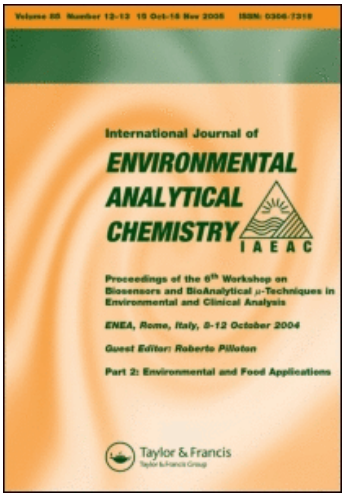

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Precision of Isolation of Aquatic Humic Matter by Xad-Resin Technology from NMR Spectroscopy's Point of View

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PRECISION OF ISOLATION OF AQUATIC HUMIC MATTER BY XAD-RESIN TECHNOLOGY FROM NMR SPECTROSCOPY'S POINT OF VIEW

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The most crucial question in the aquatic humus chemistry is a reliable and selective separation and fractionation of the humic solutes from other organic and inorganic constituents. The dilute solutions of natural dissolved organic matter must in a way or another be concentrated before fractionation and molecular characterization. The most popular method for this purpose is the application of non-ionic macroporous resins at preadjusted acidity. The routine is fractionate further at strongly acidic conditions the obtained mixture of hydrophobic humic solutes into so-called humic and fulvic acids. Quantitatively and in the light of elemental analysis and basic functional groups this fractionation seems to be reliable. However, from the point of view of the structural composition, the precision of the procedure was not so self-evident. The data of nuclear magnetic resonance spectroscopy has proved that it is slightly erratic to repeat from the standard homogeneous water sample the isolation-fractionation for obtaining structurally wholly identical humic and fulvic acids. This deviation was not solely as a result of the technique itself, but also of the highly sensitive nature of the humic solutes.

Keywords: XAD resin; humic substances; separation; nuclear magnetic resonance; structural composition

INTRODUCTION

The importance of macromolecular humic matter in aquatic systems has been pointed out in several fundamental textbooks.[1–19] In general, humic matter in water ecosystems accounts for the major part of the dissolved organic matter (DOM) and carbon (DOC). However, this essential biomaterial from various environmental sources can be defined, e.g. according to Malcolm [20], only operationally, based on the procedure applied to its isolation. The most frequently applied procedures for isolating aquatic humic solutes are based on column sorp-

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tion techniques which simultaneously concentrate and fractionate specific organic solutes (referred to humic substances) from most other dissolved constituents. The application[21–33] of non-ionic macroporous sorbents (e.g. XAD resins) has become almost a standard procedure for isolating aquatic humic solutes. The properties of most of the non-ionic sorbing solids are fairly well understood and extensive studies have been conducted to evaluate resin performance in the isolation and recovery of organic solutes from water. Non-ionic macroporous copolymers classify organic solutes in a water sample at preadjusted acidity (ca. pH 2) into different artificial hydrophobic and hydrophilic fractions according to their specific sorption. Irrespective of the fact that the exact mechanism of the so-called XAD technique is unknown, this operational classification of aquatic humic solutes based on certain hydrophobic-hydrophilic interactions between organic solutes and the sorbing solid under the preadjusted conditions is in principle clear, and thoroughly discussed in the literature. However, no information appears to be available as regards the repeatability of the whole XAD isolation procedure. Furthermore, it has been argued[34,35] that sorption under strongly acidic conditions may include certain risks for uncontrolled reactions, and utilization of non-ionic sorbing solids alone may lead to serious errors in classification of the DOM.

Nuclear magnetic resonance spectroscopy of carbon isotope 13 (^{13}C NMR), both in solution (S) and solid (CPMAS, cross-polarization magic angle spinning) states, has been extensively employed[20,31,32,36–44] to obtain structural information of carbon nuclei in different humic isolates and on the relative amount of each type of carbons. A well-known[45–47] common problem in both solution and solid state ^{13}C NMR spectroscopy of humic extracts is the correct quantitative evaluation of the different types of C in order to compare different samples with each other. Nevertheless, ^{13}C NMR spectral analysis is one of the most useful interpretative tools for the characterization of humic matter. Proton (^{1}H) NMR spectroscopy has been found[20,31,32,38,39,48–55] to be another useful tool in determining functional group concentrations for humic isolates, which together with ^{13}C NMR has provided very essential compositional information. Due to the very heterogenous macromolecular nature of various humic solutes the nuclei can experience a wide variety of chemical environments thus producing a wide range of ^{1}H and ^{13}C NMR chemical shifts. This fundamental characteristic interfere somewhat with the exact definition and quantification of specific resonance regions of different kinds of carbons and non-exchangeable protons since they can resonate within a wide shift range with numerous overlapping.

The aim of the present work was to assess the repeatability of the whole XAD isolation procedure based on three parallel experiments with the same fresh water sample. In addition to gravimetric measurements, elemental analyses, titra-

tion of acidic functional groups and determination of molecular size distributions, the uniformity of the separated hydrophobic humic (HA) and fulvic (FA) acid fractions were compared with ^{13}C and ^{1}H NMR. Because of the lack of comparable spectra in the literature, ^{13}C NMR spectra were determined both in solid and solution states on a commercial FA-reference.

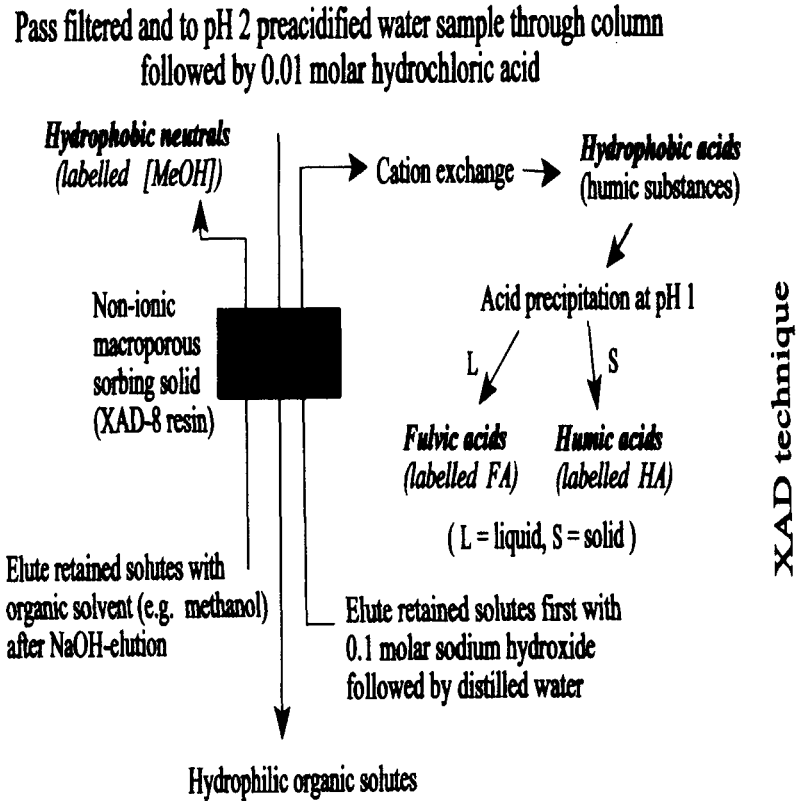
EXPERIMENTAL

Origin of fresh water samples

The fresh water sample (SS) was collected in September 1994 from Lake Savojärvi situated in a marsh region in southwestern Finland. Lake Savojärvi has very brown water (colour as cobalt-platinum units ca. 150 mg Pt/L, DOC 19 mg C/L and pH 5.8).[56]The water sample (ca. 200 L) was collected from 1 m below the surface into glass containers. The water sample was filtered (0.2 μm, Nuclepore polycarbonate filter cartridge, no. 611101) immediately after sampling, homogenized and stored in the darkness at 4°C before the XAD isolation (within four weeks) of humic solutes. The aquatic Nordic fulvic acid (No.FA), used as the commercial reference, was isolated with the XAD technique during the summer of 1986 from the runoff water (colour as cobalt-platinum units >200 mg Pt/L and DOC ca. 20 mg C/L) of a Norwegian mire lake.

Isolation of aquatic humic solutes

After filtration the homogenized water sample was divided into three aliquots of 50 L and each acidified to pH 2. After acidification each aliquot was pumped through the Amberlite XAD-8 resin column. Hydrophobic organic matter adsorbed onto the resin was eluted at pH 13 (0.1 M NaOH) directly onto the strong cation exchanger (Dowex 50W X-8). The isolated mixture (pH 2.5) of the hydrophobic acids (classified as humic substances) was further partitioned at pH 1 (acid precipitation with HCl) into separate HA- and FA-acid fractions. These hydrophobic HA- and FA-type acids were further desalted on the XAD-8 resin (at pH 2) and finally passed through the cation exchanger before freeze-drying the solutes (in the H^+-form). The hydrophobic organic matter not eluted from the XAD-8 resin with base was eluted with methanol directly onto the strong cation exchanger, before freeze-drying the solutes, and labelled [MeOH] (hydrophobic neutrals). The above XAD isolation was repeated three times for parallel sub-water samples as visualized in Scheme 1.



SCHEME 1 Analytical procedure for classification of the DOC at preadjusted acidity into hydrophobic and hydrophilic fractions

Chemical analyses

Basic parameters

The methods applied for the determination of the elemental composition (carbon, hydrogen, nitrogen and sulphur; O% = 100 − (C+H+N+S)%); acidic functional groups (carboxyl (COOH) and phenolic hydroxyl (Ar-OH)); molecular weights (number- $(\overline{M}_n)$ and weight-averaged $(\overline{M}_w)$) have been reported[29–32,56] previously.

Solid state (CPMAS) ^{13}CNMR

The spectra for the freeze-dried HA- and FA-fractions were recorded[31] on a Bruker AC 300 P spectrometer at 75.5 MHz. Operational parameters were: sample size 75–80 mg; ^{13}C pulse width (90°) 5.3 μsec; 1 msec contact time; 2.5–5

sec repetition time; spectral width 41.67 kHz; size of a spectrum 16384 (complex) points (5 Hz/point); and 5.0 kHz spinning speed. The chemical shift scale is referenced to carboxyl carbons of glycine (>C=O; δ_C = 176.1 ppm).

Solution state (S) ^{13}C NMR

The spectrum for the freeze-dried No.FA-fraction was recorded[31] on a JEOL JNM-A500 spectrometer. Operational parameters were: concentration of the sample 240 mg in 0.3 ml D_2O; internal reference for chemical shifts (sodium 3-trimethylsilyl-propionate-2,2,3,3-d_4); pulse sequence of inverse gated decoupling (SGNNE) for eliminating the NOE (nuclear overhauser enhancement) was used to acquire the spectra; spectral window 50000 Hz; pulse width 45°; acquisition time 0.6554 sec; pulse delay 6.0 sec; number of transients 26000; and line broadening 50.0 Hz.

^1H NMR

The spectra for the freeze-dried HA- and FA-fractions were recorded[32] on a JEOL GX400 spectrometer at 400 MHz under homonuclear inverse gated decoupling conditions with irradiation of the HDO peak. The sample (15 mg) was added to 0.5 cm^3 D_2O and a few μL of NaOD (1 mol/L) was added to dissolve the HA-type samples. Spectra were recorded with 45° pulse and 32K data points for 5000 Hz spectral width. Acquisition time was 3 sec with a pulse delay of 3 sec and 700 scans were collected for an acceptable signal-to-noise ratio. Chemical shifts were referenced against an internal reference (3-(trimethylsilyl)-1-propane-sulfonic acid, sodium salt), the resonance ofwhich is based on the tetramethylsilane scale. The ^1H NMR spectra were recorded as soon as possible after sample preparation (within 1/2 h) to avoid various potential hydrolytic reactions since the spectra were obtained in slightly alkaline solution.

RESULTS AND DISCUSSION

Repeatability of the isolation procedure from point of view of quantities and basic parameters

Recoveries, elemental compositions (C,H,N,S,O), acidic functional groups (COOH and Ar-OH) and molecular size distributions ($\overline{M}_n$ and $\overline{M}_w$) determined for hydrophobic aquatic humic fractions obtained in the three parallel (I-III) XAD isolations are listed in Table I. The accuracy for the determination of elemental compositions was 1.6±0.2%. Similarly, the reproducibility for the determination of acidic functional groups and molecular size distributions was quite good (RSD-value ca. 3 and 5%, respectively). The critical examination of Table I based on the recoveries, elemental compositions and acidic functional

groups indicates that no systematic deviation is found between the three parallel (I-III) isolations. In other words, the XAD procedure seems to isolate and fractionate the FA- and HA-type fractions reliably as unique aquatic humic solutes. However, the data for the molecular size distributions ($\overline{M}_n$ – and $\overline{M}_w$ – values) shows somewhat to the contrary: some systematic overlapping occurs in the $SS.FA_{III}$- and $SS.HA_{III}$-fractions. Therefore, it is presumable that the acid precipitation of the hydrophobic acid mixture at pH 1 into separate FA- and HA-acid fractions does not take place fully reliable via a given unambiguous mechanism.

^{13}C and ^{1}H NMR data measured for FA- and HA-acid fractions

Figure 1 shows the comparison between **solution (S)** and solid state (CPMAS) ^{13}C NMR spectra measured for the No.FA reference. The differences between S and CPMAS ^{13}C NMR spectra and the corresponding relative abundances calculated for different types of carbons in Table II agree fully with the results obtained[57] by Conte *et al.* These differences between the S and CPMAS spectra, according to Ref. 57, points to the pseudo-micellar model of the conformational nature of aquatic humic solutes. Table II reports the areas of the ^{13}C and ^{1}H spectral regions reflecting different types of structures.

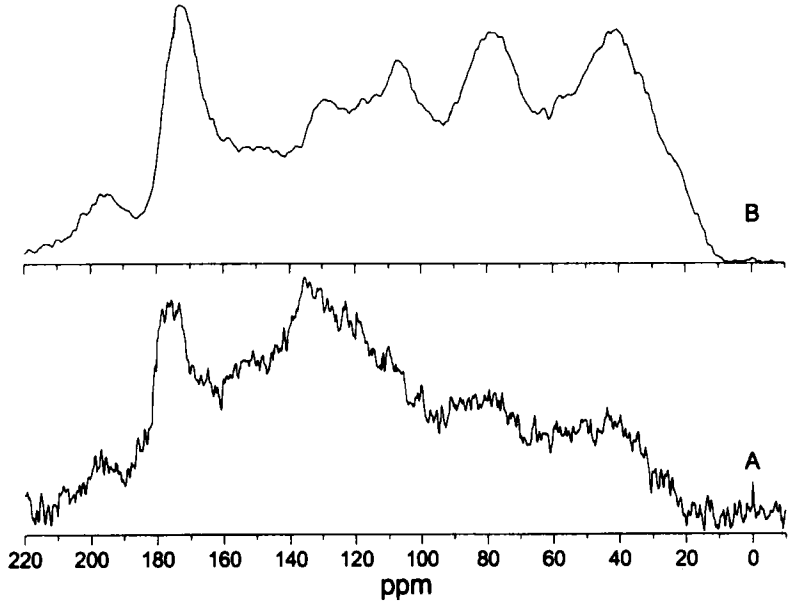


FIGURE 1 S (A) and CPMAS (B) ^{13}C NMR spectra of reference fulvic acid (No.FA)

TABLE I Precision of quantities and basic parameters determined for hydrophobic aquatic humic fractions obtained through three parallel (I-III) isolation procedures from the same water sample SS (moisture- and ash-free basis)

Fraction:	*SS.FA.I*	*SS.FA.II*	*SS.FA.III*	*RSD (%)*	*SS.HA.I*	*SS.HA.II*	*SS.HA.III*	*RSD (%)*	*SS.[MeOH]*[a]
Recovery (mg/L)	13.3	12.6	13.6	3	2.3	2.6	2.1	11	1.2
C (%)	54.9	54.5	55.3	1	55.6	56.3	55.8	1	56.3
H (%)	4.48	4.32	4.25	3	3.77	4.22	4.54	9	6.67
N (%)	0.75	0.67	0.56	14	2.09	1.73	1.26	25	1.39
S (%)	0.5	0.72	0.72	20	0.65	0.95	1.03	23	1.09
O (%)	39.4	39.8	39.2	1	37.9	36.8	37.4	1	34.6
$COOH_{tot}$(meq/g)	5.3	5.1	5.1	2	4.8	4.7	4.9	2	1.9
Ar-OH (meq/g)	1.2	1.2	1.0	9	2.2	2.7	2.4	10	0.9
$\overline{M}_n$	1980	1920	2800	22	4900	4700	3700	15	650
$\overline{M}_w$	5510	5130	6820	15	26400	24500	15600	26	3100

SS = water sample of Lake Savojärvi; FA = hydrophobic fulvic acid; HA = hydrophobic humic acid; [MeOH] = hydrophobic neutrals; RSD = relative standard deviation

a. = mean value for the combined fractions (I-III); $\overline{M}_n -$ and $\overline{M}_w -$ values have been determined[56] by the HPSEC-method.

TABLE II ^{13}C and ^{1}H NMR data measured for different FA- and HA-acid fractions. Precision of relative abundances of different types of carbons and hydrogens determined for hydrophobic aquatic humic fractions obtained through three parallel (I-III) isolation procedures from the same water sample SS

Sample	*^{13}C NMR*	*^{13}C NMR*	*^{13}C NMR*	*^{13}C NMR*	*^{13}C NMR*	*^{13}C NMR*	*^{13}C NMR*	*^{13}C NMR*	*^{1}H NMR*	*^{1}H NMR*	*^{1}H NMR*	*^{1}H NMR*
	C_A (%)	*C_B (%)*	*C_C (%)*	*C_D (%)*	*C_E (%)*	*C_F (%)*	*C_G (%)*	*C_H (%)*	*H_{A1} (%)*	*H_{A2} (%)*	*$H_{R\text{-}O}$ (%)*	*H_{Ar} (%)*
No.FA (CPMAS)	21.7	6.6	18.5	5.4	21.0	7.6	14.4	4.7				
No.FA (S)	9.9	7.5	12.0	5.5	34.0	8.4	17.6	5.3				
SS.FA.I	36.6	2.7	19.7	3.9	15.8	3.8	14.5	3.1	26.6	36.3	23.5	13.6
SS.FA.II	36.1	2.3	20.1	4.1	16.3	5.0	13.2	2.9	28.5	36.1	22.0	13.4
SS.FA.III	23.9	5.6	14.4	5.2	22.0	6.8	16.8	5.2	26.2	35.5	22.9	15.4
RSD-%	22.3	61.4	17.6	15.9	19.1	28.1	12.3	45.5	4.5	1.2	3.3	7.8
SS.HA.I	22.2	8.1	17.7	4.7	21.4	8.8	11.4	5.9	22.8	25.1	36.1	16.0
SS.HA.II	20.7	6.9	17.8	4.8	24.0	9.0	11.5	5.3	24.5	22.5	35.7	17.3
SS.HA.III	24.4	6.9	17.0	4.4	21.2	7.3	13.1	5.7	26.3	24.3	31.3	18.1
RSD-%	8.3	9.5	2.5	4.5	7.0	11.1	7.9	4.6	7.1	7.4	8.5	6.2

CPMAS = solid state ^{13}C NMR; S = solution state ^{13}C NMR; RSD = relative standard deviation; No = commercial reference; SS = water sample of Lake Savojärvi; FA = hydrophobic fulvic acid; HA = hydrophobic humic acid; C_A = aliphatic carbons (0–50 ppm); C_B = methoxyl carbons (50–60 ppm); C_C= carbons of C-O (60–90 ppm); C_D = carbons of O-C-O (90–110 ppm); C_E = aromatic carbons (110–140 ppm); C_F = phenolic carbons (140–160 pm); C_G= carboxylic carbons (160–190 ppm); C_H = ketonic carbons (190–220 ppm); H_{A1} = aliphatic hydrogens (0.2–1.4 ppm); H_{A2} = aliphatic hydrogens (1.4–2.8 ppm); $H_{R\text{-}O}$ = hydrogens of H-C-O (2.8–4.4 ppm); H_{Ar} = aromatic hydrogens (5.8–8.0 ppm).

The ^{13}C NMR spectra were divided into eight chemical shift ranges[20,31,32,36–44] (δ ppm; relative to tetramethylsilane): C_A (ca. *0–50 ppm*; aliphatic carbons); C_B (ca. *50–60 ppm*; mainly methoxyl carbons (-OCH_3)); C_C (ca. *60–90 ppm*; C-O resonances arising from, e.g. carbohydrate-type compounds); C_D (ca. *90–110 ppm*; e.g. anomeric carbons of polysaccharides); C_E (ca. *110–140 ppm*; unsubstituted and alkyl-substituted aromatic carbons); C_F (ca. *140–160 ppm*; phenols, aromatic ethers or amines); C_G (ca. *160–190 ppm*; represents largely resonances due to carboxyl carbons) and C_H (ca. *190–220 ppm*; carbonyl carbons of aldehydes and ketones). It is noticeable that the range *60–110 ppm* has been generally supposed to represent chiefly carbohydrate structures. However, it is quite evident that the majority of carbons in the *60–110 ppm* region does not merely correspond to 'normal' polysaccharide material. It has been assumed[45] that only a minor portion of the ca. *60–92 ppm* region is due to carbohydrates, and that the major part is made up, e.g. from aliphatic ethers and oximes. Furthermore, accurate integration of the 'anomeric peak' (*90–110 ppm*; C_D) is complicated. Therefore, 50% of the intensity in the ca. *95–110 ppm* region has been assigned to anomeric carbons and 50% to aromatic carbons.[45] This assignment has been utilized in this study as well.

Figures 2.a and 2.b show CPMAS ^{13}C and ^{1}H NMR spectra for $FA_{I\text{-}III}$- and $HA_{I\text{-}III}$-acid fractions obtained through three parallel isolation-fractionation procedures from the same water sample SS. The ^{1}H NMR spectra were subdivided [20,31,32,38,39,48–55] into four categories (δ ppm; relative to tetramethylsilane). The small resonance at ca. 8.3 ppm occurring in all spectra arises possibly from the proton of the formate ion (H-COO^-), a decomposition product of humic solutes dissolved in NaOD.[38] The broad resonance peak at ca. 4.4–5.2 ppm is caused by the proton of HDO. The sharp absorptions sometimes found around 0.9, 1.2, 1.9 and 3.5 ppm were probably due to the presence of some external impurities. The four main chemical shift ranges representing different types of protons in Table II were: H_{A1} (ca. *0.2–1.4 ppm*; methyl and methylene protons of carbons directly bound to other carbons); H_{A2} (ca. *1.4–2.8 ppm*; methylene and methine protons α to aromatic rings, or carboxyl and carbonyl groups); $H_{R\text{-}O}$ (ca. *2.8–4.4 ppm*; consists chiefly of protons attached to carbon atoms bound to oxygen); H_{Ar} (ca. *5.8–8.0 ppm*; protons predominantly aromatic, but not olefinic protons)[32].

Repeatability of the isolation procedure in the light of the ^{13}C and ^{1}H NMR measurements

Figures 2.a and 2.b indicate rough similarities for different types of carbon and hydrogen atoms of SS.$FA_{I\text{-}III}$- and SS.$HA_{I\text{-}III}$-fractions isolated parallelly from

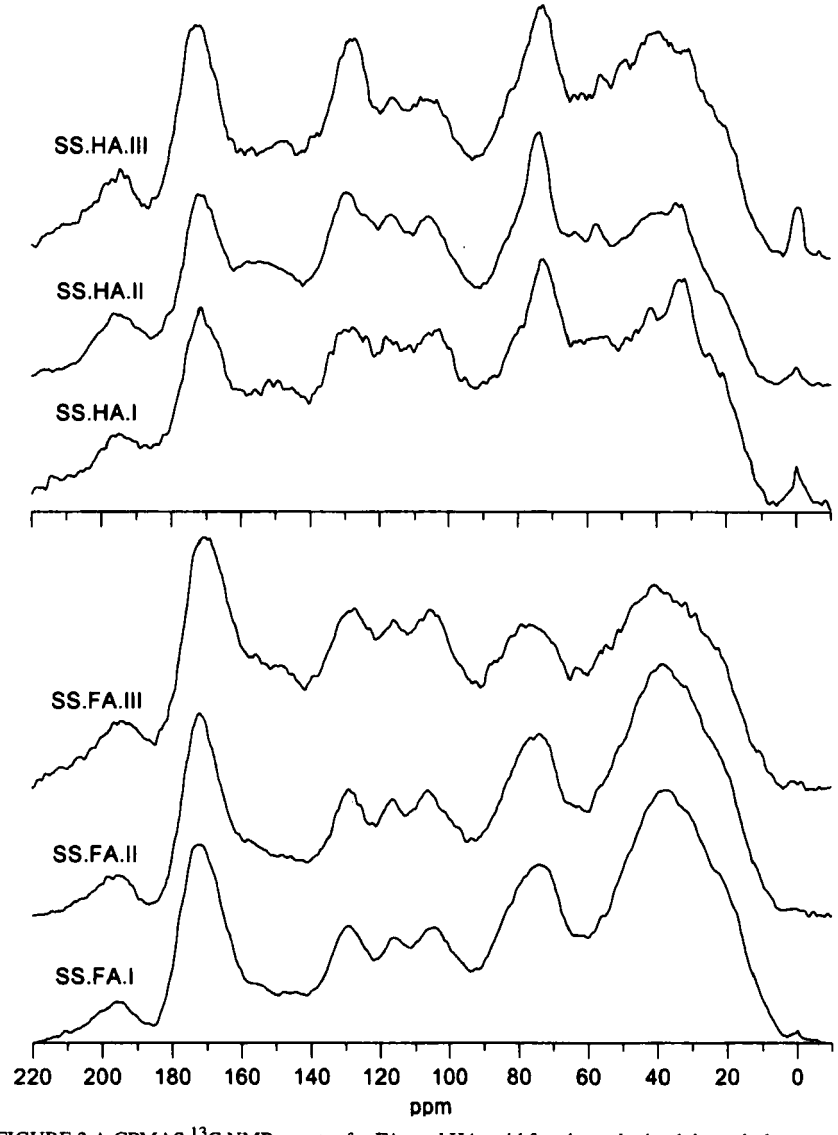


FIGURE 2.A CPMAS ^{13}C NMR spectra for FA- and HA-acid fractions obtained through three parallel (I-III) isolation procedures from the same water sample SS

the same water sample SS. The cross-correlations between ^{13}C NMR spectra (n = 2201 data points, significant at $p < 0.05$) were for FA-type solutes: $SS.FA_I \leftrightarrow S.FA_{II}0.99$, $SS.FA_I \leftrightarrow SS.FA_{III}$ 0.93, $S.FA_{II} \leftrightarrow SS.FA_{III}$ 0.93; and for

HA-type solutes: $SS.HA_I \leftrightarrow SS.HA_{II}$ 0.92, $SS.HA_I \leftrightarrow SS.HA_{III}$ 0.92, $SS.HA_{II} \leftrightarrow SS.HA_{III}$ 0.93. Similarly, ^1H NMR spectra (n = 799 data points, significant at $p < 0.05$) produced the cross-correlations for FA-type solutes: $SS.FA_I \leftrightarrow SS.FA_{II}$ 0.94, $SS.FA_I \leftrightarrow SS.FA_{III}$ 0.94, $SS.FA_{II} \leftrightarrow SS.FA_{III}$ 0.98; and for HA-type solutes: $SS.HA_I \leftrightarrow SS.HA_{II}$ 0.94, $SS.HA_I \leftrightarrow SS.HA_{III}$ 0.90, $SS.HA_{II} \leftrightarrow SS.HA_{III}$ 0.90. The correlations point out that the carbon distribution of the fraction $SS.FA_{III}$ and the hydrogen distribution of the fraction $SS.HA_{III}$ differ somewhat systematically from those of the parallel $SS.FA_{I-II}$- and $SS.HA_{I-II}$-fractions.

For obtaining better visual about the carbon and hydrogen distributions of Figures 2a and 2b, respectively, between the different samples and especially strengthen the resonances, the original irregular, but still definable, baseline of the spectra were mathematically manipulated. The new definable baseline was calculated[58] via the segments assigned to the resonance ranges of the different types of carbons and protons (cf Table II). For the carbon spectra the number of points utilized for creating the new baseline was nine (points at 0, 53, 65, 92, 111, 141, 160, 186 and 220 ppm) and for the hydrogen spectra seven (points at 0.2, 1.4, 2.8, 4.4, 5.2, 5.8 and 8.0 ppm). Figures 3.a and 3.b show the with each other directly proportional subtracted ^{13}C and ^1H NMR spectra, respectively. The resonance strengthened spectra show clearly that the carbon distribution of the SS.FA.III-fraction (Figure 3.a) and hydrogen distribution of the $SS.HA_{III}$-fraction (Figure 3.b) are systematically different from the other $SS.FA_{I-II}$-and $SS.HA_{I-II}$-fractions. On the other hand, the carbon distributions between the all $SS.HA_{I-III}$-fractions (Figure 3.a) and the hydrogen distributions between the all $SS.FA_{I-III}$-fractions (Figure 3.b) are randomly divided (as it actually should be the case for the whole data set). Apparently, there occurs an certain structural inconsistency between the all parallel samples.

The multidimensional data set of the ^{13}C and ^1H NMR spectra obtained for the different parallel samples was examined closer with a statistical-graphical principal components analysis (PCA)[59,60]. The original data set of ^{13}C (2201 data points, interval 0.1 ppm between 0–220 ppm) and ^1H NMR (799 data points, interval 0.01 ppm between 0–8 ppm) spectra were reduced, owing to the computational capacity, to smaller size (^{13}C to 221 data points, interval 1 ppm, and ^1H to 222 data points, interval 0.03 ppm) yielding enough resolving power spectra. It was computed for the ^{13}C and ^1H data sets 221x221 and 222x222 matrixes, respectively, of variances and covariances for the PCA. From these, it has been extracted 221 and 222 eigenvectors and eigenvalues for the ^{13}C and ^1H data sets, respectively. The basic utilization of the PCA is to reduce the numerous variables (resonances) and to seek for linear combinations (scores) of those variables explaining most of the variability.

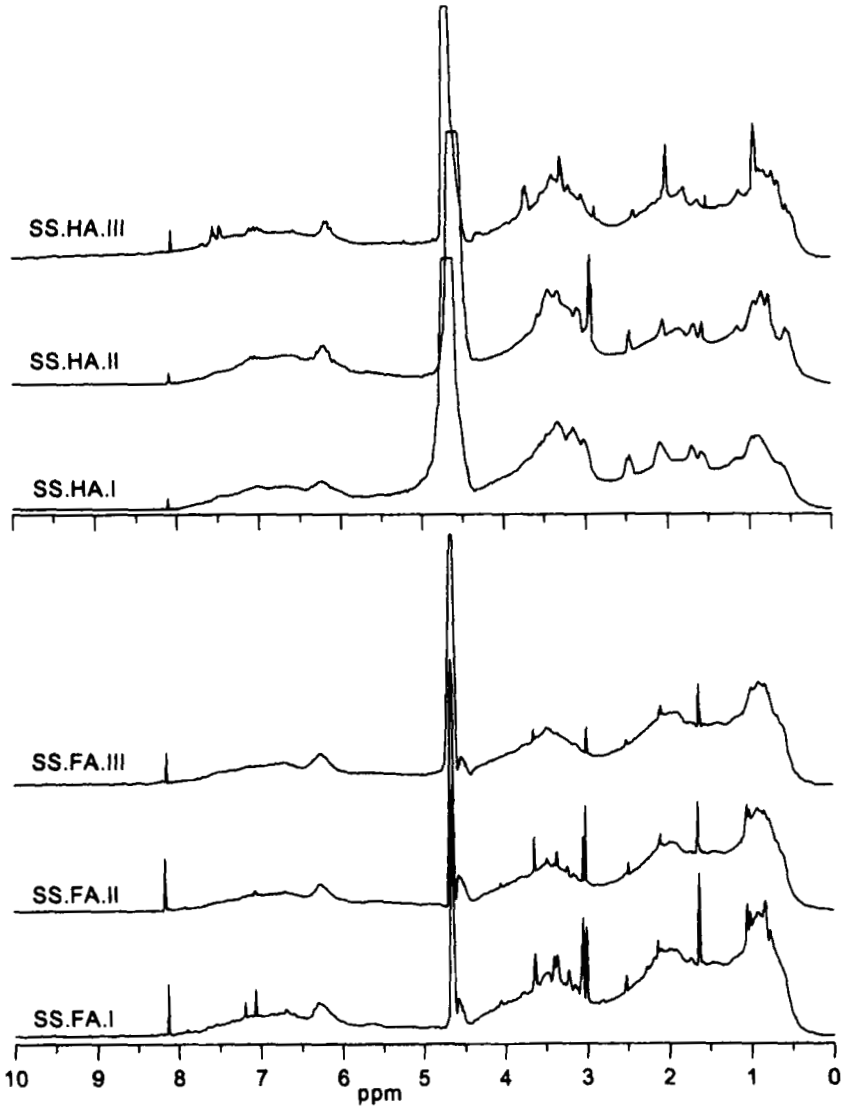


FIGURE 2.B ^{1}H NMR spectra for FA- and HA-acid fractions obtained through three parallel (I-III) isolation procedures from the same water sample SS

Figure 4.a shows the 3D scatterplot of principal component scores of the ^{13}C NMR data set ($SS.FA_{I-III}$ + $SS.HA_{I-III}$) plotted on first three principal components, level of statement 81% for the first three PC. The $SS.FA_{I-III}$- and $SS.HA_{I-III}$-fractions were divided on the PC1 (49% of the total variance) into

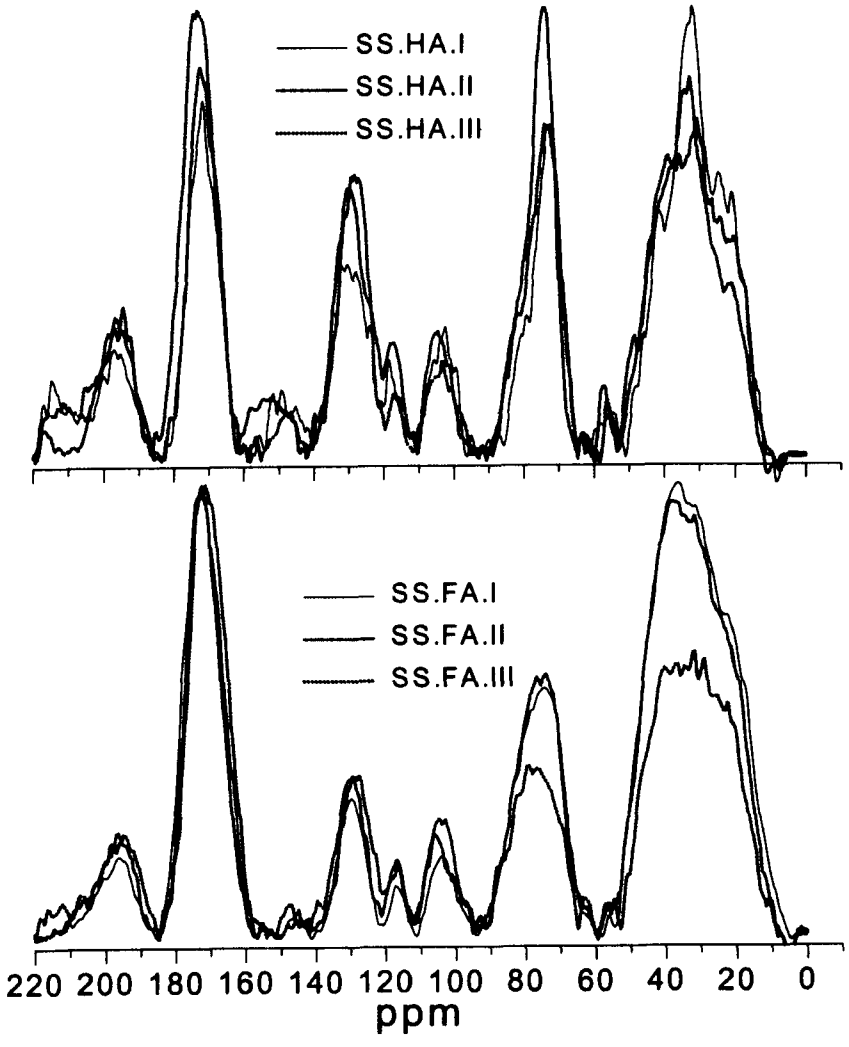


FIGURE 3.A Baseline corrected (number of points = 9) CPMAS ^{13}C NMR spectra for FA- and HA-acid fractions obtained through three parallel (I-III) isolation procedures from the same water sample SS

three main clusters based on their carbon compositions. The distribution of different kinds of carbons between the $SS.FA_I$- and $SS.FA_{II}$-fractions was extremely homogeneous also both on the PC2 and PC3, 18 and 14% of the total variance, respectively. The all $SS.HA_{I-III}$-fractions associated quite loosely on the PC2 indicating, however, a rough conformity in their carbon skeletons.

Whereas, the carbon distribution of the $SS.FA_{III}$-fraction formed its detached cluster resembling somewhat more that of HA-type solutes than other FA-type solutes.

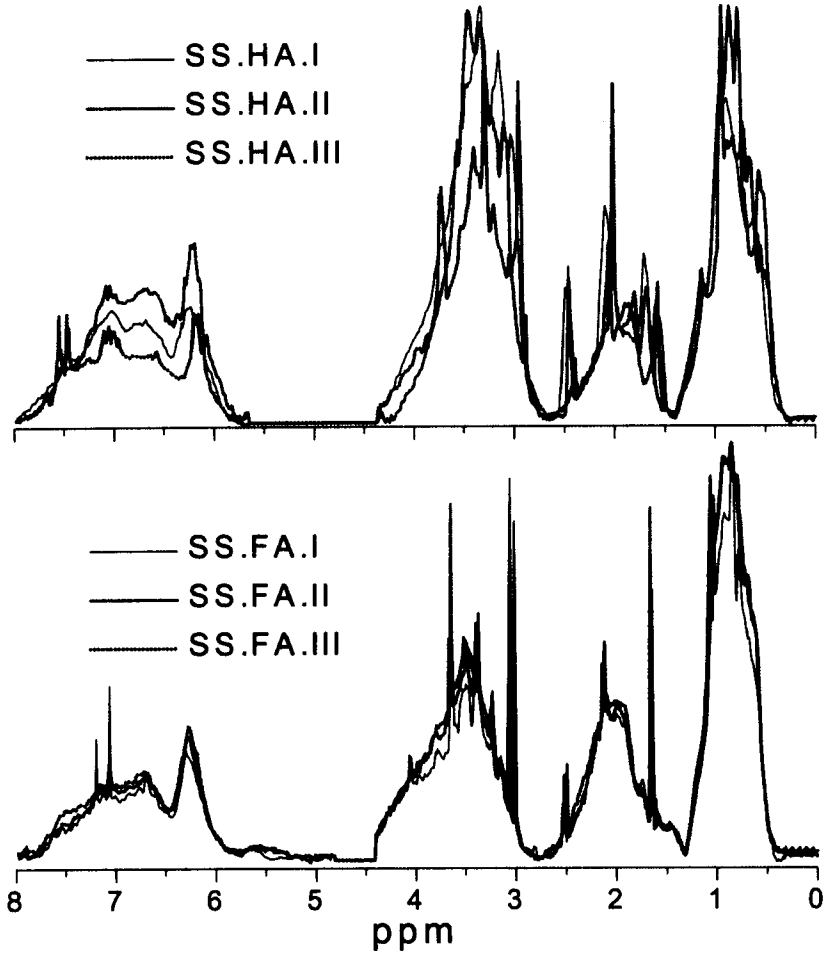


FIGURE 3.B Baseline corrected (number of points = 7) ^1H NMR spectra for FA- and HA-acid fractions obtained through three parallel (I-III) isolation procedures from the same water sample SS. The HDO resonances are omitted

The critical examination of the eigenvectors (loadings) computed for Figure 4a indicated that richer content of aliphatic carbons, carbons of C-O (e.g. carbohydrate compounds) and carboxylic carbons discriminate on the PC1 the SS.FA-type solutes from the SS.HA-type solutes. More closely, resonances

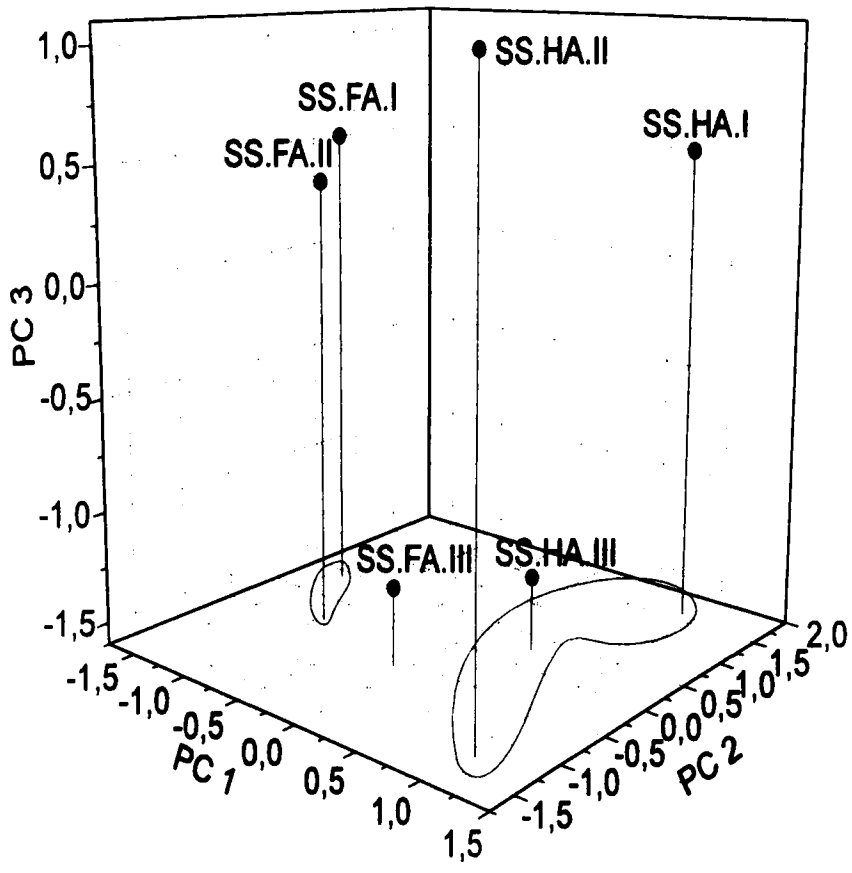


FIGURE 4.A 3D score plot of projections of ^{13}C NMR data onto their first three principal components

between 54–62, 69–78, 94–96, 98–110, 112–160 and 184–220 ppm of ^{13}C NMR spectra were more dominant for the HA-type than for the FA-type fractions. There did not occur any specific carbon resonance that would be a discriminating factor between the $SS.HA_{I-III}$-fractions on the PC2 but the carbon distribution (0–220 ppm) between the $SS.HA_{I-III}$-fractions was randomly divided. The deviation of the carbon distribution on the PC3 for the $SS.FA_{III}$- and $SS.HA_{III}$-fractions compared with the other corresponding parallel fractions was mainly caused by resonances of carbons between 190–220 (carbonyl carbons of aldehydes and ketones), 160–190 (carboxylic carbons) and 110–140 ppm (unsubstituted and alkyl-substituted aromatic carbons), also some resonances of carbons between 50–60, 60–90, 90–110 and 140–160 ppm gave a slight effect to this deviation.

Figure 4.b shows the 3D scatterplot of principal component scores of the ^1H NMR data set ($SS.FA_{I-III}$ + $SS.HA_{I-III}$) plotted on first three principal components, level of statement 89% for the first three PC. The $SS.FA_{I-III}$- and $SS.HA_{I-III}$-fractions were also divided on the PC1 (53% of the total variance) into three main clusters based on their hydrogen distributions. The all $SS.FA_{I-III}$-fractions associated extremely close both on the PC2 and PC3 (20 and 16% of the total variance, respectively) indicating practically homogeneous structural hydrogen distribution. The hydrogen distribution of the $SS.HA_I$-and $SS.HA_{II}$-fractions resembles each other on the PC2 but not on the PC3 reflecting, however, a given basic conformity in their structural compositions. Whereas, the hydrogen distribution of the $SS.HA_{III}$-fraction was totally different from that of other SS.HA- or SS.FA-fractions, indicating irregular structural composition.

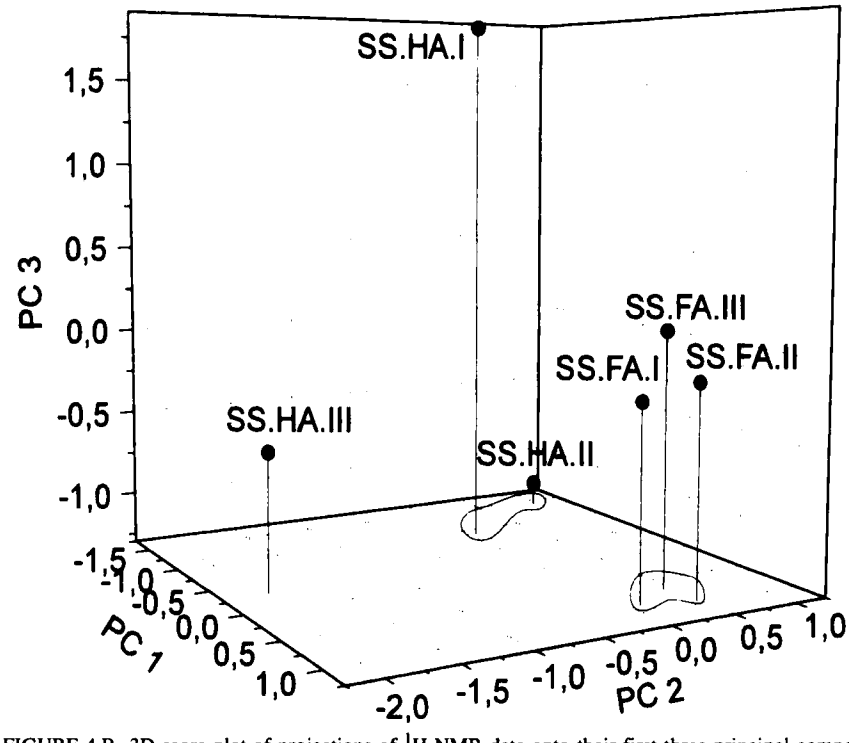


FIGURE 4.B 3D score plot of projections of ^1H NMR data onto their first three principal components

The partition into separate SS.FA- and SS.HA-type fractions based on the resonances of different non-exchangeable hydrogens occurred also on the PC1. Eigenvector1 demonstrated that the richer content of aliphatic hydrogens (both

H_{A1}- and H_{A2}-type hydrogens) was, on the whole, characteristic for SS.FA-type solutes, while $H_{R\text{-}O}$- and H_{Ar}-type hydrogens dominated SS.HA-solutes. Resonances between 5.8–7.9 and 2.7–3.8 ppm of ^1H NMR spectra were unambiguously stronger for the SS.HA-type solutes on the PC1. On the contrary, the situation in the case of aliphatic hydrogens (within ca. 0–2.8 ppm) was not so straightforward. There was some specific resonances of aliphatic hydrogens that were on the PC1 more dominate for the SS.HA- than SS.FA-solutes, namely resonances between 0.3–0.6, 1.1–1.4, 1.5–1.6, 1.7–1.9 and 2.4–2.5 ppm. The deviation of different types of hydrogens on the PC2 between the all $SS.FA_{I\text{-}III}$-fractions was very close to zero indicating practically homogeneous structural composition. The alignment of the $SS.HA_{III}$-fraction on the PC2 into detached cluster was caused by the whole resonance range of the ^1H NMR spectrum, especially resonances at 7.56, 7.47, 2.70–2.88, 2.01–2.04, 1.80–1.83 and 0.66 ppm differed mostly from those of other SS.HA-fractions. The deviation of the $SS.HA_I$-fraction on the PC3 from the other SS.HA-fractions was caused by unexplained resonances within the whole range of the ^1H NMR spectrum.

According to the statistical-graphical approach and to the relative abundances of different types of carbons and hydrogens calculated in Table II for the $SS.FA_{I\text{-}III}$- and $SS.HA_{I\text{-}III}$-fractions, it is obvious that the XAD isolation-fractionation procedure is not able to produce structurally identical parallel FA-or HA-type solute fractions. The structural composition for third part of the parallel isolates was, especially in the case of FA-type solutes, definitely different. It was possible to obtain two thirds of FA-type solute fractions within the confidence interval of 97%. Whereas, the probability to get two representative parallel HA-type isolates is certainly much accidental.

It has been strongly proposed[57,61–72] that a micelle-like or aggregate model most probably describes the physical-chemical behaviour of humic matter in aqueous solutions and that hydrophobic bondings are largely responsible for the apparent macromolecular configuration. Although, the pseudo-micellar model for the conformational nature of aquatic humic matter is not, particularly at the natural concentrations, definitely acknowledged[73–77], the hydrophobic interactions are very relevant in controlling the conformation of humic matter in solution. It has been asserted[72] that when negatively charged, fully expanded filamentous-like humic matter, is treated with mineral acids (such as HCl) the molecular configuration changes into, e.g. a spheroidal shape due to the establishment of inter- and intramolecular H-bondings upon protonation of the functional groups.

It has been reported[31,32] previously that the isolation-fractionation of aquatic humic-solute mixtures with the XAD procedure appears to occur with nearly the same mechanism independent of the molecular size or the degree of polydisper-

sity of original solutes. Furthermore, the isolated humic solutes, especially the unseparated mixture of hydrophobic acids (FA+HA), must play a role as certain definite entities in the DOM and they cannot be merely accidental products of the isolation process. Humic and fulvic acids, or their aggregated combination, are substances of different solubility properties in terms of acidity. Therefore, it can be generally supposed that there is no possibility to find two humic molecules exactly the same, i.e. this should not be a limitation of the XAD procedure but the sample.

The basic nature of the DOM is extremely labile and prone to structural alteration during any isolation procedure. Such contributory factors as autohydrolysis, decarboxylation, co-precipitation, intramolecular entrapment of low-molecular weight compounds, concentration dependant formation of metall adducts, etc. are sources of variability. These on contact time, temperature, stirring, concentration, drying method, etc. depending processes, in addition to the regeneration of the sorbent, were kept as constant as possible during the each parallel XAD isolation procedure carried out. Therefore, it would be expected that the structural composition of the parallel humic isolates obtained from the same homogeneous original water sample is practically uniform with each other. However, the truthful situation, as reported here, was not so self-evident. According to the present results and that of stated[31,32] previously, the most critical operation of the XAD procedure is the phase of the fulvic/humic acid separation at very strongly acidic conditions. The two organic constituent fractions (HA and FA) obtained as final outcome may randomly contain more or less certain structural subunits traditionally fixed on as a unambiguous parting factor. This limitation is not solely a consequence of the technique itself but also an evidence of the highly sensitive nature of the aquatic humic solutes.

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