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Review

Separation methods in the chemistry of humic substances

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Abstract

Separation methods are widely used to isolate humic substances (HSs), to fractionate them before further investigation, and to obtain information about their structure and properties. Among the chromatographic methods, techniques based on a size-exclusion effect appear to be most useful, as they allow us to relate elution data to the molecular mass distribution of HSs. The limitations of this approach are discussed in this review. Gas chromatography with mass spectrometric detection is typically used to identify the products of pyrolysis or thermochemolysis of HSs; this technique is considered most important in the structural investigation of HSs. Electrophoretic methods (especially capillary zone electrophoresis) provide detailed characterization of HSs, but it is very difficult to relate the electrophoretic data to any specific subfraction, structure or properties of HSs. The electrophoretic patterns are often called ''fingerprints'' and can potentially be used for the identification and classification of HSs. This is limited, however, by the great diversity of the procedures employed and by the low degree of harmonization—no data on reproducibility and between-laboratory comparability are available. The same holds true, to a certain degree, for most methods utilized for the characterization of HSs. Separation methods play an important role in the examination of the interactions of HSs with heavy metals and other chemical pollutants. They allow us to determine binding constants and other data necessary to predict the mobility of chemical pollutants in the environment.

Keywords: Reviews; Humic substances; Humic acids; Fulvic acids

Contents

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1. Introduction

Humic substances (HSs) are ubiquitous natural materials occurring in huge amounts in soils, sediments and waters as a product of the chemical and biological transformation of animal and plant residues. A substantial proportion of carbon-containing substances in the environment can be referred to as HSs—it is estimated that HSs form 50–90% of dissolved organic carbon (DOC) in freshwater systems [1]. Because of their ability to interact with various components of the environment, HSs play an important role in soil and aquatic chemistry and therefore have attracted the attention of researchers. Despite great effort, resulting in an enormous number of published papers, the structure and function of HSs are not well understood. Many of the published papers begin with a statement such as ‘‘ . . . HSs are complex macromolecular substances containing a variety of building blocks and various functional groups, the structure and behavior of which in the environment remain unclear’’. It is a little frustrating that a great number of these papers also end with a similar, somewhat vague and pessimistic statement.

In general, HSs are amorphous, brown or black, acidic and polydisperse, and they have molecular masses in the range from several hundreds to tens of thousands. They may be considered as consisting of substituted aromatic rings linked together by aliphatic chains. However, there is uncertainty as to whether HSs are truly polymeric, i.e. show a regular repetition of simpler structural units [2]. Various simple organic compounds are considered to be building blocks, from which the complex structure of HSs is composed, e.g. salicylic acid, phthalic acid and others (more than 50 of these building blocks are listed in Ref. [2]). The secondary structure of HSs was modelled computationally from the primary building blocks (simple organic acids) using spectroscopic measurements (circular dichroism, NMR) to confirm the proposed structure [3]. Structural concepts for HSs can be found in many reports [4–7].

A new model of the polymeric structure of HSs was suggested by Piccolo and co-workers [8–10]. They deduced, mainly from size-exclusion chromatographic measurements, that relatively small and heterogeneous humic molecules are self-assembled in supramolecular conformations stabilized only by weak forces, such as dispersive interactions and hydrogen bonding. The basic characteristics of HSs together with their properties and reactions are well described by Stevenson [11]. Among the many other monographs and reviews, Refs. [1,12,13] are mentioned.

The recent large-scale production of HSs from low-rank coals with various applications in agriculture, wastewater treatment, soil remediation, the paper and plastics industry, the building industry, and even in cosmetics and medicine, calls for a more precise and detailed description of the properties of HSs. Separation techniques, chromatographic and electrophoretic in the first place, are widely utilized to isolate and fractionate HSs before further characterization, to obtain structural characteristics of HSs from retention and migration data, to investigate interactions of HSs with environmental pollutants, etc. In this article, the role of separation methods in the chemistry of HSs is reviewed with emphasis on chromatographic and electrophoretic methods. Conventional separation schemes are also mentioned briefly, as well as other less commonly employed techniques, such as field-flow fractionation and ultrafiltration.

2. Isolation and fractionation, conventional separation schemes

Commonly, HSs are operationally subdivided according to their solubility into humic acids (HAs) and fulvic acids (FAs). HAs comprise high-molecular-mass organic substances that are soluble in alkaline media (e.g. in 0.1 mol/l NaOH) and insoluble in acidic media (at pH 1–2), whereas FAs comprise moderate-molecular-mass organic substances of non-specific composition that are soluble at all pH values. The portion of organic matter present in soils and sediments that is insoluble at any pH value is called humin [1,2,6,11–13]. HSs can be isolated as a group from aqueous solution by extraction into non-polar solvents after acidification to a pH of ca. 2 (to suppress dissociation of acidic functional groups in HSs), or, more typically, by sorption on non-ionic sorbents. Liquid–liquid extraction, or more recently solid-phase extraction, are

used to determine the total content of HSs in waters [14].

Strongly alkaline extraction agents, typically aqueous solutions of NaOH [15–17], are used for the isolation of HSs from soils and sediments, as well as from coals and peat. Sometimes, alkaline pyrophosphate or a mixture of pyrophosphate and NaOH are employed [16,18–20]. However, it has been hypothesized that the nature of HSs may be changed during extraction with pyrophosphate, and therefore NaOH is recommended, especially for the extraction of HSs from peat [16].

The International Humic Substances Society (IHSS) has published a general procedure for the fractionation and isolation of HSs. The basic and simplified separation scheme is presented in Fig. 1. Preparations of pure HAs and FAs require additional refining steps, such as re-precipitation and HCl/HF treatment for the removal of inorganic impurities, as described in detail in Ref. [15].

HSs from aqueous solutions, such as natural waters, are isolated using a standardized XAD procedure, which consists of the sorption of HSs (HAs+FAs) from acidified samples onto non-ionic macroporous sorbents—XAD (Fig. 2). XAD are styrene–divinylbenzene or methyl methacrylate polymers with various hydrophobicities and cross-linkages. Some of them exhibit size-exclusion effects. A comparison of various XAD sorbents and limitations of the general XAD procedure are given by Town and Powell [22]. The sorbent XAD-8 has been used most frequently for the isolation and purification of HSs [23–28] (the original procedure is described in Ref. [23]), whereas other XAD resins, such as XAD-1, XAD-2 [22,29–31], XAD-4 [22] and XAD-7

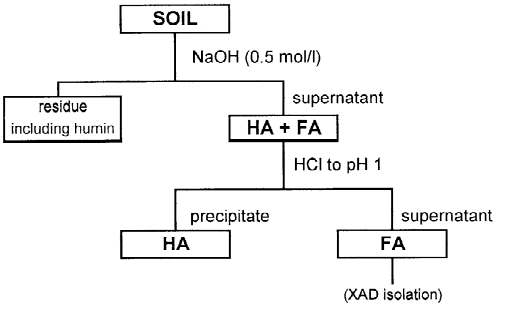

Fig. 1. Separation scheme for soil HSs (adapted from Ref. [15]).

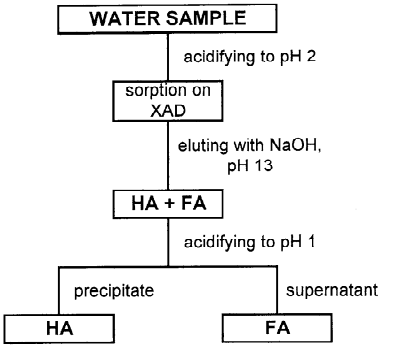

Fig. 2. Isolation of HSs from water samples (adapted from Ref. [21]).

[22,32], are used less often. A more subtle fractionation of HSs can be achieved with the aid of XAD resins using a gradient elution. HSs are desorbed from the resin with an eluent, the pH of which changes (increases) continuously [30,33,34]. This fractionation employs a wide range of acid–base equilibria, allowed by the presence of various functional groups in the HS molecule. As the pH value of the eluent increases, compounds having progressively higher pK_a values are ionized and thus desorbed. Curtis et al. [35] attempted to characterize synthetic and commercial HAs by their elution profile using a nearly linear pH gradient elution of pre-sorbed HAs from an XAD-8 column. Gradient elution has also been used in immobilized metal ion affinity chromatography for the fractionation and characterization of FAs [36].

In addition to the standard XAD procedure, methods employing other sorbents have also been tested. Sorption on a weak anion exchanger, such as diethylaminoethyl (DEAE)-cellulose, appears to be a convenient method for the isolation of HSs from large volumes of water [37,38]. This method has several advantages over methods with macroporous resins, i.e. the sorption is more rapid allowing higher flow-rates, and it does not require pre-acidification of the water sample with the concomitant possibility of sample alteration. The XAD and DEAE procedures have been compared in several papers [38–40]. In general, DEAE isolates and the main XAD fractions consist of almost identical organic compounds [40]. According to ^{13}C and ^{1}H NMR spectra, the structural composition of acidic humic solutes obtained by the

DEAE procedure appears to be a combination of certain hydrophobic and hydrophilic acidic solutes obtained by the multi-stage XAD procedure [39]. The DEAE procedure seems to be more effective for the adsorption of aquatic HSs, but only 76% of the adsorbed HSs were recovered by elution with 0.1 mol/l NaOH [38]. The DEAE-cellulose column has also been used for the pre-treatment of samples before direct chromatographic determination of HSs in environmental waters [41].

Differences in the molecular sizes of HSs can be utilized for their fractionation. For example, ultrafiltration with a series of membranes allows the division of HSs into five fractions of different molecular sizes (M_r 1000–10 000, 10 000–50 000, 50 000–100 000, 100 000–300 000, and >300 000), which differ somewhat in the content of functional groups, aromaticity, etc. [42]. It was demonstrated by NMR that the fractions obtained by ultrafiltration differ not only in the degree of aggregation, but also in primary structure [27]. Membrane separation has been used in combination with ultracentrifugation for the fractionation of soil HSs according to their molecular sizes [25].

Quite different fractionation schemes are based on aqueous two-phase systems, such as the dextran–poly(ethylene glycol) (PEG) system [43,44]. The fractionation is based on differences in the hydrophobic/hydrophilic properties of the investigated HSs and can be used for the classification of HSs of different origins by their relative hydrophobicity [44].

3. Liquid chromatography, size-exclusion/gel permeation chromatography

Reversed-phase liquid chromatography can be used to determine the total content of HSs in various samples, in addition to the standardized liquid–liquid extraction method mentioned above. It should be noted, however, that these determinations are not required very often—usually, more general parameters, such as total or dissolved organic carbon (TOC, DOC), or chemical oxygen demand (COD), are used to quantify the amount of organic matter, including HSs. Silica-based column packings with various chemically bonded stationary phases, including the common octadecyl-bonded phase, have been tested for the determination of HSs in environmental samples [45]. Detection limits at the nanogram level were achieved for the determination of HSs in coral skeletal matter, sea water, river water, soils and plants with fluorescence detection. The method was modified for the determination of HSs in aluminium-containing solutions from the Bayer process [46]. Fluorescence detection has also been used for the simultaneous determination of the concentration and molecular mass of HSs in river waters [41]. The chromatographic behavior of HSs in reversed-phase systems was studied by Hayase and Tsubota [47], and relations between hydrophobicity, molecular mass and retention were found for sedimentary FAs. Recent studies [48] have demonstrated that the chromatographic behavior of HSs (retention, fractionation) depends significantly on the amount of sample injected.

Degradation products resulting from the oxidation of HSs and lignin have been determined by reversed-phase HPLC in waters and in effluent from a kraft pulp mill [49], and in alcoholic beverages [50].

In general, reversed-phase systems do not allow an effective fractionation of HSs and the chromatograms do not give any useful information about the nature or structure of the analyzed substances [51], although certain structural features can be identified when chromatographic separation is combined with more sophisticated detection methods, such as diode-array detection [52].

Probably, the most important liquid chromatographic methods employed in the chemistry of HSs are those based on a size-exclusion effect—gel permeation/size-exclusion chromatography (GPC/SEC). Since the pioneering works [53,54] in the 1960s, GPC and SEC have frequently been used, especially for the characterization of aquatic [55–57] and soil [58–61] HSs (determination of molecular mass), or for the fractionation of HSs before further investigation [62].

Determination of the molecular mass distribution (MWD) by GPC/SEC methods is based on the key presumption that the size-exclusion effect is solely responsible for the fractionation (separation). Smaller molecules penetrate more deeply into the pores of the stationary phase and thus their pathway is longer, and this results in a longer retention time (greater

elution volume). Larger molecules, on the other hand, cannot penetrate into the stationary phase pores and they reach the end of the chromatographic column in a shorter time (with a smaller elution volume). It is evident that the separation is based rather on differences in molecular *size* (hydrodynamic size, effective diameter) than on differences in molecular *mass*. Although the term ''molecular mass (weight) distribution'' is widely used in the literature (also in this article), the term ''molecular-size distribution'' would be more correct for the values determined by GPC/SEC. This subtle difference is not very significant for substances with rigid molecules, but is crucially important for HSs with their complex three-dimensional and flexible structure.

The size of a HS molecule is naturally related to its mass, but it is strongly affected by many other factors. For example, numerous functional groups in the HS molecule are dissociated/protonated dependening on the pH. The dissociated functional groups carry negative charges. Electrostatic repulsion between neighboring negatively charged sites causes stretching (uncoiling) of the molecule. Moreover, the electrostatic forces are influenced by ionic strength, by the presence of cationic species, ion-pairing agents, etc. As a result, the same molecule with a certain molecular mass may have different sizes depending on the surrounding medium, and hence also on the GPC/SEC experimental conditions. Therefore, GPC/SEC methods are not able to determine ''true values'' of the molecular masses of HSs. The molecular masses of HSs measured by GPC/SEC are operationally defined and should rather be called ''apparent molecular masses''.

The size-exclusion chromatographic process is further complicated by the active nature of HSs. Non-size-exclusion effects, which can lead to serious misinterpretation of GPC/SEC measurements, can be subdivided into two main categories: the first caused by coulombic forces (ion-exchange or ion-exclusion interactions between the solute and stationary phase) and the second by adsorption or reversed-phase partitioning [63,64]. The most commonly used stationary phases in conventional (low-pressure) GPC are Sephadex gels. Non-size-exclusion effects on Sephadex gels, which are particularly marked when distilled water is used as eluant [64], are eliminated by the proper selection of the mobile phase composition. Borate buffer or alkaline buffers containing large amino cations are recommended as the main constituents of the mobile phase [64,65]. Possible interferences from metal cations in real samples are minimized by the addition of pyrophosphate to the mobile phase [66]. Elevated ionic strength further reduces undesirable electrostatic interactions [63,64].

Typical GPC elution curves for peat- and brown-coal-derived HSs obtained with a Sephadex column are shown in Fig. 3a. Elution curves for the main fractions from membrane separation of brown-coal HSs are shown in Fig. 3b.

To obtain values of molecular masses or MWDs from GPC/SEC elution curves, the chromatographic column has to be calibrated by macromolecular substances with known molecular masses. Globular proteins [55,56,63] or polysaccharides [64,65] are used most often. Polystyrene sulfonates have been used as calibrating standards for the determination of molecular masses using SEC and other fractionation methods [67]. Since HSs have a markedly different

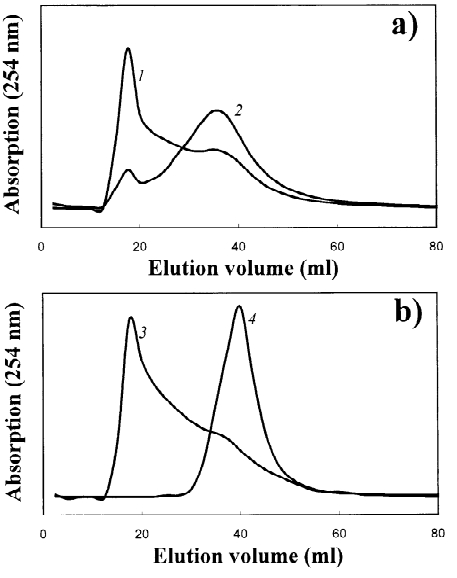

Fig. 3. GPC elution curves for HSs. Column, 40×0.9 cm packed with Sepharon G 100; mobile phase, 25 mmol/l $Na_4B_4O_7$ + 10 mmol/l NaCl + 1 mmol/l $Na_4P_2O_7$; UV detection at 254 nm. 1 = Peat-derived HSs, 2 = brown-coal-derived HSs, 3 = high-molecular-mass concentrate from membrane separation, 4 = low-molecular-mass permeate from membrane separation.

constitution from these calibration species, HA samples with a low polydispersity and a known molecular mass determined by ultracentrifugation were suggested for calibration [68]. This approach, however, does not seem to be practicable for routine measurements.

UV–Vis spectrometry remains the most important detection method in the GPC/SEC of HSs because of its simplicity and sensitivity. In the simplest arrangement, HSs are detected at a single wavelength in the eluate from the column, usually in the UV region. Useful additional information on the structure of HSs fractionated by GPC/SEC can be obtained by recording the whole UV–Vis spectra of individual fractions, or using diode-array or fast-scanning online detection in more sophisticated arrangements. The UV absorbance of the eluate from the column (the single-wavelength detector response) is proportional to the concentrations and molar absorptivities of the chromophores active at the detection wavelength. The detector response is proportional to the mass concentration of HSs (at least to a first approximation) rather than to the molar concentration of HSs. Because the spectra of fractions with different molecular masses are not entirely uniform, the shapes of the GPC/SEC elution curves recorded at different wavelengths will not be identical. Hence, the apparent MWD may be affected to a certain degree by selection of the detection wavelength. It has been shown [69,70] that the average molecular masses apparently increase with increasing wavelength. Within the range of wavelengths commonly used for the determination of the MWDs of HSs by GPC/SEC (i.e. 220–280 nm), the magnitude of the increase is not significant, as can be seen from the results obtained by Zhou et al. [69], and O'Loughlin and Chin [70].

In addition to UV–Vis detection, fluorescence detection has frequently been used to follow the GPC/SEC fractionation of HSs. A comparison of GPC chromatograms with UV, fluorescence and DOC (measurement of dissolved organic carbon) detection can be found in Ref. [71]. Both UV and DOC detection methods have been used to characterize natural organic matter (NOM) with the aid of SEC [72]. The general shapes of the MWD curves determined by each method were similar. However, it was found that UV detection overestimates the carbon content of the larger-size fractions and underestimates the smaller-size fractions.

Despite the problems associated with separation and calibration discussed above, the GPC/SEC elution curves contain comprehensive and valuable information about the MWDs of HSs. A simple visual observation of the elution curves is probably the best way for experienced chromatographers to obtain an overall picture of the character of the HSs, to estimate some average values and polydispersity, and to compare various samples. On the basis of the known MWD, the average molecular masses can be calculated according to the following general formula [73]:

$$\overline{M} = \frac{\sum N_i M_i^{r-1}}{\sum N_i M_i^{r}} = \frac{\sum W_i M_i^{r}}{\sum W_i M_i^{r-1}} \quad (1)$$

where W_i is the total mass of molecules with molecular mass M_i, and N_i is the number of molecules with molecular mass M_i; i is incrementing over all molecular masses in the sample. For $r = 0$, the respective value of the average molecular mass is called the number-average molecular mass, M_n. For $r = 1$, the respective value of the average molecular mass is called the weight-average molecular mass, M_w. M_n and M_w are the most common parameters employed to characterize polymeric substances [74]. Several other descriptors (average molecular masses and their ratios) can be found in Ref. [73]. The ratio M_w/M_n is a measure of the breadth of the molecular mass distribution, and is equal to unity for monodisperse systems.

The advantages and limitations of GPC/SEC methods are discussed in detail in Ref. [75], where the main sources of uncertainty are also identified.

More recently, chromatographic columns packed with soft gels, such as Sephadex, have been replaced by more rigid column packings compatible with HPLC instrumentation. Probably, the first paper reporting the high-performance GPC of HSs was published by Saito and Hayano [76]. The column was packed with a rigid spherical silica gel (TSK-SW). Silica-based columns are frequently used for the high-performance GPC/SEC fractionation of HSs [31,55,56,61,77,78]. Hydrophilic phases, such as polyether [77] or glyceryl propyl [63] moieties, are usually bonded to the silica support [61]. Cross-

linked polymethacrylate [16] or a hydrophilic vinyl polymer with surface hydroxyl and ether groups [57] have also been used for the determination of the molecular masses of HSs. Various high-performance GPC/SEC columns are compared in Refs. [61,79].

Although the separation efficiency is enhanced significantly in the high-performance mode of GPC/SEC, there are still problems associated with the calibration of the columns and the evaluation of the elution curves. In principle, they are the same as discussed above when the method is used for the determination of the molecular masses of HSs. Recently, the introduction of more sophisticated detection methods utilizing multi-angle (laser) light scattering (MALS) opened up new perspectives in the measurement of the MWDs of polymeric substances and allowed us to overcome some problems associated with calibration [80]. In MALS, the intensity of the scattered laser light emitted by sample molecules is measured at several different angles simultaneously and molecular masses (sizes, radii) are calculated by extrapolating to zero angle. The principle and measurement procedures are described in detail elsewhere [80–82]. In combination with SEC, MALS detection enables direct determinations of molecular sizes without calibration of the SEC column. SEC–MALS has been used to characterize loam HAs fractionated by preparatory-scale SEC [81]. MWDs of lignosulfonates were determined by SEC–MALS using a polymer-based column [82]. Various SEC columns (silica- and polymer-based) and various detection methods, including MALS, have been compared for the determination of the molecular masses and radii of aquatic HSs [83].

Some examples of the application of GPC/SEC methods to the investigation of HSs and related materials are listed in Table 1.

4. Electrophoretic methods

Techniques based on the use of the migration of electrically charged particles or ions in solution due to an applied electric field between an anode and a cathode (electrophoretic methods) have traditionally been extensively employed in investigations of natural polymeric substances. Although the principles of electrophoretic methods have been known for a long time, applications increased rapidly after the introduction of paper and gel electrophoresis in the 1950s. In 1989, Vesterberg [97], in an excellent retrospective review, called the gradual progress in applications of electrophoretic methods during the preceding 30 years a ''virtual explosion''. From today's viewpoint, this appears to be somewhat inadequate—the real explosion in electrophoresis started just a few years after the publication of that review with the introduction of electrophoretic separations in narrow capillaries (capillary electrophoresis, CE).

Many interesting papers were published on the electrophoretic separation and fractionation of HSs in the ''pre-CE period''. Separations were carried out in various supporting media, such as paper or gel, either in single-buffer solutions (zone electrophoresis), or in a more complex arrangement of discontinuous buffers (disc electrophoresis, isotachophoresis). Isotachophoresis and isoelectric focusing of soil HSs in a polyacrylamide gel are compared in Ref. [98]. Similar to the previous separations by disc electrophoresis [99], several subfractions (bands) were obtained during isotachophoretic separation—typically 10 for FAs and 13 for HAs. Amino acids and organic acids were used as spacers in gel isotachophoresis. HAs from Columbian soils were fractionated by GPC on Sephadex and the individual fractions were further characterized by disc electrophoresis and infrared spectroscopy [100]. Whereas paper electrophoresis was used only exceptionally for the separations of HSs [101], gel electrophoretic methods became rather popular and are still utilized for the separation and characterization of HSs [102–105]. Trubetskoj et al. [60,89,90] used polyacrylamide gel electrophoresis (PAGE) for checking the purity of HA fractions obtained by SEC or ultrafiltration. The electrophoretic behavior of HSs in physical gels (in capillaries filled with long-chain polyethylene glycols) was studied in Ref. [105] and the relations between electrophoretic mobility and molecular mass were determined. Very complex isotachopherograms, consisting of as many as 18 zones, were obtained by capillary (free-solution) isotachophoresis of HSs with chloride and caproate as leading and terminating ions, respectively [106]. It is, however, highly probable that most of the observed zones were mixed zones, which can hardly be

Table 1
Separation and characterization of HSs with the aid of GPC/SEC methods

Analyzed material	Column packing	Mobile phase	Detection	Aim of the study	Ref.
Commercial HAs, soil HAs and FAs	Sephadex G-50	1 m*M* phosphate buffer, pH 7–9	UV 230 nm	Measurement of MWD	[86]
Reference soil HAs, FAs	Sephadex G-100	10 m*M* $Na_4B_4O_7$ + 1 m*M* $Na_4P_2O_7$, pH 9.2	UV 280 nm	Measurement of MWD	[66]
Coal HAs and their derivatives	Sephadex G-100	25 m*M* $Na_4B_4O_7$ + 1 m*M* $Na_4P_2O_7$	UV 255 nm	Determination of M_w, M_n, M_w/M_n; study of thermal stability	[17,87]
Acid-hydrolyzed HSs	Sephadex G-10	Water	RI		[88]
Peat HSs	Sephadex G-50	Water	Measurement of COD	Comparison of isolation procedures (XAD vs. DEAE)	[38]
HSs in environmental waters	Agarose gel	10 m*M* NaOH	UV 280 nm, FL	Direct determination of M_r	[41]
UF fractionated HSs			UV 254 nm	Measurement of average M_r	[62]
Aquatic HAs and FAs	Sephadex G-25, G-75	10 m*M* Tris–HCl + 100 m*M* Na_2SO_4, pH 8.0	ICP-MS	Study of methylmercury binding to HAs and FAs	[24]
Soil HAs	Sephadex G-75	0.1 *M* Tris–HCl, pH 9, or 7 *M* urea	UV 280 nm	Comparison of fractionation techniques (UF, PAGE)	[60,89,90]
Peat and soil HSs	Sephadex G-75, G-100	25 m*M* Tris–HCl + 50 m*M* NaCl + 0.1% SDS	UV 280 nm	Determination of M_r of fractionated HSs	[44,91]
Lignite HAs	Sephadex G-50	50 m*M* Tris–HCl, pH 9.0	UV 280 nm	Determination of M_n	[92]
UF fractionated HAs	Polymethacrylate	1.65 m*M* $Na_4B_4O_7$	UV 262 nm	Determination of M_w, M_n	[16]
Organic colloids, HSs	Modified silica	4 m*M* phosphate buffer + NaCl, pH 6.9	UV	Determination of M_w, M_n, M_w/M_n	[55]
Commercial HAs, unfractionated organic matter	Modified silica	0.1 *M* NaCl + phosphate buffer, pH 6.8	UV 224 nm	Determination of M_w, M_n, M_w/M_n	[56]
Groundwater HAs and FAs	Vinyl polymer	50 m*M* phosphate buffer + 100 m*M* NaCl + 1 m*M* EDTA, pH 8.5	UV 254 nm, FL	Identification of HSs of different origin from elution profile	[57]
Soil HSs	Modified silica	50 m*M* $NaNO_3$ + 4 m*M* NaN_3, pH 7.0	UV 280 nm, RI	Determination of M_w, M_n, M_w/M_n	[61]
Solubilized coal	Glycerylpropyl bonded to silica gel	KH_2PO_4 + 10% methanol, pH 6.9	UV 280 nm	Study of polydispersity of solubilized coal (high-M_r HSs)	[63]
River FAs	Modified silica	2 m*M* phosphate buffer + 100 m*M* NaCl, pH 6.8	UV 215–280 nm	Determination of M_w, M_n, M_w/M_n at various wavelengths	[69]
Aquatic FAs	Modified silica	2 m*M* phosphate buffer + 100 m*M* NaCl, pH 6.8	UV 220–380 nm	Determination of M_w, M_n, M_w/M_n at various wavelengths	[70]
Aquatic HSs	Polyether bonded to silica	Water or 0.1 *M* Na_2SO_4, pH 5	UV 200 or 254 nm	Measurement of elution profiles with various mobile phases	[77]
Aquatic HSs	Macroporous silica gel	10 m*M* Na acetate	UV 254 nm	Determination of M_w, M_n of fractionated HSs	[78]
River HSs, reference FAs	Polyhydroxy-methacrylate, polyethylene glycol, silica	20 m*M* phosphate buffer, pH 6.5	UV 280 nm	Measurement of elution profiles using various columns	[79]
Coal HAs	Biosep SEC-S-2000	NaCl/NaN_3, pH 7.0	UV 280 nm	Determination of M_w, preparative fractionation	[93]
Soil HSs	Modified silica	10% methanol	UV	Study of association of HSs with ^{36}Cl	[94]
Humic-like substances in fog and aerosols	Hydrogel	300 m*M* NaCl + 30 m*M* NH_4Cl, pH 10.5	UV 210–280 nm, MS, DAD	Comparison of elution profiles	[95]
Coal extracts	Polymer (PS–DVB)	Mixed solvents	UV 300 nm, RI	Comparison of elution profiles	[96]
HPLC-fractionated HSs (FAs)	Sephadex G-25	10 m*M* Na_2HPO_4/NaH_2PO_4 + 200 m*M* NaCl, pH 6.8	DAD, FL	Comparison of MWDs	[71]
Aquatic HSs	Modified silica	100 m*M* NaCl + phosphate buffer, pH 6.8	UV 260 nm	Characterization of DOM in wastewaters	[84]
Chlorinated aquatic HSs	Ethylene glycol–methacrylate copolymer	30 m*M* NH_4HCO_3	UV	Measurement of MWDs of disinfection by-products	[85]

MWD, molecular mass distribution; M_w, weight-average molecular mass; M_n, number-average molecular mass; RI, refractive index; FL, fluorescence; UF, ultrafiltration; Tris, 2-amino-2-(hydroxyethyl)-propane-1,3-diol; ICP-MS, inductively coupled plasma mass spectrometry; PAGE, polyacrylamide-gel electrophoresis; SDS, sodium dodecylsulfate; EDTA, ethylenediamine tetraacetic acid; DOM, dissolved organic matter; DAD, diode-array detection; PS–DVB, polystyrene–divinylbenzene.

attributed to any particular constituent or fraction of HSs. The capillary isotachophoretic separation of HSs was improved by the utilization of discrete spacers (inorganic and organic acids and amino acids) and photometric detection [107], but the nature of the information obtained from the isotachopherograms remains the same—fingerprints.

Dunkelog et al. [32] carried out a comparative study of the separation of aquatic HSs by various electrophoretic techniques, namely free-solution electrophoresis, capillary gel electrophoresis, isoelectric focusing, micellar electrokinetic chromatography and SDS (sodium dodecylsulfate) gel electrophoresis. The best separation was achieved by isoelectric focusing in ultra-thin layers of polyacrylamide. Isoelectric focusing was also used for the characterization of soil organic matter [18]. However, as pointed out by de Nobili et al. [19], great caution is needed when interpreting the electrofocusing results—the bands cannot always be interpreted as being related to the true isoelectric points of molecules because of the polyanionic nature of HSs. Nevertheless, it was concluded that, apart from speculation about the mechanism of the electrofocusing separation of HSs, the electrofocusing profile can be a reliable source of information. Free-flow electrophoresis was used for the preparative isolation of two fractions of HAs, whereas free-flow isotachophoresis was used for investigation of metal–humate complexes [108].

With knowledge of the extraordinary separation efficiency of capillary zone electrophoresis (CZE), accompanied by the high speed of analysis and other advantageous features (as discussed, for example, in Ref. [7]), researchers expected a breakthrough in structural investigations of HSs after the introduction of CZE to the chemistry of HSs. Today, it can be stated that the expectancies were not fully satisfied. Nevertheless, a number of papers published on the CZE of HSs provided much useful information on the properties and behavior of HSs, and allowed their more detailed characterization and identification (often called by the popular term ‘‘CZE fingerprinting’’).

The typical features of CZE separations of HSs were described in the first papers dealing with the CZE of HSs in the mid-1990s [109–111]. HAs usually exhibit one very broad peak (humic ‘‘hump’’) in electropherograms, whereas FAs can give a characteristic set of sharp peaks extending from the humic hump. Based on the characteristic electropherograms (fingerprints), it is, for example, possible to differentiate between ‘‘young’’ and ‘‘old’’ groundwater FAs [109]. The shape of electropherograms is strongly influenced by the composition of the background electrolyte (BGE) as well as the ‘‘chemistry’’ of the capillary walls. Borate, which is a constituent of one of the most common BGEs in CZE, can form complexes with HSs. The observed peaks extending from the humic hump do not, therefore, necessarily indicate distinct humic fractions, but may be artifacts caused by the interaction of borate ions with 1,2- and 1,3-diols present in HSs [112]. It was shown that a significant proportion of HSs is adsorbed onto the walls of uncoated capillaries, which can affect the electrophoretic patterns. Sorption can be eliminated by adding magnesium salts to the BGE, with resultant highly reproducible electropherograms [113]. Recently, cyclodextrin- or oligosaccharide-modified BGEs were used to improve the reproducibility of the CZE of HAs. Electropherograms with a greater number of peaks were obtained for commercial and coal-derived HAs. It was hypothesized that the peaks correspond to the compounds which are liberated from the supramolecular structure of HAs by the action of cyclodextrins, forming inclusion complexes [114]. An increased number of peaks was also observed when urea was added to the BGE [32].

Many BGEs, typically consisting of tetraborate, acetate, phosphate or alanine buffers, have been examined for HS separations by CZE, as reviewed in Ref. [115]. Some operational systems are listed in Table 2.

5. Structural investigations using pyrolysis–GC–MS and other hyphenated techniques

Macromolecular materials such as HSs are recalcitrant to a direct analytical approach, unless some kind of degradation is accomplished to yield more affordable lower-molecular-mass products. Of the various approaches, the most reliable seems to be pyrolysis–gas chromatography–mass spectrometry (Py–GC–MS), which combines the high separation efficiency of GC with the identification power of

Table 2
CZE operational systems for the separation and characterization of HSs

Analyzed material	BGE	Detection	Aim of the study	Ref.
Peat FAs	50 m*M* borate buffer, pH 8.15–9.70, or 50 m*M* phosphate buffer, pH 7.10, or 50 m*M* citrate–HCl, pH 3.30 or 6.25	UV 254 nm	Characterization of FAs, comparison of extraction agents	[20]
Soil and water HAs and FAs	50 m*M* acetate buffer, pH 4.95, or 90 m*M* borate buffer, pH 8.30, or 100 m*M* phosphate buffer, pH 6.95	UV 254 nm	Characterization of HSs, comparison of ''young'' and ''old'' groundwater FAs	[109]
Commercial and standard river and soil HSs	3 m*M* KH_2PO_4 + 6 m*M* $Na_2B_4O_7$, pH 8.9	UV 214 nm	Fingerprint characterization of HSs	[111]
Standard soil and river HSs	50 m*M* acetate buffer, pH 4.95, or 40 m*M* borate buffer, pH 9.3, or 50 m*M* carbonate buffer, pH 9.3	UV 254 nm	Comparison of various HSs	[112]
Coal-derived HAs	20 m*M* rimantadine–HCl, pH 3.40 + 14–50 m*M* $MgCl_2$	UV 215 nm	Fingerprint characterization of HSs	[113]
Peat- and coal-derived HAs	50 m*M* $Na_2B_4O_7$, pH 9.6 + α-, β-, γ-cyclodextrins or maltose or HEC or dextran	UV 210 nm	Fingerprint characterization of HSs	[114]
Commercial and coal-derived HAs	Various buffers	UV 215, 235, 275 or 320 nm	Study of aggregation of HSs	[115]
Aquatic HSs	Wide range of buffers containing alanine, acetate, borate, MES, Tris, CAPS, CHES, pH 3.17–10.40	UV 220 nm	Comparison of various electrophoretic techniques	[32]
Soil-, peat- and coal-derived HAs	90 m*M* boric acid + 115 m*M* Tris + 0.75 m*M* EDTA, pH 8.4	UV 210 nm	Fingerprint characterization of HAs	[116]
Commercial HAs, metal humates	50 m*M* borate buffer, pH 8.6, or 50 m*M* borate + 25 m*M* tartrate, pH 8.6	UV 200 nm	Characterization of HSs fractionated by FF-ITP	[114]
Humic-like substances in fog and aerosol	6 m*M* $Na_2B_4O_7$ + 3 m*M* KH_2PO_4, pH 9.0, or phosphate buffers with pH ranging from 4.38 to 10.53	UV 214 nm	Comparison of humic-like substances with standard HSs	[95]

BGE, background electrolyte; HEC, hydroxyethylcellulose; MES, morpholinoethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-propane-1,3-diol; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; FF-ITP, field-flow isotachophoresis.

MS. It was demonstrated that as many as 322 compounds can be identified in pyrolysates of soil HSs [117]. Py–GC–MS provides detailed structural information, but also suffers from limitations when analysing the polar functional groups (–COOH, –OH) typically present in HSs. Interpretations of pyrolytic experiments may possibly be incorrect, as, in many instances, the naturally occurring units (building blocks) may have been altered before or after their release from the macromolecular structure. In several papers [40,118], the Py–GC–MS technique is called a ''two-edged sword'', because interpretation may lead to serious errors without knowledge of the chemical nature and thermal behavior of the studied organic matrix. The limitations and pitfalls of the analytical pyrolysis of HSs are discussed in detail in the review presented in Ref. [118]. Although not ideal, Py–GC–MS and its modifications are probably the best techniques that have been employed so far for structural investigations of HSs.

On pyrolysis of soil HAs at different temperatures, essentially distinct classes of evaporation and pyrolysis products are obtained [117–119]. A temperature of 358 °C causes the evaporation of adsorbed phenols, dialkylphthalates and lipids (alkanes, fatty acids), the pyrolysis of carbohydrates and polysaccharides, and some pyrolysis of lignin. Pyrolysis at 510 °C is a good compromise for polysaccharide- and lignin-rich humic materials,

whereas a slightly lower temperature is recommended for FAs [118]. The use of higher temperatures is justified when more in-depth insight is required (e.g. for purified HA fractions).

In order to overcome the above limitations of Py–GC–MS, Challinor [120] introduced a technique for the simultaneous pyrolysis and methylation of polar groups using tetramethylammonium hydroxide (TMAH) as derivatizing agent. It was demonstrated that strongly basic TMAH does not merely act as a pure methylating reagent, but also promotes hydrolytic ester and ether bond cleavage even at low temperatures, i.e. the overall degradation mechanism may more resemble thermochemolysis than pyrolysis. Saiz-Jimenez et al. [121] attempted for the first time to apply methylation pyrolysis to the characterization of humic fractions and compared the data with those obtained by conventional pyrolysis. The most important result was the identification of furancarboxylic, benzenecarboxylic, and aliphatic dicarboxylic acids as their respective methyl esters in the methylated pyrolysate. The presence of 3,4,5-trimethoxybenzoic acid and benzenecarboxylic acids is of interest as they represent the final steps in the oxidation of side chains of lignin units through microbial degradation. In general, methylation substantially changes the pattern of pyrolysis products when compared with conventional pyrolysis, giving a cleaner chromatogram, probably due to less structural fragmentation.

TMAH–thermochemolysis–Py–GC–MS was used for the characterization of HAs from leonardite coal [122] and from peat [123]. The mechanism of the thermochemolytic degradation of lignin biopolymers was investigated using ^{13}C-labeled TMAH [124]. An alternative derivatizing agent—tetraethylammonium acetate—allowed discrimination between the different forms of mono- and dicarboxylic acids present in the structure of HAs and humin [123].

Recently, acid-catalyzed transesterification followed by GC–MS was used to determine the chemical composition of low-M_r moeities linked to the core structure of HSs [125].

A great number of compounds have been identified in the degradation products from the pyrolysis of HSs [18,19,117–119]. A comprehensive discussion focused on the presence and composition of the main classes of compounds (aromatics, aliphatics, furans, and N, S and halogen compounds) obtained by TMAH–Py–GC–MS was published by Lehtonen et al. [40]. As an example, the distribution of the main compound classes as identified in various HAs by Py–GC–MS is shown in Fig. 4. Based on Py–GC–MS analyses at different temperatures, Lehtonen et al. [40,127] deduced that there are two kinds of main substructures in humic macromolecules, i.e. an esterified phenolic-dicarboxylic acid network and a carbon–carbon bound alkylaromatic network.

To obtain more complete information, Py–GC–MS has been combined with other advanced techniques, such as ^{13}C nuclear magnetic resonance spectroscopy [126,128,129] or isoelectric focusing [18,19]. This allowed, among other things, the evaluation of the degree of humification of organic matter in different soils [18].

Occasionally, other hyphenated techniques have also been used for the structural characterization of HSs. Various aquatic HSs were investigated by coupled pyrolysis–gas chromatography–Fourier transform infrared spectroscopy (Py–GC–FT-IR) and more than 25 compounds were identified in the pyrolysate [130]. Coal wastewaters were characterized by Py–GC–MS and Py–GC–AES (atomic emission spectroscopy). Polymers of natural and anthropogenic origin were distinguished by comparing the building blocks of dissolved organic matter [131].

The rapid progress in the instrumental implementation of atmospheric pressure ionization

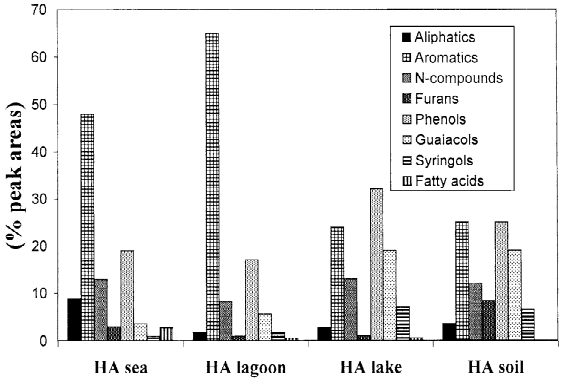

Fig. 4. Distribution of the compound classes (estimated from peak areas) determined by GC–MS in pyrolysates from various HAs. Data from Ref. [126].

mass spectrometry (API-MS) has changed dramatically the applicability of LC–MS coupling. Especially electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are widely employed in environmental analysis [132]. LC–MS is better suited for the analyses of polar compounds than GC–MS and thus it can be used for investigations of HSs on the molecular level.

ESI-MS coupled with SEC separation was used to characterize HAs and FAs [133]. However, the spectra were very complex and difficult to interpret in terms of structural elements. Quasi-continuous spectra with little diagnostic value were recorded in the single MS mode. Multistage tandem mass spectrometric (MS–MS) detection increases the amount of information that can be obtained from a single run. In this way, both molecular masses and structural information can be acquired from the chromatographic peak [134].

ESI as well as APCI in positive and negative ionization mode were used for the determination of the M_r of HSs [135–137]. The average molecular masses of FAs determined by ESI-MS were in rather good agreement with the values obtained by independent methods [137], including SEC with UV detection [136]. Recently, Kujawinski and co-workers [138,139] used ESI coupled with quadrupole time-of-flight MS and Fourier transform ion cyclotron resonance MS for the structural characterization of aqueous and soil HSs, and HAs from degraded wood. The very high resolving power of this experimental arrangement allowed the measurement of both the compound and molecular mass distributions without preliminary chromatographic fractionation.

6. Other methods

Field-flow fractionation (FFF) is one of the less common methods for M_r determination, and was first applied to FAs and HAs in the late 1980s [140]. In the experimental arrangement, FFF resembles liquid chromatography except that the separation is based on the physical forces arising from an applied field which distributes the sample components across a thin channel. A variety of fields can be used, including cross-flow, gravitation, temperature gradient and electrical fields [141].

FFF offers advantageous features for the characterization of HSs. Compared with GPC/SEC and other chromatographic techniques, the open FFF channel has a greatly reduced surface area and interactions with active groups of HS molecules (typical for GPC/SEC and often undesirable) are minimized. The lack of such interactions allows the characterization of materials that are difficult to separate by other methods. Another advantage of the open-channel FFF is a precise understanding of the separation dynamics, which allows retention times in the channel to be related directly to the physicochemical parameters that govern the sample's interaction with the applied field.

Asymmetrical flow field-flow fractionation (AsFl-FFF), an FFF subtechnique, was used to study the behavior of HSs in solution [142]. In AsFl-FFF, analyte components are swept through a ribbon-shaped channel at different rates owing to their interaction with a field composed of carrier liquid flowing across the thin dimension. AsFl-FFF separates components strictly by their hydrodynamic size, and thus the FFF profile can be used, after proper calibration, to determine molecular masses and MWDs. Globular proteins [142] or other standards similar to those utilized in GPC/SEC can be used for the calibration. The AsFl-FFF technique was applied to assess the effects of ionic strength and pH on the size of HS molecules [142]. A similar technique was used to characterize HA samples with respect to the molecular mass distribution before polarographic investigation [143]. Flow FFF and MALS were used to study the interactions of HAs and FAs with hematite [144]. Flow FFF with UV absorbance detection was used to measure the MWD of organic colloids in river waters [145]. Reference HSs (IHSS standards) were also analyzed for comparison. The FFF elution curves (fractograms) consisted of single broad peaks, from which the average molecular masses (M_w, M_n) were calculated. The M_w values determined for the river colloids (ca. 1000–1400) correspond to the values reported for FAs, whereas the M_w value of HAs determined by FFF (2090) seems to be slightly lower than the values commonly reported for HAs.

7. Interactions of humic substances with heavy metals and other chemical pollutants

As mentioned in the Introduction, the importance of HSs stems mainly from their ability to interact with heavy metals and other chemical pollutants, and thus to affect considerably their mobility in the environment. Therefore, it is not surprising that extensive research has focused on the metal-binding properties of HSs and on other kinds of interactions. As most methods are based on distinguishing between ''free'' and HS-bound metal (or other pollutant), separation methods are widely employed.

Even a simple method such as filtration (more often, however, in its more sophisticated form—ultrafiltration) has been successfully used for the determinations of stability constants of metals with HSs. A concise review of ultrafiltration and related methods was published by Burba and co-workers [146,147]. Zn–HS and Cu–HS complexes were gently size-fractionated by sequential-stage ultrafiltration and free metal fractions were discriminated. The calculated conditional stability constants exhibited clear molecular-size dependencies [26]. The so-called ''availability'' of metal–HS species in humic-rich waters was determined by ultrafiltration using EDTA as a competitive ligand [148]. Tangential-flow ultrafiltration in combination with a competitive exchange reaction with EDTA were used for the on-site examination of humic-rich colloids and their metal loading [149].

Analytical ultracentrifugation was used to study the interaction of Cu(II) [150] and other metals [151] with dissolved HSs and to measure the metal-induced aggregation of HSs.

GPS/SEC techniques have also been successfully applied for the measurement of the metal-binding properties of HSs. The method is based on the fact that complexes formed between metals and macromolecules (HSs) are excluded from the column, whereas free metal ions completely permeate the gel. A typical experimental arrangement is described in Ref. [152]. A Sephadex G-15 column was equilibrated with a flowing buffer solution containing a known concentration of the metal ion of interest. A similar buffer solution which had been equilibrated with a known amount of complexing agent (HS) was injected into the flow system. The complexing agent continuously binds the metal after the injection, forming the complex excluded from the gel. The resulting metal deficiency travels more slowly than the band of the metal complex. Monitoring of the metal concentration of the effluent from the column shows first the peak, the area of which corresponds to the amount of bound metal. This is followed by a trough (negative peak) corresponding to the metal deficiency. The stability constants can be calculated from the concentrations of complexed and free metal ions. More information on the nature of the binding can be obtained by carrying out chromatographic runs at a variety of metal concentrations. The results are usually treated by the method of Scatchard [153]. The contributions of the ''strong'' and ''weak'' binding sites were distinguished from the Scatchard plots for the binding of Cu(II), Ni(II) and Zn(II) by peat, soil and lake FAs [152]. A similar approach was used for the study of Ni(II) complexation with aquatic FAs and HAs with the aid of a modified (glyceryl propyl-bonded) silica gel column [154]. Various SEC columns were compared for measurement of the complexation capacity of natural waters [tested on the Cu(II)–FA system] [31].

Not only the binding of free metal cations, but also the binding of organo-metallic compounds (methyl mercury) to HAs and FAs can be examined by GPC coupled to inductively coupled plasma MS [24].

The classical Schubert's ion-exchange method is one of the most popular methods for metal complexation studies. The principle, theory and a brief overview of its applications for the investigation of metal–HS interactions can be found in Ref. [155]. The method is based on measuring the distribution coefficient of the metal ion between the cation-exchange resin and the solution phase, in both the presence and absence of a complexing agent. Schubert's method has been used for the determination of the stability constants of di- and trivalent metal ions with HSs [155–158]. The conditional stability constants determined for river FAs followed the sequence Cu(II)>Cd(II)>Ni(II)>Zn(II)>Ca(II) (at pH 6.0 and an ionic strength of 0.1) [155]. In some studies, chelating ion exchangers were used for the determination of metal lability in HSs [159],

or for kinetic studies of metal (Cd, Cu, Pb) speciation in river water [133]. The experimental data indicated discrete sets of rate constants and hence heterogeneous binding sites in FAs distinguishable by their rates of dissociation [160]. The macroporous weakly basic resin DEAE-Sephadex A-25 has been found to be useful for speciation studies of metal–humic complexes in natural waters [161]. In combination with the Chelex-100 cation exchanger, it was used for the quantitation of metal–HS complexes in natural waters and for the classification of metals (Al, Ba, Cd, Co, Cu, Fe, Hg, Mn, Pb, Sr, U, Zn) with respect to their binding strength to HSs [162]. The metal-binding capacity of HSs was estimated from elution volumes in immobilized metal ion affinity chromatography [36].

Electrophoretic techniques, including CE, are often utilized to examine the interactions of metals with various complexing agents [163]. However (slightly surprisingly), attempts to apply these methods to the investigation of metal–HS interactions have not been fully successful. It was demonstrated that complexation of HSs with metals influences their electrophoretic mobility [108] and can be employed to improve the reproducibility of electrophoretic patterns [113]. By including metal ions in the BGE, it was possible to relate the shapes of the electropherograms to the ability to form complexes [164], but no quantitative data (e.g. stability constants) were obtained. It was concluded in a review [165] that CE offers little benefit over chromatographic techniques for this application.

Various methods have been used to study the interactions of a wide range of organic chemical pollutants with HSs. A silica gel column with chemically immobilized HAs was used to characterize the sorption of polycyclic aromatic hydrocarbons (PAHs), and the retention characteristics were compared with those obtained with common stationary phases—octadecyl-bonded (C_{18}) and diol. Strong correlations were found between the sorption of PAHs on HAs and the capacity coefficients on the diol and C_{18} phases [166]. A reversed-phase separation technique was used to determine the binding of ^{14}C-radiolabeled organic pollutants (PAH, DDT, and others) to HSs in waters. The humic-bound pollutants were separated from the free dissolved pollutants using a C_{18} cartridge; the humic-bound pollutants passed through, while unbound pollutants were retained. The partition coefficients were determined in this way [167]. A similar approach was used to study the distribution of DDT and benzo[*a*]pyrene between water and dissolved HSs; the free and bound fractions were separated on a C_{18} cartridge and subsequently determined by GC–MS [168]. A tandem-cartridge solid-phase extraction system, combining reversed-phase separation and dynamic ion exchange (utilizing cetyltrimethylpyridinium bromide as an ion-interaction agent) followed by GC–MS, was applied to measure the partition coefficients of PAHs to HAs [169]. The partitioning of PAHs and phenols between the water phase and a HS-containing pseudophase was investigated by solid-phase microextraction, and the results were compared with those obtained by liquid–liquid extraction [170]. Partition coefficients between dissolved organic carbon and water were determined for pentachlorobenzene, hexachlorobenzene, polychlorinated biphenyls and DDT using solid-phase microextraction. The interactions of organic pollutants with HSs were studied from the point of view of possible interferences during their determination [171] (a similar study was performed for a number of pesticides [172]).

The interactions of organic model compounds of different polarity and four pesticides with water-soluble soil HSs were studied by SEC [173]. It was found that the association is strongly affected by pH; at neutral or alkaline pH, association occurred only for the most hydrophobic compounds such as atrazine.

An equilibrium dialysis technique (dialysis tubing with a molecular mass cut-off of 1000) was used to examine the binding of DDT [174] and PAHs [175] to dissolved HSs in natural waters. It was found that the extent of binding depends on the source of the HSs, the pH, the ionic strength, the presence of polyvalent cations (calcium ion), and the concentration of HSs [174].

8. Conclusions

Separation methods are employed in practically every study focused on the research of HSs, at least for their isolation or fractionation. Moreover, some

of the separation methods are also able to provide valuable information about the properties and composition of HSs. To date, most structural data have been obtained from pyrolysis GC–MS measurements. Recently, rapid progress in LC–MS instrumentation has made it possible to study the properties of HSs at the molecular level, which may allow new insights into the supramolecular arrangement of HSs.

Studying the colloidal properties of HSs and measurements of their molecular masses are the most important tasks of separation methods. However, it should be pointed out that not only separation methods are used for this purpose. In fact, almost every method of molecular mass determination has been applied to HSs, including osmometry, cryoscopy, viscometry, light scattering, X-ray scattering, the separation methods mentioned in this review, and, recently, advanced MS–MS techniques. The extremely broad range of molecular masses published for HSs (10^2–10^5 or even more [11]) is witness not only of their great diversity and dispersity, but is probably also a result of the limited comparability of the results obtained with different methods often based on quite different physical principles. Unfortunately, systematic and reliable comparisons of the various methods (applied to well-defined humic material) are only rarely found in the literature. Some recently published results are listed in Tables 3 and 4. At present, none of the commonly used methods can be regarded as superior to the others. Some of them, nevertheless, seem to be less convenient—e.g. ultrafiltration, for which the results depend strongly on the type of membrane (see Table 4).

Table 4
Molecular masses of NOM measured by different methods. Data from ''NOM-typing project'', published in Ref. [176]

Method	Sample		
	TRE	HEM	AUR
FCS	2200	3100	2000
DIF	1400	2300	1500
SEC	800	2700	400
MALLS	23 200	22 400	57 800
DAM	5000	15 300	7200
UF1	1900	1400	1300
UF2	20 800	33 200	26 600
UF3	29 800	33 600	

NOM, natural organic matter; TRE, HEM, AUR, denotation of sampling sites; FCS, fluorescence correlation spectroscopy; DIF, difussifimetry; SEC, size-exclusion chromatography; MALLS, multi-angle laser light scattering; DAM, dynamic adsorption experiments; UF1, UF2, UF3, various modifications of ultrafiltration utilizing various membranes.

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Table 3
Comparison of average molecular masses determined by different methods. Sample: Suwannee FAs (IHSS standard). Most data compiled by Leenheer et al. [137]

Method	M_n	M_w
Equilibrium ultracentrifugation	470 (acid form), 1060 (K^+ salt)	110 (acid form), 1340 (K^+ salt)
Vapor pressure osmometry	623–965 (acid form)	
Low-angle X-ray scattering	645–816 (acid form)	
GPC	1390 (Na^+ salt)	2080 (Na^+ salt)
ESI-MST-MS [137]	617 (+mode), 591 (−mode)	936 (+mode), 914 (−mode)
ESI-FTICR-MS [135]	1908 (+mode), 699 (−mode)	1924 (+mode), 714 (−mode)
ESI-MS–MS [136]	1280 (+mode), 940 (−mode)	1790 (+mode), 1420 (−mode)

ESI-MST-MA, electrospray ionization multistage tandem mass spectrometry; ESI-FTICR-MS, electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry; +mode, positive ionization mode; −mode, negative ionization mode.

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