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Review

Relevance of Organic Matter Compositions, Structures and Associations to Soil Aggregates and to Sustainable Productivity

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Abstract

Long-term cultivation practices, in which mineral fertilizers are the only amendments made to crop-supporting soils, are giving rise to the degradation of soil structures in the world's most fertile soils. This leads to erosion and to the loss of productivity and may well become a greater threat than that of global warming. Humic substances (structurally related compounds), and humin (which no longer falls within the modern definitions of humic substances), are major transformation or humification components of organic matter entering the soil, with varying resistance to biological degradation, and properties vastly contributing to soil fertility. There is considerable discussion on the macromolecular structures arising from associations or supramolecular structuring of some components of humic substances. The compositions, structures, shapes, sizes, and surface properties of these molecular components determine their intra- and inter-molecular associations, their interactions with the soil particles, and particularly with the soil inorganic colloids. Such interactions play a vital role in soil aggregates' formation, which is important for soil health and productivity. In this work, an outline is given of modern methods for the isolation of broadly defined soil organic components, of what is known of their origins (plant or microbial), compositions, sizes and shapes, of how they interact to promote soil structure and productivity, and how the materials composing the hydrophobic fraction form strong associations with the inorganic colloids. A better understanding should be sought of how these interactions and associations take place giving rise to the structured systems that are characteristic of fertile soils.

Keywords: humic substances; humin; soil fertility; humic substances structure; XAD-4 acids; fulvic acid; soil organic matter; soil aggregates



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1. Introduction

Soil organic matter (SOM) constitutes a large reservoir of global carbon. Hence, considerations of soil organic carbon (SOC) should take account of its abundances, and how its management can have relevance to carbon sequestration, depletion, and soil fertility. Cárceles Rodríguez et al. [1] and Gayan et al. [2] have emphasized the importance of soil health and sustainability for modern agricultural management, especially when facing the challenges of climate change and carbon neutrality.

The world's SOC pool represents the largest component of the terrestrial biosphere carbon, and it is estimated that it amounts to about 1550 Gt, and with an additional 850 Gt

of inorganic carbon [3]. Soil organic matter is estimated to contain three to four times more carbon than all living biomass on the Earth's surface.

Some modern studies of soil carbon disregard the vast variety of organic components and structures in SOM. A 'Soil Continuum Model' (SCM) proposed by Lehmann and Kleber [4] considers that decomposer organisms have ready access to the components of SOM, which they regard as a continuum spanning the full range from intact plant material to highly oxidized carbon in carboxylic acids. They suggest that in this 'continuum', organic fragments are continuously processed by a decomposer community from large plant and animal residues towards smaller molecular size. At the same time, greater oxidation of the organic materials increases solubility in water as well as affording the opportunity for protection against further decomposition through association with mineral surfaces and incorporation into aggregates [4]. It has been suggested that temporal changes in the spatial assemblage of SOM in relation to microbes may be an important driver of SOC persistence. This theory is based on the energy return on investment and adaptation by microorganisms and transport of organic matter over time so that, the interaction between molecular diversity of organic compounds, their spatial heterogeneity and temporal variability may dictate the persistence of SOC [5,6].

Hayes and Swift argued that the concept of a 'continuum' overlooks transformation products that exhibit inherent persistence due to their chemical composition, rather than solely from protection via interactions with soil minerals [7]. Hayes and Swift agreed that all organic materials of natural origin entering the soil will eventually decompose and referenced Jenkinson [8] who pointed out that if any material were completely resistant to decomposition, it would by now cover the Earth's surface. We must acknowledge that the degradability of materials varies, with some being significantly more resistant than others.

This article examines aspects of the composition and reactivity of the components that make SOM the essential material—alongside inorganic colloids and relatively inert mineral 'filler' materials such as sand and pebbles—involved in the development and especially in the stabilization of the aggregate or crumb structures, characteristic of fertile soils. In this context, the classical definitions are outlined for the mixtures that compose SOM and, on the basis of some more recent developments, minor changes to these definitions are suggested. The significant advances in molecular biology have relied on the isolation and fractionation of individual compounds. This approach is equally useful in the study of soil organic matter, where isolating and characterizing its principal components is a key focus. Given the uncertainties of the HSs structure, the modern methods for isolating soil organic components are discussed. The origins, compositions, sizes, and shapes of soil organic components are also examined.

There are widespread concerns about the biological degradation of SOM as the result of long-term cultivation practices for which mineral fertilizers are the only amendments made. In this work, the interactions between mineral colloids, soil organic components, and soil microorganisms—that generate the living soil systems and still remain unidentified—are also reported. The section on soil aggregates focuses both on components in SOM that are known to be active in bridging between particles and on the bridging mechanisms that are likely to be involved.

2. Soil Organic Matter and Humic Substances: Basic Definitions

In this article the terms humification, humus, humic substances (HSs), humic acids (HAs), fulvic acids (FAs), hydrophilic (HPIs) or XAD-4 acids, and humin (HN) are referred to, and are used to indicate classes of materials in SOM that have identifiable compositional properties and, in some cases, functions. *Humification* is regarded as the processes that give rise to humus as a result of the organic materials transformations in soils and composts.

Humus is a general term and refers to the products resulting from the transformation of diverse organic substrates, irrespective of their structure or chemical composition.

Humic Substances, in contrast, is a scientific term that describes specific components produced during humification that can be isolated and fractionated as described in Sections 4 and 5.

Building on Kononova's approach [9,10], Hayes and Swift [11] described soil organic matter as a heterogeneous assemblage of all organic constituents present in soil. These constituents can be classified into two groups, each with distinct morphological and chemical characteristics:

- (1) Unaltered materials including fresh organic debris and non-transformed plant or animal remnants;
- (2) Transformed products (or *humus*), which no longer exhibit morphological features of their original biological structures. These humified components comprise both humic and non-humic substances, and can be further categorized into:
 - (a) Brown-coloured amorphous humic components, differentiated on the basis of their solubility in acid and alkali into humic acids, fulvic acids, and humins.
 - (b) Other components belonging to recognizable classes, such as polysaccharides, polypeptides, altered lignins, etc. These can be synthesized by microorganisms or can arise from modifications of similar compounds in the original debris. Such materials, though components of humus, would not be regarded as HSs.

Classically, soil humic acids are defined as materials that form precipitates in aqueous base extracts acidified to pHs ranging between 1 and 2. The soluble fraction is known as fulvic acids. Insoluble materials that are either produced from plant or microbial substrates or formed via secondary synthesis routes are referred to as humin. (Because these materials contain recognizable components from the products of origin they do not qualify for inclusion in the humic category in the modern interpretations). These classical definitions do not take into account the synthesis, chemical compositions, molecular structures and sizes of the HSs. They are operational definitions not intended to imply compositional homogeneity. Instead, they provide a basis for discussion and foster the understanding of the fact that '*families*' of molecules exist broadly characterized by their composition. As will be discussed in Section 5, modern separation procedures enable more detailed compositional analysis of the HA, FA, HPI (XAD-4 acids) and HN fractions.

Likewise, other closely related '*families*' of molecules exist within soil organic matter; the broad category of '*soil polysaccharides*', which encompasses a diverse range of polysaccharides derived from both plant and microbial sources is a characteristic example. While these polysaccharides differ in their detailed molecular compositions, they share similarities in their monomer units and the modes that bind them together. They also exhibit comparable overall chemical properties (i.e., solubility, polarity, and reactivity), although their specific reactivities vary (refer to Section 5). Similar principles also apply to soil peptide structures.

It would be desirable to have chemical or structural rather than operational definitions for components of HSs. The reason why operational definitions must apply at this time is that, unlike biological macromolecules, the synthesis of humic molecules is not governed by genetic control. In short, humic substances are produced from a wide variety of aromatic and aliphatic components through various transformation stages involving biologically mediated degradation and re-synthesis processes. The diverse '*monomer*' compounds are held together via strong, non-hydrolysable C–C and C–O–C bonds giving rise to a rapidly growing number of distinct, non-repeating molecular structures. Many of these structures share similar—but not identical—compositions and configurations constituting a *family* of

closely related compounds with comparable properties and behaviour, collectively known as HSs.

It should be noted that similar operational definitions are widely applied to other complex natural materials, such as 'kerogen', which describes an organic component in mineral deposits on the basis of its solubility in specific solvents.

3. The Formation of Humic Substances and Their Recalcitrant Properties

3.1. Genesis of Products of Humification

Considerations of the roles that SOM components play in soil should primarily consider how these come into being and of their compositions, their structures, sizes, and shapes. Ideally it would be easier to understand their roles should details of the structures be known but, as Wershaw has stated [12], "that after 100 years of research we are no closer to a single structural diagram for humic acid than we were at the beginning". The same applies to some extent for all of the major reactive organic materials in soil. Burdon [13] concluded that there is neither biological nor chemical justification to support the formation of the proposed humic molecule structures in the soil environment. He asked, "why would a microorganism expend energy and resources making a material that it has no use for. Any organism that did this would become extinct because of competition by organisms that did not waste energy and resources in this way." The answer to that might be that the microorganisms obtain their energy from the breakdown of the organic materials they are presented with, and catalyzed chemical processes then give rise to the humified materials from the breakdown products.

Some consider that organic matter (OM) entering the soil degrades to CO₂ and water without giving rise to transformation or *humification processes* that give rise to *humus* materials with varying degrees of resistance to biological degradation. That concept is discredited.

In mineral soils the humus contents are affected both by internal factors, i.e., clay minerals and inorganic colloids, and by external factors including rainfall and temperature. These factors will also influence the formation (genesis) and decomposition rates of humus. The constituents of various organic residues decompose at different rates, leading to several distinguishable stages of decomposition [14]. The labile compounds, i.e., simple sugars, amino acids, most proteins, and certain polysaccharides, will be rapidly decomposed unless they become protected through sorption on mineral colloids, or by physical entrapment within soil aggregates. Contrarily, more resistant macromolecules, e.g., lignins, cellulose, and some proteins, decompose more slowly. Organic compounds like long chain aliphatic hydrocarbons, cutins, esters and fatty acids exhibit even greater resistance to breakdown.

Soil microorganisms use the organic residues that enter the soil as food sources. They break down the macromolecular residues into their constituent monomers, which they use to build their own biomass. This *degradative* pathway is one of the mechanisms suggested to explain the genesis of humus in soil. It includes solely biological processes, mostly the modifications of relatively recalcitrant plant components, such as lignins, suberins, and cutins [15]. An alternative pathway involves an initial phase of biological degradation followed by a *chemical* or *abiotic synthesis*, as previously mentioned.

Lignin is a major precursor in the formation of humic substances. Although it exhibits a notable resistance to biodegradation, it can be decomposed by fungi (mostly by white rot and less by brown rot). Although the degradation is very complex and yet remains not fully understood [16], the Hatcher group has provided strong evidence for the contributions of the lignin structures [17,18]. They analyzed a wide variety of samples and concluded that the humic acids "are distinguished by the presence of three predominant molecular components: lignin-like molecules, carboxyl-containing aliphatic molecules, and condensed aromatic molecules

that bear similarity to black carbon". These results confirm that lignin is a major source of the SOM components and surprisingly indicates how it can be a source for "condensed aromatic organic molecules often categorized as black carbon". These findings suggest an alternative and/or complementary non-pyrogenic source to account for the fused aromatic structures and black carbon in soils [19,20]. The data also indicate that a significant portion of the aliphatic components bearing carboxyl groups may originate from lignin.

DiDonato et al., and Waggoner et al. [17,18] demonstrated that in the case of soils with high humification degree the molecular formulae fitted into two main regions of the Krevelen plots [21], i.e., those including condensed aromatic molecules, and those containing aliphatic molecules with carboxyl groups exhibiting high H/C and low O/C ratios. In contrast, humic acids from a poorly humified spodosol soil showed more molecular formulae in the lignin-like region and relatively fewer condensed aromatic structures present. Additionally, the authors proposed a humification pathway involving (photo- or microbially produced) reactive oxygen species to convert the fresh organic matter—primarily lignin—into the compounds detected in their studies. Supporting this concept, Waggoner et al. [18] demonstrated that black carbon-like and alicyclic aliphatic compounds were formed through degradation of lignin assisted by hydroxyl radicals, further underscoring the key role of lignin transformations in the humification process.

Lignin peroxidase, manganese peroxidase, and laccase are three types of oxidative enzymes that are suggested to participate in the synthesis and degradation processes. In vitro experiments using lignin precursors have verified both the formation of polymers as well as their degradation [22].

It seems that phenols (originating from lignin) play a key role in the genesis of humic substances [23–26]. Flaig et al. [25] demonstrated how polyphenols could oxidize to quinones leading to the formation of humic-like compounds. Wershaw [27,28] discussed the enzymatic depolymerization of lignins and tannins by reactive oxygen species. He observed that microorganisms secreted oxidative enzymes that could not only depolymerize the plant polymers but also produce polar and hydrophilic segments, i.e., carbonyl and carboxyl groups, which transformed lignin into an amphiphilic compound with surface active properties exhibiting a distinct similarity in structure to HSs.

Research has also provided evidence for abiotic HSs' synthesis in soil [29–33]; the oxidative polymerization of phenolic compounds to produce humic-like structures can be promoted by soil inorganic compounds such as clay minerals (e.g., chlorites, smectites, vermiculite, kaolinite, halloysite, and dickite); primary minerals (e.g., olivines, pyroxenes, amphiboles, micas, and feldspars); and Fe, Al, Si, and Mn oxides or oxyhydroxides. Clay-mediated reactions are promoted mostly due to the variety of host-guest interactions based on the organization of clay lamellae to form a two-dimensional aluminosilicate framework, the self-association of guest molecules into the interlayer as well as the sorption of the guest molecules onto the soil mineral surface increasing their concentration [34].

Research conducted by Martin, Haider and their colleagues supports the biological synthesis of macromolecular substances resembling humic acids from phenolic compounds [35–46]. It is of significant importance that fungi synthesized phenols and humic-like substances from non-aromatic parent materials [36] (reviewed by Hayes and Swift [11]). In this case, clays accelerated the macromolecular synthesis [46].

Literature data has shown that some of the soil enzymes, including phenoloxidase, transform phenols into radicals, which in turn initiate polymerisations forming new compounds [42]. Laccase (widely found among fungi) is a characteristic example [47–50]. Also, phenoloxidases can catalyze the extracellular formation of macromolecular substances in soils [51]. Given the abundance in soil microorganisms and their potential in synthesis and degradation reactions [47–49], it is surprising that more focused attention is not being

paid to the role of fungi in the HSs synthesis. It seems that the incorporation of phenolic compounds bearing unsaturated aliphatic side chains into the HSs macromolecules could offer them additional protection further increasing their resistance towards microbial decomposition [42,44,45].

Biological processes may contribute to the release of reactive chemical species. A notable example is the Maillard reaction [52–54], a non-enzymatic reaction between amino acids and reducing sugars that produces melanoidins—complex polymers with properties resembling those HAs. Early work using ^{13}C -CPMAS NMR conducted by Benzing-Purdie and Ripmeester [55] indicated that melanoidins possessed structural similarities with soil organic matter compounds. However, subsequent ^{13}C and ^{15}N -NMR investigations by Knicker and Lüdemann [56], Knicker [57], and Kögel-Knabner [58] revealed substantial compositional differences between melanoidins and native organic matter. In contrast, research by Poirier et al. [59,60] identified melanoidin-like substances in the humin fraction of a ferralitic soil from southwestern France and in deep layers of a Congolese soil where the carbon was highly stabilized (ca. 8300 years old). In the latter case, melanoidin-type compounds accounted for approximately 5% of total soil carbon. Nonetheless, the possibility remains that these compounds were artefacts resulting from the harsh acid and base hydrolysis methods employed during extraction. It is plausible, however, that melanoidin formation plays a role in the generation of humic-like substances under composting conditions, where the concentration of reactants is higher and temperatures are elevated.

The work of Enders and his co-workers [61–65], focuses on the abiotic synthesis of humic substances; when glycine and amino compounds reacted with 2-oxoproponal compounds like HSs were obtained. Schuffelen and Bolt [66] and Hayes [67,68] studying the reactions between 2-oxoproponal (exhibiting high reactivity towards amino acids and amine functionalities) and glycine also verified macromolecular compounds with properties similar to those of soil HAs being synthesized.

The ligno-protein concept formulated by Waksman and Iyer [69], which significantly influenced the understanding of humic composition and structure for an entire generation, suggested that oxidized lignin and protein material can form a HA-like complex resistant to microbial decomposition. However, a complex prepared by Hayes [67] from oxidized lignin and casein (similar to that of Waksman and Iyer [69]) exhibited a differential-thermal-analysis thermogram closer to those of the parent materials, i.e., lignin and casein, than to that of a HA sample.

Wershaw [12] and MacCarthy [70] suggested that research should focus more on the humification process and not on the intermediates that were ambiguously defined. Wershaw et al. [71] demonstrated that the plant tissues oxidative degradation results in natural dissolved organic carbon and humus, and that lignin transformation products were the major precursors of HSs. Humification can be retarded under anaerobic conditions as oxygen availability plays a vital role in the synthesis of the lignin alteration products. It should also be marked, that there are some plants, which either do not contain lignin at all or contain it in only very small amounts. Mosses, liverworts, hornworts, and some of parasitic plants do not contain lignin because they do not perform their own water transport or need support.

Additional research by Wershaw et al. [72,73], consistent with findings by Simpson et al. [74], provided evidence that humics consist of mixtures of relatively refractory organic materials acquired from organic residues transformation. These materials could also sterically protect other more labile organic compounds. It is important to note that Wershaw's studies specifically focused on humic substances derived from the Suwannee River (Florida), with forest litter serving as the primary source material.

Senesi et al. [75] evaluated HS-like components at the molecular level in organic amendments of diverse origins and characteristics. Comparison between the HS-like components and the native HAs and FAs showed that the former possessed high molecular diversity, low aromaticity and structural complexity, high aliphatic and nitrogen content, modified lignin and polysaccharide structures, fewer oxygenated acidic groups, different metal-binding behaviour and elevated sulphur content. The authors concluded that applying mature, humified organic wastes to soil alters the structure and chemistry of native soil HS, particularly in the FA fraction. These changes diminish over time, returning toward the characteristics of native soil HS.

Thus, chemical reactions occur simultaneously with biological processes, highlighting the depth and complexity of interactions in SOM decomposition and synthesis of new products. The growing body of evidence suggests that the humic component of soil organic matter is a complex mixture of compounds derived from both plant material and microbial activity. It could be that no single molecular structure can represent a substantial portion of this fraction. Considering the diverse array of potential reactions and pathways discussed above, the following assessment may be useful: microbially mediated processes appear to be the primary mechanisms driving both decomposition and secondary synthesis in soils. Abiotic reactions may also contribute to these transformation processes under certain conditions—such as areas with high, localized concentrations of organic matter.

3.2. Indications of the Origins of Components of Soil Organic Matter

The relative contents of ^{13}C and ^{15}N in soil organic matter provide valuable insights into the origin and formation processes of the material. Differences between the ^{13}C contents in C_3 and C_4 plants have been utilized as in situ tracers for studying organic carbon turnover and SOM dynamics [76–78]. It has been established that in C_4 plants the $\delta^{13}\text{C}$ values lie between -9 and -17 (averaging at -12‰) while C_3 plants $\delta^{13}\text{C}$ values range from -23 to -34‰ (with an average of -26‰) [79].

The parameter $\delta^{13}\text{C}$ values can be calculated as follows:

$$\delta^{13}\text{C} = 1000(R_{\text{sample}}/R_{\text{standard}} - 1)$$

where R is the $^{13}\text{C}/^{12}\text{C}$ ratio. Similarly, in $\delta^{15}\text{N}$ determinations, R is the $^{15}\text{N}/^{14}\text{N}$ ratio.

Clapp et al. [80] reported $\delta^{13}\text{C}$ values for various soils, coals, and for HAs, FAs, and XAD-4 acid fractions isolated from soils and waters. These values were related to the type of vegetation and reflected transitions from C_3 to C_4 plant dominance in cropping systems. Fractions of microbial origin proved slightly more enriched in ^{13}C , suggesting a modest isotopic preference for ^{13}C during microbial synthesis processes in soil. Based on the above data, Huggins et al. [81] calculated higher SOC decay rates for C_4 plants (corn) than for C_3 plants (soybean). Contrarily, the corn humification rates were lower than those of soybean.

Clapp et al. [82] used the $\delta^{13}\text{C}$ parameter to investigate how different management practices affect soil carbon dynamics. In their study, corn was grown continuously for 13 years, followed by 4 years of soybean cultivation, with crop residues either removed from or returned to the soil. Tillage treatments included no-till, moldboard plough and chisel plough. Analysis of $\delta^{13}\text{C}$ data revealed that, with few exceptions—such as the surface layer (0–5 cm) under no-till and chisel plough with residue addition, and the 25–30 cm depth under moldboard plough with residue removal—the soil organic carbon throughout the profile consisted primarily of relic (old) carbon. Moreover, nitrogen fertilizer applications alone were insufficient to maintain SOC levels in the absence of residue return.

Isotopic discrimination between ^{14}N and ^{15}N seems to take place during both biological and chemical processes [83]; specifically, during nitrogen transformations in plants and soils, ^{14}N is preferentially mineralized [69] becoming more prone to leaching and plant

uptake, whereas the residual organic nitrogen becomes ^{15}N -enriched. Consequently, crops may exhibit lower $\delta^{15}\text{N}$ values than those of the total soil nitrogen pool [84].

Létolle [85] demonstrated the spread of the $\delta^{15}\text{N}$ values for terrestrial materials, e.g., for animal manures $\delta^{15}\text{N}$ ranges from +5 to +15‰, for SOM between +4 and +20‰, and for commercial fertilizers lies between −4 and +4‰. There are limitations, however, in interpretations of data because the ranges of $\delta^{15}\text{N}$ values do not have distinct boundaries. It is possible, for example, to have a sample with a $\delta^{15}\text{N}$ value of +6‰ that is made up of 50% N with a $\delta^{15}\text{N}$ of 0‰, and 50% with a $\delta^{15}\text{N}$ of 12‰. These are important in the cases where N inputs are mixed, for example mixtures of fertilizers and animal manures.

Hayes [86] measured $\delta^{15}\text{N}$ values for HAs, FAs, and XAD-4 acids extracted from drainage waters of two English soils. Both were Pelostagnogley soils, one under long-term pasture (33% fine silt, 37% clay) and the other under long-term wheat cultivation (*Triticum aestivum* L.; 39% silt, 54% clay). For drainage water passing through approximately 0.85 m of the pasture soil, $\delta^{15}\text{N}$ values decreased as follows: XAD-4 acids > HAs > FAs. The $\delta^{15}\text{N}$ values for HAs and XAD-4 acids (2.8–3.2‰) were significantly higher than for FAs (1.2‰), suggesting that commercial fertilizers applied to the grassland may have influenced FA nitrogen, while higher $\delta^{15}\text{N}$ values in HAs and XAD-4 acids were mostly due to grazing animal manure.

In contrast, $\delta^{15}\text{N}$ values for fractions isolated from runoff waters showed different patterns: HAs (1.6‰) and FAs (0.8‰) suggested strong fertilizer influence, whereas the XAD-4 acid fraction (3.6‰) appeared to include sources that diluted the fertilizer signal (fertilizers applied at $400 \text{ kg N ha}^{-1} \text{ y}^{-1}$).

From drainage waters that had passed through approximately 0.8 m of the cultivated soil, the $\delta^{15}\text{N}$ order was: XAD-4 (3.0‰) > FA (1.5‰) > HA (1.0‰), again indicating fertilizer inputs. In a separate case, samples from drainage water in a not well-drained brown forest soil under a 3-year grass–3-year arable rotation showed a different order: HA (+4.7‰) > XAD-4 (+3.6‰) > FA (+3.5‰), with all values higher than those from the grassland or arable soils, further reflecting fertilizer influence.

Fractions isolated directly from the soils at pH levels of 7, 10.6, and 12.6 also showed differences compared to those from drainage waters. XAD-4 acids generally exhibited higher $\delta^{15}\text{N}$ values than HAs and FAs. However, the highest $\delta^{15}\text{N}$ values were observed in hydrophobic organic matter not recovered from the XAD-8 resin using NaOH. These components resemble more the precursor materials (plant or microbial) than the conventional HA, FA, and XAD-4 fractions.

Skjemstad et al. [87] utilized carbon isotope ratios to investigate the persistence of SOM in continuous production systems. Their study compared SOM in the surface 15 cm of a C_4 -derived forested Oxisol with that of an adjacent soil, cleared of forest vegetation 90 years earlier and subsequently managed under C_3 grassland. By analyzing $\delta^{13}\text{C}$ values and employing photo-oxidation to selectively remove organic matter not stabilized within microaggregates, the authors demonstrated that a significant part of forest-derived carbon persisted in the grassland soil for over a century. This long-term preservation was attributed to both the intrinsic chemical characteristics of the organic matter and its physical stabilization via association with fine mineral particles or occlusion within soil aggregates.

A distinct methodological approach was employed in the long-term bare fallow experiments conducted by Barré et al. [88], in which plots were maintained entirely free of vegetation for extended durations, thereby eliminating plant-derived inputs to the soil following the start of the trials. One such study synthesized results from sites in Denmark, France, Russia, Sweden, and the United Kingdom, with experimental durations ranging from approximately 30 to 80 years. The sites varied in land-use history—some having been arable, others grassland—and encompassed a broad spectrum of soil textures, from coarse

sands to silty clay loams. As anticipated, soils with a grassland history exhibited more rapid initial losses of organic matter upon conversion to bare fallow compared to those with a long-term arable history. However, following this initial phase, the rate of OM decline converged with that of the arable soils. Across all sites, the proportion of OM remaining ranged from 79% to 35%. Sites with 30–49 years of fallow treatment retained, on average, 68% of their initial OM, while those maintained for 50–80 years retained an average of 38%. The residual SOM was composed primarily of chemically resistant, slowly decomposing, humified organic matter, along with variable amounts of pyrogenic carbon—dependent largely on historical land management practices such as residue burning or forest clearing. Notably, SOM persistence was observed even in coarse-textured sandy soils, with dynamics similar to those in high-clay soils. This finding underscores that SOM stability is an *inherent property* governed by the intrinsic properties of the organic matter itself not dependent on mineral protection (e.g., clay-OM associations).

In summary, although limited, the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ data for SOM fractions underscore the utility of stable isotope analyses in elucidating the origins and transformation processes of SOM components. These findings highlight the potential of isotopic approaches for advancing our understanding of SOM genesis and nitrogen cycling in contrasting land-use systems. The use of stable carbon isotopic ratios ($\delta^{13}\text{C}$) offers a significant advantage over radiocarbon (^{14}C) dating, as it circumvents potential contamination issues commonly associated with ^{14}C methodologies.

It also becomes evident that the composition of SOM at any given time reflects dynamic inputs that vary with vegetation type and seasonal conditions. Fresh, largely unaltered plant materials will always be present in variable amounts, but rapidly decomposable components—such as simple sugars, oligosaccharides, amino acids, peptides, proteins, and starch—are typically transient due to their rapid assimilation by soil microorganisms. As a result, these labile compounds are usually present in low concentrations, if at all. In contrast, more recalcitrant plant-derived constituents—including complex hemicelluloses, structural cellulose, lignin, cuticular and suberized materials, and pyrogenic residues—persist in the soil for extended periods and collectively contribute significantly to total SOM. The persistence and transformation of these materials reflect the interplay of biochemical resistance and physical protection mechanisms. Consequently, the study of SOM requires methodological simplification through the targeted isolation, fractionation, and characterization of specific components within this heterogeneous mixture.

4. Isolation from Soil of Humic, Hydrophilic, and Humin Components of SOM

Hayes and Swift [89] outlined two approaches for the study of SOM. The first, often referred to as the “lumpers” or holistic approach, emphasizes the examination of SOM in situ—studying the soil system as it exists in its natural habitat. The second, known as the “splitters,” advocates for the isolation and fractionation of SOM components to better resolve their chemical nature and function. Given that SOM comprises a complex assemblage of structurally and functionally diverse ‘families’ of organic molecules (among which the most chemically and functionally active are humic substances, humin, soil polysaccharides, and peptides) the latter approach is essential for elucidating the composition, reactivity, and ecological significance of these constituents. This concept is an instructive analogy with the isolation of proteins, nucleic acids, and polysaccharides from their biological matrices [90]. However, it must also be recognized that in the soil environment, these components often exist in intimate molecular association, and their ecological functions are likely to be influenced by such interactions.

Hayes [91] and Clapp et al. [90] have examined in detail the principles and methodologies involved in the isolation and fractionation of soil organic components, with particular emphasis on the role of solvation processes. Effective solvation is a prerequisite for the extraction and separation of soil organic matter constituents.

Hayes [91] provided a detailed discussion of the intermolecular forces that govern molecular associations within soil organic matter. He emphasized the roles of monopole, dipole, and quadrupole interactions, and described how polar molecules can induce dipole moments in adjacent, less polar molecules, resulting in dipole-induced dipole interactions. Dispersion forces—commonly referred to as London or van der Waals forces—are particularly relevant when considering the behaviour and aggregation of non-polar organic molecules in the soil matrix. Hydrogen bonding is especially significant in the context of the HSs secondary structures, as well as in mediating interactions among SOM components that possess functional groups capable of forming hydrogen bonds. These molecular associations underpin the supramolecular organization of SOM and influence its stability, reactivity, and interactions with soil minerals and other biogeochemical constituents.

4.1. Isolation of SOM Components in Aqueous Solvent Systems

In light of the above, Hayes [91] has reviewed key aspects of water chemistry relevant to the solvation process given the central role of water as the primary solvent in the extraction and isolation of humic substances. The major interactions between water molecules are the linear H-bond [92].

When ionized or polar solutes come into water, electrostatic interactions between the solute and solvent molecules develop resulting in solvation, i.e., an ordering of water molecules around the solute. In contrast, the incorporation of non-polar molecules into aqueous systems is governed by hydrophobic interactions. These non-polar entities preferentially interact with one another rather than with water molecules leading to the aggregation or micellization of non-polar species, a fact bearing important implications for the physical behaviour and fractionation of hydrophobic components of SOM.

4.1.1. Extractions in Neutral Salt Solutions

Since Achard (1786) [93], the extraction and isolation of HSs was carried out in alkaline aqueous solutions. Achard extracted peat using aqueous potassium hydroxide (KOH), and upon acidification of the extract, obtained a dark, amorphous precipitate—later identified as humic acid. He also observed that larger quantities of this dark-coloured material were acquired from the more humified layers of peat than from less decomposed materials, highlighting the humification process [93].

Sprenkel [94,95] concluded that the HAs were bound to minerals in soils rich in bases and were considered more likely to be in solution in soils with low base contents. This interpretation aligns with current understanding of the role of divalent and polyvalent cations in forming cation bridges between acidic functional groups on humic molecules and mineral colloids bearing a negative charge, or between adjacent humic molecules themselves, thus promoting precipitation. When the pH of the medium is such that dissociations of the acidic functionalities occur and the conjugated bases formed are not neutralized by divalent and by polyvalent cations, solvation occurs and that is the rationale behind the use of monovalent cationic bases for the isolation of humates.

Bremner and Lees [96] and later Bremner [97] noted that an aqueous solution of 0.5 M NaOH increased oxygen uptake by SOM. This caused them to investigate extractions with neutral salt solutions. Hayes and Swift [11] and Clapp et al. [90] examined the relative efficiencies of the salt solutions tested. Their findings indicated that the most effective extractants in this category form complexes with polyvalent metals bound to the

humic core, allowing sodium ions to associate with the conjugated bases in the humic structures. Of particular note was the use of 0.1 M sodium pyrophosphate, neutralized to pH 7. This reagent effectively replaces strongly bound divalent and polyvalent cations occupying charge sites, thereby promoting solvation of carboxylate and phenolic groups. The extraction yields obtained with pyrophosphate are generally lower than those achieved with dilute alkali, due to the reduced ionization of acidic functionalities at neutral pH compared to strongly alkaline conditions (e.g., pH 13).

4.1.2. Extraction with Dilute Aqueous Base Solutions

It is essential that artefacts are not formed in an extraction process, or if artefacts should be formed, it is fully understood how these are formed, and the products can be accurately linked to the structures of their origins in the materials under study. There follows an outline of procedures that may be used to obtain fractions that can be considered to provide isolates similar to those that exist in soil, or with changes that can be explained on the basis of the solvation processes used.

Lehmann and Kleber stressed structural damages caused by extractions of SOM in alkaline solutions [4]. They state that the traditional 'humification' concepts limit observations of soil organic matter to its solubility in alkaline extracts, unlike the emergent view of organic matter based on solubility in water and its accessibility to microorganisms. They wrote that HSs extracted by alkali from soil environments are artefacts of the extraction process, with humic acids and fulvic acids having an exaggerated chemical reactivity relative to "true isolates". Their position remained unchanged in a subsequent publication [98], in which the authors introduced the term "true isolate" to account for the whole SOM and not just the alkali-extractable fractions. However, the precise meaning of "true" isolate remains ambiguous. They state that extraction is always incomplete leaving 50–70% of the organic carbon unextracted which is then defined as the insoluble humin fraction. These are not consistent with many detailed studies that have been carried out with different extraction systems (including non-alkaline extractants) used for the isolation of OM components from soil and from water (e.g., Aiken [99]; Clapp et al. [90]; Green et al. [100]; Hayes [91]; Hayes et al. [101,102]; Malcolm and MacCarthy [103]; Mylotte et al. [104,105]; Ritchie and Perdue [106]; Serkiz and Perdue [107]; Song et al. [108,109]; Swift [110]).

In their considerations of extractions of SOM, Lehmann and Kleber also cited extractions with alkali as the most efficient although incomplete (with 50–70% of the SOM unextracted), selective, and prone to creating artefacts [4]. They agreed with Waksman [111] (and still do [98]) who wrote that 'humic' "*nomenclature should be dropped because the term relates only to material obtained by a specific procedure*". However, it should be noted that, similar to Lehmann and Kleber, Waksman did not present any experimental evidence to substantiate his claims.

Dilute sodium hydroxide solutions, under extended exposure and appropriate conditions, can induce limited hydrolysis of labile ester bonds facilitating minor oxidative transformations. Earlier research of Swift and Posner [112] whose experiments, conducted nearly two generations ago, demonstrated that some oxidation of humic acids actually occurred when the extract was stored in 1 M NaOH for 30 days under an atmosphere of oxygen, a process inhibited when humic acids were kept under nitrogen. These findings clearly indicated that oxidative effects could be mitigated through oxygen exclusion, minimisation of extraction duration and prompt neutralization of the alkaline extracts. Moreover, at room temperature dilute NaOH does not facilitate the formation of aromatic or aliphatic compounds or the generation of C-C linking bonds, features that are evident in the NMR spectra of humic substances. In the absence of plausible mechanisms by which simple precursor molecules could undergo reactions under such mild conditions that would give

rise to such large quantities of HSs in such a short time, the view that HSs are artefacts created during extraction by weak alkali should be ignored.

4.1.3. Extractions in Base Solutions Amended with Urea

In water, urea functions as a proton acceptor, a potent protein denaturant, and a disruptor of H-bonds. At high contents, i.e., in the range of 5 to 9.5 M, urea has been shown to effectively break non-covalent bonds within proteins [113]. Additionally, urea has been employed to disrupt lignocellulosic structures and to solubilize hemicellulose components in roughage materials [114,115].

The sequential use of an alkaline base followed by a urea-containing solvent to extract humic substances was first introduced by Song and Hayes [116]. This method is based on the facts that a broad range of pK_a values is associated with the acidic functional groups in HSs, and that the most strongly acidic components are expected to solubilize at lower pH values [117]. In this protocol, the H^+ -exchanged soil is subjected to exhaustive sequential extraction at pH 7 (adjusted with 0.1 M NaOH), pH 10.6, pH 12.6 (0.1 M NaOH under nitrogen), and finally with a solution of 0.1 M NaOH + 6 M urea (also under nitrogen). The inclusion of urea in this solvent mixture promotes the disruption of hydrogen bonding within the humic matrix and enhances the breakdown the clay-humic associations, thus, facilitating the less polar functionalities to solvate. Although classical operational definitions would classify the fraction extracted using the base/urea mixture as 'humins', its chemical composition was found to be comparable to that of the HSs obtained with 0.1 M NaOH in an exhaustive sequential extraction process [109]. This suggests that a significant portion of what is traditionally considered 'humins' may, in fact, be solubilizable under appropriately modified extraction conditions.

4.2. Extractions with Organic Solvents

4.2.1. Relevant Properties of Organic Solvents

Key factors influencing the ability of an organic solvent to dissolve a solute include its acidity or basicity, hydrogen-bonding capacity, relative permittivity, and dipole moment. Other properties including viscosity, boiling point, and density may not directly affect solute-solvent interactions or separation efficiency, they should nonetheless be considered when designing an effective solvent system. Additionally, an ideal solvent should be recoverable without undergoing significant compositional changes.

Hayes [91] and Clapp et al. [90] have set up comprehensive listings of solvent parameters relevant to the isolation of SOM components. Among these, the relative permittivity (K_r), also referred to as the dielectric constant, is particularly significant. For instance, water, with a K_r of 78.5, is a highly effective solvent for ionic compounds, as its high dielectric constant significantly reduces the Coulombic attraction between oppositely charged ions inhibiting their tendency to self-associate, thus facilitating solvation. Generally, non-polar solutes dissolve more readily in solvents with low K_r values, while polar solutes are better solvated in high K_r environments. However, specific solute-solvent interactions—particularly hydrogen bonding—can significantly influence solubility behaviour [118].

Dipolar aprotic solvents, as defined by Parker [119], are characterized by K_r values greater than 15 and an inability to donate hydrogen atoms for strong hydrogen bonding. Examples include acetone, acetonitrile, dimethyl sulfoxide (DMSO), dimethylformamide (DMF), methyl isobutyl ketone (MIBK), and N-methyl-2-pyrrolidone. These solvents, possessing exposed electronegative atoms and significant dipole moments, are well-suited for solvating polar organic molecules. However, anions are less effectively solvated. In dipolar aprotic solvents solvation primarily occurs (in the absence of hydrogen bonding) via weaker, polarizability-driven electrostatic interactions [119]. The electrostatic factor

(EF), defined as the product of a solvent's relative permittivity and dipole moment, captures the combined influence of these properties on electrostatic solvation efficiency.

Hydrogen bonding plays a vital role in solute–solvent interactions. Pimentel and McClellan [120] categorized solvents based on their hydrogen bonding behaviour into proton donors, proton acceptors (e.g., ketones, ethers, and dipolar aprotic compounds), dual proton donors and acceptors (e.g., alcohols, carboxylic acids, primary and secondary amines, and water), and non-hydrogen bonding solvents (e.g., carbon disulfide and paraffins). Taft et al. [121] defined a base parameter value, pK_{HB} , which measures the relative strength of the acceptor when a H-bonded complex is formed using any suitable hydroxyl reference acid with the exception of intramolecular hydrogen bonds. Higher pK_{HB} values indicate compounds acting as good acceptors in H bonding.

Dispersion (London) forces, another key aspect of solute–solvent interactions, are correlated with a solvent's refractive index—the ratio of the speed of light in a vacuum to that in the solvent. A higher refractive index suggests stronger dispersion interactions [122]. From a practical standpoint, solvent viscosity should also be considered as it affects the ease of handling, with lower viscosity being preferable [118]. Finally, solvent density becomes important in gravity-based separation processes. Mixtures of solvents can be used to fine-tune density.

4.2.2. Applications of Organic Solvents to Isolate Humic and Humin Soil Components

Hayes [91] and Clapp et al. [90] have conducted comparative evaluations of the physicochemical properties of organic solvents suitable for HSs extraction from soils. The abilities of a variety of solvents to dissolve the IHSS HA standard isolated from a Florida peat were compared with those for 0.5 M NaOH, and for 0.5 M NaOH at pH 9.2 (no difference was observed for the two NaOH solutions). *N,N* dimethylformamide (DMF), formamide, and DMSO were found to be good solvents. However, the difficulties in recovering the solute from these aprotic organic solvents preclude their usefulness in the extraction of HSs from soil. In contrast, the dipolar aprotic solvents DMSO and MIBK have value in studies of soil humin.

Rice and MacCarthy [123] described the separation of humin by liquid partitioning using MIBK, and Song and Hayes [116] introduced the use of DMSO for the isolation of soil humin. Later Song et al. [108,109,124–127] described the uses of urea-amended base and acidified DMSO for the isolation of humin from soil. They found that 75–95% of the HN could be recovered in the extract with DMSO (94%) and concentrated H_2SO_4 (6%). The DMSO-acid extraction followed a prior exhaustive extraction with the urea-fortified (6%) base to remove the humic components. When the DMSO-acid isolate was added to water the HN could be recovered as a precipitate. The remainder of the HN could be recovered when the silicates were dissolved in HCl/HF. The compositions of the HN materials that dissolved in acidified DMSO were effectively the same as those recovered following the HCl/HF treatment (Figure 1) [108,109,124].

4.3. Summary of Solvents and of Techniques Used for the Isolation of SOM Components

Swift and Posner [112], as well other researchers, developed recommendations for HSs extraction from soil at neutral and alkaline pHs, at room temperature (up to 298 K). These include reducing alkali concentration, limiting extraction time, excluding light exposure, and promptly neutralizing or acidifying the extract post-extraction. These precautions aim to ensure that the HA and FA fractions isolated closely resemble their native counterparts in SOM. Such considerations informed the selection of 0.1 M NaOH as the preferred extractant for isolating soil HS standards by the International Humic Substances Society (IHSS).

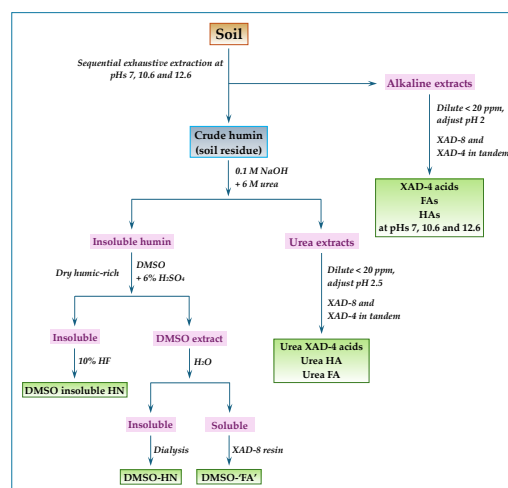


Figure 1. Summary of procedures for the isolation and fractionation of humic and humin components of soil.

Swift [110] has detailed the IHSS protocol for the isolation of humic substances from soils. In contrast, Aiken [99], as well as Malcolm and MacCarthy [103], have described resin-based techniques for isolating HSs from aquatic systems. Reverse osmosis methods have also been employed for the extraction of HSs and natural organic matter from water sources [100,107]. Additionally, Hayes et al. [101,102] applied an exhaustive sequential extraction procedure using progressively increasing pH values to fractionate humic and other soil components based on differences in charge characteristics.

Song et al. [108,109,124] removed the less polar and sterically constrained humic components employing a mixed urea-NaOH solvent, and then isolated humin using DMSO acidified with sulfuric acid. These procedures are also described by Mylotte et al. [104,105] and by Hayes et al. [128]. Extensive analyses have been carried out on the fractions obtained using both the IHSS protocol and a resin-in-tandem process in which the extracts (highly diluted and acidified) passed through XAD-8 ((poly)methyl methacrylate) and XAD-4 (styrenedivinylbenzene) resins in tandem. In this procedure, the more hydrophobic components—primarily HAs and FAs—are retained by the XAD-8 resin, while the more hydrophilic acids, representing non-humic substances associated with the HA and FA fractions, are sorbed onto the XAD-4 resin [129]. This method allows for the isolation and recovery of up to 95% of SOM, providing a comprehensive representation of its major components without introducing extraction artefacts. Olk et al. [130,131] reported no evidence that alkaline extractions, when conducted under recommended conditions, result in the formation of artefacts or significantly alter the reactivity of the isolated organic matter.

Figure 1 summarizes the extraction techniques discussed in relation to the work conducted by Hayes et al. and Song et al. [108,109,124]. Soil residues were exhaustively extracted (8 to 10 times) to obtain maximum recovery at pH 7, then the residual soil was further exhaustively extracted at pH 10.6 (under N_2) and, finally, the residual soil was again exhaustively extracted (under N_2) at pH 12.6. The extracts at the different pHs were diluted, their pH was adjusted to 2 and were passed through XAD-8 and XAD-4 resins in tandem. The humic and fulvic acids are obtained from the XAD-8 resin column and the XAD-4 acids (the hydrophilic materials) are acquired from the XAD-4 resin.

The residual organic matter remaining in the soil after sequential exhaustive extractions can be considered as a “crude” humin. The term ‘crude’ is used because additional humic material is extracted in the amended base solvent used following the extractions in 0.1 M NaOH. Finally, it is extracted with a solvent mixture of 0.1 M NaOH + 6 M urea (under N_2). This solvent mixture facilitates the cleavage of both hydrogen bonds in the humic

matrix and clay-HSs associations. In addition, it helps to solvate less polar functionalities. The base/urea extract would be a “humic” fraction according to the classical operational definitions of HSs. However, the base urea extracts proved similar to those culminating in 0.1 M NaOH in an exhaustive sequential extraction process.

The urea is washed from the residual material, which is then dried before extracting with the DMSO (94%) concentrated sulfuric acid (6%) solvent system. The humin is precipitated when the DMSO-acid soluble material is added to water and recovered by dialysis and freeze drying. A small fraction (< 15%) of the isolate which remains in solution is passed through XAD-8 and it has been shown to have all the properties of FA materials. It can be assumed that it was present in soil in association with, or trapped in the humin matrix. The DMSO material that is not extracted in the DMSO-acid is recovered from the residue of the silicates treated with HCl/HF. This material has been shown to be compositionally similar to that isolated in the DMSO acid system. The comparison between this extraction technique and the traditional ones is presented in Figure 2.

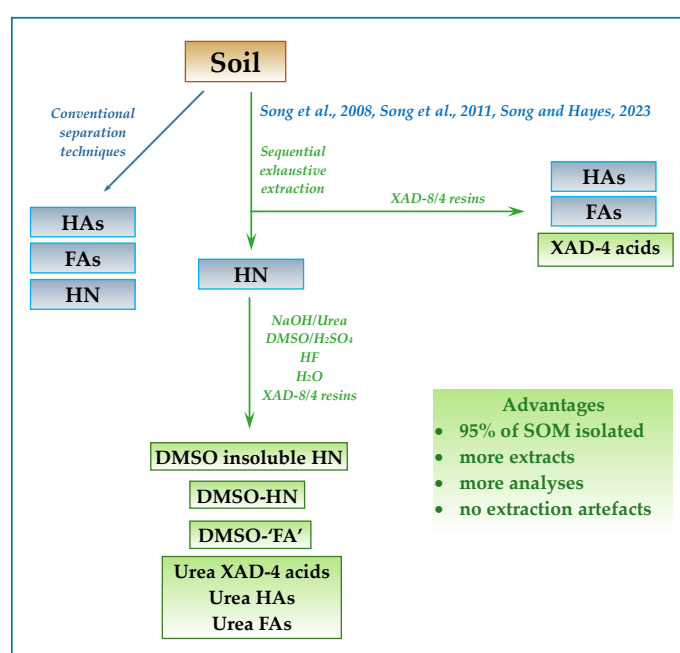


Figure 2. Comparison between the extraction techniques conducted by Song et al. 2008, Song et al. 2011 and Song and Hayes, 2023 [108,109,124] and the conventional separation methods. Using XAD-8/4 resins and NaOH/urea, multiple other extracts are obtained besides the typical HAs, FAs and HN fractions.

To conclude, although sodium hydroxide solutions have never been the only extraction option, they eventually became the predominant method despite the reservations (possible oxidation of the extractants, large portion of organic carbon unextracted, co-extraction of non-HS, ionization of the HSs’ groups [4]) about the use of NaOH for the HSs extraction. Even when multiple processes for HSs isolation were followed, NaOH solution has been employed as the first step [132,133]. This is due to the facts that (a) isolated humic substances possess chemical characteristics similar to those obtained by neutral extractants, and (b) NaOH generally provides higher extraction yields [134–136]. Most importantly, this method has been recommended by IHSS and can provide good and repeatable results for humic acids isolation, from both soil and water. Isolated preparates can be further characterized by elemental composition and spectral advanced techniques. Finally, this method is generally used and standardized, which helps both comparing samples of different origin and spectral library formation. Also, the use of XAD-8 and XAD-4 resins in tandem for

the isolation of dissolved organic matter has been extensively applied in water chemistry; the limitations have to do with the fact that the tubing line must be manually reset as the cleaning solution is changed [137]. In contrast, extraction with water and neutral salt solutions is much less effective than that of conventional alkaline solvents. Regarding the organic solvent systems, although dipolar aprotic solvents (especially DMSO) are good solvents for HSs, it is difficult to recover the solute products in the extracts. They may also alter the chemical nature and composition of extracts [124].

5. Fractionation of Soil Humic Substances

5.1. Gel Chromatography Techniques

Swift [138] referred to classical fractionation procedures that were in use at the time. These included uses of pH, of salting out, uses of metal ions, and of some organic solvents such as ethanol and diethyl ether. He outlined techniques being introduced to the humic sciences that include gel permeation chromatography, ultrafiltration, and centrifugation. He also discussed fractionation based on charge characteristics, including electrophoresis and electrofocusing, and ion exchange, and, finally, he outlined procedures involving adsorption.

Chromatographic techniques for separations based on molecular size, i.e., gel or low-pressure size exclusion chromatography, have been widely applied for the fractionation of humic substances to obtain more size-homogeneous fractions. Commonly employed stationary phases include polysaccharide-based gels like Sephadex and polyacrylamide gels with varying pore sizes. The separation efficiency can be affected by electrostatic repulsion between the negatively charged HS molecules and the gel matrix, as well as by hydrophobic interactions between dissolved HSs and the stationary phase. To minimize the electrostatic interferences, salt is often added to the mobile phase; however, ionic strengths exceeding 0.5 M may promote hydrophobic adsorption onto the gel matrix [139].

Cameron et al. [140] and Swift et al. [141] employed gel chromatography to fractionate HA materials into distinct components, for which they determined molar mass values (Section 6).

5.2. Sequential Exhaustive Extractions and XAD-8 (DAX-8) and XAD-4 Resins in Tandem

This procedure is based on the fractionation of the extracts from exhaustive extractions at increasing pH values in 0.1 M aqueous NaOH and the pyrophosphate solutions as referred to in Sections 4.1.1 and 4.1.2. The extracts are diluted to < 20 ppm, the pH is adjusted to 2 (with HCl) and the dissolved materials are passed on to the XAD-8 (or DAX-8), and XAD-4 resins in tandem. The hydrophobic (HAs and FAs) and the hydrophilic (or XAD-4/HPI acid) fractions (recovered, respectively, from the XAD-8 and XAD-4 resins) from a long-term stagnogley grassland soil have been isolated at pH 7, pH 10.6, and at pH 12.6 [101]. The XADN sample represents the material isolated in the ethanol Soxhlet-extracted material that was not recovered in the 0.1 M NaOH back elution of the XAD-8 resin following the processing of the materials isolated at the different pH values. An additional study [104] was carried out on a similar stagnogley soil under long-term cultivation to wheat (*Triticum aestivum* L.) (Refer forward to Section 5.7).

The ¹³C-CPMAS NMR spectra in Figure 3 show similarities and differences in the compositions of the fractions acquired at different pHs. In broad terms, the resonances between 0 and 40 ppm are for aliphatic hydrocarbon functionalities, those between 50 and 60 ppm can indicate peptide and methoxyl (from lignin) structures. Resonances from 60 to 90 ppm can largely be attributed to carbohydrate and this is confirmed by the resonance for anomeric C at 105 ppm. The 60–90 ppm resonance can also indicate contributions from alcohol and ether groups. Resonances in the 110–140 ppm range will relate to aromaticity,

that at 140–150 ppm, to O-aromatic structures, often indications of phenolic and methoxyl from lignin; those in the 160–190 ppm range will signify carbonyl from carboxyl, ester, and amide groupings.

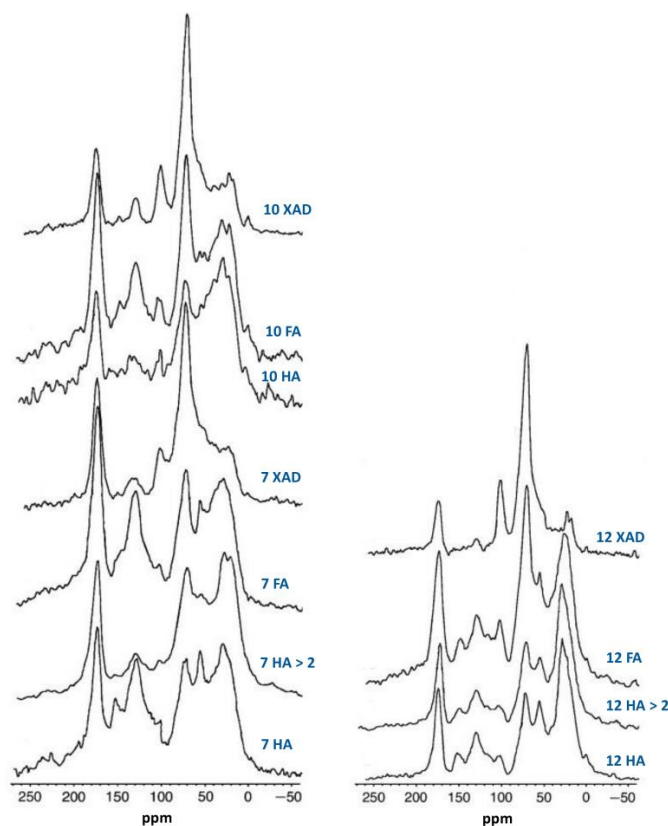


Figure 3. ^{13}C -CPMAS NMR spectra of humic acids (HA), humic acids precipitated at pH > 2 (HA > 2), fulvic acids (FA), and XAD-4 acids (XAD) at pH 7 (7), at pH 10.6 (10), and at pH 12.6 (12) obtained from a Stagnogley soil [101].

The spectra in Figure 3 highlight compositional differences between the HA and FA fractions obtained at several pHs. The carboxyl and aromaticity functionalities are greatest for the pH 7 extracts, i.e., 7HA, 7HA > 2, 7FA and 7XAD, indicating most highly oxidized materials. They also exhibit relatively large amino acids content; thus it is unlikely that components derived from lignin contributed to these XAD-4 fractions but it is more likely to arise from peptide material serving as strong evidence for microbial inputs. The 7XAD, 10XAD and 12XAD spectra representing soil XAD-4 acids at different pHs are significantly different from those for the traditional humic and fulvic acids (HA and FA spectra). They contain low quantities of aromatics, and the carboxyl functionality decreased as the pH of the extractant increased. These novel humic acid components had significant enrichments of peptide and saccharide materials that indicated origins in microbial synthesis processes. Evidence for the origins of humic and fulvic isolates at higher pH values suggested more extensive inputs from plants. The spectra 7HA > 2 and 12HA > 2 are for small amounts of materials that precipitated when the diluted extracts at pH 7 and at 12, respectively, were left standing when the pH was adjusted to 2. These were found to be rich in sugar and carbohydrate and peptides.

5.3. Polyvinyl Pyrrolidone Resin

In cases where the XAD-8 resin was not available, IHSS proposed the application of a cross-linked PVP (polyvinyl pyrrolidone) resin to isolate the HSs [142–145]. Under acidic conditions, the aromatic carbon compounds are preferentially adsorbed onto the PVP

resin. Organic molecules not adsorbed onto PVP typically include carbohydrates, proteins, amino acids, and uronic acids. PVP has proven highly effective for isolating humic-like components from freshwater samples. On this basis, Peuravuori et al. [144] demonstrated that PVP retained over 86% of the dissolved organic matter (DOM) in a freshwater sample, compared to 77% retention using DEAE (diethylaminoethyl cellulose sorbent), and 65% with XAD-8 resin.

5.4. High Performance Liquid Chromatography (HPLC)

Size exclusion chromatography (SEC) has been the most widely applied technique for the chromatographic fractionation of soil humic substances. However, as discussed in Section 5.1, SEC is subject to limitations due to adsorption effects and ionic interactions, which can interfere with accurate size-based separation. In addition, the M_r values for the different SEC fractions often lack proper calibration with suitable standards.

The HPLC technique proved more advantageous for applications to geological samples [146–149] thanks to its rapid analysis, increased sensitivity, and efficient recovery of subfractions. These attributes make HPLC a promising alternative for the fractionation of humic substances and dissolved organic matter.

Balabanova-Radonova et al. [150] demonstrated that reverse-phase (RP) HPLC provided high resolution for lignite-derived HAs. The sample was dissolved in DMF, and methanol was used as the eluent. Analysis using a Li Chrosorb RP-18 column (5 μm) yielded more than 10 distinct peaks. Similarly, Fievre et al. [151] applied the HPLC technique for the separation of the standard Suwannee River fulvic acids. Their system employed an Alltech Absorbosphere Phenyl column (5 μm particle size; 25 cm \times 4.6 mm i.d.) and a mobile phase consisting of equal volumes of methanol and water, to which 1% (*v/v*) of CH_3COOH had been added. Two fulvic acid subfractions were obtained, which were further analyzed using high-field (9.4 T) Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS). The resulting mass spectra revealed that the molecular compositions of the two fractions were similar.

A novel RP-HPLC method was developed using a stepwise gradient of dimethylformamide in an aqueous phosphate buffer mobile phase, combined with a wide-pore (30 nm) C_{18} column, to fractionate HSs and HSs-like from soil, peat, and airborne particulate matter, as well as lignins [152]. The system provided both spectrophotometric diode array and fluorimetric detections. The method demonstrated excellent reproducibility in retention times and produced well-resolved peaks. The synergistic effects of DMF's solvating and disaggregating capabilities, along with the wide-pore RP sorbent, enhanced the analyte–surface interactions and minimized any size exclusion influences.

Hyphenation of LC and NMR has been widely employed for the characterization of various natural products [153,154]. Simpson et al. [155] conducted preliminary research applying both LC-NMR and LC-SPE-NMR techniques to examine heterogeneous NOM. The LC-SPE-NMR method has demonstrated significant potential for the isolation of specific components, thereby facilitating their detection by NMR. Finally, 2D-HPLC techniques have been developed for the characterization and fractionation of HSs and their constituents [156,157].

5.5. Electrophoresis

Electrophoresis is widely employed to separate ionized and ionizable compounds, i.e., amino acids, lipids, nucleotides, charged sugars, peptides, and complex protein mixtures [158]. The application of electrophoretic techniques to the HSs fractionation and M_r estimation is owed to the polyelectrolyte nature of humic substances [159–161].

Castagnol et al. used electrophoresis to study the effect of varying urea concentrations on the disaggregation of humic molecules in soil humic acids samples. Their approach successfully separated four distinct humic subfractions [162]. Dunkel et al. [160] compared several electrophoretic methods including free solution capillary electrophoresis (FSCE), isoelectric focusing (IEF) in ultra-thin layers, micellar electrokinetic chromatography (MEKC), capillary gel electrophoresis (CGE), and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for the separation of aquatic HAs and FAs. The HSs separation was facilitated when a 5-M urea solution was added to the running buffer, and, as a result, more peaks appeared in the electrophoretogram. The best separation was achieved via isoelectric focusing (IEF), revealing approximately 20 sharp peaks. Analysis of different FA samples via SDS gradient-gel electrophoresis produced three consistent bands of molar masses of approximately 1000, 6000, and 8000 Da, respectively. For further discussion, see Section 6, including insights by Simpson et al. [74] and studies by the Piccolo and Wells groups on the self-assembly of heterogeneous molecular aggregates stabilized by dispersive hydrophobic forces and hydrogen bonding.

Schmitt et al. [163] first used capillary isoelectric focusing (CIEF) to fulvic acid separations yielding 3–4 fractions. Kovacs and Posta [161] further optimized the CIEF method for soil humic acids, i.e., enhanced resolution and used anticoagulants, and received 30 to 50 stable fractions.

Capillary electrophoresis (CE) separates ionic species within a narrow capillary on the basis of their charge-to-friction ratio. Separation efficiency depends on differences in electrophoretic mobility among analytes. However, neutral compounds or those with similar mobilities present challenges. These can be addressed by techniques such as micellar electrokinetic chromatography (MEKC), where the separation of neutral molecules is facilitated by the addition of surfactants to the electrolyte. Charged polymers like DNA can be separated via capillary gel electrophoresis (CGE), which uses a gel matrix to retard longer strands more than shorter ones, providing a high-resolution alternative to traditional slab gel electrophoresis. CE systems can also enable microscale liquid chromatography or capillary electrochromatography (CEC).

To summarize, various CE modes—including capillary zone electrophoresis (CZE), CIEF, CGE, MEKC, and CEC—can be applied in HSs separations and identifications, either independently or combined with other analytical techniques [164,165]. They give rise to the possibility of gaining an insight into the compositions of ‘members’ of the ‘families’ of molecules referred to in Section 2. Detailed compositional information that may emerge will advance awareness of structures within the framework of humic materials but may not provide significant advances in our understanding of the ways in which the humic molecular *families* contribute to their fundamental and essential roles in the soil environment. However, such detailed studies should be encouraged because the information that will emerge will enable a better understanding of the processes that are taking place.

5.6. DEAE Preparations

DEAE (diethylaminoethyl) cellulose is a weak anion exchange resin used for the isolation of aquatic HSs, with several studies comparing its performance to that of XAD resins [144,166,167]. DEAE can retain humics in water samples without pH adjustments at pHs ranging from 4 to 8 and can also be applied in FAs fractionations. Hejzlar et al. [166] reported that DEAE-cellulose adsorbed a greater proportion of HSs from peat water (94%) compared to XAD-2 (74%). However, Boulton et al. [168] noted that DEAE extraction is not selective for humic substances alone, as the DEAE column also adsorbed pure polysaccharides.

DEAE-Sephadex integrates both size- and charge-based separation mechanisms for macromolecules fractionation. Finch et al. [169] has reported the fractionation of a polysaccharide mixture extracted from soil with the use of an anion-exchange chromatography with a DEAE-A50 Sephadex column, a phosphate buffer (pH = 6), and a NaCl solution gradient (0 to 1 M) (see Section 7.4.5). The eluted-at-120 mL fraction was almost entirely removed from solution by adsorption onto H⁺-exchanged montmorillonite. Barker et al. [170] identified that the retained compound possessed one uronic acid per six to seven sugar units (see also Clapp et al. [90]).

5.7. XAD-8 (DAX-8) and XAD-4 Resin Techniques

XAD-8 (or DAX-8) and XAD-4 resins, used in tandem, have been widely applied for the separation of NOM [103,171–173]. This methodology enables the isolation and reconcentration of NOM into operationally defined *hydrophobic* (HPO) and *hydrophilic* (HPI) subfractions. Specifically, acidified samples at pH = 2 passed sequentially through the XAD-8 and XAD-4 resin columns. The material sorbed onto the XAD-8 resin is classified as HPO, while the NOM fraction that is retained by the XAD-4 resin is considered as HPI. Following adsorption, the resin columns are desalted using distilled water. The XAD-4 column is then back-eluted with 0.1 M NaOH, and the eluate was sent through an IR-120 (H⁺-form) resin before freeze-drying. The final products are referred to as XAD-4 acids (see Section 5.2 for SOM fractionation and isolation).

HSs adsorbed on XAD-8 are recovered following the same desalting and back-elution steps. The centre cut of the back elute is acidified to pH 2 to induce precipitation of humic acids, while FAs remain in solution. The precipitation is carried out at 277 K, and the fulvics are separated by syphoning and centrifugation. The fulvic acid solution is then reapplied to an XAD-8 column for desalting and elution, as described for the XAD-4 acids. The precipitated humic acids can be dialysed and freeze-dried or they can be redissolved in 0.1 M NaOH, diluted to < 20 ppm, reacidified to pH 2, and reapplied to XAD-8 in line with the FA protocol. This latter approach may lead to loss of some hydrophobic neutral components, which can be recovered by Soxhlet extraction of the resin using ethanol.

It should be noted that small methodological variations in the XAD-8/XAD-4 chromatography method—such as flow rate or sample concentration—can significantly impact the fractionation outcome, complicating inter-laboratory comparisons. For instance, exceeding the resin's sorption capacity or applying flow rates above 40 mL min⁻¹ may result in disproportionately large HPI fractions. This is because there are no clear cut-off boundaries between the hydrophobic and hydrophilic fractions. Consequently, the chemical composition of both HPO and HPI fractions may differ depending on operational parameters.

5.8. General Summary

It is agreed that soil organic matter is a gross mixture of organic molecules that can be associated with each other and with other molecules for which they will have affinities through forces such as hydrogen bonding, hydrophobic bonding and mineral cation bridging. In order to achieve maximum extraction, it is first necessary to remove the influences of mineral cationic bridging mechanisms, and the most effective way to achieve this is by H⁺-exchanging the system using mineral acids, of which HCl is preferred. Then aqueous NaOH is the solvent of choice and it will, as the Na⁺ salt, isolate the component organic acids that will include the HA, FA, the HPI (or XAD-4) acids, and the water soluble neutral organic molecules. Some soluble molecules are held by strong hydrogen bonds, or by occlusion in the insoluble core. Addition of urea (6 M) to the base solvent will cleave the restraining bonds and/or cause changes in the conformations of restraining structures allowing trapped soluble molecules to be solvated and set free. Then, when the base urea is

removed and the residual has dried, extraction with a mixed solvent (DMSO-concentrated H_2SO_4) will allow the HN fraction to be recovered.

It is desirable, in so far as is possible, to fractionate the isolates. It is realized that what might be considered to be the most reactive components of SOM arise from transformations by the soil biota. These get their energy from the biomass entering the soil environment and give rise in the transformation process to humus and especially to humic and humin materials that are resistant to degradation and carry out essential roles in the soil environment. Some polysaccharide and peptide structures are also formed and their persistence may well be through associations with the humic and HN materials, and with the soil mineral colloids. It is desirable to know which of the components of SOM are engaged, and how they are engaged, in processes such as aggregate formation and stabilities, soil cation-anion-exchange properties, stimulation of plant growth, and interactions with anthropogenic chemicals, etc. It can be seen that results from the fractionation procedures outlined in this Section indicate that it should be possible to free structures that are constrained by forces other than covalent linkages.

6. Sizes, Shapes, Compositions of Humic Structures

6.1. The Classical Concepts

Cameron et al. [140] employed gel chromatography to isolate eleven humic acid fractions from a highly humified organic muck soil. They applied $\text{Na}_4\text{P}_2\text{O}_7$ for the extraction of the first two fractions, 0.5 M NaOH (298 K) for the next seven, and 0.5 M NaOH (333 K) for the last two ones. All fractions were recycled through the gel until a relatively homogeneous product was received on the basis of the elution volume from the column. Ultracentrifugation determined M_r values of 2.4 and 4.4 kDa for the pyrophosphate-extracted fractions. For the NaOH extracts at 298 K, molar masses ranging from 12.8 to 412 kDa were obtained via gel chromatography and pressure filtration while the last two fractions extracted with NaOH at 333 K exhibited molar masses of 408 and 1360 kDa, respectively.

An outline is given in Clapp et al. [90] of the mathematical treatments used to obtain the data for Figure 4.

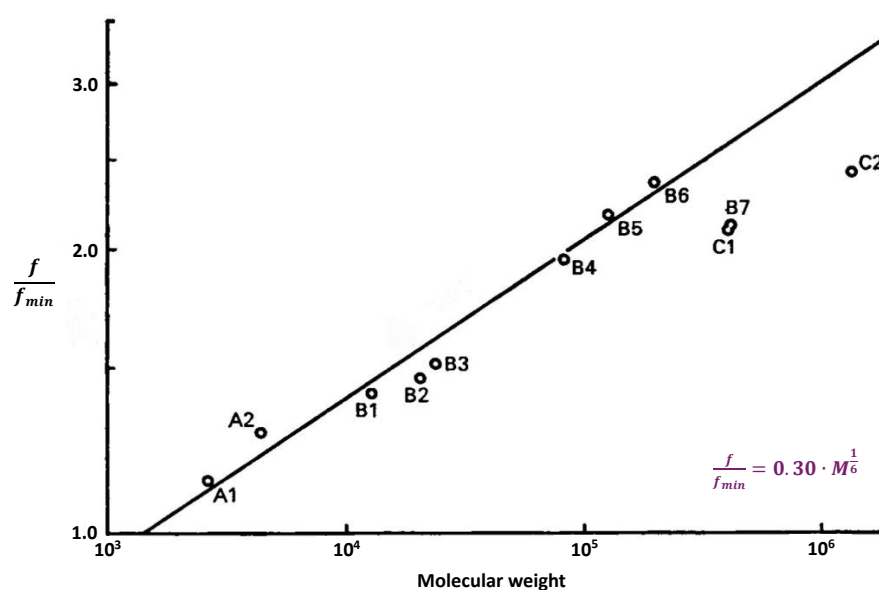


Figure 4. The experimental data (o) are plots of frictional ratio values (f/f_{min}) versus M_r values of different HA fractions from an organic soil. The solid line represents the theoretical relationship between f/f_{min} and M_r for randomly coiled molecules [127].

Flaig and Beutelspacher [24] observed that the molar masses varied significantly, ranging from 77,000 Da (without any electrolyte) to just 2050 Da upon the addition of 0.2 M NaCl. The salt served to reduce repulsive negative charges. This observation insinuates that, in the presence of the electrolyte, humic substances form aggregates.

Cameron et al. [140], under the leadership of Swift and Posner [112,174], proposed the polydisperse macromolecular model for HSs which was widely accepted [138,141,175–177]. The results of Swift et al. [141], along with the perspectives later articulated by Swift [178], presented what was then considered as an irrefutable case for macromolecularity. They used gel chromatography to separate a soil-derived humic acid into four subfractions, each of which exhibited stability in both molecular size and elution volume upon repeated chromatography. ^{13}C -CPMAS NMR spectroscopy revealed that each fraction had a unique chemical composition. Notably, aromaticity, carboxyl content, and lignin-originating structures increased, while structures related to carbohydrates decreased with decreasing molecular size.

6.2. Molecular Associations

Considerations of their shapes, sizes and the surface properties have importance in understanding how the molecules react and interact. More recently, significant attention has been directed toward the concept that small HS molecules of low- M_r associate to form self-assembling supramolecular structures. To explain the coexistence of hydrophobic and hydrophilic components in these structures, Wershaw proposed for humus a “membrane-micelle” model [27,28,179]. Engebretson and von Wandruszka [180] demonstrated that pyrene, when interacting with HSs molecules, was shielded from Br^- quenching. They suggested that HSs molecules could readily aggregate via hydrophobic interactions, thereby trapped and protected pyrene within the humic core. Similarly, Kenworthy and Hayes [181] found that pyrene was more protected in humic and fulvic acids isolated at higher pHs, as HSs contained more hydrophobic fractions.

Simpson advanced the concept that humic acids and fulvic acids are not crosslinked, macromolecular or high- M_r compounds, but rather aggregates composed of relatively simple molecules that exhibit colloidal properties in the presence of metal ions [74]. A study on soil-derived FAs showed that the components possess diffusivities consistent with relatively low- M_r species (about 1 kDa). These aggregates are likely stabilized through a complex interplay of hydrophobic interactions, electrostatic interactions, hydrogen bonding, and metal ion bridging.

Alessandro Piccolo describes the concept of supramolecular associations that he and his colleagues have championed [139,182–184]. This model envisions humic substances as self-assembled aggregates of relatively small, heterogeneous molecules held together primarily by dispersive hydrophobic forces (including van der Waals, π - π , and CH- π interactions) and hydrogen bonding. Disruption of these supramolecular assemblages is thought to occur through interactions with specific chemical agents—such as amphiphilic organic acids (e.g., stearic acid), urea, dimethyl sulfoxide, and mono- or polyvalent cations—which can dismantle the larger humic associations, yielding more homogeneous or chemically simpler fractions. In view of the influences that urea amendments to basic solvent extractants have on the breakup of hydrogen bonded associations in humified structures (Section 4.1.3), the authors can sympathize with concepts expressed by Piccolo.

Wells and Stretz [185], drawing on the “Comprehensive Hydrogen Bond Theory” [186], proposed a conceptual model for the supramolecular aggregation of organic matter based on H-bond interactions of varying strengths—including very strong ones. Martha Wells employs a striking analogy to illustrate the concept of self-assembly: “How did the elephant get into the cage, when the only entry was through a mouse hole in the cage frame?”

High-pressure size exclusion chromatography (HPSEC) offers several advantages compared with traditional low-pressure gel permeation chromatography (GPC), including higher reproducibility, extended column lifespan, faster analysis, and greater sensitivity to chemical changes. Piccolo et al. [184] employed HPSEC to size-fractionate HAs and observed that treatment with acetic acid disrupted the weak, non-covalent associations within the humic supramolecular structure. Analysis with pyrolysis-gas chromatography–mass spectrometry (Py-GC-MS) showed that acetic acid treatment altered the distribution of molecular components (unsaturated alkyl chains and aromatic moieties) shifting them from larger to smaller apparent molecular size fractions. The fractions containing the largest molecules were richest in alkyl chains, insinuating that humic supramolecular structures are primarily stabilized by multiple weak interactions among these apolar groups. Additionally, the ^1H NMR spectra of the size fractions became markedly simpler and better resolved following the acetic acid treatment, supporting the breakdown of complex aggregate structures into more discrete molecular entities.

It is widely accepted that the molar mass of soluble fulvic acids falls within the range of 400 to 1500 Da [187]. The relatively small variation in reported M_r values for FAs is attributed to their high hydrophilicity; they are small highly acidic compounds whose hydrophilic character reduces the tendency for the formation of strong molecular associations via hydrophobic interactions.

In his article entitled “*Molecular Properties of Soil Humic Substances: Fact, Fiction and Opinion*”, Swift critically examined the evidence then available regarding the supramolecular concept [178]. Referring to his earlier work [141], he argued that the observed compositional differences among the gel chromatography fractions would not have occurred if the molecules had reassembled according to the supramolecular association model. It would be amazing, he reasoned, if the reassembling did give rise to the same molecular dimensions and properties during the repeated applications to the gel columns needed in order to get satisfactorily size-homogeneous fractions.

That is a valid point. The humic materials that Swift had worked with were from an organic soil. It is of interest that the most highly oxidized and the most aromatic (lignin derived) components were in the lower M_r fractions and the highest M_r materials were richest in carbohydrates. The properties of the lower M_r fractions indicate that greater biological oxidation had taken place. The enrichment of carbohydrate in the higher M_r material could be part of the answer that explains its size because a polysaccharide component in the 0.3 M H_2SO_4 isolate by Finch et al. [169] from a cultivated Fenland organic soil had a M_r of 60×10^3 . The HAs in association with polysaccharide material could compose the higher M_r fractions. If that association included covalent bonding (e.g., as phenolic glycoside structures) then the association could be classified as a HA material.

6.3. General Inferences

The authors have been impressed and influenced by the more classical and by the recent concepts regarding the sizes and the shapes of HSs molecules. Based on the concepts put forward and the procedures used by the different authors, it must be agreed that the conclusions reached by the Swift, Hatcher, Piccolo, and Wells Groups are valid. Studies have been reported for humic materials (and humins) from different soil types in different climatic regions and subjected to different management systems, and we know that the compositions of the humic components differ (but perhaps not the humin) from these varied systems. The same will apply for HSs and DOM from waters, and their compositions will be influenced in the cases of runoff waters by the vegetation they have traversed and by the organic matter in the soils through which they have drained.

Wershaw's work is excellent, but his work referred to in this article was primarily involved with isolates from the Suwannee River. His compositional studies showed that the materials identified could be related to the compositions of the leaf litter in the watershed, and it is not likely that the materials isolated from the waters had significant residence time in the riparian soils.

Work on the Suwannee-river HSs arose from concerns about the extents to which chlorinated organic substances, in particular 'mutagen X' or MX (3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H) furanone), are formed in chlorinated waters. Concerns were raised by the US EPA, and the US Geological Survey Water Resources Division (USGS WRD) was commissioned to carry out investigative studies. Dr. Bob Averett (of USGS WRD) initiated the studies [188], and he was succeeded by Dr. Ron Malcolm, the first President of the IHSS. The HSs in runoff waters will have a different genesis and will be compositionally different when compared with those generated in the soil environment, as was seen for the compositions of the NOM isolated from the runoff and from the drainage waters of a grassland soil [101]. The same stands for comparison of HSs in soils and sediments [189]. It can be assumed that those in the drainage waters [101] had a longer residence/reaction time, were subjected to a broader range of microbial processes, and will contain materials solubilized from the resident SOM. The formation of the IHSS in 1981, led by first President, Ron Malcolm, has brought together humic scientists from the lignite, soil, sediment, and water disciplines and each group has profited from interactions and associations formed at the 21 International meetings of the Society that have taken place since then. Output from the Water scientists has been particularly proficient, and we now have data for ground waters, lacustrine, ocean, riverine and even South Pole waters.

It can be assumed that there will be compositional differences in HSs from different soil types, e.g., those formed in different climatic regions. The management that the soils had been subjected to (for example long-term grassland versus long-term cultivation) also plays a key role. Organic materials generated under aerobic conditions will have different compositions compared with those formed under anaerobic conditions.

Work by the Hatcher Group (see Section 3.1 and [15,17,190]) has shown that HSs isolated from various soils and environments exhibit different compositions. Significant compositional differences are also observed between the HSs isolated at different depths in a peat profile. Those in the deep, most humified layers will be different from those closer to the surface. The final stages in the humification of the deep, dark, highly humified materials are likely to result from anaerobic processes and with little or no evidence for structures characteristic of origins in lignin. This contrasts with the humic components in the less humified materials closer to the surface.

Much emphasis is given to the vast amounts of organic matter and of methane locked in tundra soils and on the environmental impact that the release of greenhouse gases will have (arising from biological oxidation resulting from global warming). It will be appropriate to have in-depth studies of the organic materials compositions at increasing depths in these soil profiles. That should apply also for studies of the deep Midlands peat bogs (especially) of Ireland. One of the authors (M.H.B.H.) considers that the decision to flood the Irish Midland peat bogs was taken in haste before a comprehensive appraisal was made of the compositions and properties of the component substances and of the materials formed as the result of humification processes.

The study by Cameron et al. [140] was made on a highly organic soil with a high degree of humification. The possibility should be recognized that combinations of biological transformations and synthesis, and of chemical reactions over time would allow high-M_r structures to form. We do not know when the HAs that persisted in that soil were formed, but it can be assumed, if the soil had not been subjected to long-term cultivation manage-

ment, that they had a prolonged residence in the soil, long enough for slow transformations to take place to give rise to humic products of high molar mass.

Regarding the molecular structure of the SOM components, the dominant theories of macromolecular and supramolecular associations were analyzed. On the other hand, according to the SCM concept mentioned above [4], organic matter exists as a continuum of organic fragments that are continuously processed by the decomposer community towards smaller molecular size. Although certain experimental techniques suggest that HSs contain a large fraction of small molecules, HSs molecules are clearly sizable and fractions that exhibit the highest molar masses are expected to be of macromolecular construction [134,191]. It has been reported [192] that spontaneous abiotic non-catalyzed oxidation of tannins can induce polymerisation and this may occur at 298 K by simple exposure of these substances to atmospheric oxygen. Also, HSs themselves can react to form long molecular chains under the action of laccases [193].

It should be possible to resolve the questions of molecular size and shape. For this it might be suggested that samples be taken from the most humified layers of a peat that formed over marl, and another from an upland (blanket) bog. In the same way samples might be taken from mineral grassland and forest soils that had been under grass and under forest for 100 years or more, and from paired soils that had been under cultivation for similar time periods. Isolation might use procedures outlined in Section 4 and the inclusion of urea in the extraction solvent and in the fractionation processes (Section 4.1.3), and consideration might be given to the high resolution electrophoretic fractionations outlined in Section 5.5. Then the fractionation and M_r determination procedures used by Swift and colleagues and by Piccolo and Wells and their colleagues might be applied. The results might lead to definitive solutions.

What is needed to be resolved, however, is whether or not the macromolecular or the molecular associations' properties make differences in so far as the essential roles that humic molecules play in the soil environment. If it is unambiguously shown in laboratory studies that the humic components isolated from soil can be separated into associations of smaller molecules, the fact will remain that they will be present in the field as supramolecular associations and/or as macromolecules. If they should be in associations then there is the possibility that the association processes may involve functionalities that we know (from studies of humic molecules in solution) are relevant for the interactions and reactivities of these molecules in soil. Could, therefore, the association process alter our views about how humic materials behave in the soil environment?

The resolution of the sizes, shapes, and associations of humic molecules in the soil and those isolated from it is academically challenging and can be rewarding. The major reward will be if the resolution will enable a better understanding at the molecular and practical levels of how HAs, in particular, contribute in the field to soil structure and to soil fertility.

7. Soil Structure, Aggregates, Compositions, Stabilities

7.1. Soil Structure: General Considerations

Soil structure refers to the arrangement of soil particles—sand, silt, and clay—along with organic matter into aggregates. Thus, the soil structure results from the complex interactions between soil texture, plant roots, soil biota, and soil organic matter. A well-developed 'crumb' structure is considered ideal, as it promotes effective gas exchange, water retention, and the circulation of both water and essential nutrients within the soil environment. Because of their extensive surface areas (up to $750 \text{ m}^2 \text{ g}^{-1}$) and (predominantly) negative charges (generated from isomorphous substitutions in both tetrahedral and octahedral layers of the 2:1, 2:1:1, 1:1 clays and mixed lattice structures (e.g., ref. [194]), clays and other colloid-sized (hydr)oxides and inorganic particles are the reactive mineral

components in soils. The dimensions of microbial cells are such that they would cover a major proportion of the soil particles available to them if all were sorbed by the soil colloids. However, steric constraints would allow their entry only to transmission ($> 50 \mu$ equivalent spherical diameter (ESD)) and to storage (0.5 to 50μ ESD) pores. The entry into pore structures of macromolecules formed from microorganisms may be important for the stabilization of aggregates [195].

Physical forces such as wetting and drying and freezing and thawing have a role because these give rise to fracturing along planes of weakness leading to the development of voids in the soil mass. As a result, networks of connected pores of varying dimensions develop, regulating the circulation of gases, liquids, solutions, particulates and organisms. These networks play key roles in governing soil functions [196,197].

Edwards and Bremner subjected a wide variety of soils to different intensities of sonic vibration and concluded that soils are composed of macroaggregates ($>250 \mu\text{m}$) and of microaggregates ($<250 \mu\text{m}$). Additionally, they proposed that macroaggregates are composed of weakly associated microaggregates and, therefore, macroaggregates are less stable than microaggregates, show faster turnover rates in soil, and are more susceptible to soil management practices [198,199]. Tisdall and Oades [200] regarded as microaggregates particles in the range of 20–250 μm ; thus, lower microaggregate particles fall into the silt-size range ('silt' includes particles of $<50 \mu\text{m}$).

Totsche et al. provided a comprehensive review of microaggregates in soil [201]. It is proposed to focus in this treatise on the organo-mineral associations and on reactions especially involved in the synthesis and stabilization of microaggregates. All organo-mineral associations can be considered to be 'building blocks' for aggregates, and especially for microaggregates. This will involve organo-clay interactions, including clay-microbial associations.

In short, soil is a highly complex system. We know the compositions, structures, and most of the associations of mineral colloids, but it is the ways by which these interact with the soil organic components and with the soil microbial population that generate the living and highly reactive mineral soil systems. A better understanding should be sought of how the interactions and associations take place that give rise to the structured systems that are characteristic of fertile soils.

7.2. The 'Building Blocks' of Microaggregates

Clays, oxide and (hydr)oxide colloids are the inorganic compounds exhibiting the highest reactivity within the microaggregates. The surface properties of the mineral components in microaggregates allow interactions to take place between the mineral colloidal components. Cation bridging enables negatively charged clays to associate via coulombic attraction forces with the positive charges generated below the point of zero charge (p.z.c.) on the (hydr)oxides of iron, aluminum and manganese species. Quirk [202] has considered that clays associate in parallel to form quasi-crystals and domains incorporated in microaggregates which further bond to form the larger aggregates. The most reactive organic components in the soil environment are negatively charged at ambient soil pH values. These will sorb to (hydr)oxide surfaces positively charged below their p.z.c. and in this way give rise to clay, (hydr)oxide, and organic building block associations.

Significant contributions are made by the soil microfauna (bacteria, fungi, viruses) which can number 1×10^7 to $1 \times 10^{10} \text{ g}^{-1}$ of dry weight of soil—the living tissue of microbial biomass is estimated to be from 17 to 22 g m^{-2} of soil [203,204]). Protozoa, eelworms, earthworms, soil insects, and burrowing animals are also expected to be involved.

Soil microorganisms bear a negative surface charge and Stotzky [205] has pointed out that considerations of electrical double layer effects will apply for interactions be-

tween clays and bacteria, virus, and fungal cells. In the next chapter in that publication series, Burns [206] was first to give a comprehensive treatise on microbial adhesion to soil surfaces. Sulphates and carbonates also contribute to the adhesion processes. Chenu and Stotzky [207] and Kögel-Knabner et al. [208] indicated how mineral colloid organic association 'conglomerates' contribute to soil structure.

Two organic mixtures of macromolecules rich in water and with gel-like properties, i.e., the extracellular polymeric substances (EPS) exuded by microbes and the mucilage secreted by both microorganisms and plant roots, play important roles to the formation and stabilization of soil microaggregates. Mucilage primarily consists of polar glycoproteins and polysaccharides [209], whereas EPS comprises not only carbohydrates but also significant amounts of lipids and proteins, along with minor quantities of nucleic acids [210]. In incubation experiments, Morel et al. [211] observed that mucilage induced "a spectacular and immediate increase in stability" of water-stable aggregates smaller than 2000 μm . They proposed that freshly secreted mucilage rapidly adheres to soil particles, offering effective protection against disintegration by water.

The importance of mucilage in considerations of soil structure and for crop production is recognized, but its composition and structures are imperfectly understood. That may well be because of the difficulties in isolating and observing the relevant interactions of the different components in the mixtures. It could be, however, that the most desirable effects are achieved by the different components in the mixtures working in harmony. A relevant review by Agniuhotri et al. [212] has outlined the contribution of glycoprotein materials from arbuscular mycorrhiza for carbon sequestration in soils.

Significant amounts of the proteinaceous fraction in EPS can be lost during the extraction processes [213] and that may lead to an overestimation of the significance of polysaccharides in the formation of soil microaggregates. The work of Omoike and Chorover [214] indicated that EPS-derived proteinaceous compounds were preferentially bound by goethite. These are in line with McGill and Paul [215] who proved that nitrogen associates preferably with sesquioxides than with phyllosilicates. They suggested that amorphous Fe and Al compounds located onto the clay surface were responsible for the organic N-inorganic colloids association.

Interest is steadily increasing in the role of black carbon (BC) in soil [19,216–219]. BC arises from the incomplete combustion of woody and herbaceous materials in forest and grassland fires and is composed of a range of mainly aromatic materials and especially in fused aromatic structures [220–222]. Charcoal, possessing varying chemical composition, is generated from both anthropogenic and natural fire events and is a major contributor to organo-mineral complexes formation. Aged charcoal particles, characterized by oxidized and reactive surfaces [223], can function as effective binding agents contributing to the stabilization of soil aggregates [224]. Charcoal addition induced enhanced nutrient retention and bacterial diversity to new microaggregates [225]. These effects are attributed to charcoal's high microporosity and hydrophobic properties, which facilitate the microorganisms, and adsorption phenomena, respectively, within the soil matrix.

Research conducted by the Hatcher Group [18,190] has shown that a non-pyrogenic route (from the oxidation of lignin precursors) is possible for the generation of condensed aromatic structures. The process involves the hydroxyl radical-initiated oxidation of lignin which can produce black carbon-like condensed aromatic compounds. Additionally, interactions of ^{15}N -labelled peptide with model quinone produced NMR signals resembling those of the solid lignin [226]. These results are valuable for assessing nitrogen incorporation into SOM and demonstrate how proteinaceous molecules may be protected and sequestered through covalent bond formation.

Sparling and Cheshire [227] compared microaggregate stabilities in rhizosphere soils and in bulk soils. They found that polysaccharides contributed less to microaggregate stability in the rhizosphere soil even though that soil possessed a high mucilage-polysaccharides content. This unexpected difference was attributed to the large quantities of plant remains and debris in the rhizosphere soil, in contrast to the more degraded and chemically transformed components in the (bulk) soil.

7.3. Roles of Microbial Polysaccharides and Peptide Structures

In 1945, Martin [228,229] established that the slimy bacterial products—previously shown by Waksman and Martin [230] to promote aggregation in sand–clay mixtures—were, in fact, polysaccharides. Around the same time, two other research groups, working independently and being unaware of Martin’s work, were examining the application of dextran and levan polysaccharide slimes for improving soil structure: Haworth et al. [231], and Geoghegan and Brian [232]. Geoghegan and Brian showed that proteins as well as carbohydrates influenced the aggregation of soil particles [233].

Significant amounts of proteins, peptides, and amino acids will always exist in soils as a result of the inputs from plant roots, from plant materials directly added to the soil, or entering the soil as senescent matter from vegetative cover (introduced by organisms such as earthworms), and from microfauna. While most of these proteinaceous substances are recycled by soil biota, a portion persists—protected within soil aggregates and humic substances. McLaren [234] demonstrated that proteinaceous materials were adsorbed by kaolinite clay and McLaren et al. [235] showed for the first time how enzymes could interact with clays and insert into the interlayer space. Also, McLaren et al. [236] proved that enzymatic activity can be preserved via associations with humus.

The pioneering studies by Geoghegan and Brian [232,233] showed that fungi and bacteria provided glueing agents that inferred considerable silt and clay particles aggregation. Half of the aggregation effect induced by fungi was considered to arise from materials produced by the cells and the rest could be attributed to the binding by the fungal mycelium. Mycelial binding has later been attributed primarily to the binding of macroaggregates by the hyphae. The hyphal structure enables physical enmeshment of soil particles, and bacteria, *Bacillus* species especially, were considered to have relevance to the formation and stabilization of microaggregates [237–239].

7.4. Sorption of Saccharides and of Peptides by Clays

7.4.1. Interactions of Uncharged Polysaccharides with Clays

Clapp et al. [90] have provided details of the compositions, structures and macromolecular conformations relevant to the interactions between polysaccharides and the soil mineral colloids that lead to the formation and the stabilization of soil aggregates. The glycosidic linkages (α or β) uniting the component sugar units determine the conformations of the polysaccharide structures, and hence the nature and the extent of interactions with the mineral surfaces.

The β (1→4) glycosidic bonds that link the glucose molecules (in the ‘chair’ conformation) in the cellulose polymer give rise to linear helical conformations allowing the polymer strands to be held together by strong hydrogen bonding. Thus, cellulose is insoluble in water and resistant to biological breakdown because the hydrolysing enzymes have restricted access to the glycosidic linkages. In contrast, amylose bears a more open random coil configuration and the α (1→4) glycosidic bonds can be readily hydrolyzed.

The Na^+ -exchanged cellulose xanthate (in which the C-6 –OH group of the cellulose structure is replaced by $-\text{OCS}_2^-$) is water soluble and capable of diffusing inside soil structures. It was shown by Page to be an excellent stabilizer of aggregates [240]. Clays su-

perificially modified with xanthan gum biopolymer tested as substrates promoted ryegrass growth, raising the shear strength and cohesion and decreasing the internal friction [241]. Harrison [242] observed that when the xanthate is adsorbed by clays, CS_2 is given off and the cellulose remains sorbed onto the clay as a neutral polymer.

Clapp et al. [243] examined the adsorption of various polysaccharides by clays on the basis of on the clays properties and of the nature of the glycosidic linkages (α or β) joining the sugar residues in the polymers providing information relevant to the interactions involved in the formation and stabilization of soil aggregates.

Clapp et al. [244] and Olness and Clapp [245,246] examined the retention of extracellular rhizobial polysaccharides and commercial dextrans (B-512F and Polytran) by Na^+ -montmorillonite. Exopolysaccharides play critical roles in the formation of soil microaggregates, as well as in regulating soil moisture and nutrient bioavailability [247]. B-512F, a polyglucose derived from a *Leuconostoc mesenteroides* strain, contains 95% α -(1→6) and 5% α -(1→3) glycosidic linkages. In contrast, Polytran consists of 75% β -(1→3) and 25% β -(1→6) linkages. Both polymers exhibit M_r values about 2×10^6 . Both polymers exhibited high-affinity adsorption isotherms (H-type) according to the Giles et al. classification [248]. However, while the isotherm for Polytran initially levelled off—indicating surface saturation—it then exhibited an inflexion point, followed by a renewed increase in adsorption [246] indicating that Polytran was retained in higher quantities than B-512F. Parfitt and Greenland [249] also observed H-type isotherms for the adsorption of B-512F onto Ca^{2+} - and Al^{3+} -montmorillonite but the amount of polymer retained was half of that adsorbed onto Na^+ -montmorillonite. X-ray diffraction patterns acquired by Olness and Clapp [245] indicated that only a monolayer of each polymer had entered the interlamellar space. The rest of the Polytran retained was accommodated onto the clay surfaces. When a random coil polymer comes into contact with an adsorbing surface, it loses its three-dimensional spatial configuration as individual segments attempt to maximize interaction with the surface. The greater the intimacy of this contact, the higher the adsorption energy. As adsorption proceeds the adsorbent surface is decreased and parts of the adsorbate (polymer) will remain in solution, thus, reducing the adsorption energy. Microcalorimetry data supports this reasoning [250]; it is safe to assume that Polytran anchored to the external clay surfaces and then extended as loops into the bulk solution so that more polysaccharide would be adsorbed than in the case of the globular, randomly coiled B-512F.

Burchill et al. [250] estimated that a fully extended polysaccharide chain bearing only β -(1→6) bonds would span between 6.2 and 7.4 μm (the repeating unit measures approximately 0.5 to 0.6 nm). β -linkages promote an extended structure while α -glycosidic bonds favour a more compact random coil conformation decreasing the clay-sugar contacts. Consequently, an intermediate conformation (between the fully extended linear chain and the random coil) would be more realistic. As all $-\text{OH}$ and bulky substituents occupy equatorial positions, the pyranose groups could associate with the mineral (clay) phase.

7.4.2. Adsorption by Clays of Charged Polysaccharides

The polyanions' conformations in solution are affected by the degree of dissociation of their ionizable groups. For example, when ionizable groups are present into the cellulose backbone—as in cellulose xanthate—the energy of solvation associated with the conjugate bases is sufficient to disrupt internal hydrogen bonding, leading to dissolution.

Parfitt and Greenland [249] examined the adsorption of (poly)galacturonic acid (PGA), a linear α -(1→4)-linked polysaccharide with axial $-\text{OH}$ groups on C-4, by Na^+ -, Ca^{2+} -, and Al^{3+} -exchanged montmorillonite. At pH 6, adsorption was weak for Na^+ - and Ca^{2+} -clays and nonexistent for Al^{3+} -clay. However, at lower pH values (< 6), adsorption onto the various clays increased in the order: $\text{Na}^+ < \text{Ca}^{2+} < \text{Al}^{3+}$. X-ray diffraction indicated

that the adsorbate had not entered the interlayer space. Therefore, only a fraction of the polymer segments associated with the external clay surface; the rest of the polymer extended as loops into the bulk solution. At pH 6, PGA is fully dissociated, and electrostatic repulsion between carboxylate groups drives the polymer into extended conformations that minimize intramolecular charge repulsion. In contrast, at lower pH values, uronic acids are protonated, allowing PGA to behave more like a neutral molecule. This enables hydrogen bonding with the siloxane surface or, more plausibly, coordination with exchangeable cations [251,252].

Further insights were provided by Burchill et al. [250], who, using microcalorimetry, reported for PGA an adsorption energy of 37.5 kJ mol^{-1} per segment of anhydrogalacturonic acid adsorbed onto Al^{3+} -montmorillonite. This relatively high interaction energy supports a water-bridging coordination mechanism. However, the axial orientation of the C-4 hydroxyl groups in PGA limits its ability to establish maximum contact with the clay surface. Its comparatively rigid structure likely prevents intercalation into the interlayer regions, particularly in Ca^{2+} - and Al^{3+} -clays. Thus, adsorption is mainly restricted to external surfaces, involving coordination to charge-neutralizing cations, while the bulk of the polymer projects into solution.

Moavad et al. [253] provided further evidence for cation bridging in the adsorption of anionic microbial exopolysaccharides on clays exchanged with monovalent, divalent, and trivalent cations. Their surface coverage estimates—assuming flattened conformations—ranged from $60\text{--}70 \text{ m}^2 \text{ g}^{-1}$ for Na^{+} - and K^{+} -kaolinite, $100\text{--}140 \text{ m}^2 \text{ g}^{-1}$ for Ba^{2+} -, Ca^{2+} -, and Mn^{2+} -clays, to as high as $\sim 500 \text{ m}^2 \text{ g}^{-1}$ and $2000 \text{ m}^2 \text{ g}^{-1}$ for Al^{3+} - and Fe^{3+} -exchanged clays, respectively. The H^{+} -exchanged clays showed intermediate coverage of $\sim 170\text{--}180 \text{ m}^2 \text{ g}^{-1}$.

Finch et al. [169] studied the retention of a soil polysaccharide onto (Na^{+} -exchanged) kaolinite. The strongly retarded periodate oxidation of the adsorbed polysaccharide implies that the polysaccharide was tightly bound to the clay surface, with less protection for loops extending into solution. These findings align with the results from Moavad et al. [253] for Na^{+} - and K^{+} -kaolinites. The substantial increase in adsorption observed for Fe^{3+} -clay likely arises from cation bridging and possibly (hydr)oxide formation, although the extent of the latter remains unclear.

Finally, it has been shown that bacterial synthesis of anionic polysaccharides during sucrose/glucose fermentation—when applied to clay–sand mixtures—significantly reduced crack ratio, total crack length, and cumulative erosion ratio [254]. These findings highlight the multifunctional role of microbial polysaccharides in improving soil structure and mitigating physical degradation.

7.4.3. Adsorption of Polysaccharides by (Hydr)oxides

Polysaccharides are held via electrostatic attraction forces to (hydr)oxides [255] with acidic polysaccharides exhibiting particularly strong interactions at pH values below the p.z.c. of the minerals. Further investigations into the interactions between polysaccharides and Fe and Al-(hydr)oxides (which can play pivotal roles in soils) are expected.

7.4.4. Soil Polysaccharides

There is evidence in the literature to indicate an abundance of polysaccharides in SOM, but we await the isolation from soil of what might be considered a pure or compositionally homogeneous polysaccharide. That is not surprising in view of the vast variety of potential microbial and mucigel sources.

Swincer et al. [256], Greenland and Oades [257], Hayes and Swift [11], Cheshire [258], Cheshire and Hayes [259], Stevenson [187] and Clapp et al. [90] have referenced procedures used for the isolation and fractionation of such polysaccharides. Häusler and Hayes [260]

demonstrated that significant amounts of saccharides were retained by the HA fraction of a Sapric Histosol. The fraction dissolved in DMSO/HCl passed on to an XAD-8 resin column, which retained HAs while the saccharides were washed through.

In Figure 4, Section 5.2, it is seen that when a diluted soil extract in base was passed through XAD-8 and XAD-4 resins sequentially, the fraction obtained from the XAD-4 resin was highly enriched in carbohydrates. The soil polysaccharide materials studied by Finch et al. [169] and by Barker et al. [170] were extracted in 0.3 M sulphuric acid from the Sapric Histosol employed by Häusler and Hayes [260].

7.4.5. Adsorption of Soil Polysaccharides by Clays

Finch et al. [169] investigated the reaction between H^+ -exchanged montmorillonite (prepared from Na^+ -montmorillonite through ion-exchange) and a polysaccharide material isolated from a Sapric Histosol soil (as referred to above) in 0.3 M H_2SO_4 —details of the isolation and fractionation are given in Clapp et al. [90]. The polysaccharide material, before and after reaction with the clay was eluted from DEAE-A50 Sephadex. The pH was adjusted to 6 using a phosphate buffer and a 0 to 1 M NaCl gradient was applied. Figure 5 shows that H^+ -montmorillonite effectively retained the saccharide mixture. At around the same time Barker et al. [170] demonstrated that the adsorbed component contained one uronic acid per six to seven sugar units. Adsorption of soil polysaccharides on clay materials has also been studied by Cheshire et al. [261].

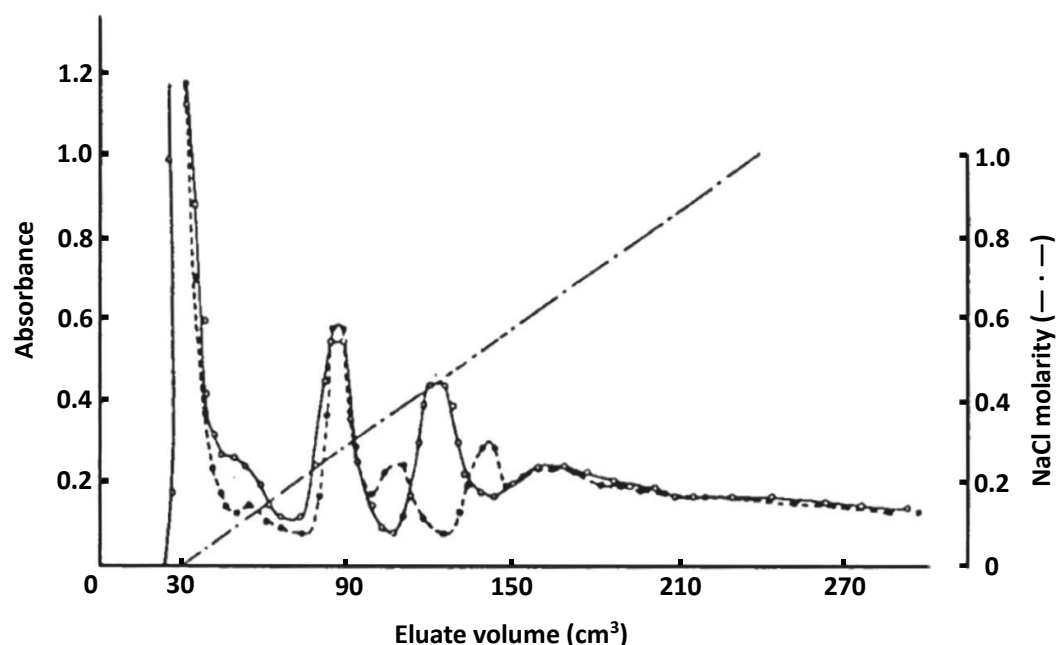


Figure 5. Anion-exchange chromatography of a soil polysaccharide mixture before (open circles) and after (filled circles) interaction with an H^+ - or H^+/Al^{3+} -montmorillonite [161].

7.5. Role of Humic Substances in Soil Aggregate Stabilization

Chaney and Swift [262] correlated the stabilities of aggregates in 26 agricultural soils with their sand, silt, clay, cation exchange capacity and organic matter contents. Very important correlations were found between aggregate stabilities and SOM contents. None of the other contents was related to the aggregate stability. In further studies with 120 soils, the total organic matter, total carbohydrate, and the humic material contents gave highly or very highly significant correlations with aggregate stability.

In an expansion of that study Chaney and Swift [263] crushed soil aggregates and stable aggregates were not formed when subjected to freezing and thawing cycles, or when

incubated in the absence of amendments. A transient stability, which declined over a period of 12 weeks, was formed when the crushed soils were incubated with a glucose amendment. The authors considered that this stability could be explained by the formation of extracellular polysaccharides which subsequently were decomposed. The addition of microbial polysaccharides gave rise to aggregate formation but these also degraded on incubation as the amendments were utilized by the soil microorganisms.

In their final publication in the series, Chaney and Swift [264] isolated a soil humic acid and incubated it as the solid material and as an adsorbate on mono-ionic (Na^+ or Ca^{2+})-exchanged crushed mineral soil. The product from physical addition after incubation with glucose gave aggregates exhibiting low stabilities. Increased stability was observed, which persisted over time, when the reformed aggregate-humic acid sorbed material was incubated. The stability of the re-formed aggregates was even greater when the samples were incubated with glucose.

This study proved that humic substances could stabilize soil aggregates, with the stabilization exhibiting long-term persistence. Also, they clearly outperformed the tested polysaccharides in terms of durability.

Silt particles (2–50 μm), particularly those at the upper end of this size range, fall within the lower boundary of the microaggregate size class. Thus, clay aggregates can fall in the silt classification. Figure 6 presents the CPMAS ^{13}C -NMR for humic acids obtained from a Mollisol silt and clay fractions to which maize (a C_4 plant) residues were added or were removed after harvesting during an 8-y period [265]. The results indicated that the clay HAs were more oxidized than the silt ones. Resonances in the 25 to 35 ppm range indicate aliphatic C, likely associated with methylene groups. The peak at ca. 56 ppm is most likely due to methoxyl/ethoxyl groups; amino groups may also resonate in this region. Signals appearing between 65 and 90 ppm suggest aliphatic C compounds linked by O (e.g., ether and ester linkages), and/or C associated with secondary alcohol-type structures, including saccharides. The small shoulder between 100 and 105 ppm mostly represents the anomeric C not excluding the contribution of the carboxyl spinning side band.

Resonances in the 110 to 140 ppm range indicate aromaticity. The contribution from char-derived materials is insinuated by the peak's relative symmetry. Mao and Schmidt-Rohr [266] developed a ^{13}C -MAS NMR method to distinguish between the fused aromatic rings in charcoal and those derived from lignin—common HSs constituents. This technique relies on long-range dipolar dephasing so that unprotonated carbons having two or more nearby protons (within two bonds) dephase significantly faster than aromatic carbons separated from the nearest proton by three or more bonds. Consequently, char exhibits slower dephasing than lignin-derived aromatic structures. This method may be useful in determining whether char-derived components contribute to the aromatic signatures observed in spectra of Mollisols and other similarly profiled soil HSs. However, as noted in Section 3.1, fused aromatic structures can also originate from lignin, complicating interpretation.

There is a distinct difference in chemical shift in the 145–150 ppm range for the spectra for HAs isolated from the silt and from the clay fractions. That resonance, characteristic of O-containing aromatic substituents (such as $-\text{OH}$ and/or $-\text{OCH}_3$), is not evident in the spectra of the clay humic acids rather indicating of a more pronounced lignin signal in the silt-sized separates fraction. This interpretation is also consistent with the sharp peak observed at 56 ppm in silt HAs spectra indicating methoxyl groups originating from lignins. In contrast, the corresponding resonance in the clay-associated HAs is more rounded, suggesting a greater contribution from amino or peptide functionalities. Resonances between 160 and 180 ppm are primarily attributed to carboxyl, ester, and amide carbon atoms, while those between 230 and 250 ppm likely correspond to carbonyl groups. The $\delta^{13}\text{C}$ measurements further indicated a reduced contribution from maize-derived carbon in the HAs isolated

from silt-sized separates. Thus, the silt-sized fraction consists of clay aggregates that had previously sequestered and protected the organic matter to maize cropping. This organic matter appeared less humified compared to that associated with the clay-sized material.

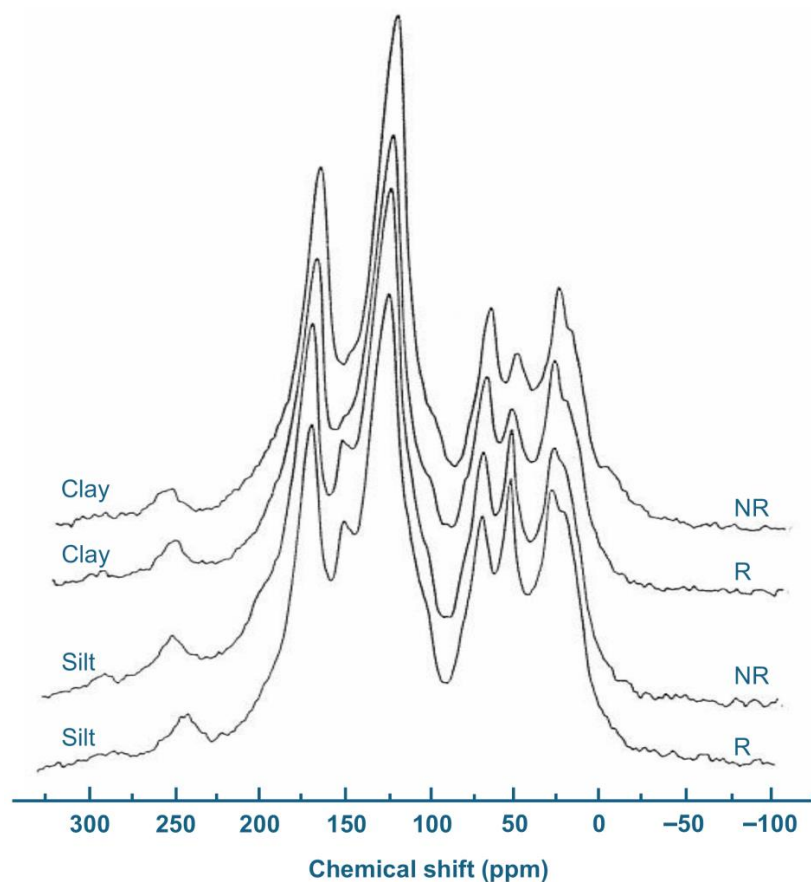


Figure 6. ^{13}C -CPMAS NMR spectra of HAs obtained from a Mollisol silt and clay fractions with (R) or without (NR) the annual addition of maize residues for 8 y [254].

This study proposes that both SOM and its humic fraction influence the aggregate wetting and slaking by altering porosity, shifting it from micropores (5–30 μm) and mesopores (30–75 μm) toward ultramicropores (0.1–5 μm). Moreover, hydrophobicity increased due to variations in humic composition—hydrophobic compounds were predominantly associated with complex HSs molecules. However, models of rapid wetting dynamics indicate that hydrophobicity may not be the dominant factor contributing to aggregate stability—particularly in soils with high organic carbon inputs [267].

In addition to their role in aggregate stabilization, HSs and clays—whether acting synergistically or independently—may be highly effective in the removal of chemical contaminants from both soils and water systems [268,269].

7.6. Possible Roles of Humic

As stated, humin was considered in the classical definitions to be within the humic classifications. Modern analytical procedures have identified the major components of HN to be, to a very considerable extent, made up of hydrophobic molecules, largely of plant origins, and with a considerable resistance to biological degradation. These include components such as cutin-cutan and suberin structures, long chain aliphatic hydrocarbon, long chain fatty acid esters and waxes. Details of structures are given in Hayes et al. [128].

Recently, procedures involving extractions in acidified DMSO (94%) and concentrated H_2SO_4 (6%) (see Section 4.3 and Figure 1), provide reliable methods for the isolation of

the HN component of SOM. For this (and for any) procedure to be effective, HSs should be removed from SOM. This, as has been pointed out, can be achieved by carrying out exhaustive extractions with urea-fortified base solutions.

Almost all the spectra for the HN material isolated in acidified DMSO from clays have shown it to be effectively identical to that isolated when the clays were removed by the HCl/HF treatment (for ease of description that HN has been called DMSO-insoluble DIHN). Figure 7c indicates that this DIHN isolated in the study in question is somewhat different. It has a significant enrichment in carbohydrate (resonances at 72 and 103 ppm), and the resonance between 55 and 60 ppm could suggest the presence also of peptide structures [270]. The clear evidence for carbohydrate in the HN materials isolated is contrary to what would be expected for HN materials dominated by hydrophobic functionalities. The organic materials in intimate associations with mineral colloids are predominantly HN structures because these hydrophobic structures have high affinities for the clays.

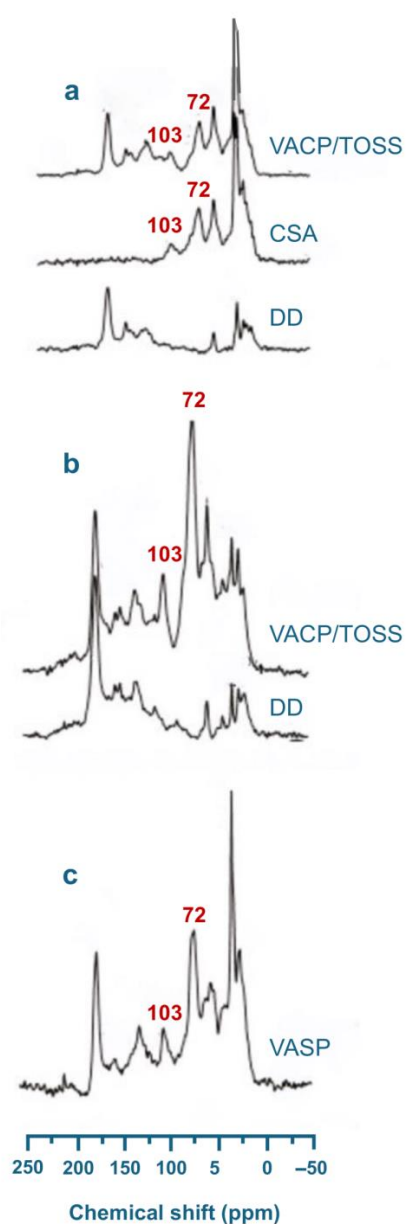


Figure 7. VACP/TOSS ^{13}C NMR spectra of DMSO humin from an Irish Brown Earth soil (a); DMSO FAs (b); and VACP/MAS ^{13}C NMR spectrum of DMSO insoluble humin (DIHN) (c) [270].

8. Overview and Suggestions for the Future

Most of world's highly fertile soils are used either for long-term cultivation or in mono-cropping systems. To a significant extent, mineral fertilizers remain the primary—often the sole—amendments applied to soils. Numerous researchers, including Lal [271,272], have emphasized the long-term consequences of relying exclusively on such inputs. While conservation practices like no-till can slow the loss of soil organic matter carbon to some degree, they cannot fully prevent it. The 28th Conference of the Parties (COP 28, 30 November to 12 December 2023), with emphasis on Climate Change, stressed how the burning of fossil fuels is increasing the concentrations in the atmosphere of the greenhouse gas CO₂ (now close to 420 ppm, as opposed to the 278 ppm level in 1850). The increased CO₂ emitted from the biological oxidation of indigenous SOM (which can be regarded as a store of fossil C) arising from non-conservational tillage practices, deserves as much, perhaps even more emphasis. It is possible that the food needs for the current world population could not be met if the levels of CO₂ in the atmosphere should be at pre-industrial revolution levels. There is sufficient data available to give good indications of the extents of such emissions, and these are quantitatively significant.

Plant responses to atmospheric carbon dioxide are of growing concern, as CO₂ levels are projected to continue rising in the future. Elevated CO₂ concentrations enhance photosynthesis, resulting in increased carbohydrate production and biomass accumulation in plants [273]. There is debate about the extents to which the climate changes that we are experiencing are attributable to increases in greenhouse gases (GHG), particularly CO₂, and with possible contributions to variations in the composition and intensity of incident solar radiation (considered by some to be minor at this time). We cannot control outputs from the sun, but we can control outputs of CO₂ from the burning of fossil fuels. It will be important to establish the levels of GHGs that are tolerable in order to avoid damaging increases in global temperature without decreasing photosynthesis capabilities.

The importance of soil organic matter for agriculture has been recognized from antiquity, and adequate maintenance practices have ensured that SOM levels have been maintained over the centuries [274] without diminishing crop productivity. In the preface to *Soil Colloids and Their Associations in Aggregates* [275], Professor Maurice Stacey wrote “*All that comes from the soil that is not used for food and fibre should be returned to the soil*”.

That is not being done, though it was in the past. Ash [276] cited the works of Columella (0–70 AD) who recognized the significance of organic wastes management (essentially composting) prior to their application as soil amendments.

A major challenge is to make aggregates resistant to degradative forces while allowing access to soil biota, plant roots, air, and water. The Piccolo Group has given an indication of at least a partial solution [277]. By applying a 10 kg ha⁻¹ of a water-soluble iron porphyrin, and with sunlight as a catalyst, they showed that SOM loss was arrested during three years of conventional tillage, and crop yields were maintained.

It was not until the late 19th century that the importance of the soil biota was recognized for the transformations of soil amendments. It became clear throughout the 20th century that microorganisms were the sources of the humus products that include humic substances, polysaccharides, peptides and other materials essential for the formation and maintenance of soil structure and fertility.

Isolation and fractionation processes for microbially transformed components of SOM and for microbial metabolism products have been reviewed and the view is taken that a search for detailed structural assignments of the component molecules is not feasible. Instead, it is suggested that the focus might be on ‘*families*’ of molecules or components with relatively similar functionalities, and perhaps shapes, that confer reactivities that are important for the functions responsible for a productive soil environment.

8.1. Sizes and Shapes of Humic Components

Awareness of the sizes and shapes of the major components of SOM are central to understand the mechanisms by which these operate in the soil environment. The classical interpretation of macromolecularity and the random coil solution conformations of humic molecules is challenged, and a concept has emerged of supramolecular associations that give rise to humic aggregates. For that concept to be validated it would be essential to deal with humic fractions that are freed from molecules to which they have associations in the soil environment. These associated non-humic components include soil polysaccharides and soil peptides (Section 2) which, in associations with HSS, have important roles, among other things in the formation and stabilization of soil aggregates. Polysaccharides can have an independent existence in the soil environment and can be held to the humic and HN components by hydrogen bonding, by weak physical forces, and by physical entrapment in humic and HN structures. They also can be part of the humic core through covalent linkages such as phenolic glycoside structures. It is known that soil polysaccharide components of 60×10^3 Da (and more) can be present among soil humus components [169]. These can be separated from the humic materials (the XAD-4 acids, as indicated in Figure 4).

8.2. 'Families' of Molecules

The concept of 'families' of molecules was outlined in Section 2. This refers to associations of molecules which exhibit broadly similar properties such as solubilities, functional groups, and reactivities. The 'family' term can include 'members' with a range of molecular sizes but with a distribution of functionalities that confer on each similar properties such as solubilities in specific media, sorption properties, etc. Fractionation procedures that allow the 'families' to be separated are outlined in Section 5. In that Section there is reference to electrophoretic separation in which the addition of 5 M urea to the carrying buffer gave rise to a significant increase in the number of peaks in the electrophoretogram of the humic molecular aggregates (Section 5.8). That is to be expected based on the additional humic material that was released when 6 M urea was included in the basic solvent prior to the isolation of HN (Section 4.2.1).

There is reference in Section 5.8 to the fact that 30–50 stable fractions could be obtained by using a variety of refined forms of electrophoresis, and the additions to the carrier buffer of various amendments, including urea. That would suggest that it should be possible to isolate family members that are closely 'related' in terms of the distribution of reactive functionalities within their structures. It should, in principle, be possible to isolate these closely related members. It will, however, be challenging to achieve such isolations and detailed characterizations will be difficult, though academically rewarding. The immediate objective should be to isolate and to characterize the 'family members' that play major roles in the formation and the stabilization of soil aggregates, that enhance plant growth and interact with the anthropogenic chemicals that enter the soil environment (by design or accident).

8.3. The Composition and the Role of Humin in the Soil Environment

There still is not a general consensus about the role of soil humin. It has been considered that HN is the major component of SOM, but it is clear that its relative abundance has been over emphasized somewhat. That is because the humic components entrained in the HN structures were part of the material contained in the classical classification of HN. The addition of urea to the base extractant can be relied to free the HN from humic contaminants (Section 4.1.3).

The NMR spectra of materials that can be regarded as 'true humin' clearly emphasize that its major components are aliphatic hydrophobic compounds, many of which are

sourced in cuticular plant components, and in long chain hydrocarbons and fatty acid esters [128]. The spectra of HN materials from a wide variety of different soil types have broadly similar compositions, and invariably there is evidence for saccharide and peptide contents. The hydrophobic HN structures have high affinities for the soil mineral colloids. Some saccharide and peptide structures also have strong affinities for such materials. Thus, a question arises with regard to the presence and roles of saccharide and peptide components in HN extracts. It is conceivable that these biological molecules are sorbed on the mineral colloid surfaces and covered by the hydrophobic HN components, or they could be entrained in the HN matrix.

8.4. Soil Aggregates: Formation and Stabilization

For the most part soil aggregate studies have been carried out on mature soils where soil aggregates had formed over prolonged periods of time and aggregate degradation had taken place as the result of soil mismanagement. There should be immediate focus on the need to prevent destruction of soil aggregate (crumb) structures; increases in agricultural output will be required to feed a world population predicted to reach 10 bn by 2050.

A case can be made for studying the formation and degradation of aggregates starting from a pristine, but relevant simulated soil system. Besides the soil constituents, it would be essential to have a comprehensive inoculum of the soil microorganisms. A mixed-forage cropping system might be used and the experiments would be long term, extending to several decades. This could probably help monitoring over a prolonged period the organic materials formed and their associations with each other and with the mineral components

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