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Biochemical origin and refractory properties of humic acid extracted from the maize plant

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Abstract. Humic acids (HA) contribute to soil fertility because of their chemical, physical, and biological properties. The origin of HAs in soils has puzzled scientists for decades, and what HAs are and what their origin is remain unclear. The isolation of HAs in plants, which have characteristics close to soil HAs, suggests the probable origin of soil-HA is the preservation of plant tissue, indicating biochemical origin. In this paper HA from maize plant at different stages of maturity is isolated, from which it was found that the evolution of this fraction depends on and is derived from cell wall formation. Evidence was also found that HA was above all composed of lignin and cutin residues, and was characterized by low surface area. After 8 months of incubation in both mineral-artificial and natural soils, humic acid isolated form maize plant could be recovered intact.

Introduction

Soil quality depends on the quantity, quality, and dynamics of the soil organic matter (SOM) (Lal 2000). Up to 70–80% of SOM in mineral soil is composed of humic substances (HS) (Piccolo 2002). HS are composed of chemically complex, non-biochemical organic components, which are largely hydrophilic, amorphous, dark colored, and resistant to chemical and biological degradation (Schintzer 1991; Hedges and Oades 1997).

The definition of HS is only operational, being based on the properties of solubility in the aqueous solutions used as extractants (Piccolo 2002). The term humic acid (HA) is used to indicate the HS soluble in dilute alkali but insoluble in dilute acid (pH $<$ 1) (Piccolo 2002). The HA is negatively charged colloid recalcitrant to biodegradation so it can be stored in soil for a long time (Qualls 2004). These characteristics mean HA plays an important role in determining soil characteristics by influencing its chemical, physical, and biological properties. As a result, the balance of this fraction in soil is a key factor in maintaining soil fertility and preserving fixed carbon.

The classical route for formation of humic acid hypothesize firstly that biomacromolecules break up into small constituents, and then subsequently recombine to form chemically complex ''geopolymer'' (Senesi and Loffredo 1999). These mechanisms appear to be unlikely and have lost credibility

nowadays (Hedges and Oades 1997). On the other hand, many studies conducted in both soil and marine ecosystems (Kögel-Knabner et al. 1992; Hedges and Keil 1995; Hedges et al. 2001) indicate that it is the preservation and modification of biopolymer that provides the humification pathway. For example, sedimentary HS exhibit distinct compositional characteristics when compared to soil HS, compositional characteristics resulting from a predominantly planktonic rather than higher plant origin (Hedges and Oades 1997). Recent isolation of humic acid from both marine (e.g., *Pilayella litoralis*) (Radwan et al. 1997a, b) and terrestrial plants (e.g., *Brumasia sanguinea; Eucalyptus camaldulensis; Zea mais*) (Harper et al. 2000; Adani and Ricca 2004) seems to confirm this hypothesis. As plant residues are an important source of carbon for soil (Lal 2000), the isolation of HA from plant raises questions about its contribution to soil humus and so to both soil fertility and carbon (C) storage. In particular, as HA is recovered in plant residue, could plant represent the first step in the humification of the organic matter?

In order to answer this question, HA was extracted during maize plant (*Zea mays*) growth, and then its contribution to the soil HA was studied by using plant residue (shoot) incubation in both artificial (no other organic matter except plant residue present) (sandy–clay soil) and natural (soil on which maize was cropped) soils.

Materials and methods

Maize plant cropping

Maize plants (*Zea mays*), (cv. Eleonora, Pioneer) were grown in 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}, $CaCO_3$ 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 and 3 days respectively, ground to 0.5 mm, and stored for subsequent incubation tests and analyses.

Macromolecules determination

Forage fiber analyses were performed for neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) (14). Hemicelluloses, cellulose, lignin, and soluble cell materials (g kg^{-1}) were then calculated as:

(NDF–ADF), (ADF–ADL), (ADL), and (1000-NDF) respectively (van Soest et al. 1991).

Elemental analysis

Elemental analysis of HAs (C, H, N, S, and O), were measured with an elemental analyzer (NA-2100 – CE Instruments – Rodano, Milan – Italy).

Incubation test

The artificial soil used for the incubation tests was a sandy mineral substrate composed of sand (particle size $\emptyset = 0.5$–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 $\emptyset < 1$ 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 ± 2 °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 g. 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.

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 and O'Neil (1987).

Humic acids 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} $Na_4P_2O_7$ and successive precipitation of the suspension to pH < 1.5 (Adani

and Ricca 2004). All HAs extracted were quantified by organic carbon determination (Adani and Ricca 2004).

^{13}C-CP-MAS-NMR analysis

^{13}C-cross polarization-magic angle spinning-nuclear magnetic resonance (^{13}C-CP-MAS-NMR) spectra on solid samples were obtained using a Bruker AMX 300 spectrometer (Bruker BioSpin GmbH, Rheinstetten, D) operating at 75.47 MHz. The conditions used were: 4.5 kHz spinning rate, 0.033 s acquisition time, 4 μs pulse time, and line broadening of 50 Hz. For each spectrum, 3600 cycles were accumulated and a recycling delay of 1 s was used. A contact time of 1 ms was obtained after the VCT experiments. The error in signal acquisition caused by the use of the average contact time was determined by comparing the signal intensity in the absence of carbon relaxation – I_0, and the intensity of the signal I_{tcp} measured at the optimal contact time. In these conditions it was shown that ^{13}C-CP-MAS-NMR provides a quantitative representation of the C content in humic substances (Conte et al. 2002). Spectra were elaborated by using 1D-WIN-NMR software (Bruker BioSpin GmbH, Rheinstetten, Germany).

Surface area and porosity analyses

Surface area and porosity were determined by gas adsorption analysis of dried samples using a sorptometer apparatus (Sorptometer KELVIN Mod. 1042, COSTHEC, Cernusco S.N., Italy).

Analyses were preceded by degassing procedure performed at 80 °C for 60 min. Analyses were carried out by using N_2 and He as adsorptive and carrier gasses respectively. Full isotherm (overpressure) modality was used to perform the analysis.

Statistical analysis

Chemical analyses were performed in triplicate. Average and standard deviation values from chemical analyses were calculated according to standard procedure and results analyzed by ANOVA. The Tukey test was used to compare mean values and to assess the significance of the differences between mean values in the case of elemental analysis. All statistical analyses were carried out using SPSS statistical software (SPSS, Chicago, IL). Since elemental analyses were performed on three analytical samples withdrawn from composite bulk samples, standard deviation values calculated from the data for three replications were estimates of the variability caused by both the analytical method and bulk sample homogeneity.

Results and discussion

The content of humic acids in maize plant at different stages of maturity is reported in Table 1. HA content in young plant is higher than in mature plant. This is probably due to the solubility of part of the cellular material in alkali (e.g. cell membrane lipids, protoplast proteins, etc.) (see later). Plant maturation led to a stable HA content, and plant residue (shoot part) showed a relevant content of HA.

^{13}C-CP-MAS-NMR carbon (C) assignments corresponding to nominal 'alkyl' (0–45 ppm), 'O/N-alkyl' (45–110 ppm), 'aromatic' (110–165 ppm), and 'carbonyl/amide' (165–190 ppm) (Figure 1, Table 2) together with elemental analysis (Table 3) indicate that HA changes composition during plant growth. HA extracted from young plant (4–5 leaves) shows a high content of cellular material, such as membrane lipids (high content of alkyl C), and protoplast molecules (e.g. proteins) (high content of N and S). On the contrary, at the stage of plant senescence (plant residues), HA mainly seems to be composed of fragments of plant polymers as lignin (high aromatic-C content and well resolved peak at 56 ppm representing methoxy groups of lignin) and carbohydrates (hemicelluloses and cellulose) (high O-alkyl-C content), with fewer lipids (cutin) (alkyl-C), and fewer protein (e.g., structural proteins) (N-alkyl-C) moieties, as previously reported (Adani and Ricca 2004). Therefore, plant maturation, i.e. primary (cellulose and hemicelluloses deposition) and secondary (lignification) cell wall formation affect HA composition. The good correlations found between the HA contents of O-alkyl C (polysaccharides carbon), aromatic C (part of lignin carbons), alkyl C, and the plant contents of cellulose plus hemicelluloses ($r = 0.93$; $p < 0.05$), lignin ($r = 0.99$, $p < 0.01$)

Table 1. Yield of humic acids (HA) extracted from plant, cell wall and, soil before and after incubation.

Plant sample	HA (g kg ss^{-1})		Soil sample	TOC[a] (g kg ss^{-1})	HA (g kg ss^{-1})
Plant[b]	127.1 ± 5.7b	Artificial soil	0 month	15.43 ± 1.6b	1.65 ± 0.08a
			8 month	8.08 ± 0.36a	1.41 ± 0.28a
Plant[c]	73.5 ± 1.5a	Natural soil	0 month	16.75 ± 0.15b	3.97 ± 0.30a
			8 month	14.19 ± 0.26a	3.93 ± 0.02a
Plant[d]	74.1 ± 2.7a	Natural soil plus maize	0 month	28.29 ± 0.45b	6.19 ± 0.17b
			8 month	20.91 ± 0.71a	5.48 ± 0.11a
Cell wall[e]	71.1 ± 0.5a (49.8)[f]	Natural soil plus maize calculated[g]	0 month	32.18	5.62
			8 month	22.27	5.34

[a]Total organic carbon contents for soils.
[b]Plant post emergency (4–5 leaves).
[c]Plant maturity.
[d,e]Plant senescence.
[f]HA expressed as percent of the entire plant
[g]Data obtained as the sum of the yields of HA extracted from artificial soil plus maize, and natural soils.

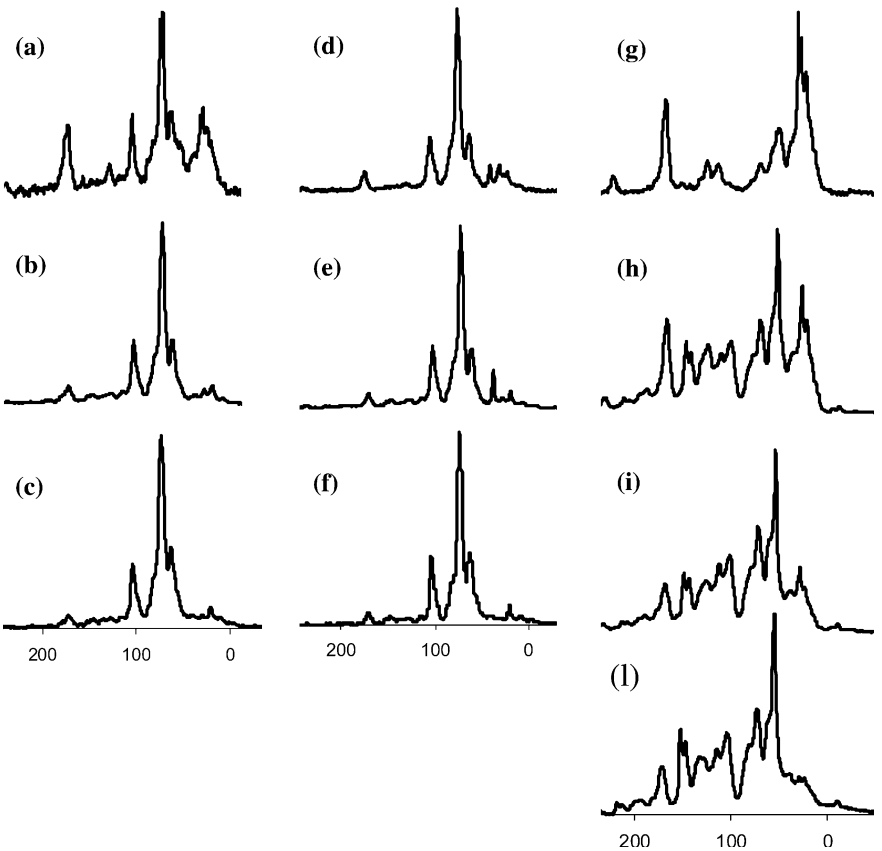

Figure 1. ^{13}C-CP-MAS-NMR of maize plant, maize cell-wall, and HAs at post emergency (a, d, g), waxy maturity (b, e, h) and senescence (c, f, i, l). Plant (a–c); cell wall (d-f); humic acid from plant (g–i); humic acid from cell wall (l).

and soluble cell material ($r = 0.99$, $p < 0.01$) (Table 4), respectively, confirm the above mentioned findings. The close relationship between HA and cell wall formation suggest that it is worth considering the maize-HA as a fraction derived from the cell-wall as previously indicated (Adani and Ricca 2004). The extraction of HA from pre-purified maize cell wall at the plant-senescence stage (plant residue) provides a fraction whose composition was very close to HA extracted directly from plant, supporting the HA cell wall-derivation hypothesis (Figure 1; Tables 2 and 3). Nevertheless, HA yield from cell wall accounted for 67% of yield obtained extracting HA directly from maize plant (Table 1). This difference could be due to the plant pretreatment used to obtain cell wall in which the potential alkali-soluble fraction is lost.

Physical measures such as the surface area and the total porosity can be useful in improving understanding of the architecture of plant biopolymers in

Table 2. ^{13}C-CPMAS-NMR integrated area of different carbon type of plant, cell wall, and humic acids, and soil HA extracted from soil before and after incubation.

Plant sample	Carbonyl 220–160[n] (Area %)	Aromatic 160–110[n] (Area %)	O/N-Alkyl 110–50[n] (Area %)	Alkyl 50–0[n] (Area %)		Soil sample	Carbonyl 220–160[n] (Area %)	Aromatic 160–110[n] (Area %)	O/N-Alkyl 110–50[n] (Area %)	Alkyl 50-0[n] (Area %)
Plant[a]	13.64	10.44	51.12	24.8	Artificial	HA 0 months	13.32	25.01	45.43	16.23
Plant[b]	8.54	11.41	70.49	9.57	soil plus maize	HA 8 months	17.54	25.80	41.29	15.37
Plant[c]	5.33	8.88	73.93	11.86	Natural soil	HA 0 months	18.09	21.04	31.10	29.77
Cell wall[d]	5.46	6.77	73.14	14.63		HA 8 months	18.58	20.94	30.29	30.19
Cell wall[e]	5.92	9.47	73.49	11.13	Natural soil	HA 0 months	16.04	24.47	34.32	25.18
Cell wall[f]	3.23	7.07	79.46	10.25	plus maize	HA 8 months	18.31	23.97	31.76	25.96
HA-plant[g]	18.58	13.18	21.21	47.03	Natural soil	HA 0 months	16.78	22.27	35.24	25.74
HA-plant[h]	17.06	23.94	36.78	22.22	plus maize calculated[m]	HA 8 months	18.33	22.27	33.27	26.28
HA-plant[i]	13.62	25.21	45.13	16.03						
HA-cell wall[l]	11.65	27.48	45.83	15.04						

[a, d, g]Plant post emergency (4–5 leaves).

[b, e, h]Plant at waxy maturity.

[c, f, i, l]Plant at senescence.

[m]Data obtained as weighted mean of the areas of HA extracted from artificial soil plus maize, and natural soils.

[n]Signal intensity within the indicated chemical shift intervals (in parts per million) expressed as a percentage of total signal intensity between 0 and 220 ppm.

Table 3. Elemental composition of plant, cell wall, and humic acids, and soil HA extracted from soil before and after incubation.

Plant sample	C (g kg^{-1} dry matter) (ash free)	H (g kg^{-1} dry matter) (ash free)	N (g kg^{-1} dry matter) (ash free)	O (g kg^{-1} dry matter) (ash free)	S (g kg^{-1} dry matter) (ash free)	Soil sample	C (g kg^{-1} dry matter) (ash free)	H (g kg^{-1} dry matter) (ash free)	N (g kg^{-1} dry matter) (ash free)	O (g kg^{-1} dry matter) (ash free)	S (g kg^{-1} dry matter) (ash free)
Plant[a]	323.8 ± 3.2	46.3 ± 0.2	38.4 ± 0.5	260.5 ± 1.7	2.4 ± 0.1	Artificial soil HA 0 month	542.3 ± 7.2	59.8 ± 0.1	23.7 ± 0.5	312.6 ± 0.5	1.6 ± 0.4
Plant[b]	376.7 ± 2.1	52.9 ± 1.2	10.1 ± 0.3	363.9 ± 0.8	1.1 ± 0.3	HA 8 month	541.6 ± 2.3	56.9 ± 0.5	25.2 ± 0.3	316.9 ± 0.3	3.1 ± 0.1
Plant[c]	391.9 ± 4.6	55.6 ± 0.6	7.6 ± 0.1	401.2 ± 1.0	0.9 ± 0.1	Natural soil HA 0 month	478.5 ± 0.4	48.4 ± 2.2	45.1 ± 0.5	254.5 ± 1.2	5.6 ± 0.4
Cell wall[d]	384.5 ± 1.0	57.8 ± 2.1	17.2 ± 0.4	364.8 ± 0.4	16.1 ± 0.0	HA 8 month	499.6 ± 1.1	51.9 ± 1.4	47.5 ± 0.7	292.9 ± 0.5	5.4 ± 0.5
Cell wall[e]	399.6 ± 0.3	56.3 ± 0.4	8.3 ± 0.2	373.8 ± 1.1	26.1 ± 1.6	Natural soil plus HA 0 month	516.6 ± 0.2	53.4 ± 0.4	37.8 ± 0.6	309.8 ± 0.4	4.7 ± 0.3
Cell wall[f]	405.0 ± 3.8	54.9 ± 0.1	3.9 ± 0.1	407.8 ± 0.6	0.7 ± 0.3	maize HA 8 month	512.1 ± 7.9	53.5 ± 0.5	41.5 ± 0.7	279.6 ± 0.6	4.6 ± 0.5
HA-plant[g]	539.0 ± 3.0	71.7 ± 0.8	86.6 ± 0.0	239.0 ± 0.2	3.9 ± 0.3	Natural soil plus HA 0 month	497.4	51.8	38.8	271.7	4.4
HA-plant[h]	545.6 ± 5.1	65.0 ± 0.6	43.2 ± 1.0	288.0 ± 0.8	2.3 ± 0.2	maize calculated[m] HA 8 month	511.5	53.3	41.6	299.8	4.8
HA-plant[i]	542.8 ± 7.2	59.7 ± 0.1	23.3 ± 0.5	311.4 ± 0.5	1.3 ± 0.4						
HA cell wall[l]	542.1 ± 0.2	56.2 ± 1.2	17.6 ± 0.1	323.3 ± 1.4	1.6 ± 0.3						

[a,d,g]Plant post emergency (4–5 leaves).

[b,e,h]Plant at waxy maturity.

[c,f,i,l]Plant at senescence.

[m]Data obtained as weighted mean of the elemental analysis of HA extracted from artificial soil plus maize, and natural soils.

Table 4. Macromolecular composition for plant at different stages of maturity.

Plant sample	Hemicellulose[d] (g kg ss^{-1}) (ash free)	Cellulose[e] (g kg ss^{-1}) (ash free)	Lignin[f] (g kg ss^{-1}) (ash free)	soluble cell material[g] (g kg ss^{-1}) (ash free)
Plant[a]	254.6 ± 6.3a	154.9 ± 2.7a	17.7 ± 0.6a	572.9 ± 6.3b
Plant[b]	318.9 ± 13.2b	306.7 ± 9.7b	20.5 ± 4.3a	354.0 ± 13.9a
Plant[c]	313.5 ± 29.6b	306.3 ± 14.6b	41.0 ± 0.9b	339.3 ± 29.6a

[a]Plant post emergency (4–5 leaves).
[b]Plant maturity.
[c]Plant senescence.
[d]Hemicellulose: neutral detergent fiber (NDF)-acid detergent fiber (ADF).
[e]Cellulose: acid detergent fiber (ADF)-acid detergent lignin (ADL).
[f]Lignin: acid detergent lignin (ADL).
[g]Soluble cell wall: 100-neutral detergent fiber (NDF).

cell wall, and so in understanding the HA derived from it. Cell wall porosity (Table 5) is represented by mesoporosity whose sizes varied in average diameter from 2.94 to 50 nm, and is due to spacing between individual polysaccharide chains which are modified by the presence of other polymers (e.g., pectin, lignin, and cutin) (Chesson 2002) (Table 2). Maize-HA is characterized by both lower porosity volume and surface area than cell-wall, but is identical to data for HA-cell wall (Table 5), again supporting the HA cell wall-derivation hypothesis. The extraction of HA from cell wall, determined the loss of 70% in porosity (Table 5), which can be ascribed to the loss of part of the cell wall-polysaccharide chains because of the nature of the porosity (Chesson 2002). This data coincides with the fact that HA is richer in lignin (aromatic-C) and cutin (alkyl-C), and poorer in cellulose and hemicelluloses (O-alkyl-C) than cell wall (Figure 1) (Table 2).

On the basis of the above reported results it seems that the alkali treatment selectively extracts a fraction of the cell wall, which in turn results in small domains formed by cross-linked polymers, mainly represented by lignin and cutin, leaving part of insoluble polysaccharide polymers and partially conserving the original cell wall-like structure. From a chemical point of view the solubilization of HA could be a result of the breakdown of some of the ferulate ester linkages (non alkali resistant bonds) that bind lignin to cell wall carbohydrates in maize tissues (Grabber et al. 1995; Carpita 1996) solubilizing part of lignin, determining the enrichment of HA in acid functional groups (carboxylic groups). As deduced from ^{13}CP-MAS-CNMR analysis, the enrichment

Table 5. Total surface area and porosity determined for plant, cell wall, and humic acids.

	Plant	Cell wall	HA-plant	HA-cell wall
Total surface area (m^2 g^{-1})	3.03	2.05	0.58	0.63
Porosity (mm^3 g^{-1})	3.25	3.72	1.0	1.11

of maize-HA with carbonyl-C compared to cell wall, confirms this hypothesis (Table 2).

Plant residue was incubated for 8 months in an artificial mineral soil, and resulted in HA being extracted at the same yield and with identical chemical composition to the HA extracted at the time the soil and plant residues were mixed (Tables 1–3; Figure 2), which means that HA extracted from maize is recalcitrant to biodegradation. This result was obtained despite 500 g kg^{-1} of total organic carbon (TOC) degradation during incubation time, which indicates high microbial activity (Table 1). Low HA degradability is caused by both chemical and physical properties of HA. High lignin content (Table 2) gives refractory properties to HA (Scobbie et al. 1993). On the other hand, compared to the plant residue and cell wall (Table 2), the low surface area that characterizes HA limits enzyme activity. Moreover, not all HA-surface area is available to enzymes because pores of diameters under 5 nm, representing 19% of the total surface area of HA, are not accessible to enzymes (Chesson 2002).

The refractory properties of HA suggest that this fraction could actively contribute to the soil HA balance by simply adding itself to the native soil HA.

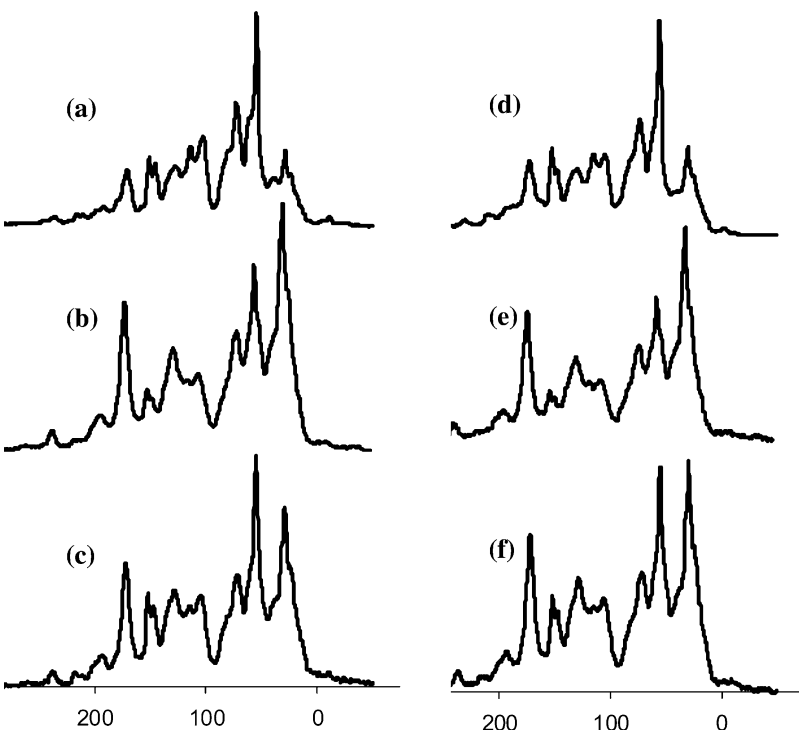

Figure 2. ^{13}C-CP-MAS-NMR spectra of HAs extracted from soils before and after incubation. Humic acids from artificial soil at time 0 and after 8 months (a, d); humic acids from natural soil at time 0 and after 8 months (b, e); humic acids from natural soil amended with maize at time 0 and after 8 months (c, f).

In order to confirm this hypothesis, maize residues were incubated for 8 months in the soil on which maize plant were cropped, at the same rate as mineral soil (see M & M). Moreover, we considered the same soil but without plant residue addition. After 8 months of incubation ^{13}C-CP-MAS-NMR analysis, elemental analysis, and mass balances show that the HA yield and composition for soil amended with residual plant was exactly the weighted mean of the yield and composition of HA extracted from artificial mineral and natural soils, that were, respectively, amended and non amended with plant residues (Tables 1–3). This confirms that the contribution of maize-HA to native soil HA fraction occurs by simply adding it to native HA.

In conclusion, our findings corroborate the hypothesis that the preservation of biomacromolecules resistant to degradation plays an important role in the 'humification process'. Moreover, as plant maturation is important in determining the refractory properties of HA, it assumes a real role in the 'humification process' and so in the preservation of fixed carbon in soils.

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