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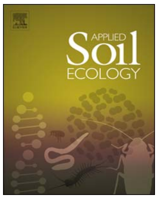

Short communication

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 (BF_3-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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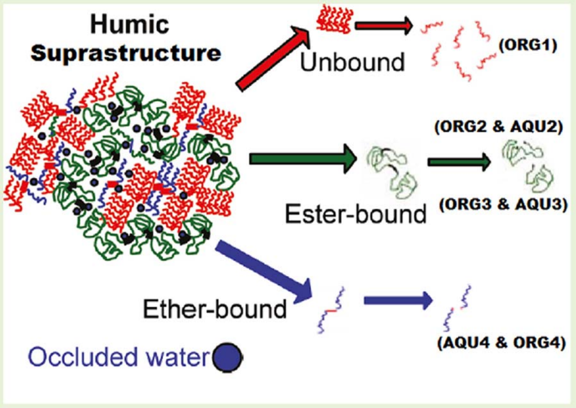

Scheme 1. Humeomics Fractionation.

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 ± 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 organosolublehumeomic 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 alkanoic 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 organosolublehumic 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 alkanoic, unsaturated, hydroxylated and hydroxy-unsaturated acids, as well as