THE SUPRAMOLECULAR STRUCTURE OF HUMIC SUBSTANCES

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The scientific understanding of the molecular size and shape of humic substances (HS) is critically reviewed. The traditional view that HS are polymers in soil is not substantiated by any direct evidence but is assumed only on the basis of laboratory experiments with model molecules and unwarranted results produced by incorrectly applying either analytical procedures or mathematical treatments developed for purified and undisputed biopolymers. A large body of evidence shows an alternative understanding of the conformational nature of HS, which should be regarded as supramolecular associations of self-assembling heterogeneous and relatively small molecules deriving from the degradation and decomposition of dead biological material. A major aspect of the humic supramolecular conformation is that it is stabilized predominantly by weak dispersive forces instead of covalent linkages. Hydrophobic (van der Waals, π-π, CH-π) and hydrogen bonds are responsible for the apparent large molecular size of HS, the former becoming more important with the increase of pH. This innovative understanding of the nature of HS implies a further development of the science and technology for the control of the chemistry and reactivity of natural organic matter in the soil and the environment. (Soil Science 2001; 166:810–832)

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Humic substances (HS), natural organic substances that are ubiquitous in water, soil, and sediments, are of paramount importance in sustaining plant growth and controlling both the fate of environmental pollutants and the biogeochemistry of organic carbon (OC) in the global ecosystem (Piccolo, 1996). Despite their role in the sustainability of life, the basic chemical nature and the reactivities of HS are still poorly understood. The scientific community of humic scientists has so far failed to provide a unified understanding of this field of science, and there is still, therefore, a poor awareness of fundamental aspects of humic structures and reactivities. Nevertheless, the implications of the relevance of awareness of HA structure should extend far beyond the interests of a few chemists; HA structures affect the ways that the soil ecosystem work, as well as the bioavailability of organic substances (including potential pollutants) in the soil environment (Tate, 1999).

Most of the difficulties encountered in chemically defining the structures and reactivities of HS derive from their large chemical heterogeneity and geographical variability. The substances are undoubtedly mixtures that develop randomly from the decay of plant tissues, from microbial metabolism-catabolism, or from both. Thus, the chemistry is not only highly complex, but it is also a function of the different general properties of the ecosystem in which it is formed, such as vegetation, climate, topography, etc. It is not surprising that, despite the efforts of many excellent scientists in the distant and recent past (Kononova, 1961; Stevenson, 1994), there is still much to be done to achieve an appropriate awareness of humic chemistry.

The objective of this contribution is to report on recent experimental findings leading to a new understanding of the conformational nature of HS and to mention the profound implications that this may have on our understanding of soil organic matter (SOM) functions and reactivities.
Traditional Paradigms in Humus Chemistry

The amount of HS in soils is several times greater than the amount in waters. Up to 70–80% of the OM in mineral soils may be composed of humic material, with recognizable plant remains constituting a small percentage of the organic matter (OM) of mineral soils. Even though the abundance of OC in HS is from 2 to 3 times greater than that in the terrestrial biomass, the latter greatly influences the OM dynamics in soils (Insam, 1996). Biological activity rapidly decomposes labile plant materials on entering aerobic soil environments with adequate water supplies, but more resistant components transform slowly in the same environment. The compositional diversities and the differences in the modes of transformation of the components make it extremely difficult to define accurately the gross mixtures that compose SOM, or the dissolved (DOM), or the particulate organic matter (POM) of waters.

Because of the heterogeneity of humic mixtures, simplification and reductionism must be adopted. Stevenson (1994), in summarizing previous reports and definitions, stated that humus includes a broad spectrum of organic constituents, many of which have their counterparts in biological tissues. He distinguished between non-HS and HS, the former of which consists of compounds belonging to the well known classes of organic chemistry such as amino acids, carbohydrates, lipids, lignin, and nucleic acids, whereas the latter are unspecified, transformed, dark colored, heterogeneous, amorphous and high molecular weight (MW) materials.

The classical definitions of HS are operational only and are based on solubility properties in the aqueous solutions used as soil extractants. The generalized terms humic acids (HAs), fulvic acids (FAs), and humins cover the major fractions still used to describe HS components, but the boundaries between these fractions have not been yet clarified in chemical terms. Because modern analytical techniques for organic compounds were not available during the first half of the 20th century, the data that were available from elemental analyses (C, H, O) and determinations of acidic functional groups suggested that HAs and FAs from many different soils were relatively similar (Kononova, 1961). Such simple correlations, although not based on any molecular understanding, encouraged scientists to consider humic fractions to be chemical entities having specific properties rather than complex mixtures of nonspecific compounds.

Many of the modern concepts of HS derive from theories illustrated in the book of Kononova (1961). In a review of hypotheses advanced predominantly by Russian scientists, Kononova introduced the concept of HS as a system of polymers, based on the observation that elemental composition, optical properties, exchange acidities, electrophoretic properties, and MW characteristics varied consistently with soil classes. Using this concept, the various fractions of HS obtained on the basis of solubility characteristics are imagined to be part of a heterogeneous mixture of molecules, which, in any given soil, range in MW from as low as several hundreds to perhaps over 300,000 Da, and exhibit a continuum of any given chemical property (Stevenson, 1994).

It must be noted that despite the existence of data showing that the molecular dimensions (as measured by osmometry, viscometry, and diffusion) of some HS were scarcely beyond one or two thousand Da (Scheffer and Ulrich, 1960; Schnitzer and Khan, 1972), more reliance was placed on the early work of Flaig and Beutelspacher (1958) who showed, using the ultracentrifuge, that MW values were in the range of 30,000 to 50,000 Da for HAs and about 10,000 Da for FAs. One reason for such bias towards high molecular weight (HMW) structures may be found in the historical hypotheses that considered HS to be products of biologically-assisted syntheses from compounds derived from degradations of lignin, polyphenols, cellulose, and amino acids. Evidence for this polymeric assumption was researched in many classical laboratory experiments that indicated possibilities for either abiotic or biotic condensations of simple molecules into humic-like materials (Kononova, 1961). Some of these early laboratory studies have been researched in more carefully defined conditions in recent times (Haider and Martin, 1967; Martin and Haider, 1969; Flaig et al., 1975; Flaig, 1988; Hedges, 1988). However, no direct evidence has ever been provided for the occurrence of such polymer build-up processes in natural soil systems.

The polymeric view of HS has included the general concept of polydispersity (Dubach and Metha, 1963), in which the HS are made of polymers with different MW values, similar to that which applies for other natural biological macromolecules such as proteins, polysaccharides, and lignin. The experimental difficulties encountered in isolating chemically homogeneous fractions of HS were compared with those observed for bio-
polymers with varying molecular dimensions synthesized in the living cell. This unwarranted similarity supported the polydisperse polymeric concept of HS and justified the observation that these are mixtures of compounds. The concept of HMW and polydisperse polymers became a paradigmatic part of the descriptive definitions of HS proposed thereafter (Schnitzer and Khan, 1972; Aiken et al., 1985; Malcolm, 1990; Stevenson, 1994).

The assumption that HS are polymers, which has no sound molecular basis, has promulgated the use of simple physical-chemical measurements to characterize HS. An example is the E4/E6 index, the ratio of the absorbance of HS at 465 nm to that at 665 nm, introduced by Welte (1955) and proposed by Kononova (1961). This ratio supposedly indicates a reverse relationship with progressive humification as well as increased condensation assumed to produce large amounts of polycondensed aromatic-ring structures. Despite its widespread and continued application, the E4/E6 ratio has repeatedly been shown not to hold the predicted relationship with MW. At variance with the hypotheses related to this ratio, Campbell et al. (1967) had already indicated that humic material with the lowest mean residence time in the soil had the highest E4/E6 ratio, and Anderson and Hepburn (1978), using gel permeation chromatography, showed that humic fractions with large molecular sizes and low E4/E6 ratios were mainly aliphatic, whereas those with molecular size had the highest aromatic contents. Piccolo (1988) compared the E4/E6 ratios of various HS with their gel permeation chromatograms and found that the results were comparable only when the HS had been subjected to extensive purification. Summers et al. (1987) reported on the limitations of E4/E6 ratios and found that the ratio values varied considerably with the concentrations of ultrafiltered fractions of HS. By comparing HS extracted from soils and soil particles before and after long term amendments with organic wastes, Piccolo and Mbagwu (1990) found that the E4/E6 ratio values did not account for the increase in molecular sizes in amended samples as observed by gel filtration.

Despite a lack of evidence, various reasons led the scientific community to accept the polymeric concept for HS. One of these reasons lies in the acceptance of the description by Staudinger (1935) of macromolecular polymers occurring in living cells. Based on the Staudinger concept, it seemed convenient to assume that HS were also polymers biologically synthesized from plant tissues components, notwithstanding that HS arise from cell death rather than cell biosynthesis, as occurs with other biomolecules. The rapid degradation and decomposition in soil of biopolymers liberated from cell lysis after death is now a well accepted process from both a biological and a thermodynamic perspective (Jenkinson, 1981; Haider, 1987; Clapp and Hayes, 1999; Spaccini et al., 2001). Surprisingly, Staudinger’s view with regard to macromolecularity is still advocated by defenders of the polymeric nature of HS (Swift, 1999).

A second reason for acceptance of the polymeric concept is that a stable polymeric structure would account for the refractory characteristics of HS in soil, rather than considerations of physical and chemical protection, as provided, for example, by interactions with inorganic soil particles. The OC in stable humic materials is known (Stevenson, 1994) to have extended residence times (from 250 up to 3000 years) in soil. In addition, the classical hypothesis of HS formation through condensation between amino acids and components of degraded lignin promoted the assumption that HS had a polymeric structure similar to that of lignin (Waksman, 1936). Lignin is known to be polydisperse in MW, with values ranging from <1000 to several million Da (Goring, 1971), and its resistance to microbial degradation in soil has been attributed repeatedly to its macromolecular structures (Waksman, 1936; Amalfitano et al., 1992). Similarly, polymerization processes carried out in laboratory or in confined conditions led to other classical hypotheses of HS formation, such as the polyphenol theory (Flaig et al., 1975) and the melanoidin theory based on the Maillard reaction (Maillard, 1912). Another aspect of HS contributing to the polymeric view is their colloidal properties, which were related to those of polyelectrolytes in aqueous media (van Dijk, 1972; Flaig et al., 1975). Most of the properties of polyelectrolytes, such as processes of flocculation and dispersion, responses to electrolytes, and double-layer behavior were also observed for HS, and thus concepts of HMW properties were readily attributed to HS.

The macromolecularity of HS, despite the wide acceptance of the concept, has never been demonstrated unambiguously in chemical and physical-chemical terms for soil-extracted humic fractions. The instrumentation for organic and physical chemistry analyses was not generally available in the past, nor was it completely reliable. This situation has changed dramatically in the present world of sophisticated biophysical
technology. Thus, the polymeric paradigm of HS should either be proven beyond any doubt or abandoned in favor of another description.

CONCEPTS OF CONFORMATIONAL MODELS FOR HUMIC SUBSTANCES

Determination of the true MW of a humic fraction is central to HS research. Conformational aspects of structures of HS (i.e., shapes and sizes), and ultimately their reactivities in soil and the environment, are determined by MW. It is quite evident from reviews that different methods for measuring MW values of HS do not give the same answers (Wershaw and Aiken, 1985; Swift, 1989; Clapp et al., 1989; Stevenson, 1994). Generally, the differences are not slight but can be several orders of magnitude. The great differences in values have been attributed either to the variability of the HS or the intrinsic limitations of the methods when applied to polydisperse systems. However, the polymeric paradigm has not been questioned in analyses of the differences, and very few attempts have been made to decrease significantly polydispersity by classical chemical methods.

Much of the confusion with regard to MW determinations of HS has arisen as a result of sedimentation-velocity and diffusion methods that became fashionable with the upsurge of biochemistry research in the 1950–1970 era. Despite the clear indication that such semiempirical methods are not suitable for polydisperse systems because of the multiple diffusion coefficients and sedimentation constants for different size particles (Wershaw and Aiken, 1985), studies were carried out with whole HA extracts, and MW values ranging from 25,000 to more than 200,000 Da were reported (Stevenson et al., 1953; Flage, 1958; Piret et al., 1960). Interestingly, Flage and Beutelspacher (1968), when working with polydisperse humic solutions, found that MW values varied from 77,000 Da, when 0.2 M NaCl was added to depress repulsive negative charges, to only 2050 Da in the absence of the background electrolyte. Though the latter is commonly added to polyelectrolytes to reduce interferences from charge repulsion in monodisperse systems, a jump of about two orders of magnitude in MW for the same humic polydisperse system could suggest a molecular association rather than an extreme interference in sedimentation-velocity ultracentrifugation. The fractions obtained were more homogeneous than the unfractionated solution, but these could still not be considered to be monodisperse. They reported MW values for the fractions ranging from 2600 to a surprising 1,360,000 Da. It is most likely that they measured associations of smaller molecules randomly aggregated by different forces during the fractionations procedures. Moreover, Cameron et al. (1972a) overlooked the difficulties in assessing sedimentation coefficients for associations of molecules such as HS. Measurement of sedimentation coefficients of polydisperse materials that include subunits invariably leads to erroneous values of MW (Laue and Rodhes, 1990). Other studies using sedimentation-velocity ultracentrifuge studies (Ritchie and Posner, 1982) and the even more mathematically sound equilibrium centrifugation (Posner and Creeth, 1972; Reid et al., 1990) also showed polydispersity in HS, and, for this reason, confirmed the ambiguity of the MW values obtained by ultracentrifuge methods.

There is more reliability in MW obtained for FAs, or for OM dissolved in waters. There is a general agreement that the MW of these humic molecules is in the 400–1500 range, as determined by a variety of methods (Aiken and Gillam, 1989; Wershaw, 1989; Stevenson, 1994). As will be seen below, the smaller discrepancies among MW values obtained for FAs can be ascribed to their larger hydrophilicity (small very acidic molecules), which prevents strong molecular associations by hydrophobic forces. However, a process of molecular associations in solution can produce a polydisperse system of apparently HMW also for FAs. In fact, FA MWs when analyzed by ultrafiltration and gel filtration methods, were consistently higher than when measured with osmometry or cryoscopy (Thurman et al., 1982).

Similar contradictions (as described for the MW values of HS) apply for the concepts of shapes attributed to humic polymeric macromolecules. Globular shapes (Visser, 1964), flexible linear configurations (Mukherjee and Lahiri, 1959), ellipsoidal shapes (Orlov et al., 1975), spheroid polyelectrolytes (Ghosh and Mukherjee, 1971), and randomly folding long chains (Cameron et al., 1972a) have all been proposed to describe the elusive polydisperse humic system. Ghosh and Schnitzer (1980) reconciled the different views by measuring surface pressures and viscosities of HS at different pH values and neutral salt concentrations and adapting the results to relationships (the Flory and Fox and the Staudinger equations) that
had been developed for real polymers. They explained the observed behavior of HS (uncharged matter at low pH and polyelectrolytes at high pH) on the basis of the polymeric theory and they proposed that HS are rigid spherocolloids at high sample concentration and ionic strength and at low pH, whereas at high pH values, low sample concentrations, and ionic strength, they behave as flexible linear polymers. This understanding had two major flaws: it was based on studies with whole humic extracts with full polydispersity and, despite the lack of a direct knowledge of the real molecular structure, the data were arbitrarily used in equations specifically derived for polymers. Nevertheless, this reversible coiling model for humic configurations soon became the most widely used to describe HS, although it does not explain all of the behavior of HS.

In applications of small-angle X-ray scattering to determine the particle sizes of whole HAs in solution, or of fractions separated by adsorption chromatography using cross-linked dextran gels (see Wershaw, 1989), it was found that HS formed molecular aggregates in solution and their sizes were a function of pH. It was concluded that the various fractions were different chemically and that the differences in aggregation behavior were a reflection of the interactions of different bonding mechanisms. These findings, coupled with other results that showed that humic fractions from different sources have surface active properties (Hayase and Tsubota, 1983), have led to a description of HS that is an alternative to the random coil polymeric structure. Wershaw (1986, 1993) proposed that HS consist of ordered aggregates of amphiphiles, composed mainly of relatively unaltered plant polymer segments possessing acidic functionality. In this model, HS are aggregates held together by hydrophobic (π-π and charge-transfer bonds) and H-bonding interactions, and the hydrophobic parts of the molecules are in the interiors with the hydrophilic parts make up the exterior surfaces. Ordered aggregates of humus in soils were depicted to exist as bilayer membranes coating mineral grains and as micelles in solutions.

Wershaw’s model represented a major breakthrough because it introduced the concept of aggregation of different particle sizes of humic constituents in contrast to the traditional view of polydisperse linear humic polymers. Nevertheless, the micelle-like model did not yet solve the issue of MWs of HS. The spontaneous aggregation of humic molecules into micellar aggregates was advocated by other authors (Engebreton and von Wandruszk, 1994, 1997) to explain fluorescence quenching of pyrene, but the explanation of the results was still based on the polymeric nature of the aggregating humic molecules. However, the classical concept of an ordered micelle is hardly applicable because of the heterogeneity of HS: the CMC (Critical Micelle Concentration) reported in the literature for HS, in the range of 1 to 10 g L⁻¹ (Hayase and Tsubota, 1983), is much higher than those found for surface active compounds giving regular micellar structures (Tanford, 1980).

Despite its limitations, the concept of aggregation of hydrophobic parts of HS could explain:

1) results from light-scattering which showed that addition of Cu ions to dilute soil FA increased the amount of light scattered by the solution (Ryan and Weber, 1982);
2) the increased solubility of nonpolar compounds in humic solution because of partition/adsorption in the hydrophobic interior of HS (Carter and Suffet, 1982); and
3) the further release of humic matter through dialysis bags from an already extensively dialyzed HS when this is treated with an amphiphilic compound such as acetic acid (AcOH) or an electrolyte (De Haan et al., 1987; Nardi et al., 1988).

The advances that the molecular aggregation model made for an understanding of the environmental behavior of HS (compared with the polymeric paradigm) did not prevent recent hypotheses, based on pyrolytic analyses of HS, indicating macromolecular structures for HAs with MW values up to about 100,000 Da (Schulten et al., 1998). Despite the many limitations inherent in the analytical pyrolysis of HS (Saiz-Jimenez, 1995, 1996), compounds identified by pyrolysis-mass spectrometry techniques were arbitrarily linked together by covalent bonds in computer molecular models to give graphical representations of large branched polymers. Such large macromolecules are even being proposed as models of humic structures and used to explain the behavior of HS in soil (Schulten and Leinweber, 2000).

SIZE EXCLUSION CHROMATOGRAPHY OF HUMIC SUBSTANCES: HISTORICAL PERSPECTIVE

Gel permeation chromatography or low-pressure size exclusion chromatography on Sephadex cross-linked dextran gel columns, a method de-
vised to desalt and purify proteins, has been applied extensively to humic fractions to evaluate molecular sizes and to obtain more size-homogeneous materials (see reviews of Wershaw and Aiken, 1985; De Nobili et al., 1989). It soon became clear that separation by GPC is not a pure size fractionation (Determann and Walter, 1968) and that interferences may occur with HS, namely ionic exclusion and adsorption chromatography. In the first process, electrostatic repulsion between the negative charges present on both dissolved HS and the dextran gel enhances the chromatographic velocity, whereas in the second process, hydrophobic interactions between HS and the stationary phase retard the chromatographic elution of HS (Lindqvist, 1967). The ionic strength of mobile phases should be sufficiently high to prevent electrostatic interactions but not too high (>0.5 M) to drive hydrophobic interactions (Chicz and Regnier, 1990).

Much of the inconsistency found in HS behavior in gel permeation has been ascribed repeatedly to either ionic (electrostatic) exclusion or to hydrophobic gel-solute interactions. Swift and Posner (1971), by eluting a Sephadex G-100 with distilled water, showed that increasing concentrations of HS produced shifts of peaks from high to low molecular size ranges. They disregarded the possible hydrophobic retardation and qualitatively attributed the behavior they observed to the repulsion between HS and the negatively charged Sephadex. They also assumed that repulsion would become stronger if a decreasing HS concentration lowered the ionic strength. Using a classical polyelectrolytic (polymeric) view, they claimed that “charged double layers on the solute and the gel extend further into the solution, resulting in effectively larger solute molecules and smaller pore sizes. Charge repulsion effects therefore occur at great distances leading to increased exclusion with decreasing sample concentration.” However, in order to corroborate these theoretical assumptions, the authors did not carry out measurements of actual charge density at different sample concentrations or in the gel bed. Notwithstanding the theoretical and experimental inconclusiveness of HS behavior in gel permeation, buffers of high ionic strength were repeatedly recommended as mobile phases to suppress interferences caused by ionic exclusion (Swift and Posner, 1971; Swift, 1989; De Nobili et al., 1989).

High ionic-strength buffers reduce the molar volume of HS in solution and, by thermodynamically favoring the hydrophobic associations of humic molecules, invariably produce chromatograms with a bimodal distribution. However, they also enhance hydrophobic adsorption on the gel solid phase (Specht and Frimmel, 2000). Yonebayashi and Hattori (1987) showed that the addition of 2 M urea to either phosphate or borate buffers eliminates the hydrophobic adsorption observed during the GPC of HS. An explanation for the urea effect will be given below. Adsorption of HS on gel columns has traditionally been accounted for either by salinity or by changes in ionic strength (Lindqvist, 1967; De Nobili et al., 1989). Anderson and Hepburn (1978) attributed the appearance of new HS elution bands to adsorption when sodium acetate was added to the mobile phase and regarded these bands as unspecified artifacts created by adsorption effects. However, De Haan et al. (1987) demonstrated for the first time that HAs in saline solution were able to diffuse more freely across a dialysis membrane than when in solutions of lower ionic strength. After comparing dialysis and gel filtration experiments, they suggested that changes in elution profiles caused by ionic-strength variation had to be attributed to real alterations of humic conformations rather than to any interactions between the gel and HS. This was at variance with the classical explanations.

Further understanding came from the work of Yonebayashi and Hattori (1987) who studied several HS by gel filtration through Sephadex G-75. The eluent was 0.1 M phosphate or borate buffers in 2 M urea, and they thereby avoided interferences by both ionic exclusion and adsorption on the column. They first found that by increasing the pH of the buffer eluent from 4.7 to 11.2, the molecular sizes of the HS increased progressively, although the fraction excluded at high molecular-size (Vo) was larger at pH 4.7. Interestingly, the elution pattern at pH 4.7 showed adsorption of humic material beyond the total volume (Vt) of the column. They attributed the behavior at low pH to an aggregation process of humic molecules but still advocated ionic repulsion as the reason for the size increase at high pH. However, their experimental conditions (large ionic strength and urea) should have excluded an electrostatic effect.

Yonebayashi and Hattori (1987) also showed that for both phosphate and borate buffers and for all pH values from 4.7 to 11.2, the molecular sizes of HS increased significantly with time of standing (from 5 min to 24 h) in the buffer solution before injection into the gel column. To explain these findings, they had to invoke again a
process of molecular association into micelle-like aggregates. In order not to contradict the polymeric paradigm of HS, they assumed that association occurred between humic molecules of large and small molecular sizes. However, this explanation did not account for what they further observed when HS were treated with ethanol-benzene, 6 M HCl, 0.5 M H2SO4, or 5 M NaOH before dilution in the buffer and separation by GPC. After the ethanol-benzene or acid treatments, the gel chromatograms were almost the same as those for the original HA, but the fraction excluded increased with an increase of standing time in the buffer solution. Conversely, the excluded fraction disappeared after the alkaline treatment, and the gel chromatogram remained unchanged regardless of the standing time in the buffer solution before injection. Although they invoked only micellar aggregation to explain their findings, these results were clearly a sign of conformational changes caused by the establishment of hydrophobic interactions (see discussion below). The conformational rearrangement could take place in the buffer solution for all pretreated samples but not in 5 M NaOH, where a full humic dispersion (disruption of all hydrogen bonds) and hydrophobic rearrangement had already taken place.

In an attempt to clarify the formation of micellar aggregates, Yonebayashi and Hattori (1987) also measured the surface tension of HS. They found that the surface tension of the whole HS mixture in neutral phosphate buffer containing urea decreased with an increase in sample concentration. This suggested that molecular associations increase the surface activities of humic materials. In fact, four fractions isolated by GPC revealed that only the first HMW size fraction behaved as a surface active agent, whereas the three smaller fractions showed low surface activity. A cluster analysis grouping the gel behavior of several HS with their chemical and physical-chemical properties showed that HS with high surface activities (and a large fraction excluded at Vo) had a high content of -COOH groups, and a low content of -OCH3 groups. Based on the results of Yonebayashi and Hattori (1987), which were similar to those of Anderson and Hepburn (1978), it may be concluded that the associative nature of HS was proven by gel filtration studies. Fractions had different chemical compositions, and hydrophobic attractive forces between aliphatic components favor their association into apparently large and surface-active fractions.

The work of Ceccanti et al. (1989) has provided new evidence that HS behavior in GPC where changes in ionic strength occurred cannot be ascribed to simple electrostatic interactions (ionic exclusion), as for a polyelectrolyte (a polymer holding multiple charges). They fractionated HS by ultrafiltration and gel filtration using both water and salt solutions as mobile phases and found that 100% of the humic C was in the lowest molecular size fraction (less than nominal 10,000 Da) when HS were ultrafiltered in 100 mM of pyrophosphate/HCl buffer at pH 7.1. However, no humic C was in the same fraction when ultrafiltration was in water only, when 43% of the C was in the highest molecular size fraction (greater than nominal 100,000 Da). Contrary to previous qualitative research, Ceccanti et al. (1989) did measure by isoelectric focusing the negative net charge of HS fractions isolated by gel filtration. Despite the charge being very similar, refractionation of fractions in the Sephadex G-50 column in water gave distinctly different elution patterns. Moreover, fractionation by water elution from the polydextran Sephadex, which has some residual negative charges, gave a molecular size distribution in accordance with that obtained on the uncharged PM-10 (polysulfone) ultrafiltration membrane. They concluded that gel filtration occurred by a true size fractionation rather than by a charge-dependent elution. The work of Ceccanti et al. (1989) also showed that the strength and the shape of humic associations controlled the elution volumes of HS fractions.

A major problem in using size exclusion chromatography to determine the sizes of humic fractions is the lack of adequate standards to calibrate the gel column. A calibration of Sephadex columns with presumably size-controlled humic fractions differed substantially from that obtained with globular proteins (Cameron et al., 1972b). This indicated that the hydrodynamic behavior of HS is different from that of globular macromolecules, and their molecular size may be more apparent than real and could be ascribed to molecular aggregation phenomena. Hayes (1997) reported that the ability to obtain humic fractions of homogeneous size by repetitive gel fractionation is not effective because reprocessing of fractions results in the separation of smaller sized components.

The difficulty of obtaining MW values of HS by gel-filtration was noted by Reuter and Perdue...
They measured by osmometry a value of 1231 Da for the number-average MW of the HA fraction excluded from G-50. The published globular-protein exclusion limit for the gel is 30,000 Da. Again, the reason for the exclusion of the humic material at such an apparent high MW cut off may be attributable to the molecular aggregation of small molecules into apparent large sizes. This interpretation is substantiated by the lack of differences between the IR spectrum of the original HA and the spectra of fractions isolated by means of gels with different MW cutoff values (Reuter and Perdue, 1981). The association of small humic molecules into different aggregate sizes may have also been the reason why Cornel et al. (1986) could not find sufficient similarity between the diffusion behavior of HS and that of synthetic polymers of known compositions such as polyethylene oxides and polystyrene sulphonates. Further questions arose when Summers et al. (1987) did not find differences in the IR spectra of HS size fractions isolated by ultrafiltration, a separation technique that is slower than the velocity by which humic molecules aggregate or disaggregate in solution.

EVIDENCE FOR SUPRAMOLECULAR ASSOCIATIONS OF SMALL HUMIC MOLECULES

Using low-pressure size exclusion chromatography (SEC), Piccolo et al. (1996a and b) reported that the 280 nm absorption of HAs was reversibly shifted from high to low molecular size ranges (column total elution volume, _V_t_ ) when organic acids were added to lower the pH of a humic solution from 9.2 to 2 before the elution in an 0.02 M alkaline borate buffer. To explain their results they suggested that, instead of being stable polymers, HS at neutral or alkaline pH values are supramolecular associations of relatively small heterogeneous molecules held together by weak dispersive forces, such as van der Waals, π-π, CH-π, interactions. The addition of organic acids altered such unstable humic conformations through the formation of energy rich hydrogen bonds, and the subsequent chromatographic elution separated the resulting smaller subunits and prevented the reassociation that would have occurred in static conditions.

Supporters (Swift, 1999) of the traditional polymeric model of HS have criticized the above results, not on the basis of an experimental replication but on theoretical and qualitative interpretations of gel-solute interactions and interferences caused by a supposed polymeric form of borate. However, the results of Piccolo et al., (1996a and b) could not be attributed to a buffering action of the organic acid toward the alkaline eluent because the amount of the different organic acids varied by two orders of magnitude, but the shift to larger elution volumes remained the same for all acids. Nor could an elution delay caused by a solid deposition on the gel and subsequent resolubilization by the eluent have been the cause of the shift since the treated samples remained soluble at low pH and entered the chromatographic elution immediately after deposition on the column. Nevertheless, if this had been the case, a progressive neutralization of the acidic buffering capacity by the alkaline eluent would have caused a smearing out in the column of the polydisperse humic mixture, and a diffuse chromatographic band from lower to larger elution volumes, instead of the sharp peak at the total volume (_V_t_ ) of the column. Furthermore, the ionic strength effect (De Nobili et al., 1989) could not be invoked for the reversible peak shift because elution in an ionic strength quencher, such as a borate buffer 10 times more concentrated, gave the same chromatographic change upon AcOH and KOH additions.

Piccolo et al. (1996a and b) considered their findings to be an expression of the associative nature of relatively small humic molecules that self-assemble only into apparent high-molecular size materials. This interpretation was in accordance with previous research, which showed that HS behaved as molecular associations when studied by SEC (De Haan et al., 1987; Yonebayashi and Hattori, 1987; Ceccanti et al., 1989; Piccolo et al., 1990a). Moreover, laboratory observations have indicated that when AcOH is added to HS that have been already extensively dialyzed, further small size components are released during subsequent dialysis (Nardi et al., 1988). These low molecular size fractions are a product of a conformational rearrangement and a chemical composition different from the bulk HS. The separated fractions were found to stimulate specific biological properties in plants and were more biologically active than the whole humic materials from which they were separated (Piccolo et al., 1992).

An experimental replication of the work of Piccolo et al. (1996a and b) that applied high performance SEC was reported by Varga et al. (2000). They obtained the same results as Piccolo et al., (1996a and b), but they observed that by using organic acids to lower the pH of humic solutions before injection from 9.2 to 1–3, the shift of
absorption to lower molecular size ranges may have been caused by hydrophobic interactions on the column upon protonation of the humic matter and the consequent change in ionic strength. A contribution to SEC of hydrophobic interactions on the column has been long recognized in the gel-filtration studies of HS (Lindqvist, 1967) and of other biomolecules (Chicz and Regnier, 1990). Nevertheless, hydrophobic interactions on size-exclusion gels have often been usefully exploited to fractionate humic aggregates into simpler molecular associations that were characterized chemically more easily than the bulk humic material (Anderson and Hepburn, 1978; De Haan et al., 1987; Yonebayashi and Hattori, 1987).

It was necessary to verify the innovative explanation of the conformational changes of HS using additional experimentation, and High-Pressure Size Exclusion Chromatography (HPSEC) was adopted for further investigation. HPSEC is, for a number of reasons, a more valid chromatographic system than low-pressure GPC (Piccolo and Conte, 2000; Piccolo et al., 2001a). The major advantages of HPSEC are:

1) high reproducibility of chromatograms (5%);
2) relatively higher rapidity of analysis (about 60 minutes per chromatogram);
3) longer column life (more than 1000 injections per HPSEC column); and
4) higher sensitivity to chemical changes in injected samples because of a much lower loading mass.

Conte and Piccolo (1999a) have compared two commercial HPSEC columns with respect to their capacity to measure the molecular sizes of HS accurately and precisely.

In the HPSEC mode, Piccolo et al. (1999) reproduced the same shift of chromatographic peaks observed earlier by low-pressure gel filtration (Piccolo et al., 1996a and b) when the pH of humic solutions was adjusted from 7 to 3.5 by using only small amounts (<0.5 × 10⁻³ M) of a number of monocarboxylic acids and, thus, without significantly changing the ionic strength of the sample. The larger resolution and reproducibility of HPSEC columns, in comparison with the low-pressure gel phases, allowed the authors to assess whether the alteration of the HPSEC molecular size distribution was dependent on the number of C atoms in the organic acids and on the hydrophilic/hydrophobic (HI/HB) C ratio of HS, as measured by CPMAS ¹³C-NMR spectroscopy. It was found that the higher the C content of organic acids and the lower the HI/HB ratio of humic materials, the larger the decrease in the average molecular size of HS.

Conte and Piccolo (1999b) conducted a second HPSEC experiment in which, instead of lowering the pH of humic solutions before injection, they allowed the HPSEC eluent to be slightly modified. A control eluent at pH 7 (0.05 M NaNO₃, not absorbing light at 280 nm) was modified by addition of 2.0 × 10⁻⁶ M of either methanol, HCl, or AcOH to pH 6.97, 5.54, and 5.69, respectively, without changing the ionic strength (I = 0.0504 M). In this way the hydrophobic adsorptions, which may occur at increased ionic strengths (Lindqvist, 1967; Chicz and Regnier, 1990; Specht and Frimmel, 2000), were avoided. Moreover, sodium humate and fulvic solutions were previously titrated to pH 7 so that their dissolution in the mobile phase at pH 7 would have prevented any random occurrence of negative charges. This was to avoid any uncontrolled formation of negative charges on the solute, ascribed to changes in the ionic strength of humic solutions, thereby affecting the volume of sample elution (Swift and Posner, 1971). UV-Vis and Refractive Index (RI) detectors were used to record the chromatograms indicating the molecular-size distributions of the humic materials with the objective of comparing the chromatographic behavior of the chromophores (UV at 280 nm) with that of the real humic mass (RI). Both UV and RI detectors showed major alterations of the humic molecular-size distributions and dramatic decreases in the weight-average MW values in the modified eluents. The decrease in molecular size was revealed by the shift toward increasingly larger elution volumes for both the UV and RI detectors and by the concomitant reductions in peak absorbance at the UV-Vis detector. In the constancy of ionic strength, the observed changes could be ascribed to the interactions of the added chemicals with the weakly associated molecules giving rise to an apparent macromolecular structure of the humic matter. The slight modifications of the mobile phases may have caused the collapse of the heterogeneous humic aggregate into molecular associations of smaller dimensions but of greater thermodynamic stabilities than for the control solution. This was attributed to the gain in energy obtained by the formation of intermolecular hydrogen bonds (from 10 to 20 kJ mol⁻¹ for each hydrogen bond) among humic molecules in the modified mobile phases (Schwarzenbach et al.,...
The large decrease in molecular size suggests that the weak association of apparently high molecular size, as observed for the HA in the control solution, must, therefore, have been due predominantly to weak intermolecular hydrophobic forces such as van der Waals, π–π, and CH–π (Nishio et al., 1998) bonds, which hold small molecules together.

The work of Conte and Piccolo (1999b) provided further direct evidence for the conformational model based on the reversible self-association of small humic molecules rather than on the macropolymeric random-coil concept. Molecular size decreases caused by the breaking of ester and other covalent linkages by such small amounts of modifiers could not be proposed considering the customary strongly alkaline and acidic conditions to which the HS had been subjected during isolation and purification. Furthermore, using HPSEC, it was shown that the molecular size distribution of HS must be interpreted by a combination of two factors: the elution volume, and the molar absorbptivity of the chromatographic peaks. Earlier research had failed to address the combination of these factors either because closely similar HAs were analyzed by UV detection only and in less sensitive low-pressure size-exclusion systems (Swift and Posner, 1971) or because weakly UV absorbing FAs and/or (aqueous) dissolved organic matter (DOM) samples were used in studies with HPSEC systems without the support of RI detector systems (Becher et al., 1985; Berden and Berggren, 1990; Chin and Gschwend, 1991; Chin et al., 1994). More recent HPSEC investigations which coupled UV, RI, and MALS (Multi-Angle Light Scattering) detectors, have also indicated different molecular size distributions for HS according to the detector employed (von Wadruszka et al., 1999).

When ionic-exclusion interferences were carefully eliminated in the work of Conte and Piccolo (1999b), permanent adsorption of HS on the column was not found to occur (Conte and Piccolo, 1999a; Piccolo et al., 2001c), in accordance with other HPSEC studies in similar conditions (Becher et al., 1985; Mueller et al., 2000). However, a modification of the mobile phase by addition of methanol, HCl, and AcOH, although in very small amounts, may have produced pore-size changes that influenced non-SEC. In order to verify that mobile phase modification did not alter the exclusion properties of the HPSEC column, Piccolo et al. (2001b) subjected polymeric standards of known MW, such as the negatively charged polystyrenesulphonates (PSS) and neutral polysaccharides (PYR), to SEC in the same mobile phases used by Conte and Piccolo (1999b) for the HS studies. The undisputed polymeric nature of the covalently linked units of both nonionic PYR and polyelectrolytic PSS made a comparison of their chromatographic behavior (using the same experimental conditions) with that of HS relevant. Regardless of the charge density of the polymer, the similar chromatographic behavior of each in different mobile phases indicated that the slight variations in the compositions of the mobile phases were not sufficient to alter either the stability provided by strong covalent bonding or their interactions with the stationary phase. Conversely, the size-exclusion chromatograms, based on UV or RI detector systems, of three different HAs (HA1 from a volcanic soil, HA2 from an oxidized coal, and HA3 from a lignite) varied dramatically (in both peak absorbance and elution volumes) with the compositions of mobile phases.

Piccolo et al. (2001b) concluded that, whereas slight modifications in the mobile phase did not affect the column capacity for size exclusion, the substantial difference between the response to HPSEC of the polymeric standards and that of HS in the very same chromatographic conditions proved that humic materials have different structural assemblies. As proposed earlier, this may well be a self-assembling association of relatively small and heterogeneous molecules rather than a coil of polymeric macromolecules. To verify that such variations were not specific for the wavelength used to record the chromatograms (280 nm), and thereby simply accountable to shifts of peak maxima, Piccolo et al. (2001b) also recorded UV spectra of HS solutions over a range of wave lengths. Their results showed that the three humic materials also produced different absorbance values upon modification of their solutions over a wide range of wavelengths. This was regarded as confirming that the molar absorptivity of the bulk HS varied with the composition of the solution. The absorptivity was decreased by adding chemicals which disrupt their weakly-stabilized molecular associations.

Piccolo et al. (2000a) also compared the chromatographic behavior of HS with that of neutral PYR and polyelectrolytic PSS when solutions (in 0.05 M NaNO3) before injection into the HPSEC system were titrated from pH 7 to 3.5 with either HCl or AcOH, as was done for HS in the work outlined above (Piccolo et al., 1999). Contrary to the work of Piccolo et al. (2001b),
the mobile phase was kept constant while the solutions under analysis were modified. It was found that plots of retention volumes (obtained using either the RI detector for PYR and PSS polymers of known MW or the UV detector for only PSS standards) versus Ln (MW) did not show significant differences between the control and samples added with either HCl or AcOH. This result indicated that the capacity of the HPSEC column in aqueous media to exclude by size was not altered by injecting the polymer solutions brought to pH 3.5 with a very small amount of acid. However, when HS solutions were similarly modified by lowering pH and chromatographed in the same mobile phase, the elution of the acid-added humic samples differed significantly from the control humic solution. As in previous experiments, these results were considered to be evidence that a humic association, stabilized only by weak dispersive forces at pH 7 (mainly hydrophobic forces), varies considerably with respect to the arrangements of its component molecules and its size distribution when treated with an acid. In contrast, covalently linked polymers have more stable molecular arrangements and their HPSEC behavior is not affected by an interaction with either a mineral or an amphiphilic acid such as AcOH.

Cozzolino et al. (2001) studied the effect of organic acids of plant, microbial, or anthropic origins on the molecular size distribution of dissolved HS. They used a Phenomenex Biosep S2000 column and eluted with a 0.05 M NaN0₃ solution to evaluate size changes in four different HS upon addition of hydroxy- (glycolic and malic), keto- (glyoxylic), and sulfonic (benzenesulfonic and methanesulfonic) acids. All HS showed a decrease in peak absorbance when humic matter was dissolved in the HPSEC mobile phase at pH 7, and the pH of the solution was lowered to 3.5 by acid addition before analysis. This effect was generally accompanied by an increase in peak elution volumes. The overall decreases of the total areas of the chromatograms compared with the control HS solutions was again explained with a disruption of supramolecular humic aggregates into smaller sized but more energy-rich associations brought about by the formation of mixed intermolecular hydrogen bonds after treatment with acid. The hydroxy-bicarboxylic malic acid was the most effective in disrupting the original humic associations. This was attributed to its greater capacity to form new hydrogen bonds with complementary functions of HS. Malic acid is a bicarboxylic acid (HOOC-CH₂-CHOH-COOH) with two carboxyl groups (pKₐ₁ = 3.4; pKₐ₃ = 5.11) that can either be protonated or partially dissociated at pH 3.5, thereby allowing a larger number of mixed hydrogen bonds to form on its oxygen-containing functions than could be formed either by the strong hydrochloric acid (which is a proton donor only) or by the more weakly acidic monocarboxylic glycolic and glyoxylic acids. The extent of molecular association variation was related not only to the pKa values of the acids but also to the chemical and stereochemical affinities of the humic components that would allow penetration of acids into the inner humic domains, depending on the acid structures. For example, the strongly acidic methanesulfonic and benzenesulfonic acids showed effects that varied with the humic properties. Methanesulfonic acid was more effective in the disruption of the molecular associations of HS that contained predominantly aliphatic and alkyl moieties, and, equally, benzenesulfonic acid disrupted the distribution of the aromatic-rich humic material because of the probable larger π-π interactions with aromatic humic components.

The indications of the supramolecular nature of HS provided by the analytical HPSEC studies has caused Piccolo et al. (2001c) to employ a preparative HPSEC column to separate humic size fractions that might be more homogeneous chemically, and they have subjected these fractions to chemical and spectroscopic characterization. A HPSEC fractionation was carried out on the same HS before and after adjusting the pH of the humic solution (0.05 M ionic strength) from 7 to 3.5 with 0.5 × 10⁻³ M AcOH. Six fractions were collected from the HPSEC separation of the HA solution at pH 7.0, whereas eight fractions were obtained from the separation of the humic solution treated with AcOH to pH 3.5 before HPSEC injections. Fractionation was run continuously using an auto-sampler and a fraction collector, and the chromatographic pattern monitored by means of a UV detector. Reproducibility was excellent (CV < 5%) for more than 100 runs for each fractionation series. Moreover, elemental analyses on the sum of fractions from both untreated and AcOH-treated HS excluded the possibility of any significant adsorption of humic matter on the column stationary phase. The molecular size distribution of the untreated HS was significantly different from that of the AcOH-treated HS. The latter showed much less peak absorbance (280 nm), and several additional peaks appeared at higher elution times.
(120–180 min). Size-fractions were analyzed by a Curie point (610 °C) Pyrolysis–Gas Chromatography–Mass Spectroscopy (Py-GC-MS) technique and by 1H-NMR spectroscopy. Total ion chromatograms by Py-GC–MS showed that fractions had a significantly different chemical composition after AcOH treatment, thereby confirming that AcOH had caused rearrangement of the humic associations in solution and that preparative HPSEC is adequate for HS fractionation. Py-GC–MS spectra showed that the AcOH addition altered the distribution of humic molecular components in the size-fractions. The unsaturated alkyl chains were moved from size-fractions of larger molecular size into those of lower molecular sizes. Most of the aromatic moieties, which were found in larger molecular size fractions for the untreated HA, were spread into fractions of lower molecular size after AcOH addition to HA. Carbohydrates, which were undetectable in any fraction of the untreated HA, appeared instead in the pyrogram of the lowest molecular size (and the most hydrophilic fraction) after treatment with AcOH. Our results suggested that AcOH disrupted the weakly bound association of humic constituents and HPSEC elution separated size-fractions of different compositions. The fractions with the largest apparent molecular sizes were the richest in alkyl chains, suggesting that humic molecules were stabilized into supramolecular associations by multiple weak interactions among apolar groups such as alkyl chains and aromatic moieties. 1H-NMR spectra of HPSEC fractions not only confirmed the findings by Py-GC–MS analyses but also showed that the chemical compositions of fractions were less complex after AcOH treatment. This was attributed to a less complex molecular association in the size separates, and that allowed a larger solubility in the NMR solvent and more favorable spin-lattice relaxation times.

Piccolo et al. (2001c) showed, by combining fraction-separation by HPSEC with chemical and spectroscopic characterization, that the fractions with larger apparent molecular sizes were composed predominantly of a mixture of alkyl compounds of relatively low molecular sizes, whereas fractions of lower apparent molecular sizes contained small aromatic systems and hydrophilic compounds. These conclusions were in harmony with previous research which characterized HS fractions separated by either low-pressure GPC or ultrafiltration (Anderson and Hepburn, 1978; Yoneyabashi and Hattori, 1987; Piccolo et al., 1990a).

SOLUTE-GEL-ELUENT AS AN INTERACTIVE SYSTEM IN EXCLUSION CHROMATOGRAPHY

Although the above results by HPSEC should be regarded as free of interferences from ionic exclusion and adsorption (coefficient of variation for more than five runs was consistently less than 5%, and there was a lack of adsorption of humic matter on the columns), a further check for possible hydrophobic adsorption phenomena was conducted. Seven molecules that might be considered monomeric constituents of HS (Stevenson, 1994) were subjected to HPSEC separately and in a full mixture (1 g L⁻¹). Elution was with a phosphate buffer at pH 3. The individual molecules that were size-excluded gave the elution volumes reported in Table 1, whereas the mixture of the seven molecules produced an elution profile with only two peaks (Fig. 1).

It must be noted that the retention volumes of the two peaks for the mixture did not correspond to any of the elution volumes found for the single molecular species (Table 1). The first peak for the mixture eluted (23.07 mL) before any of the single species, with the exception of the highly hydrophilic glucosamine, and the more intense second peak was eluted before all other monomers except dihydroxyphenylacetic and gallic acids. These results suggest that when the single molecular species are mixed together in solution, they form weak mutual associations giving rise to hydrodynamic radius values larger than when they are eluted alone. Based on the larger single elution volumes of the more hydrophobic monomers (hydrocaffeic acid, resorcinol, and catechol), it can be inferred that their elution is retarded by adsorption on the stationary phase of the column. Conversely,

<table>
<thead>
<tr>
<th>HPSEC retention volumes (mL) of different monomers and mixtures of the same monomers on a column Polysep GFC-P3000 eluted with a phosphate buffer at pH 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomers/Mixtures</td>
</tr>
<tr>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>Gallic acid</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
</tr>
<tr>
<td>Hydrocaffeic acid</td>
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<tr>
<td>Glucosammine (RI)</td>
</tr>
<tr>
<td>Resorcinol</td>
</tr>
<tr>
<td>Catechol</td>
</tr>
<tr>
<td>Mixture of the above (0 h)</td>
</tr>
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<td>Mixture of the above (after 144 h standing)</td>
</tr>
</tbody>
</table>
when the same compounds are in mixtures with other monomers the thermodynamic drive to decrease exposure of hydrophobic components to the aqueous medium increases the mutual attraction of monomers. This interpretation is substantiated by the observation that the retention volumes of mixture peaks decreased in value with time of standing before injection (Table 1), thereby indicating a further hydrophobic strengthening of the monomer associations. A consequence is that the hydrophobic interactions of single monomers with the column matrix are reduced while exclusion of the monomer association is expedited.

Evidence that hydrophobic adsorption on the column is not irreversible and affects only elution volumes was obtained by eluting progressively less concentrated solutions of the mixture. The absorbance decrease of both peaks given by the mixture gave a highly significant linear correlation with the decrease in the concentration of the mixture, as expected by the Lambert and Beer law (Fig. 2). This suggests that there were no losses attributable to adsorption on the column of chemical species that might be regarded as representative of monomeric structures in HS.

The behavior of the monomer mixture in HPSEC may have similarities with that of associations of humic molecules. When the apparently large-size, weakly bound humic associations are altered by the action of organic acids or by other species (uncharged compounds, electrolytes, cations), smaller humic clusters are separated and their elution is retarded by size exclusion; in addition, the hydrophobic interactions with the column matrix will have changed.

Other evidence that humic molecules are suprastructural associations in aqueous solutions is given by the elution patterns of HS (in HPSEC experiments) in 8 M urea. Concentrated urea is employed to disrupt protein-protein interactions and to solubilize aggregating hydrophobic proteins for their further separation (Hjelmeland, 1990). Concentrated urea has also been proposed for low-pressure size exclusion and polyacrylamide gel electrophoresis (PAGE) separations of HS because it is considered able to produce elution patterns similar to 0.1 M Tris-HCl buffer at pH 9 (Trubetskoj et al., 1997).

The elution profiles of three different purified HS (0.2 g L⁻¹) dissolved in 8 M urea and eluted in the same solution are shown in Fig. 3. The chromatograms were detected by both UV and RI detectors in a series. This experiment is illustrative of the capacity of urea to separate the hydrophobic from the hydrophilic components of HS. The chromatograms show a net separation of humic matter: a high molecular size fraction at the column V₀ visible only by the UV detector.
and a low molecular-size fraction eluting at the column V_t detected only by the RI detector. The hydrophobic humic molecules are strongly associated through hydrophobic effects induced by the highly concentrated urea. The urea interacts more favorably than hydrophobic molecules with the network of the water structure. Moreover, since urea is known to form complexes with nonionic detergents (Hjelmeland, 1990), it may also be possible that complexes between urea and nonionic humic hydrophobic compounds increased the hydrodynamic radius of the hydrophobic associations, causing these to elute at the earliest exclusion volume of the column.

Differences among HS could also be noted. The HA from an oxidized coal was the only one to show two well separated peaks in the RI detector system (Fig. 3, I-RI), whereas only one very intense peak (>1000 mV) at the V_v column was shown by the UV detector (Fig. 3, I-UV). This indicates that while light-absorbing chromophores (280 nm) with high molar absorbivities were excluded rapidly from the column, their concentrations in the humic samples were relevant and of a similar order of magnitude as the nonabsorbing hydrophilic, probably ionized, compounds detected by the RI detector at the total exclusion volume of the column. The HA from lignite gave a similar complete separation between the large-size hydrophobic chromophore fraction (Fig. 3, II-UV) and the small-size ionized and hydrophilic components shown by the RI detector (Fig. 3, II-RI). However, although the molar absorbivity of the excluding chromophores was still high (as suggested by an absorption intensity > 800 mV), the lack of corresponding peaks at the RI detector indicated that their concentrations in the sample were much less than those of the hydrophilic components excluded at large elution volumes. Identical behavior was shown by the HA extracted from an agricultural soil. Also for this sample, the chromophores association, excluded at the column void volume (Fig. 3, III-UV), was hardly representative of the mass of humic sample since the corresponding peak at the RI detector was almost irrelevant compared with the signal of the

Fig. 3. UV- and RI-(Refractive Index) detected HPSEC chromatograms of humic acids dissolved and eluted in 8M urea solution. I. humic acid from an oxidized coal; II. humic acids from a lignite; III. humic acid from a Danish agricultural soil.
small size, nonabsorbing, hydrophilic constituents (Fig. 3, III-R1).

The two experiments reported above suggest that in the HPSEC analysis of associations of molecules such as HS, the stationary and mobile phases lose the properties associated with specific single molecules alone. Rather, they become part of an interactive system involving the mixture of solutes. It is the whole solute-gel-eluent system that produces the size-exclusion of the association under analysis. Moreover, it is the humic conformational structure, and the degree of potential hydrophobic interaction between the associated molecules and either the stationary or the mobile phases, that determines the elution volume of a humic association in aqueous solution. This applies more than ionic exclusion, so often considered in the past to explain the SEC behavior of HS. These results also point out that the molecular sizes of HAs are not related to the molar absorptivity of chromophores in the humic samples, and they should be assessed by detection means that are related to the total mass of the HAs.

POLYMERIZATION OF HUMIC SUBSTANCES BY OXIDATIVE CATALYTIC REACTIONS

To understand humus as a supramolecular association of small molecules it is necessary to overcome the limitations imposed by the paradigmatic polymeric model. If HS are seen as weakly bound supramolecular associations their unstable conformations could then be stabilized in real polymeric structures. This could be achieved by increasing the number of intermolecular covalent bonds via an oxidative coupling reaction catalyzed by oxidative enzymes such as the phenoloxidases. This class of enzymes has been shown to promote, through a free-radical mechanism, oligo- and poly-merization of phenols and anilines, and, hence, is believed to contribute to soil detoxification from related organic contaminants (Kim et al., 1997).

Piccolo et al. (2000b) turned a loosely bound humic superstructure into a covalently linked polymer by treating a humic material dissolved in 0.1 M phosphate buffer at pH 7 with horseradish peroxidase (HRP) and hydrogen peroxide (oxidant). They used HPSEC to evaluate the changes in molecular size distribution brought about by the oxidative reaction with HRP catalysis. Moreover, AcOH was added to the reacted humic mixture to bring the pH to 4, and HPSEC injection was then used to assess the stability of the humic conformation following the polymerization reaction. The polymerized HS had HPSEC absorptions of larger intensities, and the elution volumes were shifted to lower values than the control. Moreover, treatment with AcOH did not alter significantly the chromatographic appearance of the polymerized HS, whereas it produced disruption of the loosely bound association of the untreated HS resulting from a significant reduction of the intensities of the peaks and their shifts to larger elution volumes. These changes indicated a significant increase in the molecular sizes of the humic materials resulting from oxidation catalyzed by HRP, which was attributable to a true polymerization of humic molecules via the formation of C-O or C-C bonds. Furthermore, DRIFT (Diffuse Reflectance Infrared Fourier Transform) spectroscopy was used by Piccolo et al. (2000b) to verify the effect of the oxidative polymerization reaction on the molecular structure of the HS. In comparison with the control, the DRIFT spectrum of the humic material subjected to oxidative coupling showed a substantial change in the 1500–900 cm\(^{-1}\) frequency interval with the appearance of three main bands at 1247, 1097, and 947 cm\(^{-1}\) and a decrease in the 1400 and 1227 cm\(^{-1}\) bands. The absorption shown at 1247 and at 1097 cm\(^{-1}\) was reasonably assigned to bond deformation of aryl and alkyl ethers, respectively, which were formed during free-radical coupling reactions catalyzed by HRP and, hence, confirm the interpretation of HPSEC measurements.

The HPSEC and DRIFT results of Piccolo et al. (2000b) suggest that the small heterogeneous molecules present in HS, as in weakly associated superstructures, can be covalently bound into true oligo- or polymers by an oxidative coupling reaction catalyzed by a peroxidase enzyme. The extent of covalent polymerization should be a function of the amount of humic molecules, mainly phenolic or benzenecarboxylic acids derived from lignin and microbial biosynthesis, which may undergo oxidative coupling reactions. However, it should be imagined that other classes of compounds may become assimilated into the macromolecular structures of polymerized humus.

Cozzolino and Piccolo (2001) extended the polymerization catalysis by HRP to other HS and studied the effects of solution pH (4.7 and 7) and compositions of humic associations. By HPSEC experiments they confirmed that an increase in weight-average MW occurred invariably for all HS subjected to oxidative polymerization. More-
over, a comparison of chromatograms and of MW values obtained by treating humic solutions with AcOH to pH 3.5 before HPSEC injection confirmed that the increase in molecular size by HRP catalysis was stable and caused by the formation of covalent bonds in the reacting humic molecules. However, covalent polymerization of humic molecules was found to proceed to a greater extent at pH 7 than at pH 4.7, despite the fact that HRP is most active at the latter pH. The difference in reactivity was attributed to the large mobility of reacting molecules in the hydrated and relatively smaller humic associations stabilized only by weak dispersive (hydrophobic) forces at pH 7. Reactive humic molecules are more mobile at neutral pH, and the polymerization via a free-radical mechanism is more efficient. Conversely, intermolecular hydrogen bonds formed at pH 4.7 confer a larger size and rigidity to humic associations, whereas the mobility as well as the reactivity of small molecules are thereby decreased.

Synthetic complexes formed with Al-(hydr) oxide preparations of montmorillonite were made using HAs from an oxidized coal and a lignite (Violante et al., 1999) to model organo-mineral complexes of soils using humic matter similar to that of soil. In a separate experiment, these synthetic clay–humic complexes were subjected to the oxidative reaction with HRP as a catalyst and H₂O₂ as an oxidant. No changes in the OC contents were observed for the oxidative conditions applied. Extraction of the complexes with alkaline-pyrophosphate solutions allowed the determination of the amounts of HS solubilizable before and after the treatment with HRP. Figure 4 shows that the yields extracted in the cases of both humic–clay complexes decreased significantly after the oxidative coupling reaction, ranging from about 42 to 32% and from 40 to 29% for the complexes made of HA from oxidized coal and lignite, respectively. These results indicate that polymerization of humic molecules occurred also in the solid phase of the clay–humic complexes and the increase in molecular sizes of the humic materials was the most probable cause for the reduction in extraction yields. General similarities to soil HS of the humic material were used to form the model clay–humic complexes, and, thus, it would also seem to be possible to induce the polymerization of HS in natural soil samples in order to control or change the properties of native SOM.

The evidence shown here that humic supramolecular associations can be turned into more stable covalently linked structures of larger molecular size can be interpreted as additional evidence that HS may be present in soils as associations of relatively small molecules.

CHEMICAL AND SPECTROSCOPIC EVIDENCE OF SUPRAMOLECULAR ASSOCIATIONS

The model of self-assembling supramolecular association of HS is related to mutual affinities of certain molecules in aqueous solutions. Molecules tend to associate by intermolecular forces (Israelachvili, 1994), and the strength of the association depends on their molecular structures. Particularly strong associations are formed by apolar compounds via the hydrophobic effect (Tanford, 1980). A humic supramolecular association in solution is thus formed by the self-organization of hydrophobic and amphiphilic compounds. The associations are isolated progressively from the network of water structure. Such separation results in an increase in the entropy of the system and in the overall energy stabilization as the different humic molecules form into a superstructure.

The importance of hydrophobic humic components in phenomena of aggregation in solution and on surfaces, as well as in controlling the reactivity of HS, is well documented (Wershaw, 1999). Investigations by fluorescence quenching
techniques provided evidence of hydrophobic microdomains in loose humic associations (Morra et al., 1990; Engebretson and von Wandruszka, 1994). By following the diffusion of 1,2-dichloroethane into humic matter, Aochi and Farmer (1997) showed that there are discrete microregions of different polarities in humic structures. Chien et al., (1997) studied by 19F-NMR spectroscopy the interactions of a trifluoromethylated atrazine with a soil HA by measuring the NMR relaxation of atrazine in the presence of both hydrophilic and hydrophobic paramagnetic probes. They confirmed the existence of hydrophobic domains by showing that atrazine occupied a domain of HS accessible only to neutral hydrophobic molecules. Similar results were obtained by Piccolo et al. (1998) who studied the isotherms for the adsorption of atrazine by HS extracted from two soils with four different extractants. The most hydrophobic humic extract, isolated from soil by an acetone-HCl solution, adsorbed more and desorbed less atrazine than the more hydrophilic humic materials extracted by either an alkaline or an alkaline-pyrophosphate solution. Since the acetone-HCl extract represents only a small fraction of soil HS (Piccolo et al., 1990b), the study of Piccolo et al. (1998) inferred that humic microdomains of different polarities are also present in soil and their distributions determine their reactivities.

Direct evidence for the presence of hydrophobic domains in HS were further given by Kohl et al. (2000), who studied the sorptive uptake of hexafluorobenzene by two peat samples using solid-state 19F-NMR spectroscopy. They found that the sorption process was rapid and related to the soil lipid content, whereas removal of the lipids decreased significantly the sorptive capacity of SOM. Hu et al. (2000), using solid-state NMR and wide-angle X-ray scattering (WAXS) detected crystalline domains composed of poly-(methylene) chains in several samples of SOM, humins, and HAs from soil and coal. Their results indicated a crystallite thickness equivalent to approximately 25 CH₂ units to give hydrophobic crystalline domains. A comparable amount of noncrystalline and more isotropically mobile poly (methylene) chains were also found and, together with the noncrystalline materials, larger aggregates were formed. In accordance with the supramolecular association model described here, Hu et al. (2000) concluded that the crystallites are expected to be resistant to environmental attack and, thus, inert in the soil and likely to have long residence times, whereas amorphous regions may play a role in the sorption of nonpolar molecules in soil.

Disruption of humic supramolecular associations as a result of the formation of hydrogen bonds stronger than the hydrophobic forces stabilizing the original conformation was shown by Miano et al. (1992). They used fluorescence and infrared spectroscopy to investigate the effects of the addition of glyphosate [N-(phosphonomethyl) glycine] herbicide to a purified humic solution to bring the pH from 9 to progressively lower values. The excitation spectra revealed increasing fluorescence quenching effects with increasing glyphosate contents at high wavelengths. Synchronous spectra showed a decrease in the main peak intensity with increasing additions of herbicide. These results were interpreted in terms of a disaggregation of the humic supramolecular association as the result of the formation of multiple hydrogen bonds between glyphosate and the small humic molecules. This implied that a decrease in electron delocalization, attributable to the apparent large molecular size of the HS, was responsible for the bands at high wavelengths. The molecular and conformational structures of HS were confirmed as determinants of the adsorption of glyphosate on different humic materials (Piccolo et al., 1996c). The high content of aliphatic components and the large and flexible molecular size increased adsorption of the herbicide on HS, possibly because of the involvement of dispersive hydrophobic interactions, together with hydrogen bonds.

Conte et al. (1997) showed that the formation of tightly bound hydrophobic domains in solutions of HS could alter the quantitative evaluation of humic C distribution in 13C-NMR spectra recorded in the liquid state. A comparison between liquid-state NMR spectra and solid-state CPMAS-NMR spectra of several HS showed that the amounts of alkyl groups measured by the former technique were invariably lower than for the latter method. The authors attributed this result to the separated phase that the humic hydrophobic components create to decrease the total solvation energy in solution and to the consequent limited spin-lattice relaxation time of hydrophobic carbons. Although liquid-state spectra may, therefore, show lower signals in the alkyl-C region, this does not occur in the solid-state mode, where signal recording occurs by polarization transfer from protons near the carbons measured.

In another approach, Kenworthy and Hayes (1997) used the fluorescence quenching of pyrene by bromide to investigate the nature of
humic associations. They found that pyrene in a HS solution was protected from bromide quenching. This protection was lost, however, when AcOH, followed by a base, was added to the medium. The authors considered that hydrophobic associations of the humic molecules protected the pyrene from the bromide. Treatment of the humic solution with AcOH, as proposed by Piccolo et al. (1996a and b), removed that protection because of disaggregation of the loose humic superstructure. This suggested that the HS in solution were associations of low MW masses held together by hydrophobic bonding and in which pyrene was enveloped.

Supramolecular associations of HS were found by Haider et al. (2000) and Ricca et al. (2000) to be disrupted into smaller components by the action of derivatizing reagents (trimethyl silyl or halogenated alkyl compounds) to silylate or methylate acidic oxygenated functions. Such simple derivatizations, which could not break ether and ester linkages, disaggregated the weakly assembled humic supramolecular structures into smaller entities that readily dissolve in organic solvents, elute in the low molecular size ranges by HPSEC (Haider et al., 2000), and produce better resolved NMR and FTIR spectra (Ricca et al., 2000). Wanner et al. (2000) used the concept of disaggregation of humic supramolecular associations to explain the shift in gel permeation to larger elution volumes of the radioactivity of a 14C-labeled dithianon fungicide bound to HS extracted from soil after 64 days of incubation together with straw. The same material extracted from soil without incubation with straw indicated that the radioactivity was mainly in the large molecular size fraction of the HS. The authors reasoned that organic acids or other amphiphilic compounds produced during microbial degradation of maize would have disrupted the association of humic molecules as proposed by Piccolo et al. (1996a and b).

Data from Simpson et al. (2000), who used Diffusion Ordered Spectroscopy (DOSY) NMR, which has enhanced sensitivity because the probe circuitry is kept at 22 °K, support the supramolecular model proposed by Piccolo and coworkers. In their preliminary study, the authors observed that the Nuclear Overhauser Effect (NOE) of a supramolecular association of a large undefined number of components into a specific phase having more or less well-defined microscopic organization and macroscopic characteristics depending on its nature (such as films, layers, membranes, vesicles, micelles, mesomorphic phases, solid state structures, etc.).

CONCEPTS OF SUPRAMOLECULAR ASSOCIATIONS OF HUMIC SUBSTANCES

The results of the experiments described that used either analytical or preparative SEC cannot be explained by analytical interferences or by the traditional polymeric model of HS. Rather, they can be interpreted by the concept of loosely bound humic supramolecular associations. In this concept, one can imagine HS to be relatively small and heterogeneous molecules of various origins that self-organize in supramolecular conformations. Humic superstructures of relatively small molecules are not associated by covalent bonds but are stabilized only by weak forces such as dispersive hydrophobic interactions (van der Waals, π–π, and CH–π bondings) and hydrogen bonds, the latter being progressively more important at low pH values. Hydrophilic and hydrophobic domains of humic molecules can be contiguous to or contained in each other and, in hydration water, form apparently large molecular size associations. In humic supramolecular organizations, the intermolecular forces determine the conformational structure of HS, and the complexities of the multiple noncovalent interactions control their environmental reactivity.

The definition given by Lehn (1995) may well be applied to HS: “Supramolecular assemblies (are) molecular entities that result from the spontaneous association of a large undefined number of components into a specific phase having more or less well-defined microscopic organization and macroscopic characteristics depending on its nature (such as films, layers, membranes, vesicles, micelles, mesomorphic phases, solid state structures, etc.).”

On consideration of the concept of supramolecular associations, the classical definitions of
HAs and of FAs should be reconsidered. The FAs may be regarded as associations of small hydrophilic molecules in which there are enough acidic functional groups to keep the fulvic clusters dispersed in solution at any pH. The HAs are composed by associations of predominantly hydrophobic compounds (polymethylene chains, fatty acids, steroid compounds) that are stabilized at neutral pH by hydrophobic dispersive forces (van der Waals, \(\pi-\pi\), and CH-\(\pi\) bondings). Their conformations grow progressively in size when intermolecular hydrogen bonds are increasingly formed at lower pH values until they flocculate.

**FUTURE PERSPECTIVES IN RESEARCH AND TECHNOLOGY**

A clarification of the aggregate structures of HS represents a major innovation in humus chemistry. The concepts presented here emphasize that HS are not macromolecular polymers, as they have been described for so long, but are rather superstructures of apparent large size only and are self-assembled from relatively small heterogeneous molecules held together mainly by hydrophobic dispersive forces. These concepts provide a new opportunity to enlarge knowledge of both the detailed chemistry and the management of HAs in soil and in the environment.

Chromatographic methods of separation such as HPSEC were found to produce reproducible and more homogenous fractions of humic superstructures. Awareness of the weak forces that cause the self-assembling of humic molecules allows methods to be devised based on interactions with chemical species, such as amphiphilic organic acids, urea, mono- and polyvalent cations, that can disrupt the apparently large humic associations and obtain fractions that are chemically simpler and more homogeneous.

The combination of chromatographic methods with spectroscopic techniques such as NMR, IR, and ESR spectroscopy, together with the different modern variations of mass-spectrometry, has increased enormously the potential to derive a complete picture of the secondary molecular structure of HS. The time is near when there will be a good grasp of the molecular structures and associations of HS, based on chemical methods rather than on computer models. Our ability to obtain a full molecular structure of any humic material or natural organic matter (NOM) molecule from any environment and ecosystem will then be limited by only the advances in analytical automation. When we consider the rapidity with which certain biological research fields can advance, this limitation could easily be overcome, provided there is the necessary public and private interest to pursue knowledge in a field that is vital for the well-being of our planet and the biological life on it.

The novel understanding of HS as supramolecular associations as described here has great implications in soil and environmental management. One example is the possibility of turning the loose humic superstructures into real covalently linked polymers by catalytic technology that can ensure polymerization of humic molecules in both water and soil environments. Such technology can improve our capacity to control SOM management, reduce the risk of erosion, and limit soil desertification with the obvious improvement of soil productivity. Another example of the potential of the polymerization of soil humic molecules in situ is the possibility of controlling CO\(_2\) emission from agricultural soils by sequestering the organic C in more stable polymerized humus. Finally, a large number of observations indicate that humic molecules released from large supramolecular associations can influence nutrient uptake by plants and increase crop yields significantly. Combinations of refined chemical analyses of humic molecules with physiological studies on their effect on plants may clarify the mechanism(s) by which soil HS increase crop yields and, possibly, revive the former interests in humus based fertility. The use of humic molecules, either native or exogenous, in combination with inorganic fertilizers, to maximize plant nutrient uptake and final yields, may also have a tremendous impact on increasing the economic efficiency of fertilizers and protecting the environment from the pollution caused by excess uses of fertilizers.

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**REFERENCES**


