

Biochemical origin and refractory properties of humic acid extracted from maize plants: the contribution of lignin

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Abstract The soil organic carbon (SOC) pool is the largest terrestrial reservoir of carbon and plant residues play an important role in its maintenance. Up to 70–80% of SOC in arable soil is composed of humic substances (HS). In these soils post-harvested residues, left in arable soil after harvesting the crops, are the basic source of humus. Previous research indicated that maize plants residue contain a humic acid (HA) fraction possessing recalcitrant compounds that contributed to soil-HA fraction. This study presents updated results obtained using Pyrolysis-Gas Chromatography/Mass Spectrometry (Py-GC/MS) to provide an indication of the contribution of the lignin to the soil HA. Results obtained indicated that the HAs from maize plants were mainly composed of lignin-derived moieties that were likely derived from the partial hydrolysis of *p*-coumaric and ferulic acid that are linked to lignin, polysaccharides or other biopolymers of the cell wall. Lignin composing the HAs derived

from plants and incubated in soil was substantially preserved. Nevertheless the modification of the syringyl/guaiacyl ratio and the oxidation of the side-chains of lignin, suggested a turnover of lignin-derived molecules in soil-HA fraction. This fact indicated an involvement of the alkali insoluble fraction of maize plant residue (humin) in the soil-HA formation, up-dating our previous knowledge.

Keywords Cell wall · Maize plant · Humic acid · Py-GC/MS

Introduction

With increasing emissions of CO₂ into the atmosphere and the possible consequences for global climate change, there has been a growing interest in studying the contribution of sequestering carbon (C) in terrestrial ecosystems, i.e. the soil (Wang et al. 2004). The soil organic carbon (SOC) pool is the largest terrestrial reservoir of carbon and plant residues play an important role in its maintenance (Lal 2001). The amount of C stored in plant residue on heart surface is about 55 PgC and contribute to more than half of the CO₂ emission from terrestrial decomposition (Wang et al. 2004). In addition SOC is perhaps the most important determinant of soil quality (Rasmussen 1998). Therefore the comprehension

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of the mechanisms of the stabilization of plant residue-C in soil is important to terrestrial and global C accounting, as returning higher amounts of crop residues to soil has increasingly positive effect on soil C (Rasmussen et al. 1998).

Up to 70–80% of SOC in arable soil is composed of humic substances (HS). In these soils post-harvested residues, left in arable soil after harvesting the crops, are the basic source of humus (Gonet and Debska 1998).

Humic substances are composed of chemically complex, non-biochemical organic components, which are largely hydrophilic, amorphous, dark colored, and resistant to chemical and biological degradation (Schitzer 1991). While previously thought of as being high molecular weight polymers, today HS are thought to be compounds of relatively low molecular weight that form (supramolecular) associations (Piccolo 2001; Sutton and Sposito 2005).

Humification, the process of transforming plant residues into humic substances, was extensively studied in soils and forest litter (e.g. Zech et al. 1997). The main routes for humic substances formation include the lignin theory, the sugar-amine theory and the lignin-protein theory (Stevenson 1994).

These theories hypothesize that biomacromolecules first break up into small constituents, and then subsequently recombine to give more complex organic matter (Hedges and Oades 1997). On the other hand the many studies conducted in both soil and marine ecosystems (Kögel-Knabner et al. 1992; Hedges and Keil 1995) indicate that it is the preservation and modification of plant tissue that provides the humification pathway. On reviewing the origin of soil organic matter, Kögel-Knabner (2002) indicated humification to be the prolonged stabilization of organic substances, countering biodegradation, and to involve plant biopolymers such as lignin, cutin and suberin.

Preservation of biomacromolecules is due to biochemical recalcitrance caused by the inherent property of the molecular structure (Kögel-Knabner 2002). The chemical composition of plant material is, also, one of the basic factors determining the process of humic substances development in soils (Gonet and Debska 1998).

The stabilization/humification of carbon in soil derived from maize plants has received much attention in the past because the larger amount of ^{13}C accumulated in maize (a C_4 plant) than in a C_3 crop (Ludwig et al. 2003).

Adani and Ricca, (2004) studying the humification of maize plant residues in the mineral soil, concluded that the humic acid (i.e. HA), i.e. the alkali soluble/acid insoluble fraction of HS was derived from the preservation of the alkali-soluble/acid-insoluble fraction of maize residues. In force of these results, new research was initiated into the involvement of the alkali-soluble fraction of maize plants in forming the humus-stable fraction of soils. The initial results of this research were recently published (Adani et al. 2006) and indicate that the alkali-soluble/acid insoluble part of the cell wall consist of small domains formed by cross-linked polymers, mainly represented by lignin and cutin, representing the HA-like (HA-like terms is sometimes used to indicate the organic fraction obtained by using the same analytical procedure used to extract HA from soil) fraction of plants. This HA-like fraction was preserved in soil for a long time (8 months), demonstrating recalcitrant properties and contributing to the stable HA soil fraction.

These results were mainly based on experimental observations from using ^{13}C CPMAS NMR. Compared to other approaches, this spectroscopic technique has many advantages: ^{13}C CP-MAS NMR spectroscopy is non-destructive and so bulk samples can be analyzed. Moreover, it is potentially semi-quantitative as it can provide complete and unbiased average chemical structures because each type of ^{13}C nucleus gives rise to the same signal strength (Conte et al. 2002).

In spite of these advantages, ^{13}C CP-MAS NMR only provides average structures of organic compounds that occur in complex mixtures such as soil organic matter and HAs (Fabbri et al. 1998). On the other hand, the Pyrolysis-Gas Chromatography/Mass Spectrometry (Py-GC/MS), provides an indication of the individual moieties present in organic matter, and therefore complements ^{13}C CP-MAS NMR (Fabbri et al. 1998; González-Vila et al. 2001). In particular as Py-GC/MS is a very powerful tool to study the

lignin composition (González et al. 2003), it is well suited to investigate the lignin transformations during the incubation of plant-residues amended soils.

Lignin is one of the most abundant classes of biological polymers (Wang et al. 2004). Lignin is composed of three different monolignols: coniferyl alcohol, sinapyl alcohol and paracoumaryl alcohol. Depending on the taxonomical affinity of the plant, three main types of lignin can be distinguished (Van Bergen et al. 2000). Gymnosperm lignin consists mainly of coniferyl alcohol moieties, while lignin of angiosperms contains considerable amounts of sinapyl alcohol units in addition to coniferyl alcohol. Within the angiosperms, the lignin of monocotyledons (e.g. maize) also contain paracoumaryl alcohol.

Lignin contain no hydrolytic bonds but only aliphatic-, alcyaryl- and biaryl-bonds (Lützow et al. 2006). Because of its molecular composition and complexity, lignin is recalcitrant to enzymatic attack and acts also as protective for the more labile fraction (Webster et al. 2005). Therefore lignin is a major determinant of the decomposition and humification of plant material (Dignac et al. 2005; González et al. 2003; Page et al. 2002).

The aim of the study presented in this paper is to use the Py-GC/MS technique to update the previous findings concerning the biochemical origin and refractory properties of humic acid extracted from the maize plant (Adani et al. 2006) posing particular attention to the contribution of lignin as precursor of humus in soil.

Materials and methods

Maize plant cropping

Maize plants (*Zea mays*), (cv. Eleonora, Pioneer) were grown in an open field on a Calcaric Fluvisol soil (FAO classification) (pH 7.4, sand 610 g kg⁻¹, silt 240 g kg⁻¹, clay 150 g kg⁻¹, carbon 16.7 g kg⁻¹, nitrogen 2.6 g kg⁻¹, CaCO₃ 30.7 g kg⁻¹, CEC 14.67 cmol⁺ kg⁻¹). Maize was chosen because it is the most widely cultivated crop in North Italy and plant residues represent an important input of organic material in agricultural soils (15 Mg ha⁻¹

as dry matter), influencing humus balance and so the soil fertility.

Plants were collected without the root system at the stages of post emergence (4–5 leaves), waxy maturity (dry matter, d.m., of 350 g kg⁻¹), and at senescence (plant residue at a d.m. of 900 g kg⁻¹).

The plant samples were dried at 45°C and 65°C for 2 days and 3 days respectively, ground to 0.5 mm, and stored for subsequent incubation tests and analyses.

Cell wall extraction

Cell wall was obtained by sequential extraction of no-cell wall materials using an aqueous solution of sodium laurylsulphate (1.5% w/v), a mixture of phenol-acetic acid-water (2:1:1, w/v/v), and an aqueous solution of dimethyl sulphoxide (90% v/v) such as reported by Selvendran et al. (Selvendran and O'Neil 1987).

Humic acids and humin extraction

The HA was directly obtained from the plant and soil samples by alkaline extraction at room temperature by using 0.1 mol L⁻¹ NaOH plus 0.1 mol L⁻¹ Na₄P₂O₇ and successive precipitation of the suspension to pH < 1.5 (Adani and Ricca 2004). The fraction insoluble in both alkali and acid represented the humin (HU). All HAs extracted and HUs were quantified by organic carbon determination (Adani and Ricca 2004).

Incubation test

The artificial soil used for the incubation tests was a sandy mineral substrate composed of sand (particle size \varnothing = 0.5–0.8 mm, pH = 7) 910 g kg d.m.⁻¹, and clay 90 g kg d.m.⁻¹ (bentonite-montmorillonite-like mineral- sieved at \varnothing < 1 mm; pH = 7; CEC = 65 cmol⁺ kg⁻¹). Plant residue (plant at senescence stage) was added at a rate of 35 g kg⁻¹ d.m.. An artificial medium was chosen in order to avoid any contamination from soil organic matter. In addition to artificial medium, soil on which plants were cropped was both incubated as it was, and by adding plant residue at the same rate as mineral soil (35 g kg⁻¹ d.m.).

For all theses, incubation tests were carried out in 3 replicates of 5000 g of soil. The soils were inoculated with water soil extract and maintained at 60% (w/w) of the maximum water holding capacity. Water content was gravimetrically corrected every 3 days. Furthermore, the pots were incubated in a chamber, in the dark, at $20 \pm 2^\circ\text{C}$ for 240 days (useful season for microbial activity at 45° parallel). During the incubation tests the soils were sampled at times 0, 60, 120, 180, and 240 days. Each sample, formed by sub-samples taken from each replicate, weighed about 300 grams. Following the taking of the sample, the soils were dried at 65°C under vacuum and then used for analytical determination. Lastly, the analyses were performed in triplicate.

Pyrolysis-gas chromatography/mass spectrometry

A Horizon Instruments Curie-Point pyrolyser was used to carry out the pyrolysis. Samples were heated for 5 s at 600°C . The pyrolysis unit was connected to a ThermoQuest Trace GC 2000 gas chromatograph and the products were separated in a fused silica column (J & W, 30 m, 0.32 mm i.d.) coated with DB-1 (film thickness 0.50 μm). Helium was used as the carrier gas. The oven was initially kept at 40°C for 1 min, then heated at a rate of $7^\circ\text{C}/\text{min}$ to 320°C and maintained at this temperature for 15 min. The column was coupled to a Finnigan Trace MS mass spectrometer (mass range m/z 45–600, ionization energy 70 eV, cycle time 1 s) (Nierop and Verstraten 2003).

Compounds were identified from their mass spectra using a NIST library or by interpreting the spectra, from their GC retention times, and/or by comparing them with data in the literature.

The syringyl-to-guaiacyl ratio of lignin is the sum of all peak areas of syringols divided by all summed peak areas of guaiacols, with the exception of 4-vinylguaiacol as this compound is partly derived from ferulic acid, which only partially contributes to the lignin macromolecules.

Samples analyzed were represented by plant, cell wall, the derived HAs, and HA extracted from soils at the end of the incubation period (240 days).

Results and discussion

The pyrolysate of the maize plant (Fig. 1) was dominated by lignin-derived products. Other compounds, included those of the polysaccharides (e.g. 2-furaldehyde, 5-methyl-2-furaldehyde, 2,3-dihydro-5-methylfuran-2-one, 2-hydroxy-3-methyl-2-cyclopenten-1-one, levoglucosan) and few alkanolic acids (C_{14} , $\text{C}_{16:1}$, C_{16} , C_{18} , $\text{C}_{18:1}$), although their intensity is very low compared with that of the lignin-derived compounds.

The dominance of the lignin-derived product is in sharp contrast with NMR data obtained in previous work (Adani et al. 2006), which showed a large contribution of O/N-alkyl carbon, indicative of a large amount of polysaccharides. However, it is a common for plant material to show such an apparent discrepancy between pyrolysis and NMR data, i.e. a relatively large abundance of aromatic compounds compared to polysaccharides (O-alkyl C) and aliphatic compounds (alkyl C) (e.g. Poirier et al. 2000; Nierop et al. 2001). Solid state CP/MAS ^{13}C NMR strongly underestimates lignin versus cellulose due to the aromatic and highly cross-linked nature of the former. In contrast, cellulose is markedly underestimated by conventional pyrolysis (Gauthier et al. 2003). In the previous our work (Adani et al. 2006) the wet analysis determined that both lignin and cellulose + hemicellulose plant contents were well correlated with NMR data (Adani et al. 2006), and from this and the fact that the lignin content in maize plants was only 40 g kg^{-1} at full maturity (Adani et al. 2006), it can be concluded that Py-GC/MS is indeed effectively less sensitive to compounds that contribute to O/N-alkyl carbon fractions in NMR spectra of HA.

Therefore, the Py-GC/MS gives a great opportunity to study more in depth the contribution and the role of maize lignin in the humification process, adding new data to our previous paper (Adani et al. 2006).

The dominance of the 4-vinylphenol and 4-vinylguaiacol is typical of grass lignin (Fig. 1) (Ralph and Hatfield 1991; van Bergen et al. 1997; Nierop 2001), and likely reflect decarboxylized *p*-coumaric acid and ferulic acid, respectively (Mulder et al. 1992). Other important lignin-derived compounds include guaiacol,

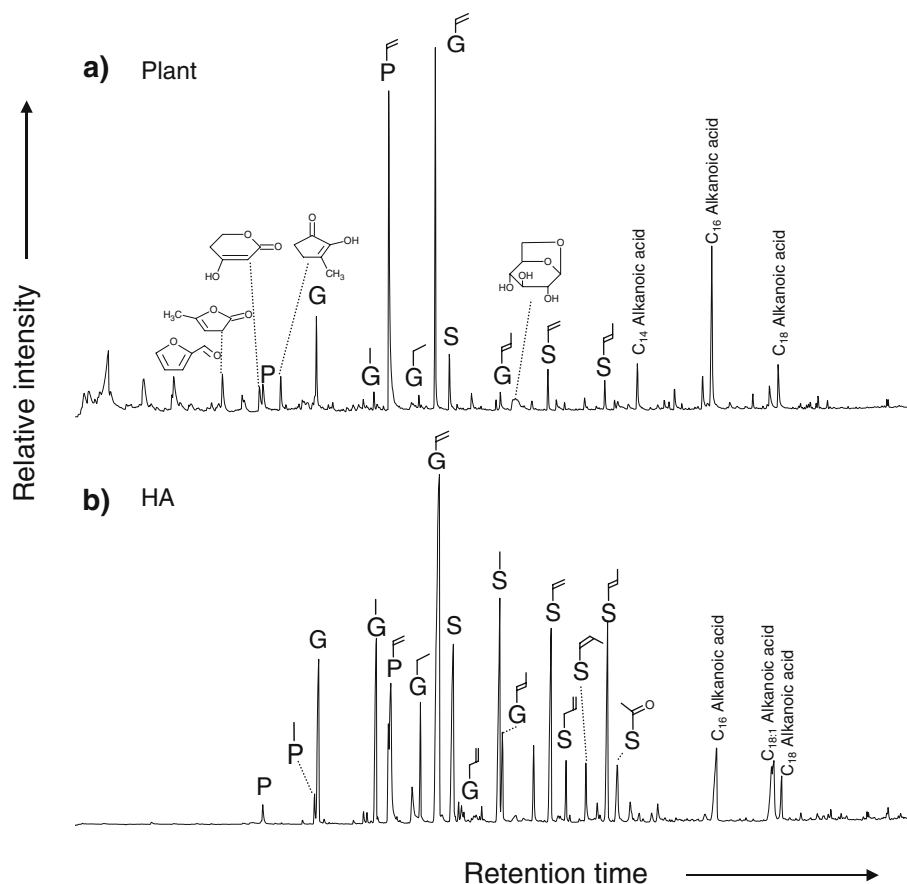


Fig. 1 Gas chromatogram of pyrolysis products of (a) maize plants, and (b) HA from maize plants. P = phenol, G = guaiacol (2-methoxyphenol), and S = syringol

(2,6-dimethoxyphenol). Indicated side-chains are at the 4-position of P, G or S

4-methylguaiacol, syringol, 4-methylsyringol, 4-vinylsyringol, and *trans* 4-(prop-2-enyl)syringol, but these are all present in much smaller amounts.

Pyrolysis determined that the HA derived from plants was composed of aromatic compounds derived mainly from lignin (Fig. 1). Polysaccharide-derived products were virtually absent and, similar to the NMR spectrum, the aromatic fraction was enriched compared to that of the whole plant (Adani et al. 2006). Furthermore, similar to the whole plants, compounds contributing to the O/N-alkyl fraction of HA by NMR are less sensitive to Py-GC/MS, and NMR data should be considered more quantitatively compared to the overall composition of HA. The higher S/G ratio indicates that the HA fraction was relatively enriched by syringyl-lignin (Table 1).

4-Vinylphenol and 4-vinylguaiacol were far less abundant in HA than in the pyrolysate of the plant residue, the most likely cause of this being (partial) hydrolysis of *p*-coumaric acid and ferulic acid. These two acids are mainly present as esters linked to lignin, polysaccharides or other biopolymers (Kögel-Knabner 2002). These results agree with the hypothesis formulated by Adani et al. (2006) in which it was supposed that the solubilization of HA from maize plants was the result of the breakdown of some of the ferulate ester linkages (alkali hydrolysable bonds) that bind lignin to cell wall carbohydrates in maize tissues.

Pyrolysis revealed that the main products from the cell wall were still 4-vinylphenol and 4-vinylguaiacol (Fig. 2), while in comparison with the

Table 1 Syringyl-to-guaiacyl (S/G) ratio of samples

	Sample	S/G
	Plant	1.04
	HA from plant	1.87
	Cell wall	1.22
	HA from cell wall	1.31
Artificial soil	0 month	1.87
	8 months	2.41
Natural soil	0 months	0.63
	8 months	0.72
Natural soil plus maize	0 months	0.95
	8 months	0.95
Natural soil plus maize ^a	0 months	0.89
	8 months	0.97

^a data obtained as the sum of the yields of HA extracted from artificial soil plus maize, and natural soils

plant material, other lignin products were less abundant. The cell wall fraction was only slightly enriched by syringyl-lignin (Table 1). A remarkable high peak assigned to *trans* 4-(prop-2-

enyl)syringol was observed. In contrast to lignin-derived compounds, a relatively high and broad peak attributed to levoglucosan, which is a main pyrolysis product of cellulose, was noted (Pouwels et al. 1989). Other polysaccharide-derived compounds included 2-furaldehyde, 5-methyl-2-furaldehyde, 4-hydroxy-5,6-dihydro-(2H)-pyran-2-one, and 1,4-dideoxy-D-glycero-hex-1-enpyranos-3-ulose.

The pyrolysate of HA from cell walls (Fig. 2) did not contain levoglucosan, which means that cellulose is more or less completely removed during HA preparation remaining in the alkali-insoluble/acid insoluble fraction i.e. the humin fraction (Genevini et al. 2002; 2003). This result contrasts with NMR data which indicated O/N-alkyl fraction makes a large contribution to HA composition (Adani et al. 2006). The relative abundance of 4-vinylphenol in relation to 4-vinylguaiacol is much lower than that in the

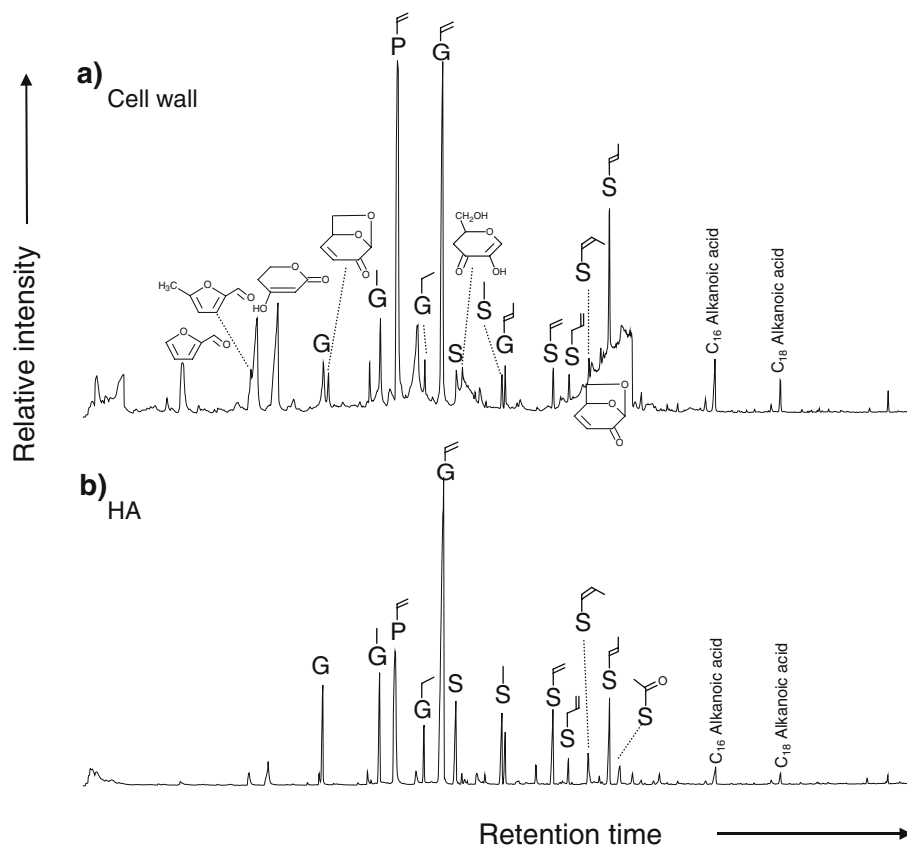


Fig. 2 Gas chromatogram of pyrolysis products of (a) cell wall from maize plants, and (b) HA from cell wall. For symbols, see Fig. 1

pyrolysate of the whole cell wall, which suggests that 4-vinylphenol was present to a much greater extent as *p*-coumaric acid in the cell wall than as 4-vinylguaiacol as ferulic acid. Compared to the HA from plants, the cell wall HA had a much lower S/G ratio and a much higher contribution of 4-vinylphenol and 4-vinylguaiacol, which in contrast to NMR data suggests that their detailed composition is not similar (Adani et al. 2006). The fact that the HA yield from cell walls accounted for 67% of the yield of HA extracted directly from maize plants (Adani et al. 2006) suggests that the pretreatment of the plant prior to obtaining cell walls from it probably removed part of the potential alkali-soluble fraction (HA), thereby altering the final HA composition.

Py-GC/MS performed on plants, cell walls, and their derived HAs seems to confirm the hypothesis of the cell wall derivation of HA from maize plants, as suggested in previous work (Adani et al. 2006). Nevertheless, Py-GC/MS is more sensitive to the lignin-derived molecules than to those contributing to the O/N-alkyl fraction in NMR spectra which seems to contribute a great deal to the overall HA composition. Moreover, in agreement with previous findings, it seems most likely that the chemical mechanism by which HAs are obtained from cell walls are caused by (partial) hydrolysis of *p*-coumaric and ferulic acids, which are mainly present as esters linked to lignin, polysaccharides, or other biopolymers in the plant.

Eight months after incubation of the artificial soil plus maize plant (Fig. 3), the pyrolysate of the HA contained lignin-derived compounds, which is similar to the HA of plants. Compared to the HA from plants, the S/G ratio of the HA from the artificial soil was remarkably high. Such an increase of syringyl-lignin contrasts with generally observed trends (e.g. Kögel 1986; van Bergen et al. 1997; Nierop 2001). During decomposition and humification of lignin, a frequently observed phenomenon is the apparent preferential removal of syringyl (sinapyl based) moieties (Van der Heijden and Boon 1994; Van Bergen et al. 1997; Nierop 2001). Such degradation will lead to a decrease of the syringyl-to-guaiacyl (coniferyl based moieties) (S/G) ratio, which seem to contrast with our results. However, the increase we see was found for the HA fraction: during incubation lignin degrades. Unaltered lignin is hardly extractable by NaOH and remains in the NaOH insoluble fraction (humin), while altered lignin will be present in the HA fraction (Nierop et al. 1999). As a result, syringyl lignin, which is more easily degraded due to the less extent of cross-linking compared to guaiacyl lignin (Hedges 1990), becomes more extractable than guaiacyl lignin yielding into an increase of the S/G ratio in the HA fraction upon incubation. This result suggested the degradation of the lignin present in the starting HA and the successive contribution of the insoluble lignin (HU-lignin) to form new HA fraction (Genevini et al. 2003). Humic acid

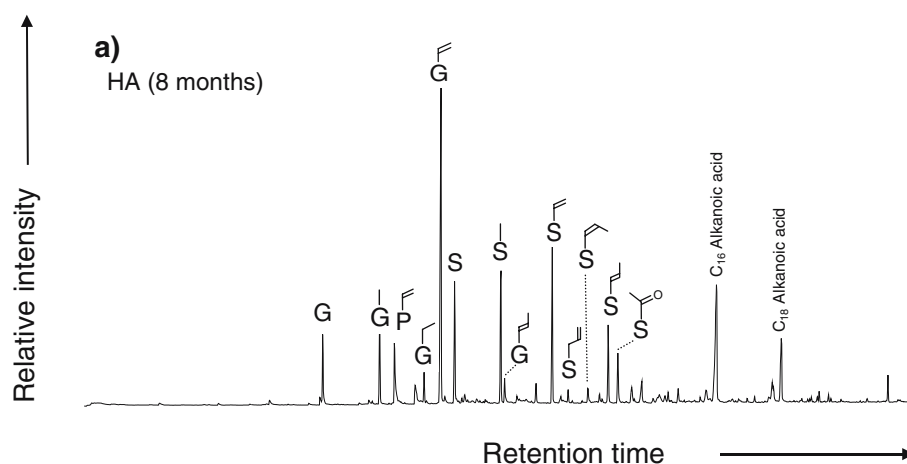


Fig. 3 Gas chromatogram of pyrolysis products of artificial soil amended with maize plants after 8 months of incubation. For symbols, see Fig. 1

Table 2 Yield of humic acids (HA) and humin (HU) extracted from artificial soil before and after incubation

Soil sample	HA ^a (g kg ss ⁻¹)	HU ^a (g kg ss ⁻¹)
<i>Artificial soil</i>		
0 month	1.65 ± 0.08a ^b	8.41 ± 1.60c
2 month	1.39 ± 0.01a	8.94 ± 0.39c
4 month	1.64 ± 0.05a	6.61 ± 0.05b
6 month	1.50 ± 0.06a	6.23 ± 1.28ab
8 month	1.41 ± 0.28a	4.73 ± 0.66a

^a expressed as carbon HA and carbon HU

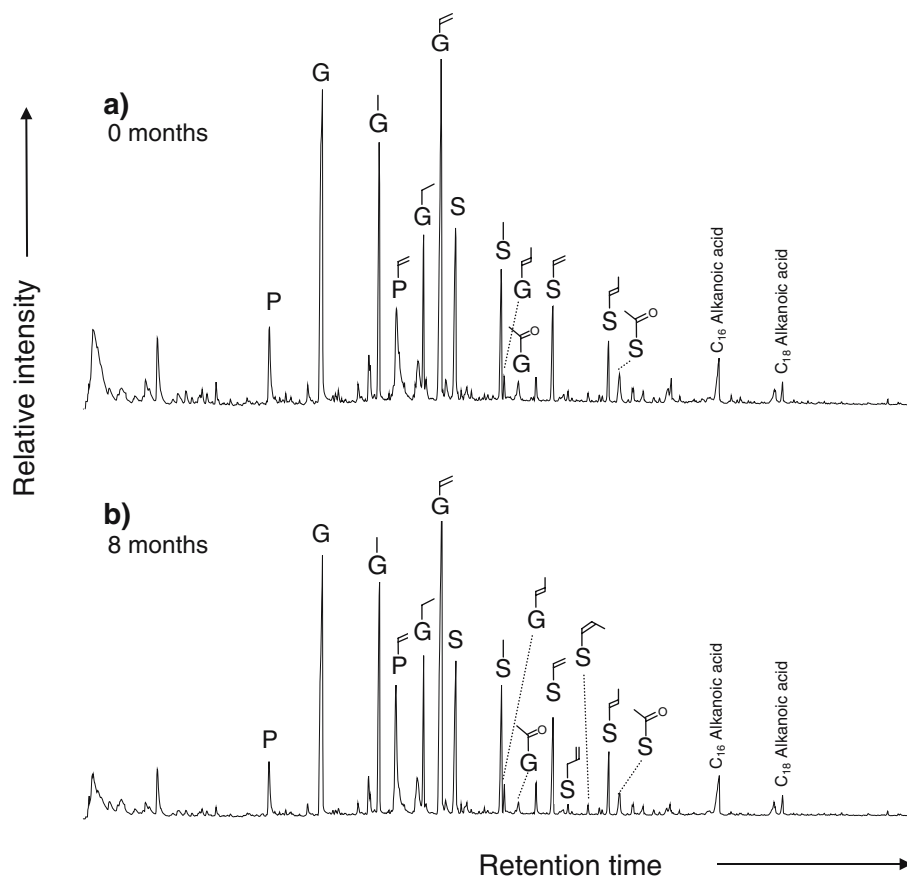
^b Means followed in the same column by the same letter are not statistically different ($P < 0.05$) according to Tukey test

and HU mass balances during incubation confirmed how above indicated, i.e. after 2 months of incubation the HA content decreased to re-increase in correspondence of the decrease of the HU fraction (Table 2).

The distribution of the lignin-derived compounds was also very different, i.e. less methoxyphenols with an intact C3 chain, and more with oxidized side-chains.

The modification of the syringyl/guaiacyl ratio and the oxidation of the side-chains of lignin, suggested a degradation and a turnover of the lignin-derived molecules in soil-HA; this is in contrast with our previous work (Adani et al. 2006) that indicated the humification as the simple preservation of the alkali-soluble lignin. Probably a more complicated mechanism, involving both lignin degradation and solubilization, could better explain the contribution of maize-lignin to the soil HA formation.

Dignac et al. (2005) studying the contribution of maize plant residue to the soil organic matter after 9 years of maize crop, concluded that there is no long-term storage of lignin in soil. Again,

**Fig. 4** Gas chromatogram of pyrolysis products of (a) natural soil, and (b) natural soil after 8 months of incubation. For symbols, see Fig. 1

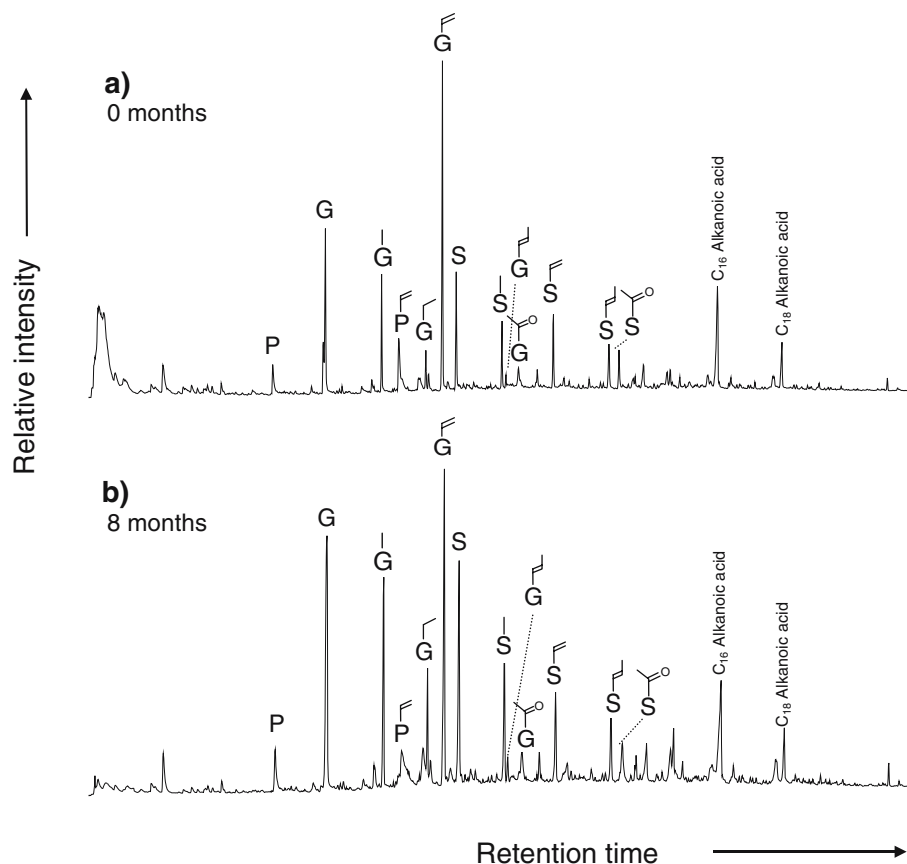


Fig. 5 Gas chromatogram of pyrolysis products of (a) natural soil amended with maize plants and (b) natural soil amended with maize plants after 8 months of incubation. For symbols, see Fig. 1

Lützow et al. (2006) reviewing available literature concluded that lignin is decomposed rapidly and does not appear to be stabilized in the long term in any soil fraction. On the other hand the same Authors indicated humic polymers as a class of organic molecules that contributes to the stabilized SOC. Humic acid are known to be a class of recalcitrant molecules that persisted in soil for thousand of years (Piccolo 2001), in which aromatic moieties derived from lignin are well represented (Stevenson 1994; Page et al. 2002; González et al. 2003).

The pyrolysates of the HAs from the natural soil contained lignin-derived compounds and HA before and after 8 months of incubation were very similar (Fig. 4). A very small increase in the S/G ratio was seen, but this represents no real overall degradation of biomacromolecules in the HA fraction during the course of the incubation

(Adani et al. 2006). Furthermore, the S/G ratio was lower than in the maize-HA. This can be attributed to the fact that natural soil has a HA fraction which is more complex than HA from artificial soil. Moreover, the parent material from which HAs were derived is different for the artificial soil, in which the only source of C was the maize plants, compared to the natural soil in which roots, microorganisms, and any other organic molecules are able to contribute to the stable soil-C. Nevertheless, the small increase in the S/G ratio agrees with those found in HA extracted from the artificial soil.

A relative decrease of 4-vinylphenol and 4-vinylguaiacol was found in the pyrolysates of the HAs during the 8-month incubation of natural soil amended with maize (Fig. 5). The S/G ratio of the natural soil was higher because of the contribution of HAs from maize plants (Table 1), and in

contrast with the natural soil and above all with the artificial soil, the S/G ratio did not change.

Furthermore, increased oxidation of lignin was found, suggesting that the lignin was degraded during the incubation. As the natural soil without maize amendment hardly showed any change in HA composition during incubation, the changes observed in HAs from maize amended natural soils must only be caused by transformations in the maize plants themselves.

Conclusion

In focusing on lignin-derived molecules, which were the main products of pyrolysis, Py-GC/MS results confirm previously reported NMR work concerning HA derivation from maize plant and the contribution of lignin. The modifications of the S/G ratio and oxidation of the lignin side-chains during the incubation of plants suggested a turnover of lignin-derived molecules forming the soil-HA fraction. This fact indicated an involvement of the alkali insoluble fraction of maize plant residue (humin) in the soil-HA formation, up-dating our previous knowledge.

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