Molecular Understanding of a Humic Acid by "Humeomic" Fractionation and Benefits from Preliminary HPSEC Separation

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Abstract A mild stepwise fractionation of molecular components of a humic acid (HA) is here aimed to address the need of a systematic and reproducible method of separation and characterisation of humic substances. This approach may be defined as "humeomics" in analogy with genomics and is aimed at (1) characterising natural humic molecules and (2) clarify their relations with ecosystem functions. Moreover, the same HA was further processed with high-performance size exclusion chromatography (HPSEC) in three size fractions. Both HA and its three size fractions underwent the "humeomic" chemical fractionation to extract non-covalently bound organosoluble compounds (ORG1), weakly ester-bound organosoluble (ORG2) and hydrosoluble constituents (AQU2), strongly ester-bound organosoluble components (ORG3), and final unextractable residues (RES4). Structural identification of initial and final material, separated organosoluble and hydrosoluble fractions, and subfractions was conducted by GC-MS (gas chromatography–mass spectrometry), HPSEC-ESI-MS (high-performance size exclusion chromatography electrospray ionization mass spectrometry) (high resolution, Orbitrap), and solid- and liquid-state NMR. GC-MS revealed in organosoluble unbound fractions the presence of both saturated and unsaturated, linear and branched, alkanoic, hydroxyalkanoic and alkandioic acids, n-alkanes, and n-alkanols. Quantisation of analytes showed that (I) the sum of compound classes in separated fractions was greater than that for the initial HA and (II) that analytical yields of identified compounds in size fractions were invariably larger than for the unfractionated HA, thereby showing that both size exclusion and stepwise chemical fractionation increased significantly the analytical identification of humic molecules. Our results suggest this "humeomic" approach as a valid path for mapping humic molecular composition and assess humus origin and formation.

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Introduction

Several experimental evidences have been discovered during last decades about the supramolecular nature of humic substance (HS) (Piccolo 2001). Size exclusion chromatography (SEC) of soluble humic acid's (HA's) shows a strong correlation between profile of chromatogram and analysis conditions. Adding acetic acid or other organic carboxylic acids produces a general increase of retention times (RT) and quenches the AUC's (areas under curve, Piccolo et al. 1999). The RT of an analyte in SEC is inversely related to molecular volume. It is therefore clear that HS, unlike polymers (Piccolo et al. 2001), are organised in such a supramolecular architecture to be broken down in smaller aggregates as a result of the acid, and, subsequently, their chromophores are separated from one another, thus quenching the molar specific absorbance.

According to the supramolecular model, HS are small molecules (PM $<$ 1,000 Da) self associating and stabilised by weak interactions such as van der Waals forces and hydrogen bonding. According to principle of minimum internal energy, the introduction of little amphiphylic substances like acetic acid results in a different organisation of the mentioned interactions, without interfering with covalent bondings. The substances of well-established macromolecular structure show a SEC profile that is not influenced by organic acids (Piccolo et al. 2001). NMR (Simpson 2002; Šmejkalová and Piccolo 2008a), and MS (Piccolo and Spiteller 2003; Stenson et al. 2003) techniques have strengthened such findings.

The supramolecular model implies that humic molecules can be separated with appropriate methods and characterised in a comprehensive way. Such a mapping, defined as *Humeome*, in relation with genetics, would contribute significantly to the research in both agriculture and environmental chemistry. In fact, HS play a role in carbon sink and delay significantly mineralisation of organic material derived from plant and microorganism decay (Spaccini et al. 2000), thus contributing to the continuity of carbon cycling in the environment. Moreover, they stimulate plant growth (Nardi et al. 2002) and are involved in natural mechanisms of pollutant neutralisation (Fava and Piccolo 2002; Halim et al. 2003; Šmejkalová and Piccolo 2008b).

The following contribution investigates the effectiveness of the "humeomic"-like fractionation of an HA and subsequently the benefit of coupling a preliminary preparative high-performance size exclusion chromatography (HPSEC) separation to this approach.

Materials and Methods

Humic Matter

A humic acid (HA) was isolated from a volcanic soil (Allic Fulvudand) at Vico (near Rome (Italy)) and purified according to International Humic Substance Society (IHSS) recommendations. This humic material (RES0) was oven-dried overnight at 40°C before being submitted to sequential chemical fractionation. All reagents used here were by Sigma-Aldrich 99.9% pure and used without further purification. All measurements were carried out in triplicate.

Sequential Chemical Fractionation

Unbound humic molecules (ORG1) were extracted by stirring for 24 h at room temperature in a 2:1 *v*/*v* dichloromethane (DCM) and methanol (MeOH) solution. The supernatant was separated by centrifugation and filtered. The residue was suspended in a Teflon tube overnight with 12% BF3-MeOH under a N_2 atmosphere at 90°C. The supernatants were centrifuged. The solution was extracted three times with chloroform/water mixture. The organic phase was separated (ORG2) and rotoevaporated. The aqueous phase (AQU2) was ultrafiltered against distilled water until it was chloride-free and freeze-dried. The residue was suspended with 1 M KOH MeOH solution and refluxed for 2 h at 70°C under a N_2 atmosphere. After cooling, the reaction mixture was centrifuged. The supernatants were then liquid extracted with a DCM/water mixture. A suspension in 47% HI aqueous solution of residue was stirred for 48 h at 75°C under a N_2 atmosphere. The solid humic residue (RES4) was dialyzed against water and freeze-dried.

Liquid Chromatography-Mass Spectrometry (LC/MS)

RES0, AQU2, and RES4 samples were dissolved using a 0.01 M NH3 solution and injected in a HPSEC system connected to the LC/MS system. HPSEC comprised a Phenomenex Bio-Sep SEC-S 2000 column (300 mm × 7.8 mm) and precolumn (30 mm × 7.8 mm), both thermostatted at 30°C. Mass spectra were obtained with a LTQ Orbitrap (Thermo Electron, Waltham, MA) and negative ESI, 100–1,000 *m*/*z* mass range, and 1.0 s scan time.

NMR Spectroscopy

Solid-state CPMAS-^{13}C NMR (cross-polarisation magic angle spinning-13C nuclear magnetic resonance) spectra were acquired with a Bruker AV 300 instrument equipped with a 4-mm-wide bore MAS probe. Samples were fitted in 4-mm Zirconia rotors with Kel-F caps and spun at 13,000 Hz. A recycle time of 1.0 s and an acquisition time of 20 ms were used, and 1,510 points were acquired for each spectrum. The scan number ranged between 500 and 700. Data were processed with Mestre-C software 4.9.9.9, and all FID spectra were transformed with 100-Hz line broadening exponential type filter function and 2-k zero filling.

Liquid-State NMR

Liquid-state NMR spectra were acquired with a Bruker AV 400 instrument equipped with a 5-mm inverse broadband, z-gradient coil, actively shielded probe. Monodimensional spectra were acquired with presaturation of water signal and 1,000 scans. Bidimensional spectra (COSY, TOCSY, HSQC, and HMBC) were acquired with the conditions described in detail in literature (Nebbioso and Piccolo 2011, 2012).

Results and Discussion

We conducted a mild stepwise fractionation of molecular components of a humic acid (HA) suprastructure and their structural identification by advanced analytical methods. This procedure may be the basis of a "humeomic" approach to characterise natural humic molecules and clarify their relations with ecosystem functions. Sequential fractionation includes: (1) organic solvent extraction, (2) transesterification with boron trifluoride in methanol (BF_3-CH_3OH), (3) methanolic alkaline hydrolysis (KOH-CH_3OH), and (4) cleavage of ether and glycosidic bonds with HI. Structural identification of initial and final material, separated organosoluble and hydrosoluble fractions, and subfractions was conducted by GC-MS (gas chromatography–mass spectrometry), HPSEC-ESI-MS (high-performance size exclusion chromatography electrospray ionization mass spectrometry) (high- resolution, Orbitrap), and solid- and liquid-state NMR. GC-MS revealed in organosoluble unbound fractions the presence of both saturated and unsaturated, linear and branched, alkanoic, hydroxyalkanoic and alkanoic acids, n-alkanes, and n-alkanols. These components decreased progressively in fractions obtained after weak and strong ester cleavage. Unsubstituted alkanoic acids with variable chain length were ubiquitously detected in all fractions, thereby suggesting their fundamental function in the architecture of humic suprastructures. An important role in differentiating supramolecular

associations should also be attributed to substituted alkanoic acids that were detected in variable amounts in different fractions. The content of aromatic acids and steroids was only noticed in the latter fractions. HPSEC-ESI-MS of initial and final solid fractions showed similar compounds, as indicated by GC–MS, whereas the hydrosoluble fraction after transesterification revealed fewer of these compounds but noticeable nitrogen-containing acids. A large amount of "cyclic" acids were identified by MS empirical formula in initial HA, and, to a lesser extent, in the final fractionation residue as well as in the hydrosoluble fraction. The predominant alkyl NMR signals in organosoluble extracts and those of CH−N, CH−O, and O−CH−O groups in hydrosoluble fraction confirmed mass spectrometry results. Homo- and hetero-correlated liquid-state NMR spectra indicated spin systems interactions varying with separated fractions. Solid-state and dipolar-dephasing NMR spectra of final residue showed predominance of sp2 carbons, 66% of which were quaternary carbons, and a significant increase in conformational rigidity with respect to initial HA. Separated fractions accounted for 60% of initial HA weight, and losses were attributed to hydration water, liberated volatile compounds, and decarboxylation. Quantisation of analytes showed that the sum of compound classes in separated fractions was greater than that for the initial HA, thereby showing that stepwise fractionation increased significantly the analytical identification of humic molecules. Our results suggest this "humeomic" approach as a valid path for mapping humic molecular composition and assess humus origin and formation.

We subsequently size-fractionated the same soil HA by preparative HPSEC in order to evaluate the potential beneficial impact of humeomics to isolate and identify humic molecular components in the separated size fractions. HA and its three size fractions were chemically fractionated to extract non-covalently bound organosoluble compounds (ORG1), weakly ester-bound organosoluble (ORG2) and hydrosoluble constituents (AQU2), strongly ester-bound organosoluble components (ORG3), and final unextractable residues (RES4). According to their solubility, the extracts were characterised by either GC-MS or on-line thermochemolysis/GC-MS techniques. The humeomic sequence showed that the analytical yields of identified compounds in either ORG or AQU extracts of size fractions were invariably larger than for the unfractionated HA. This was attributed to a weaker conformational stability of humic suprastructures obtained by HPSEC fractionation, thereby enabling an improved separation and identification of single humic molecules. In line with the supramolecular understanding of humic substances, we found that hydrophobic compounds were mainly distributed in the largest size-fraction, while hydrophilic components were eluted in the smallest size-fraction. Furthermore, compounds with linear chains or stackable aromatic rings associated in regular structures were more abundant in the former fraction, whereas irregularly shaped compounds that hindered association in larger size were mostly found in the latter fraction. Thus, the structural characteristics of single humic molecules determined their mutual association in humic suprastructures, as well as their conformational strength and shape. The lack of de novo synthesised macropolymers in the unfractionated soil humic matter was confirmed by the absence of RES4 fractions in the separated size fractions. Such

results indicate that humeomic capability to reveal the complex molecular composition of humic suprastructures was significantly improved by subjecting humic matter to a preliminary HPSEC fractionation.

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