

Short communication

Humeomics: A key to unravel the humusic pentagram

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ABSTRACT

Humeomics is a sequential chemical fractionation that applies organic solvent extraction, transesterification with boron trifluoride in methanol, methanolic alkaline hydrolysis, and cleavage of ether and glycosidic bonds with HI. The procedure revealed a series of unique humic fractions with specific molecular composition. The technique can provide molecular identification compound classes that are found inside the soil humus, thus revealing concomitant information on the original conformation of these substances in the humus matrix. Thus, humeomics can serve as an analytical tool to unfold the complexity of the soil humeome, peering into parts of organic matter that were previously unexplored.

Humic Substances are ubiquitous natural compounds arising from the chemical and biological degradation of plant and animal residues (Piccolo, 1996). They significantly influence soil chemistry, plant nutrition, heavy metal binding, and sorption of organic contaminants. The traditional and now obsolete thermodynamically unrealistic macropolymeric description of the physicochemical properties of Humics (Stevenson, 1994) has been substituted by the supramolecular understanding of humus characteristics. A general consensus, views humic substances as a random self-assembly of a large number of heterogeneous relatively low-mass molecules held together by hydrogen and hydrophobic bonds in solution (Nebbioso and Piccolo, 2013), and stabilized in soil by metal-bridges and hydrophobic adsorption on clay minerals surfaces (Conte et al., 2006, 2007; Nebbioso and Piccolo, 2011; Piccolo et al., 1996, 2002; Piccolo, 2001).

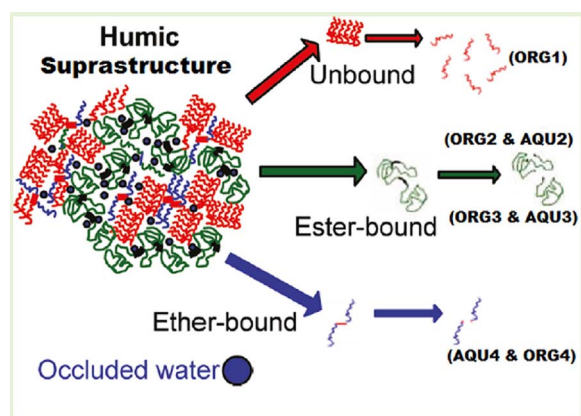
To identify the structure of humic molecules in the environment, fractionation methods have been developed (Barber et al., 2001; Brown et al., 1999; Canellas et al., 2010; Christl et al., 2000; Conte et al., 2006, 2007; Curtis et al., 1981; Li et al., 2009; Nebbioso and Piccolo, 2011, 2012; Otsuki and Hanya, 1966; Tombacz, 1999; Wershaw and Pinckney, 1973). One methodology achieves fractionation according to polarity (Barber et al., 2001; Li et al., 2009), and a second one according to molecular size (Brown et al., 1999; Canellas et al., 2010; Christl et al., 2000; Conte et al., 2006, 2007; Curtis et al., 1981; Nebbioso and Piccolo, 2011, 2012; Otsuki and Hanya, 1966; Tombacz, 1999; et al., 1973). Leenheer (2009) and Drosos et al. (2014) managed to combine these two methodologies to purify and fractionate humic matter from soil and lignite. However, all these techniques were not able to provide information at molecular level.

Since the novel concept of supramolecular structure implies that heterogeneous relatively small humic molecules (< 1000 Da) are held together by weak linkages, it is suggested that the humic molecular complexity could be reduced by breaking the inter- and intra-molecular interactions of progressively greater strength, but leaving untouched the covalent C–C bonds. This approach would allow single humic molecules to be isolated and then structurally identified by advanced analytical methods. This novel sequential chemical fractionation based on the supramolecular concept was called “Humeomics” and was firstly introduced by Nebbioso and Piccolo (2011).

The humeomic fractionation begins with an organic solvent extraction of free or unbound humic molecules associated with the suprastructuralhumic matrix only by weak dispersive interactions (Bull et al., 2000; Guignard et al., 2005; Naafs et al., 2004), and a fraction called ORG1 is separated. The next step includes the cleavage of covalent bonds in weakly-bound esters by a mild boron trifluoride-methanol (BF₃-MeOH) transesterification followed by a liquid/liquid extraction, thereby providing two fractions, one organosoluble called ORG2 and one hydrosoluble called AQU2. More-strongly bound esters are then extracted from the previous residue after an alkaline (KOH-MeOH) solvolysis followed by liquid/liquid extraction, and 2 more fractions are again obtained, called ORG3 and AQU3. Finally, a fraction called AQU4 is obtained by cleaving both strong ether (Grasset and Amblès, 1998) and glycosidic (Almendros et al., 1998) bonds from the residue by treatment with hydroiodic acid (HI), following the classic mechanism of the oxygen protonation in the organic ether and subsequent nucleophilic substitution (S_N) by iodide, being the resulting hydroxyl the good leaving group. The residue is then extracted by

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Scheme 1. Humeomics Fractionation.

diethyl ether, providing a fraction called ORG4 (Scheme 1), and a final residue called RES4 (Nebbioso and Piccolo, 2011). Quantitative and qualitative identification of humic molecules released during this stepwise fractionation were conducted by GC/MS for ORG fractions and LC/MS for AQU samples and RES.

The bulk unfractionated humic matter Humic Acid (HA) on which Humeomics was developed (Nebbioso and Piccolo, 2011), named RES0, was isolated from a volcanic soil (AllicFulvudand) at Vico (near Rome, Italy) and purified as described earlier (Nebbioso and Piccolo, 2009). According to humeomic findings, $55 \pm 0.5\%$ of the organic carbon of RES0 was obtained and identified after fractionation (Nebbioso and Piccolo, 2011). GC/MS analysis revealed the presence of fatty acids, di-, tri-, ω -, and α,β -hydroxyacids, and α,ω -diacids in ORG fractions. From these, fatty acids were the main constituent of the unbound fraction (ORG1), while the ester bound ORG2 and ORG3 mainly comprised di- and tri-hydroxyacids. Some linear hydrocarbons and steroids as a result of breakdown of plant root cells (Kolattukudy, 1980; Fuller and Nes, 1987) were also detected. Hydrocarbons were found mainly in ORG1 as unbound species, whereas, steroids appeared in ORG2 and ORG3, indicating that they were tightly bound to the humic matrix and liberated only after ester hydrolyses (Nebbioso and Piccolo, 2011). LC/MS analysis for RES0, AQU2 and RES4, revealed that the main group of compounds were cyclic acids containing either oxygen or nitrogen atoms (Nebbioso and Piccolo, 2011). The detected hydroxylated acids originated from either the decay of plant biopolyesters such as cutin (Kolattukudy, 1980) and suberin, or from bacterial metabolism (Almendros and Sanz, 1989). Polyhydroxylated compounds such as carbohydrates, aminosugars, or their derivatives are commonly associated with humic matter (Guggenberger et al., 1999). The decay of plant cellulose or fungal chitin may yield these hydrophilic substances, which may then become protected from further biodegradation in the humic hydrophobic domains (Piccolo et al., 2004). In the case of RES4, the found cyclic acids were either highly condensed aromatic structures or π - π stacked fully substituted compounds (Nebbioso et al., 2014a), which are components also observed in other humic materials (Knicker, 2007; Jiahai et al., 2010). A partial application of humeomics fractionation in a compost HA (Spaccini and Piccolo, 2007) showed a more abundant content of dioic acids and alkanols in ORG3 than for HA from the Vico soil. This difference, suggests that the humeomic fractionation enables a distinction among humic matters of different origin and formation.

Nebbioso and Piccolo (2012) moved a further step ahead with preliminary fractionation of the bulk HA from the Vico soil by preparative high performance size exclusion chromatography (HPSEC) and obtained 3 fractions (F1, F2, F3), where F1 had the largest nominal molecular size and F3 the lowest one. Both the bulk HA (F0) prior to the HPSEC fractionation and the three separated size-fractions were subjected to humeomics fractionation (Nebbioso and Piccolo, 2012). In this

study, the organosoluble humeomic fractions (ORG1-3) were detected by GC/MS, while the hydrosoluble ones (RES0, AQU2 and RES4) were detected by on-line thermochemolysis GC/MS. The humeomic sequence showed that the analytical yields of identified compounds in either ORG or AQU extracts of size-fractions (F1-F3) were invariably larger than for the unfractionated HA (F0). This was attributed to HPSEC fractionation that reduced the complexity of fractions, thereby enabling an improved release and identification of single humic molecules (Nebbioso and Piccolo, 2011). In particular, the major group of compounds found in the organosoluble fractions (ORG1-ORG3) were *n*-Alkanoic acids derivatized as methyl esters, whose abundance in the F1-F3 size-fractions was from 2-fold to 5-fold larger than in ORG1-ORG3 of F0. However, the case for the hydrosoluble fractions was different. The RES0 of unfractionated F0 revealed that the major group of compounds was *p*-Hydroxyphenols, while the major group of RES0 for F1, F2 and F3 was *N*-heterocyclic compounds, *n*-Alkanoic acids (as in the case of organosoluble fractions), and hydrocarbons, respectively. In the case of AQU2, for both F0 and F1, the main group of compounds was found to be aromatic, while for F2 and F3 the *N*-heterocyclic compounds were the most abundant components. Finally, in the residual materials (RES4) the aromatic compounds were large in the unfractionated material F0 and all F1-F3 fractions, whereas *N*-heterocyclic compounds were also abundant in RES4 of both F2 and F3. The distribution of aromatic structures over all the size-fractions (F1-F3) suggested their multiple role in stabilizing the humic supramolecular associations.

The Humeomics conducted on HPSEC fractions not only increased the analytical detection of molecular constituents in humic separates, but also revealed that the HPSEC fractions separation was influenced by the distribution of alkanolic acids (Nebbioso and Piccolo, 2012). Polyhydroxylated compounds were released in ORG2, but not in ORG3, and were mainly constituted by pentose/hexose derivatives and their oxidized equivalents. Due to their hydrophilicity, they hardly contributed to hydrophobic association of humic molecules, being found mainly in smaller humic aggregates ($F3 > F2 > F1$) and aqueous phase extracts (AQU2-3). Unlike ORG1, the polyhydroxylated substances of ORG2 showed a prominent yield increase in F3, thus suggesting a greater role in stabilizing humic aggregates with ester bonds and possible water molecules bridging (Schaumann and Bertmer, 2008).

Thermochemolysis of RES0, AQU2 and RES4 showed the presence of saccharide-like compounds, which must have resisted conversion to aromatic structures under the high temperature pyrolysis. Polyhydroxylated substances were also shown by thermochemolysis to be mostly abundant in F3, similarly to what found by GC/MS for ORG2 and ORG3 extracts. The content of polyhydroxylated compounds in RES0 of F1 was similar to that found in ORG1, thus confirming the entrapment of unbound hydrophilic compounds within hydrophobic organosoluble humic matrices (Nebbioso and Piccolo, 2012). This work on humic matter preliminary separated by preparative HPSEC indicated that humic substances are composed by heterogeneous molecules that randomly associate as a function of size, shape, chemical affinity and hydrophobicity, and the structural characterization of single molecules is limited by the strong intermolecular interactions that stabilize their supramolecular associations. Thus a reduction of the humic conformational stability by HPSEC separation in size-fractions prior to Humeomics improved significantly the structural information that humeomics may provide on the molecular composition of natural organic matter.

The final RES4 residual material obtained at the end of humeomics fractionation of Vico HA was successfully solubilized in alkaline conditions and further subjected to a preparative HPSEC, to separate 10 size-fractions (Nebbioso et al., 2014a). The ten separated size-fractions then underwent analytical HPSEC hyphenated with a high-resolution electrospray mass spectrometer (HR-ESI-MS) that allowed identification of empirical formulae of separated molecular masses. Most empirical formulae were easily associated with linear alkanolic, unsaturated, hydroxylated and hydroxy-unsaturated acids, as well as

cyclic acids, but some compound structures were unclear. Tandem MS fragmentation of some linear and cyclic compounds allowed identification of structures as hydroxyl-unsaturated hexanoic acids, furane rings and norbornane-like rings. The latter two structures were never reported before for terrestrial humic acids, but resembled the carboxyl-rich alicyclic molecules (CRAM), which had been previously proposed for dissolved organic matter (Hertkorn et al., 2006). Quantitative measurement of components indicated that long-chain saturated acids were present in large-sized fractions more than in short-chain homologues, whereas unsaturated, hydroxylated and most cyclic acids were more abundant in small-sized fractions. This suggests that long, saturated and unsubstituted linear acids allow formation of large superstructures, probably as a result of favorable intermolecular packing, compared with the irregularly shaped cyclic, unsaturated or hydroxylated compounds. The molecular elucidation of such highly unsaturated and oxidized chemical structures was unprecedented for terrestrial humic matter, and suggested that extensive application of tandem mass spectrometry may help to enhance the knowledge on the elusive quaternary-carbon components of humic matter (Nebbioso et al., 2014a).

These fractions were then characterized by different NMR techniques (Nebbioso et al., 2014b). Diffusion-ordered spectroscopy (DOSY)-NMR spectra showed that the homogeneity of RES4 was significantly changed by the HPSEC separation, which weakened the aggregation of humic molecules, thereby allowing less tight molecular associations in the hydrophobic domains of the recalcitrant end product of humeomics. Nominally large size fractions, rich in lipidic signals, had significantly lower and almost constant diffusivity, due to stable supramolecular associations promoted by hydrophobic interactions among alkyl chains, suggesting that lipid components do re-aggregate after HPSEC separation. Conversely, diffusivity was gradually increased with the content of aromatic and hydroxylaliphatic signals, which accompanied the reduction of fractions sizes and was related to smaller superstructures, indicating a molecular stabilization of assemblies through H-bonding and aromatic π -stacking forces (Spaccini and Piccolo, 2007). This study confirmed the occurrence of supramolecular structures in the recalcitrant humic residue of humeomics (RES4), and highlighted that more homogeneous size fractions were more easily characterized by NMR spectroscopy, after they were separated from the complex humic matrix.

Since humeomics can overcome the limitation of natural organic matter recalcitrance, introducing a progress in its molecular understanding, the next step for Piccolo's group was to unveil the molecular composition of soil humin, using humeomics (Nebbioso et al., 2015). The application of humeomics fractionation to the soil humin (soil from which humic and fulvic acid was previously extracted, HUM1) and its derivative humin after the removal of minerals by an HF/HCl treatment (HUM2), revealed that the nature, amount, and relative distribution of humic molecules among the humeomic fractions of humin (HUM2) were similar to those found for the HA extracted from the same soil and subjected to humeomics (Nebbioso and Piccolo, 2011; 2012). This suggests that the composition of the humic fractions is less important than the spatial organization and the mutual interaction of molecules in determining the operational differences among the humic materials. While the unaccounted mass was attributable to losses of occluded water, volatile compounds, and organic matter oxidation, the poor molecular characterization of HUM1 was mainly attributed to the physical and chemical protection by its large mineral content, which hindered the reactivity, the solubility and the detectability of the humic molecules (Nebbioso et al., 2015). When the humic molecules were partially liberated from minerals by the HF/HCl treatment, the unaccounted organic matter, attributable to clay physical and chemical protection, decreased significantly in HUM2, enhancing the analytical detection of all classes of humic molecules, with respect to HUM1 by nearly one order of magnitude. Water-soluble fraction AQU2 and solid materials RES3 and RES4 were subjected to on-line thermochemolysis

MS and organosoluble fractions ORG1-ORG3, were measured by GC-MS. The largest class of compounds for ORG1 and ORG2 were *n*-Alkanedioic acids, while for ORG3 the higher content was found for *n*-Alkanoic acids. AQU2 contained alkyl compounds with significant contributions by aromatic and carbohydrate-like compounds, while aromatic moieties were predominant in RES materials (Nebbioso et al., 2014a). The results of the soil humin agreed not only with those for humeomics applied to HA extracted from the same soil (Nebbioso and Piccolo, 2011, 2012), but also with those reported after a fractionation of soil HA and humin based only on solubilization (Nebbioso et al., 2014b). These similarities suggest that HA and humins are closely related in composition and seem to differ more by their supramolecular conformation rather than by their molecular composition (Nebbioso et al., 2015).

Since humeomics fractionation revealed the capacity to unravel the molecular composition and assembly of humics and humin, the next step that is currently developed is to use this powerful tool directly on soil, to research the composition of soil organic matter, to enhance the molecular significance of research in SOM dynamics and carbon stabilization processes in soil ecosystems. Moreover, within the intensification of sustainable agriculture expected in the next decades, the identification of soil Humeome (Drosos et al., 2017) appears to enable the respond to the essential challenge of building up a rigorous relationship between the organomineral assemblies and the structure of humic molecules in soil and their bioactivity towards plant growth.

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