR spectrum which definitely differed from other was obtained for the special $J_{[Dow]}$ faction which is, according to the XAD nomenclature, equal to the so-called hydrophilic bases. The broad absorbance bands positioned at 1633, 1395, 1100 and 802 cm^{-1} speak for the high contents of carbohydrates and peptides in this fraction, in addition to small amounts of aromatics. The functionality of this $J_{[Dow]}$ fraction was surprisingly similar to the IR spectrum reported earlier for an analogous hydrophilic base-fraction [18]. The coarse structural similarity–dissimilarity, in the light of applied isolation procedures, of the other fourteen IR spectra of Fig. 2 was proved in Fig. 4 using the statistical–graphical analysis.

Fig. 4 proves the power of the PCA in solving complicated problems. For the PCA, from the IR dataset, a 14 × 300 matrix (14 objects (samples) and 300 variables, intensities of IR spectra between 1900 and 400 cm^{-1}; bandwidth, 5 cm^{-1}) was generated for calculating variances and covariances. From this, eigenvectors and eigenvalues have been extracted. Fig. 4 shows the three-dimensional scatterplot of projections (referred also to scores) of the multi-dimensional IR dataset on the first three principal components (arbitrary units). The overall level of the statement of the structural functionalities was 95% on the first three PCs. The discriminating effect of the PC1 between different samples/isolation procedures was very high (level of the statement was near 76% of the total variance). The most effective on the PC1 were the multiple functionalities between 1760 and 1350 cm^{-1} and two more specific functionalities centred on near 1224 and 1054 cm^{-1}. The most effective remaining unexplained specific variations between the samples on the PC2 were the functionalities arising from the intensities around 1700, 1500 and 1200 cm^{-1} and from a distinct intensity centred on near 1054 cm^{-1}. The remaining unexplained variation between certain samples on the PC3 was quite specific attached to the

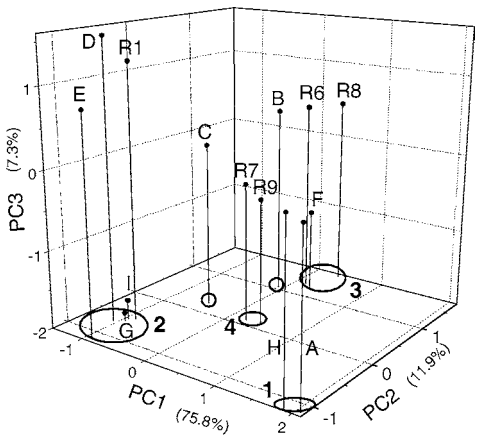


Fig. 4. Graphical three-dimensional perspective of projections of the multidimensional FT-IR dataset (in the zone, 1900–400 cm^{-1}) on the first three principal components for the distribution of the different humic-type isolates obtained by several isolation procedures. For symbols of samples, see Table 1.

single functionalities centred on near 1724, 1630, 1383, 1224 and 1554 cm^{-1}.

Fig. 4 demonstrates that the PCA divides the different samples according to their IR functionalities into distinct clusters. The greatest dissimilarity appears between the cluster 1 (samples $A_{[Orig.Wat]}$ and $H_{[HM]}$) and cluster 2 (samples $D_{[HM]}$, $E_{[HM]}$, $R1_{[HM]}$, $G_{[IRA]}$ and $I_{[IRA]}$). In view of the total reliability (95%, including the PC3), the structural functionality of the samples $D_{[HM]}$, $E_{[HM]}$ and $R1_{[HM]}$ is quite similar but totally different from that obtained for the special samples $G_{[IRA]}$ and $I_{[IRA]}$. This is in agreement with the situation in Fig. 3 (cluster I). On the other hand, the structural similarity–dissimilarity in Fig. 4 among the three actual HM-type isolates and the samples $A_{[Orig.Wat]}$ and $H_{[HM]}$ is clearly different from that obtained by means of elemental analyses and other simple parameters in Fig. 3. This is a natural result of the chemical, even relative mild, treatment of the original DOM. Figs. 3 and 4 verify also that it is possible to isolate by the same procedure from the same fresh water sampling source reasonably similar HM-type isolates ($D_{[HM]}$ versus $R1_{[HM]}$) and that the seasonal variation is quite marginal. The variation of the many functionalities dominating the PC1 was quite minor between the samples positioned in the clusters 3 and 4, and certain specific functionalities typifying the PC2 were the most significant difference between these samples. The samples $R6_{[HM]}$ and $R8_{[HM]}$, representing so-called FA-type fractions of the XAD-8 technique, formed their own cluster 3, thus indicating similar uniformity as that obtained in Fig. 3. On the other hand, the sample $F_{[XAD]}$, representing a specific fraction separated by the XAD-8/2 resin, also positioned in cluster 3 unlike in Fig. 3 where its most important discriminating factor was the molecular size distribution. This is a quite sensible result because the isolation mechanism of the XAD resins (or analogues) is based on certain functionalities of the DOM prevailing in the preadjusted conditions. The variation among the $R6_{[HM]}$, $R8_{[HM]}$ and $F_{[XAD]}$ samples was practically attached to certain single minor functionalities dominating the PC3. The HA-type samples $R7_{[HM]}$ and $R9_{[HM]}$ also formed their own cluster 4 thus being consistent with the results in Fig. 3. The sample $B_{[HM]}$, representing a total amount (HA + FA) of so-called hydrophobic humic substances isolated by the DAX-8 resin, formed its own group quite close to the combined cluster 3 (FA-type isolates). The structural similarity of the $B_{[HM]}$ sample with these FA-type isolates is in agreement with that in Fig. 3 ($B_{[HM]}$ versus $R2_{[HM]}$), with the environmental impact on the quality of structural composition as well as with the previous results [15–17]. The sample $C_{[MeOH]}$, which, according to the XAD nomenclature, represents the so-called very hydrophobic fraction, also formed a separate cluster but without any close relationship to other clusters or samples. On the other hand, the IR spectrum of the $C_{[MeOH]}$ sample resembles, excepting certain minor functionalities, those obtained for FA- or HA-type isolates, or their primary mixture, separated by XAD-8 or DAX-8 resins. This is quite evident since it has been previously verified [49,50] that the different hydrophobic FA-, HA- and MeOH-type functional fractions contained qualitatively the same principal building blocks and only the relative content of which was the most powerful discriminating factor.

3.4.2. Possible ester hydrolyses during isolation by FT-IR analysis

The FT-IR technique has been intensive adapted for determining the carboxylic acid content of humic-type constituents and analogues [62–64]. Nevertheless the carboxylate groups generate different absorption bands within a large frequency region and also other functional groups will absorb at the same wavelengths (overlapping), the main interest is directed to spectral ranges at about 1580–1630 and 1710–1730 cm^{-1} generally assigned to the carboxylate anion ($-COO^{-1}$) and protonated –COOH groups, respectively. It is noteworthy that traces of carboxylate bands are present in all FT-IR spectra of humic-type constituents, even those at pH ≈ 2, indicating the presence of very strongly acidic groups—as also remarked by Cabaniss [64]. Maurice et al. have recently reported [65] that a small shoulder being comprised in the absorption band of the protonated –COOH groups, at higher frequency with a maximum near 1770 cm^{-1}, corresponds to the ester carbonyl groups (–COOR). The separation of this hardly visible small shoulder (cf. Fig. 2) as a distinct absorption band from the spectral profile is not an easy task requiring different kind of data manipulation, e.g. linear Gaussian/Lorentzian band shapes and/or special multi-point method for baseline correction [66,67]. All chromatographic isolation techniques contain the basic extraction (pH ≈ 13) of the organic matter retained onto the sorbing solid thus permitting a possibility for potential base-catalyzed ester hydrolyses of the HM [65,68,69].

Table 2 proves that the loss of ester groups (–COOR) in concentrating (20-fold) the original water by the tangential UF technique (1 kDa of NMW cutoff) was quite minor ($A_{[Orig.Wat.]}$ versus $H_{[HM]}$). The $I_{[IRA]}$ fraction obtained from $H_{filtrate}$ (cf. Scheme 1) contained still a significant amount of –COOR groups (ca. 2% of original), and it was possible to find even some traces from the $J_{[Dow]}$ fraction after the two-fold base extraction of OM indicating the relatively permanent nature of some –COOR groups. It was possible to obtain about 70% of the original –COOR content by the DAX-8 technique ($B_{[HM]}$ + $C_{[MeOH]}$) and 72% by the DEAE technique ($D_{[HM]}$) thus speaking for the fact that the effect of the acidic pretreatment of the original water on the ester hydrolysis is practically negligible. The PAP technique alone separated from the original SSS-water about 81% of the original –COOR content and its enlarged application ($E_{[HM]}$ + $F_{[BAD]}$ + $G_{[IRA]}$) as much as about 92%. The above mass balance estimation expresses that the base-catalyzed ester hydrolysis of the DOM is not as disadvantageous factor in the chromatographic isolation of the HM as speculated (e.g. [65,68,69]).

Table 2 also indicates that only about 3% of carboxylic groups of the DOM were in their protonated form in the orig-

Table 2
Selected FT-IR data for different isolated humic-type samples[a]

Sample[b]	Method	─COOR (cm^{-1})[c]	─COOH (cm^{-1})[c]	$─COO^{-1}$ (cm^{-1})[c]	─COOR (rel. area)[d]	─COOH (rel. area1)[d]	$–COO^{-1}$ (rel. area2)[d]	─COOR (percent of $A_{[Orig.Wat.]}$)	─COOH (percent of Σrel. areas 1 and 2)
$A_{[Orig.Wat.]}$	F.D.	1770	1715	1585	237.2	33.3	1189.8	100.0	2.7
$H_{[HM]}$	UF	1770	1715	1585	229.9	29.9	1179.1	96.9	2.5
$I_{[IRA]}$	UF	1770	1725	1630	4.3	21.3	5.5	1.8	79.6
$J_{[Dow]}$	UF	1770	1735	1630	0.1	0.3	11.0	0.02	3.0
$B_{[HM]}$	DAX-8	1780	1720	1615	163.1	358.5	214.9	68.8	62.5
$C_{[MeOH]}$	DAX-8	1780	1720	1620	3.1	9.1	18.2	1.3	33.3
$D_{[HM]}$	DEAE	1785	1710	1605	170.0	408.4	126.7	71.7	76.3
$E_{[HM]}$	PVP	1780	1725	1625	191.4	721.7	108.8	80.7	86.9
$F_{[XAD]}$	PVP	1775	1715	1620	19.1	99.7	73.9	8.1	57.5
$G_{[IRA]}$	PVP	1770	1725	1630	7.4	98.6	29.1	3.1	77.2

[a] For symbols of samples, see Table 1.
[b] All isolates were separated in parallel from the same SSS-water.
[c] Maximum frequency of the absorption band.
[d] Relative areas of estimated distinct absorption bands are standardized proportional to their quantities in the original DOM.

inal acidity (pH 5.8) of the water. This outcome is in line with the heterogeneity of different acid constants (pK_a) of HM reported in the literature (e.g. [26,70,71]). All chromatographic HM isolates were finally treated by strongly acidic cation exchanger before freeze-drying for protonating the $–COO^{-1}$ groups. Despite that the frequency range between 1580 and 1630 cm^{-1} does not merely represent the absorption of carboxylic groups, it is well justifiable to attribute, at least, a small part to remaining carboxylate anions.

4. Conclusions

It is evident that no ideal system is available for isolating pure hypothetical humic substances from a water sample, and during each isolation procedure based on a chemically assisted sorption–desorption technique, certain changes in the structural composition of the DOM take place, as shown in this study and also verified [11] previously. Most important is that researchers choosing between different isolation procedures do consider the purpose of the isolation, i.e. whether humic fraction is desirable, as it has also been emphasized [65]. According to this study, the following conclusions can be drawn:

- The DEAE cellulose serves as a practicable choice for fresh water studies. The overwhelming advantage of this sorbing solid is that any adjustments of the original acidity of the water are not needed, and it is possible to isolate a significant amount (about 75–80% of DOM) of organic solutes as humic matter. A minor disadvantage is the somewhat tedious back-elution of the adsorbed organic solutes.
- The PVP resin seems to be very useful in isolating humic-type constituents from the fresh water sample, especially in relation to their original form. The ability of the PVP resin to retain organic humic solutes was exceptionally high (≥80% of DOM), and when connected in sequence with a non-ionic sorbing solid about 90% of the DOM was retained. The critical disadvantage of the PVP procedure is that the original acidity of the water sample must be adjusted to the pH 2 for protonating the acidic functional groups of the DOM.
- The mass balance estimation of ester carbonyl carbons speaks for the fact that the base-catalyzed ester hydrolysis of the DOM, possibly taking place during the chromatographic isolation phases of the HM, is not as critical disadvantage as speculated.
- To better characterize the different isolated humic-type samples in relation to applied isolation procedures more sensitive analyses (e.g. ^{1}H and ^{13}C NMR and thermal degradation) are in progress.

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