Biol Fertil Soils (2015) 51:443–451
DOI 10.1007/s00374-014-0991-y

ORIGINAL PAPER

Unveiling the molecular composition of the unextractable soil organic fraction (humin) by humeomics

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Received: 19 September 2014 / Revised: 23 December 2014 / Accepted: 30 December 2014 / Published online: 20 January 2015

Abstract We applied humeomics to a soil unextractable humin fraction (HUM1) and its derived humin (HUM2) after removal of minerals by an HF/HCl treatment. Humeomics implies progressive separation and structural characterization of molecules released from complex humin matrices as (i) unbound, (ii) weakly ester-bound, (iii) strongly ester-bound, and (iv) ether-bound. Molecular characterization of fractions was achieved by GC-MS, thermochemolysis-GC-MS, and ^{13}C-CPMAS-NMR. Both weight and chromatographic yields were higher for the clay-depleted HUM2 than those for HUM1, and this increased molecular detection in HUM2. Saturated and unsaturated alkanoic, α,ω-alkanedioic, hydroxyalkanoic acids, alkanols, and hydrocarbons were found in both HUM1 and HUM2. Abundant odd-C numbered *n*-alkanoic acids in unbound fractions indicated accumulation of free microbial metabolites, whereas plant-derived acids remained in fractions more tightly bound to the humin matrix. Unsaturated, *n*-alkanedioic, and hydroxyalkanoic acids were detected after hydrolysis of complex esters. The aromatic character in humin residues progressively increased with humeomics sequential steps, while alkyl and hydroxy-alkyl compounds were reduced. Humins contained similar components as a humic acid extracted from the same soil, implying that traditional humic pools differed in supramolecular arrangement rather than in molecular composition. The humeomic approach enables the determination of the molecular composition of humic matter and may improve knowledge of the structure-activity relations of organic matter in soil.

Keywords Soil · Humin · Humeomics · GC-MS · Pyrolysis · NMR · Thermochemolysis

Introduction

Soil organic matter (SOM) is composed of biomolecules and their metabolites that derive from the microbial degradation of plant and animal biomasses and become stabilized within the soil solid matrix. SOM is commonly referred to as humic matter, conformationally described as the self-assembling of relatively small heterogeneous molecules held together in supramolecular associations by multiple weak interactions (Piccolo 2002). Although humic matter plays an essential role in several environmental and global processes, its complexity has so far prevented its exhaustive molecular characterization. This is also true for the unextractable fraction of SOM, traditionally named humin.

An advanced molecular understanding can be reached by extensively fractionating humic material prior to instrumental analysis (Amblès et al. 1996; Almendros et al 1998; Naafs and van Bergen 2002; Fiorentino et al. 2006; Piccolo et al. 2010). A combination of chromatographic and chemical separation was recently developed as a humeomic fractionation strategy. This consists in applying efficient instrumental analyses to humic molecules which are preliminarily separated in less compositionally complex fractions. Humeomics was originally described for a humic acid (HA) isolated from a forest soil, whose molecular composition, in either the bulk material or the chemically derivative fractions, was analyzed by mass spectrometric techniques and NMR spectroscopy (Nebbioso

Electronic supplementary material The online version of this article (doi:10.1007/s00374-014-0991-y) contains supplementary material, which is available to authorized users.

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and Piccolo 2011, 2012; Nebbioso et al. 2014a, 2014b). The application of this approach to the humin fraction is complicated by the presence of inorganic components which limit the solubility of humin molecular components. Hence, hydrolyses of ester bonds in complex humic matrices (Naafs and van Bergen 2002; Fiorentino et al. 2006), as envisaged by humeomics, are essential to release humin molecules from the clay-humic aggregates and allow their identification by instrumental analysis. A traditional approach to overcome poor solubilization of humin in aqueous solutions consists in the preliminary removal of mineral soil components. This can be achieved by soil treatment with hydrofluoric acid (Preston et al. 1989; Mathers et al. 2002; Piccolo et al. 2005; Rumpel et al. 2006) or by soil extraction with acidic dipolar aprotic solvents (Spaccini et al. 2006; Song et al. 2011). Studies on isolation, purification, and characterization of humin increased markedly in recent times. However, most findings were based on poorly resolved solid-state NMR spectra of purified humin extracts and suggested a molecular composition similar to that of humic and fulvic acid fractions, despite an apparent greater content of alkyl chains and carboxyl groups (Piccolo et al. 2005; Spaccini et al. 2006; Simpson et al. 2007; Song et al. 2008, 2011).

The aim of this work was to investigate the molecular composition of soil humins by applying the sequential humeomic fractionation either before or after clay removal by an HF treatment. The molecular composition of humin samples was then determined by ^{13}C NMR spectroscopy, while gas chromatography-mass spectrometry and thermochemolysis-mass spectrometry were used for the analyses of organosoluble and nonorganosoluble humeomic fractions, respectively.

Materials and methods

A soil under forest (*Fagus sylvatica*), classified as Allic Fulvudand, was sampled (0–20 cm) in the volcanic area of lake Vico, near Rome, Italy. The detailed procedure to obtain crude soil humin (HUM1) and a derived humin without silicates and oxides by an HF/HCl treatment (HUM2) is described in Supplementary Material.

Two grams of both HUM1 and HUM2 in triplicate samples was subjected to the humeomic procedure described earlier (Nebbioso and Piccolo 2011, 2012) and reported briefly in Supplementary Material. Elemental composition was determined in triplicate for bulk soil and both humins by Fisons Instruments EA 1108 elemental analyzer. Percent carbon was 14.0, 4.2, and 13.8 in bulk soil, HUM1, and HUM2, respectively. The HF treatment produced a loss of 2 % of OM in HUM2 with respect to HUM1. Ash content in triplicate was also assessed by gravimetry in a crucible after heating 4 h at 500 °C in muffle. Ash content accounted for 76, 93, and 77 % for bulk soil, HUM1, and HUM2, respectively. Calculation of total organic matter (TOM) was based on ash content. Standard deviations for elemental analyses and ash gravimetric content were well within 5 %. Organosoluble ORG and water-soluble AQU fractions separated by humeomics were weighed to reach gravimetric yields, whereas yields for OM in residual RES solids were based on percentage of carbon and ash content.

The water-soluble fraction (AQU2) and solid materials (RES3–4) were subjected to on-line thermochemolysis with a Pyrojector II (SGE) pyrolysis accessory connected to GC–MS. Approximately 1.0 mg of dry sample was loaded in a quartz capillary, wetted with 10 μL of tetramethylammonium hydroxide (TMAH, 25 % in methanol), and left to dry for 24 h. The sample was then placed in the injector, heated at 600 °C under He flow, and the pyrolysates analyzed by GC–MS.

Analyses by GC/MS were conducted with a Perkin Elmer Autosystem XL gas chromatograph, equipped with a Perkin Elmer Turbomass Gold mass spectrometer. Solid-state ^{13}C-CPMAS-NMR (Cross Polarization Magic Angle Spinning-Nuclear Magnetic Resonance) spectra were acquired in the CP mode with a Bruker AV 300 instrument equipped with a 4-mm Wide Bore MAS probe. Detailed instrumental settings for GC-MS analyses and CPMAS-NMR spectra are described in Supplementary Material.

Results and discussion

Removal of minerals by HF/HCl treatment produced a 3-fold increase of TOM passing from 70 mg g^{-1} (referred to total bulk weight) in HUM1 to 230 mg g^{-1} in HUM2. This rearranged TOM affected both weight yields and mass spectrometric responses of humeomics fractions and residues (Table 1). In ORG1 fraction, the content of unbound components from HUM2 was 2.5 times greater than from HUM1, while analytical detectability in HUM2 was twice as much as in HUM1. Conversely, while ORG2 weight yield was reduced passing from HUM1 to HUM2, the MS detection of humin constituents was three times greater in HUM2. Both weight yield and analytical detection for ORG3 increased by around 10 % from HUM1 to HUM2 (Table 1). These variations showed that unbound humic molecules were more soluble after the HF/HCl treatment, whereas humic components involved in ester linkages were still tightly bound to the mineral fraction in both HUM1 and HUM2. However, the following humeomic specific hydrolyses induced a more abundant release of humic components from HUM2 and a greater characterization of chemical structures than for HUM1. The weight yields of AQU2 fraction and both RES3 and RES4 residues increased markedly in HUM2, and even greater was

Table 1 Weight and total ion chromatogram (TIC) yields (mg g^{-1}) and relative percentage of analytical detection (AD) of humin fractions HUM1 and HUM2 after separation by humeomics in respect to total organic matter (TOM)

	HUM1			HUM2		
Fractions	Weight mg g^{-1} $_{TOM}$	TIC mg g^{-1} $_{TOM}$	AD[a]	Weight mg g^{-1} $_{TOM}$	TICmg g^{-1} $_{TOM}$	AD[a]
Extracts						
ORG1	64±2.2	20.8±0.6	32.5	162±7.9	115.7±2.3	71.4
ORG2	179±8.9	41.7±1.7	23.3	135±5.5	100.8±3.9	74.7
AQU2	138±5.9	33.0±1.3	23.9	170±6.1	123.0±5.7	72.4
ORG3	10±0.3	4.2±0.2	42.0	13±0.3	6.6±0.2	50.8
Residue						
Before HI (RES3)	180±8.1	24.2±1.1	13	225±10.6	85.7±3.5	38
After HI (RES4)	195±8.8	26.6±1.0	14	211±8.3	79.0±1.8	37
Total (after HI)[b]	576	126.3	21.9	691	425.1	61.5
Unaccounted[c]	424	872.7	NA[d]	309	574.9	NA[d]

[a] Relative analytical yields (%) are calculated as amount of compounds detected in TIC and divided by weight of the same fraction

[b] Calculated as sum of all separated fractions and residue after the HI treatment (RES4)

[c] Calculated as the complement to 1000 of total (after HI)

[d] Not applicable

the mass spectrometric response in HUM2 (Table 1), thereby revealing a more detailed molecular composition.

The nature, amount, and relative distribution of humic molecules among HUM2 fractions (Table 1) were similar to those found for HA extracted from the same soil and subjected to humeomics (Nebbioso and Piccolo 2011, 2012). This suggests that composition of humic fractions is less important than spatial organization and mutual interaction of molecules in determining operational differences among humic materials. While the unaccounted mass is attributable to losses of occluded water, volatile compounds, and OM oxidation, the poor molecular identification in HUM1 is mainly attributed to the physical and chemical protection by its large mineral content, which hinders reactivity, solubility, and detectability of humic molecules (Table 1). When humin molecules were partially liberated from minerals by the HCl/HF treatment, the unaccounted OM, attributable to clay physical and chemical protection, decreased significantly in HUM2 (Table 1).

Unbound fraction (ORG1) Extraction with organic solvents yielded an unbound fraction (ORG1) from both HUM1 and HUM2. *n*-Alkanoic acids with carbon numbers varying between 9 and 30 were abundant in both humin samples (Fig. 1a). In plants, these acids are positioned in cell membranes, contributing to their hydrophobicity, and in exocellular epicuticular waxes, while after plant cell lysis, their amphiphilic character ensures formation of hydrophobic domains in humic suprastructures (Nebbioso and Piccolo 2011, 2012). In ORG1, both soil humins showed a greater amount of unsaturated *n*-alkanoic acids (Table 2) than for that separated from an HA from the same soil (Nebbioso and Piccolo 2011, 2012). This discrepancy may be due to a different supramolecular organization in humin residues, whereby the nonlinearity of unsaturated acids reduced solubilization in alkaline solutions used to extract HA and FA from soil. Removal of minerals greatly increased the analytical yield of *n*-alkanoic acids in HUM2, with respect to crude HUM1 (Table 2), thereby suggesting that soil minerals considerably limited solubility of unbound compounds. Moreover, similarity in the content of unsaturated homologs in HUM1 and HUM2 (Table 2) indicates that despite the larger general extractability from HUM2, the extracted molecules did not vary greatly between the two humin samples. *n*-Alkanoic acids with odd numbered chains were present in both HUM1 and HUM2 (Fig. 1a) and were probably of bacterial origin (Kaneda 1991).

n-Alkanedioic acids were also detected in ORG1 of both HUM1 and HUM2, and the length of carbon chains was shorter than for *n*-alkanoic acids (Table 2), ranging between 4 and 9 units in HUM1, and up to 26 units in HUM2. *n*-Alkanedioic acids were more abundant in HUM2 than those in HUM1 (Table 2), due to an easier release in organic solvents or increased oxidation of unsaturated compounds (Grasset and Amblès 1998; Naafs and van Bergen 2002; Rochus and Schreiber 2007), following removal of fine minerals by the HF/HCl treatment. Concomitantly, a loss of hydroxyalkanoic acids (Fig. 1b, Table 2) by acidic dehydration may partially explain the increase in unsaturated acids and their subsequent oxidation leading to *n*-alkanedioic acids.

Hydroxyalkanoic acids were also important unbound components of HUM1 and HUM2. Their content and structural diversity were greater in HUM2 (Table 2), whereas HUM1

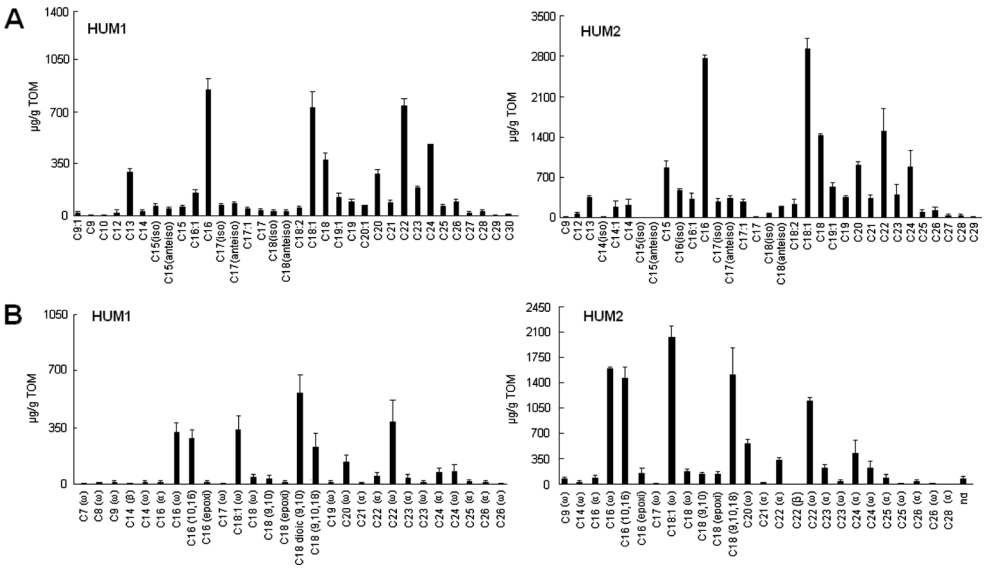

Fig. 1 Content in ORG1 (μg g^{-1} TOM, total organic matter of humin) from HUM1 and HUM2 of: **a** saturated and unsaturated alkanoic; **b** hydroxyalkanoicacids, as measured by GC-MS

was richer in some homologs, such as ω-hydroxyoctadecenoic, ω-hydroxyoctadecanoic, 9,10,18 tri-hydroxyoctadecanoic, and ω-hydroxydocosanoic acids (Fig. 1b). Moreover, the presence of short-chained odd-numbered hydroxy acids in both HUM1 and HUM2 (Fig. 1b) suggests that they may derive from hydrolysis of epoxy alkanoic acids, which were identified in ORG2 of both soil humins (Fig. 2).

Even-numbered *n*-alkanols were detected in both humin samples, with homologs ranging between 10 and 28 carbons (Table 2). Both HUM1 and HUM2 showed a similar

Table 2 Content (μg g^{-1} TOM) of compound classes and ranges of carbon-chain lengths (for linear molecules only), as measured by GC-MS in ORG1, ORG2, and ORG3 fractions separated from HUM1 and HUM2

Product	HUM 1			HUM 2		
	ORG1	ORG2	ORG3	ORG1	ORG2	ORG3
n-Alkanoic acids	5341 (C_9-C_{30})	1886 (C_{13}-C_{30})	939 (C_{13}-C_{30})	16216 (C_9-C_{29})	3475 (C_{13}-C_{29})	1928 (C_{10}-C_{32})
% unsaturation	24	7.5	4.2	27	6	11
n-Alkanedioic acids	7474 (C_4-C_9)	25132 (C_9-C_{16})	1531 (C_4-C_9)	79574 (C_4-C_{26})	62508 (C_9-C_{16})	1681 (C_4-C_{16})
% unsaturation	0	2	0	0	39	0
Di, tri-hydroxyacids	1756 (C_{16}-C_{18})	5210 (C_{16}-C_{18})	335 (C_4-C_{18})	3426 (C_{16}-C_{18})	16125 (C_{16}-C_{18})	598 (C_4-C_{18})
α, β- hydroxyacids	221 (C_{14}-C_{26})	370 (C_7-C_{28})	14 (C_{14}-C_{24})	1359 (C_{16}-C_{28})	978 (C_6-C_{28})	49 (C_6-C_{25})
ω- hydroxyacids	1424 (C_7-C_{26})	1246 (C_6-C_{26})	50 (C_9-C_{24})	5853 (C_9-C_{26})	3088 (C_9-C_{26})	176 (C_{14}-C_{26})
% unsaturation[a]	25	45	18	34	52	31
n-Alkanols	2768 (C_{10}-C_{28})	1580 (C_7-C_{28})	904 (C_5-C_{28})	5734 (C_{13}-C_{28})	3468 (C_7-C_{30})	1126 (C_5-C_{28})
n-Alkanes	134 (C_{16}-C_{31})	1359 (C_{16}-C_{31})	28 (C_{18}-C_{30})	49 (C_{30})	689 (C_{23}-C_{31})	162 (C_{23}-C_{31})
Aromatic compounds	818	4698	285	1948	10166	668
Sterols	896	221	107	1900	316	211
TOTAL	20830	41702	4199	115734	100816	6599

Standard deviations were well within 5 %

[a] With respect to ω-hydroxyacids

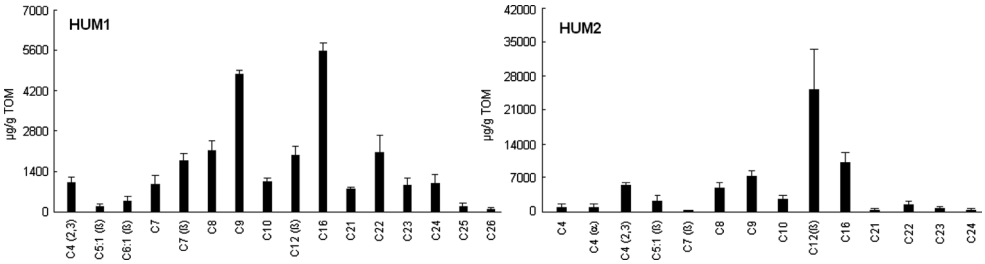

Fig. 2 Content (μg g^{-1} TOM, total organic matter of humin) of alkanedioic acids in ORG2 from HUM1 and HUM2, as measured by GC-MS

distribution of *n*-alkanol compounds, albeit in greater amount in HUM2 (Table 2).

Other compounds identified in ORG1 included hydrocarbons, aromatic acids, and sterols. Hydrocarbons such as alkanes and alkenes were found in both humin samples, although only squalene could be identified in mass spectra of HUM2 (Table 2). However, hydrocarbons in ORG1 decreased by one third from HUM1 to HUM2, while aromatic compounds were more abundant in HUM2 (Table 2). Aromatic molecules derive from degradation of plant macromolecules, such as suberin (Bernards 2002) and lignin (Nierop et al. 2003; Spaccini and Piccolo 2009), and benzoic and cinnamic rings with variable substitution are commonly found in humified matter (Nebbioso and Piccolo 2011, 2012; Spaccini and Piccolo 2009).

Weakly ester-bound fraction (ORG2) The content of *n*-alkanoic acids was greater in ORG1 than that in ORG2 and about three times as much in HUM2 as in HUM1 (Table 2). This indicates that not only *n*-alkanoic acids were further hydrolyzed from polyesters adsorbed on or occluded by soil minerals, but they were more easily solubilized in the HF-treated HUM2. The number of carbons in *n*-alkanoic acids ranged from 13 to 30 units, with main homologs being C_{16} and C_{18} (Table 2). Homologs with *iso* and *anteiso* configurations reported for ORG1 (Fig. 1a) were not found in ORG2 for either HUM1 or HUM2 (Fig. 2). This suggests that *n*-alkanoic acids released in ORG2 derived from complex plant polyesters, whereas unbound acids found in ORG1 were of bacterial origin (Kaneda 1991).

n-Alkanedioic acids are produced from either ω-oxidation of hydroxyalkanoic acids or breakdown of unsaturated alkanoic acids, or direct decay of plant material (Kolattukudy 1980; Bernards and Lewis 1998; Rochus and Schreiber 2007). Alkanedioic acids were most abundant in ORG2 from both humins (Table 2), and with a broader range of homologs than for ORG1 (Fig. 2). This indicates that molecules in soil humin were so extensively esterified to be only solvated after hydrolysis in ORG2. However, it is plausible that complex humin matrices hindered reactivity of plant polyesters and prevented completeness of hydrolysis (Kolattukudy 1980). Such an incomplete hydrolysis may explain the different solubility of alkanedioic acids in ORG1 and ORG2. In fact, after removal of minerals by HF/HCl treatment, saturated and unsaturated C_{16} alkanoic and C_{18} *n*-alkanedioic acids were more abundant in HUM2 than those in HUM1 (Fig. 2). This implies a relationship between humic/clay interactions and structural features of carbon chains, such as length and/or unsaturation. Moreover, the greater abundance of odd-numbered and unsaturated alkanedioic acids in hydrolysate from ORG2 than from ORG1 infers that these acids were preferably involved in complex biopolyesters, being recalcitrant to solubilization by organic solvents in ORG1.

Hydroxyacid structures in ORG2 were the same as in ORG1 for both humins, although their overall content in HUM2 was 2–3 times greater than that for HUM1 (Table 2). In particular, 10,16 dihydroxyhexadecanoic acid and 9,10 dihydroxyoctadecandioic acid were in greater amount in HUM2 (Fig. 2), the latter possibly being an intermediate product of ring opening of an epoxy group formed after a double bond oxidation (Regert et al. 1998).

In general, ω-hydroxyhexadecanoic, ω-hydroxyoctadecanoic and β-hydroxyoctadecanoic acids were the most abundant homologs in ORG2 of both soil humins (Fig. 2). Comparing with results of humeomics applied on the HA extracted from the same soil (Nebbioso and Piccolo 2011, 2012), it is noted that epoxy alkanoic acids were only present in humins (Fig. 2). These compounds have already been identified as intermediates in oxidation of unsaturated humic matter (Almendros and Sanz 1991), indicating that oxidation of unsaturated acids produced hydroxyalkanoic acids.

n-Alkanols were present in larger amounts in ORG2 than in ORG1 (Table 2). This may suggest that weakly bound esters hydrolyzed by the methanolic-BF_3 treatment were preferentially composed by hydroxyalkanedioic acids rather than by *n*-alkanols. The latter were more present in HUM2, although their carbon chain distribution similarly ranged from 7 to 28–30 units in both soil humins (Table 2).

Contrary to other humic molecules, alkanes in ORG2 were less abundant in HUM2 than those in HUM1 (Table 2). Conversely, alkenes were only found in HUM1, and their absence in HUM2 (data not shown) may be due to HF/HCl treatment, which favored oxidation of double bonds and their consequent transformation in alkanoic acids. Aromatic compounds, mostly *p*-, *m*-, and *o*-hydroxycinnamic, di- and trihydroxymandelic and benzenacetic acids (data not shown), generally attributed to lignins from grasses, increased markedly in HUM2, resulting about twice greater than in HUM1

from both ORG1 and ORG2 (Table 2). This further indicates that aromatic compounds are stabilized in complex polyesters in soil humins, such as those deriving from grass lignins, and can be released only after hydrolysis of ester bonds.

Strongly ester-bound fraction (ORG3) Compounds from strongly bound esters were released in ORG3 only after a methanolic alkaline hydrolysis. For both HUM1 and HUM2, *n*-alkanoic acids were found in ORG3 in smaller amount than in ORG1 and ORG2 (Fig. 3a). However, unsaturated homologs for carbon chains ranging between C_{14} and C_{22} were abundantly released in ORG3. The degree of unsaturation in *n*-alkanoic acids was slightly higher in HUM2 than that in HUM1 (Table 2), thereby implying that some dehydration of hydroxylated chains may have occurred during HF/HCl treatment of HUM1. This is consistent with results for ORG1 from HUM2 (Fig. 1a–b and Table 1) and previous findings for HA extracted from the same soil (Nebbioso and Piccolo 2011, 2012). While a considerable degree of unsaturation in alkanoic acids may occur due to dehydroxylation of hydroxyacids, our results highlight that a variety of unsaturated acids were esterified in recalcitrant humic polyesters and can only be separated by alkaline methanolic hydrolysis. Furthermore, it must be noted that ORG3 of both soil humins and HA from the same soil showed a similar composition (Nebbioso and Piccolo 2011, 2012). Such molecular similarity suggests a further doubt on the traditional distinction between these two humic pools (Piccolo et al. 2005; Spaccini et al. 2006; Simpson et al. 2007), which seem to simply differ in supramolecular conformations of similar humic molecules (Piccolo 2002).

n-Alkanedioic acids were the second most abundant compounds in ORG3 (Table 2), though their content was smaller than in ORG1 and ORG2 for both humins. Moreover, *n*-alkanedioic acids in ORG3 resulted of shorter chains than in ORG2 for both crude HUM1 and treated HUM2 (Table 2, Fig. 2), possibly because of oxidation of pristine double bonds (Regert et al. 1998). Hydroxyalkanoic acids were also found in ORG3 of both humins (Fig. 3b), although longer chain homologs were more abundant in HUM2 (Fig. 3b). The ω-hydroxyhexadecanoic acid was the most abundant in ORG3 for both humins, followed by ω-hydroxynonanoic acid for HUM1 and 9,10 dihydroxyoctadecandioic acid for HUM2 (Fig. 3b). In particular, 9,10 dyhydroxyoctadecanoic acid was observed in both ORG1 and ORG2 (Fig. 3b), as possible result of ω-oxidation of 9,10,18-trihydroxyoctadecanoic acid and further transformation in shorter C_9 chains after oxidative cleavage in the C_9-C_{10} position. The increase of ω-hydroxynonanoic acid in HUM1 supports the occurrence of this process. Conversely, a greater amount of 9,10,18-trihydroxyoctadecanoic acid was found in HUM2, where ω-hydroxynonanoic acid was concomitantly smaller (Fig. 3b). The fact that this oxidation occurred more in HUM1 than in HUM2 may be due to the HF/HCl treatment, which may have facilitated desorption of hydroxynonanoic chains from HUM2 into ORG1. Conversely, when this compound was protected by minerals, as in the untreated HUM1, stronger conditions should be required for its extraction. *n*-Alkanols were abundantly released in ORG3 from strongly bound esters, though not as much as alkanoic and *n*-alkanedioic acids (Table 2). The broad interval of *n*-alkanol saturated chains, ranging from C_5 to C_{28}, suggests plant tissues as their main source (van Bergen et al. 1997). Interestingly, short-chain polyols are peculiar of this humeomic fraction, whereas similar homologs were not found in ORG1 and ORG2. This suggests that hydroxide ions, effective in methanolic alkaline hydrolysis to produce ORG3, favor hydrolysis of sterically hindered complex esters. Conversely, short polyolic esters in reciprocal proximity may not be as easily released in ORG2 by the bulkier BF_3 hydrolyzing reagent. Thus, not only short-chain polyolic

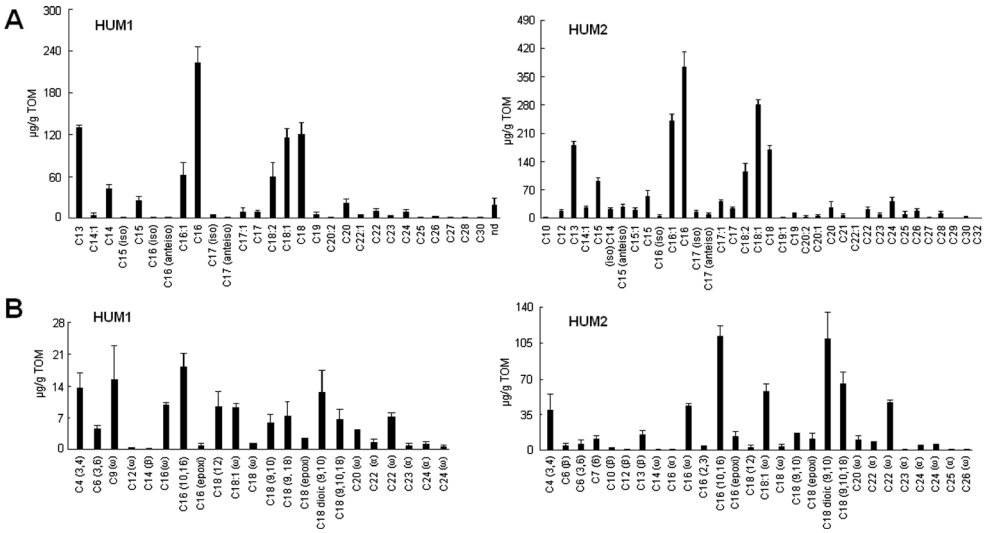

Fig. 3 Content in ORG3 (μg g^{-1} TOM, total organic matter of humin) from HUM1 and HUM2 of: **a** saturated and unsaturated alkanoic and **b** hydroxyalkanoicacids, as measured by GC-MS

esters can be distinguished from other esters by humeomics, but they may represent useful biomarkers to differentiate soil humic materials, as noted earlier (Nebbioso and Piccolo 2011). The content of *n*-alkanes in ORG3 was significantly greater in HUM2 than that in HUM1, though with different and longer chain lengths (Table 2). It is noteworthy that although *n*-alkanes cannot be part of esters, they were solubilized from HUM2 when esters in complex matrices were hydrolyzed (Kolattukudy et al. 1975; Fiorentino et al. 2006; Nebbioso and Piccolo 2011, 2012). This hydrolysis of covalent bonds simplified humic molecular associations with a consequent weakening also of the noncovalent interactions, which prevented solvation of saturated hydrocarbons in previous humeomic fractions. Thus, ester hydrolysis in clay-deprived humins enabled liberation of hydrocarbons from encapsulated humin superstructures (Gobé et al. 2000; Nebbioso and Piccolo 2012).

Thermochemolysis of hydrosoluble extracts (AQU2) and solid residues (RES3 and RES4) Hydrosoluble fractions as well as solid residues after alkaline and HI hydrolyses (RES3 and RES4, respectively) from HUM1 and HUM2 were analyzed by on-line pyrolysis-GC-MS after methylation with TMAH (Nierop 2001; Nierop et al. 2001; Santos Bento et al. 2001; Spaccini and Piccolo 2009). The total ion chromatograms (TIC) for thermochemolysis of AQU2 fractions and both RES3 and RES4 residues of HUM1 and HUM2 are shown in Supplementary Material Fig. SM1, while compounds identified in TIC are listed in Table SM1. Compounds belonged to the following classes: (i) alkyl compounds in methyl esters; (ii) aromatic compounds in alkylbenzenes, phenol or alkylphenol methyl ethers, benzoic or mono-/di-methoxybenzoic acid methyl esters and condensed rings, (iii) carbohydrate-dehydration products like furanes (Nierop 2001), and (iv) nitrogenated compounds as methylated aminoacids, heterocyclics, and aromatic amines (Table SM1).

While AQU2 extracts prevalently contained alkyl compounds with significant contributions by aromatic and carbohydrate-like compounds, aromatic moieties were predominant in RES materials (Fig. SM1, Table SM1). These compositions were found for HA extracted from the same soil, whereby humeomics highlighted a progressive aromatic enrichment in residues (Nebbioso and Piccolo 2011). Again, this similarity between HA and humins suggests that their operational separation may be determined more by spatial conformational arrangement than by specific structural features of their molecular components.

Methyl esters of alkyl compounds were present in both AQU and RES fractions (Table SM1). The distribution of alkanedioic acids considered a molecular fingerprinting for plant biopolyesters such as cutins and suberins (Guignard et al. 2000). The alkyl molecules in thermochemolysis TIC of both HUM1 and HUM2 revealed the importance of these biopolyesters in soil humin (Fig. SM1). Other plant constituents resistant to ester hydrolysis and hardly affected by microorganisms are alkanes and alkenes chains as in cutans and suberans and become stable constituents of soil organic matter (Nip et al. 1986; Tegelaar et al. 1989). Comparable distributions of alkanes and alkenes homologs were found in pyrograms of both humins (Fig. SM1A-B). This suggests that pyrolysis of these soil humins was hardly affected by soil mineral interference in the detection of HUM1 components, as noted in previous works (Buurman et al. 2007).

The abundance of aromatic compounds in pyrograms of RES3 and RES4 residues from both humins (Fig. SM1C-F) indicates that aromatic components were strongly associated to the more complex humin matrix rather than to the easily extractable/hydrolyzable alkyl materials. Aromatic structures, such as p-coumaryl, guaiacyl, and syringyl structures and relative pyrolysis by-products (Table SM1), originated partly from lignin degradattion and partly from thermogenic transformation of other compounds into polycondensed rings (Dittmar and Koch 2006). While methyl derivatives of hydroxybenzoic acid were present in RES3 and those of benzoic acid in RES4 for both soil humins, HUM2 was found richer in naphthalene and 4-methoxy-3-methylisobenzofuranone (Fig. SM1, Table SM1), probably due to greater detectability of these components after the HF/HCl treatment. The nitrogenated compounds (Table SM1) in pyrograms of AQU2 (Fig. SM1A-B), RES3, and RES4 (Fig. SM1C-F) might have derived from N-containing biopolymers, such as chitin, a constituent of fungi cell wall (Windig et al. 1982), which may be preserved due to incorporation in stable humic matrices (Zang et al. 2000; Spaccini et al. 2002).

A modest amount of pyrolytic by-products typical of carbohydrates was found in both AQU and RES fractions of both soil humins (Fig. SM1). Conversely, carbohydrate-like components were abundant in AQU2 separated from HA extracted from the same soil (Nebbioso and Piccolo 2011), thus suggesting that carbohydrates, which become protected from degradation in humic superstructures, are largely solvated in extractable humic fractions of SOM.

Solid-state NMR spectra ^{13}C CPMAS-NMR spectra of both soil humins and their RES3 and RES4 residues after KOH and HI hydrolyses, respectively, are shown in Figs. SM2 and SM3. Major resonances were those of alkyl, *O*-alkyl, aromatic, and carboxyl carbons at around 35, 75, 130, and 180 ppm, respectively. In contrast to pyrograms of humins, resolution of CPMAS spectra did not show detailed molecular differences, but enabled to evaluate how humeomics modified carbon distribution in humins. The alkyl signal (30–35 ppm) was strongly reduced from bulk humins to residues deprived of ester- and ether-bound molecules. The great contribution of aromatic structures in RES spectra agrees with the aromatic character in RES3 and RES4 pyrograms (Fig. SM1 C-F). Moreover,

CPMAS spectra of RES3 for both humins (Fig. SM2B and SM3B) showed methoxyl carbon signals at 55 and 57 ppm, probably due to previous BF_3-MeOH transesterification, which was no longer visible after the HI treatment (Fig. SM2C and SM3C). The broad resonance centered at 75–77 ppm, attributable to C-N and C-O carbons in carbohydrate-like, amino-sugars, aminoacids and other nitrogenated compounds, was progressively reduced from bulk humins to RES3 and RES4 residues (Fig. SM2B and SM3B). This is in line with pyrograms, where carbohydrate-like molecules were absent in the final RES residue of humeomics (Fig. SM1), and with previous studies conducted on HA and humin, which produced residues comparable to RES3 and RES4 after treatment with a similar fractionation sequence (Almendros et al 1998).

Conclusions

The array of molecules released by humeomics from soil humins is an evidence of their complex composition and intermolecular arrangement. Unbound, ester-, and ether-bound molecules in complex humin matrices may only be released and characterized as single compounds through their progressive separation from humins. Removal of minerals from soil humin by the HF/HCl treatment enhanced the analytical detection of all classes of humic molecules, with respect to the untreated crude humin, by nearly one order of magnitude. Our results for soil humins agree not only with those for humeomics applied to a HA extracted from the same soil (Nebbioso and Piccolo 2011, 2012), but also with those reported after a fractionation of soil HA and humin only based on solubilization (Zang et al. 2000). These similarities suggest that HA and humins are closely related in composition and seem to differ more by supramolecular conformation than by molecular composition.

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