Studies of the Compositions of Humic Acids from Amazonian Dark Earth Soils

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The compositions of humic acids (HAs) isolated from cultivated and forested "Terra Preta de Índio" or Amazonian Dark Earth soils (anthropogenic soils) were compared with those from adjacent non-anthropogenic soils (control soils) using elemental and thermogravimetric analyses, and a variety of solid-state nuclear magnetic resonance techniques. The thermogravimetric index, which indicates the molecular thermal resistance, was greater for the anthropogenic soils than for the control soils suggesting polycyclic aromatic components in the former. The cultivated anthropogenic soils were more enriched in C and depleted in H than the anthropogenic soils under forest, as the result of the selective degradation of aliphatic structures and the possible enrichment of H-deficient condensed aromatic structures. The combination of variable amplitude cross-polarization (VACP) and chemical shift anisotropy with total suppression of spinning sidebands experiments with composite π pulses could be used to quantify the aromaticity of the HAs from the anthropogenic soils. From principal component analysis, using the VACP spectra, it was possible to separate the different constituents of the HAs, such as the carboxylated aromatic structures, from the anthropogenic soils and plant derived compounds. The data show that the HAs from anthropogenic soils have high contents of aryl and ionisable oxygenated functional groups, and the major functionalities from adjacent control soils are oxygenated functional groups from labile structures (carbohydrates, peptides, and with evidence for lignin structures). The anthropogenic soils HAs can be considered to be more recalcitrant, and with more stable reactive functional groups which may, in part, explain their more sustainable fertility due to the organic matter contribution to the soil cation exchange capacity.

Introduction

Most of the soils in the Amazon are acidic, with low cation-exchange capacity (CEC), low fertility, and low production potential. The dominating soil types are Ferralsols, Acrisols, and Sesquisols that cover 70% of the region. In this environment, where soil fertility is a limiting factor for sustainable agricultural development, the "Terra Preta de Índio" soils occur. These are also known as Amazonian Dark Earths, Anthropogenic Dark Earths (ADE), Indian Black Earths, or Archaeological Dark Earths. ADE are soils that have an archeo-anthropedogenic horizon; that is a surface horizon of varying depth in soil profiles that feature elevated organic matter (OM) contents and ceramic pieces or lithic artefacts (*1*). These occur in isolated patches, most of which do not exceed 2 ha, but larger sites, with areas up to 350 ha, have also been reported (*2*). ADE soils have developed on virtually all soil types (Ferralsols, Acrisols, Sesquisols, Podzols, Arenosols, etc.), and can be found in all eco-regions and landscapes of the Amazon basin. ADE soils are commonly found near rivers and streams, but due to the lack of interior road systems, it has been difficult to access areas remote from rivers. For that reason, although the abundance of ADE sites is high, there are no reliable data for the total ADE area in the Amazon.

It is now generally accepted that ADE soils are of pre-Columbian origin (*3*), although there are several hypotheses with regard to the processes of their formation. It is not clear whether the soils resulted from intentional soil improvement processes, or were byproducts of the agricultural and household activities of the indigenous populations. The dwelling places in the pre-Columbian past gave rise to accumulations of plant and animal debris, as well as to large amounts of ashes and of bonfire residues (charcoal) and several chemical elements, such as P, Mg, Zn, Cu, Ca, Sr, and Ba, which represent a geochemical signature of human occupation (*4*). These accumulations are likely to be major contributors to the formation of the fertile soils.

Pyrogenic carbon derived substances are found in humified materials in tropical soils where there are historical records of vegetation burning, and pyrogenic carbon is also an important component in Brazilian Savanna soils (*5*), and in areas of high altitude black soils (*6*). The organic components in these soils have the common characteristics of high resistance to thermodegradation, and have charge characteristics to which the high soil CEC values are attributed. Given its high stability and reactivity, pyrogenic carbon is of great importance for the conditioning of tropical soils subjected to climatic conditions that favor OM mineralization, and where the clay fraction has low CEC values.

Pyrogenic carbon is not, of course, restricted to tropical soils. In general, pyrogenic carbon is thought to represent between 1 and 6% of the total soil organic carbon (OC) (*7*), and in general, estimates are less than 10% of the soil organic matter (SOM) (*8*). It can reach 18% in native prairie in the U.S. (*9*), 35% in some U.S. agricultural soils (*10*), up to 30% in Australian soils (*11*), up to 45% in German Chernozems (*12*), and up 65% in Canadian Chernozems (*13*). However, these values can be overestimated because of problems in obtaining accurate data (*14*–*16*).

The high fertility of ADE, and especially its sustainability, is attributed to the high levels of OM and to the physical-chemical properties of the OM. ADE have carbon contents up to 150 g C kg^{-1} soil compared to 20–30 g C kg^{-1} in the surrounding soils (*3, 17, 18*). Additionally, the depth to which C enrichment occurs may be greater than 200 cm (with average values of the order of 40–50 cm), whereas OM in the

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surrounding soils is in the top 10−20 cm. Therefore, the total C stored in ADE soils can be an order of magnitude higher than in adjacent soils and this additional C is mainly in the form of pyrogenic carbon (*18*).

ADE are characterized by higher P contents (200−400 mg kg^{-1}), higher CEC, pH, and base saturation values, and with higher stable OM contents than the surrounding soils (*17, 18*). This OM is up to six times more stable than that in adjacent soils that do not have an archeo-anthropedogenic horizon (*18*).

The contribution of pyrogenic carbon to soil fertility and to its sustainability is attributed mainly to its composition and to its molecular structures (*18*). Pyrogenic carbon is highly resistant to thermal, chemical, and photo-oxidation (*19, 20*). Its contribution to soil fertility is attributed to the chemical and biochemical transformations of carbonized residues that resulted from natural or induced burning of the biomass that led to the partial carbonization of ligno-cellulosic materials (*14*). Its core consists of polyaromatic units of different sizes and with different organizational levels (*21*). Although pyrogenic carbon does undergo some natural degradation in soil (*22*), it has a high resistance to degradation and its incorporation in the soil is an important mechanism of carbon sequestration (*23, 24*). Because of partial oxidation, the peripheral aromatic units contain acidic (carboxyl) substituents (*21, 25*) that give rise to high CEC values. This is very similar to the organic C in Mollisols of the mid-west U.S. thought to be formed by the burning of vegetation over prolonged periods (*26*).

The humic components derived from pyrogenic carbon are also characterized by hydrogen-deficient, condensed aromatic structures, and with high charge density due to carboxylic groups linked principally to the aromatic core (*21*).

It is now being recognized that the production systems of former, native cultures may provide knowledge that could serve as a basis for the development of modern sustainable management systems in agriculture and the ADE of the Amazon basin are remarkable examples of sustainable ancient agricultural management systems in tropical ecosystems. Thus, by investigating the compositions of the components responsible for the sustainable fertility of ADE soils, it may be possible to reconstruct the conditions that gave rise to the phenomenon (*27*) and to gain an understanding of technologies that could generate improved fertility in other soil systems.

Toward that end we have, using solid-state nuclear magnetic resonance (NMR), elemental (CHN), and thermogravimetric analyses, compared the compositions of humic acids (HAs) isolated from the ADE soils with those from adjacent non-anthropogenic soils. Additionally, the methodology proposal by Mao and Schmidt-Rohr (*28*) was adapted to quantify the aromatic groups using chemical shift anisotropy (CSA) filters. Small modifications were made to this methodology in order to adapt the procedure for the quantification of pyrogenic carbon, which has characteristics very different from those of the usual components of SOM.

Experimental Section

Composite samples from 10 sub-samples were taken from surface (0−20 cm) Amazonian soils having archeo-anthropedogenic horizons. In the case of ADE the presence of ceramic artifacts is the most defining factor. All soils defined as ADE in this paper had ceramic artifacts. Six of the soils were under forest (anthropogenic soil under forest—AS-F) and twelve were cultivated (cultivated anthropogenic soils—AS-C). Additionally, samples were taken from four adjacent non-anthropogenic control soils under forest (non-anthropogenic soils—control soils). Samples were sieved (2 mm) and air-dried. The HAs were extracted using the method recommended by the International Humic Substances Society (*29*). Details about localization of the samples site (Figure SI-1) and extraction procedure can be found in the Supporting Information.

A TGA-50 SHIMADZU thermogravimetric analyzer was used to obtain thermo-decomposition curves for 3.3 ± 0.1 mg samples of HAs in static air. Samples were stabilized at 303 K and heating was carried out at 5 K min^{-1} to 378 K, and then held for 10 min, followed by heating at 5 K min^{-1} up to 923 K (*6*). The weight loss at 378 K was considered to be from sample moisture. The residue at the end of the heating was attributed to the ash content. Weight losses between 378 and 623 K, and between 623 and 923 K were determined, and the ratios of these two peak areas represent the resistance of the humic substances to thermal degradation. This is defined as a thermogravimetric index (TGI) (*6*).

The C content of the soil samples and the elemental compositions of the HAs were determined in two replicates using a Perkin-Elmer 2400 CHN analyzer. The C, H, and N values were corrected for moisture and ash using the amounts of moisture and ash given by the thermogravimetric analysis. The amount of O was determined by difference from the corrected data. The H:C, C:N, and O:C atomic ratios were also calculated.

Solid-state ^{13}C NMR experiments were carried out using a VARIAN INOVA spectrometer at ^{13}C and ^{1}H frequencies of 100.5 and 400.0 MHz, respectively. The techniques used were variable amplitude cross-polarization (VACP); chemical shift anisotropy (CSA) with total suppression of spinning sidebands (TOSS); and recoupled dipolar dephasing (DD) experiments (details are in the Supporting Information).

Principal component analysis (PCA) was carried out using the full VACP spectra obtained, after mean-centering of the data, and principal component regression (PCR) data were obtained from thermo-gravimetric and elemental data.

Results and Discussion

On average, the C recovery in the HA fraction from the total soil organic C was 30%, and minimal and maximal recoveries were 16 and 46%, respectively. The control soils presented the smaller values (Table SI-1). Based on the thermal analyses data, the HAs had low ash contents, in the range of 0.1−9.2%, averaging 4.5% (Table SI-1). Representative thermo-decomposition curves of HAs extracted from AS-F, AS-C, control soils are shown in Figure SI-2. The thermogravimetric index (TGI) of HAs extracted from anthropogenic soils (AS-F and AS-C) was larger than those for the control soils (Table SI-1).

According to the review by Benites et al. (*6*) and that of Francioso et al. (*30*), the first exothermic peak (378−623 K) arises from the thermal combustion of polysaccharides, the decarboxylation of acidic groups, and the dehydration of aliphatic hydroxyl structures. The second exothermic peak (623−923 K) is related to the combustion of aromatic structures and cleavages of C−C bonds.

That indicates that the HAs from anthropogenic samples were more aromatic than those in the controls, and suggests the occurrence of polycyclic aromatic nuclei, which have greater thermal resistance.

The HAs from the AS-C were more enriched in C and depleted in H (Table SI-1), indicating that the cultivation of these soils had led to the selective degradation of aliphatic structures and a possible relative enrichment in hydrogen-deficient, condensed aromatic structures from the older HA precursors, the pyrogenic carbon. The HAs extracted from the anthropogenic soils presented lower N contents and higher atomic C/N ratios (Table SI-1); however, these ratios are in the range for biologically stabilized materials (*26*). Additionally, these HAs had a lower atomic H/C ratio indicating high aromaticity and/or aromatic ring condensation (Figure SI-3). The O/C atomic ratio values were variable,

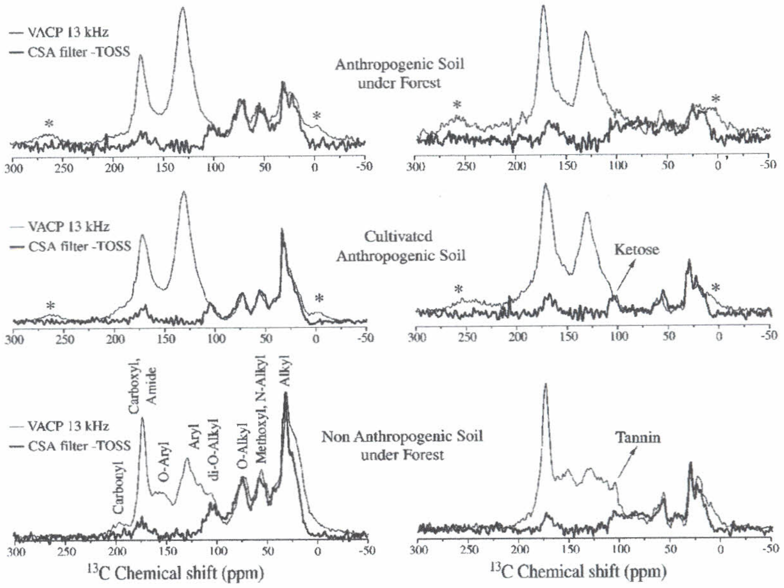

FIGURE 1. Left: Full VACP (13 kHz MAS) spectra of a humic acid sample extracted from Amazonian soils (thin line) and the corresponding VACP-TOSS spectrum (5 kHz MAS) after 35 μs CSA filter (thick line). Right: Corresponding VACP (13 kHz MAS) spectra after dipolar dephasing with 67 μs of gated decoupling (thin line) and corresponding VACP-TOSS spectrum (5 kHz MAS) after 35 μs CSA filter and 40 μs of gated decoupling (thick line). The symbols $*$ indicate the spinning sidebands.

and there was not a clear tendency for these for the different sample groups.

Although DP is probably the most quantitative ^{13}C NMR technique (*28, 31*), CP is used routinely because it requires less instrument time (*16, 31*). Among the different cross-polarization (CP) semiquantitative methods (single-amplitude with MAS−CP/MAS; CP with total suppression of spinning sidebands—CP/TOSS, and VACP with high MAS−VACP/MAS) VACP with high-speed MAS (VACP/MAS) is preferred (*31*). That is so because the radio frequency ramp used during the Hartmann−Hahn contact provides a more uniform magnetization transfer between ^{1}H and ^{13}C nuclei, making the excitation profile similar in ^{1}H rich or poor and mobile or rigid segments. This opens the possibility of replacing the time-consuming DP/MAS experiments by VACP/MAS for comparison purposes (*16, 31, 32*).

The method proposed by Mao and Schmidt-Rohr (*28*) for quantifying aromaticity by NMR combines the use of high speed MAS DP experiments and low speed MAS CSA filter experiments incorporating TOSS acquisition. However, based on our previous results, that showed that the spectra acquired using an optimized VACP excitation and DP are essentially identical for a sample rich in pyrogenic carbon (*32*), and reports in the literature (*16, 31*), we opted to replace the DP experiment by the VACP in order to decrease the experimental time. We are aware that the data obtained are not truly quantitative, but these are adequate for the comparisons made in this study.

When trying the calibration of the pulses in a sample rich in pyrogenic carbon, it was not possible to provide a simultaneous inversion of the nuclear spins of the carbons in different chemical groups (*32*). The presence of graphitic-like structures can induce local magnetic susceptibility heterogeneities (*33*) and lead to a "reflection" of the excitation pulse: the "skin effect" (*33, 34*). The problem is enhanced when carrying out the CSA filter and TOSS experiments required for the quantification method of Mao and Schmidt-Rohr (*28*) because these experiments involve the application of many cascaded π pulses that enhance the effect of the pulse imperfections. To overcome this problem we replaced all the π pulses of the CSA filter-TOSS by the composite π pulses, as suggested by Raleigh et al. (*35*) and Hagemeyer et al. (*36*) and recommended by Cook (*31*). Details of this procedure are in Novotny et al. (*32*).

The VACP spectra in Figure 1 show patterns that are representative of HAs from the different soil groups. These depict typical signals from alkyl C groups (0−46 ppm), probably of plant origin, such as cutin, suberin, wax, and dehydroxylated lignin side chains with high resistance to degradation (*37*), or from microbial neo-synthesis (*38*). The persistence of signals at 21 and 30 ppm in the DD spectra (Figure 1) indicate the presence of terminal CH_3 and long-chain mobile CH_2, respectively (*39, 40*). The signal for methoxyl C is expected at 56 ppm. It overlaps with resonances from N-alkyl C groups with chemical shift in the range 46−60 ppm; DD experiments confirmed contributions from methoxyl C. This signal shows lower intensity for anthropogenic soils than for the control soils (Table SI-2), indicating a greater incorporation of lignin and lignin-like residues to the HAs (*41*) extracted from control soils. These compounds contribute to the clear signals and shoulders in the chemical shift region of aryl C between 109 and 143 ppm and to the O-aryl signal, while the HAs of the anthropogenic soils present the typical aryl featureless signals of pyrogenic carbon derived HA (*16*). Additionally, the aliphatic region (carbohydrates, methoxyl, N-alkyl, and alkyl) is more prominent in control soils than in anthropogenic soils.

Although the signals are not well resolved, the spectra indicate that the chemical shift region of O-aryl C (142−164 ppm) contains contributions not only from phenyl C, but also from syringyl and guaiacyl units, and from other lignin O-aryl signals. That was especially evident for the control samples that contain greater lignin character. The dominating signals in the chemical shift region of O-alkyl C (60−90 ppm) and di-O-alkyl C (90−109 ppm) derive predominantly from carbohydrates, although the contribution to the signal in this region of ethers and of alcohols from the propenyl side chains of lignins and tannins cannot be ignored (*37*). A hydrolysis effect from the HF/HCl treatment may be responsible for the relatively low contents (Table SI-2) of functionalities at these resonances. The clear signal in the carboxyl C region (156−186 ppm) indicates the high degree of oxidation of the samples.

The signal at 105 ppm, observed in the DD spectra, cannot be attributed exclusively to aryl-C in condensed tannin, as is cited in the literature (*40*), but also to ketose anomeric carbon. However, the latter is preserved after application of the CSA filter (Figure 1, right: AS-C) while the former is suppressed (Figure 1, right: control soil). This example shows the importance of different spectral editing techniques to allow the correct assignments of NMR signals.

The HA samples from anthropogenic soils presented higher contents of aryl C, both total and substituted-C, of O-aryl C, and of carboxyl C (Table SI-2). Additionally, the contribution to the control soils HA samples of the methoxyl C from lignin was greater, indicating that the aromatic region (aryl and O-aryl C) of control soils samples had larger contributions from lignin residues. Therefore, a larger proportion of O-aryl C of the control soils samples is not from phenolic groups, and consequently, is not contributing to the total acidity. The same conclusion can be drawn in relation to the carboxyl signal; that is, the control soils HAs have greater contributions from amide groups of protein, as indicated by the smaller C/N atomic ratio (Table SI-1) and the greater N-alkyl composition (Table SI-2). In this way, the ionisable oxygenated functional groups signals, which could contribute to CEC (carboxyl and O-aryl minus N-alkyl and methoxyl C), were larger for the anthropogenic samples (Table SI-2).

The high carboxyl C content in pyrogenic carbon derived HAs is well-known. However, artifact formation from the isolation and fractionation methods cannot be ignored, because the alkali-soluble fraction of these highly aromatic HAs requires the presence of hydrophilic groups (*21*).

The contribution of carbohydrates (O-alkyl and di-O-alkyl C) was larger for the control soils (Table SI-2). These carbohydrates, that resisted the HF/HCl treatment, were mainly in ketal form (Table SI-2), since the anomeric C signal remains in double filtered spectra (CSA + DD), indicating that it is an unprotonated C (*28*). The C substituted aryl groups in anthropogenic samples could be underestimated due to polycyclic aromatic structures undetected by CP-NMR (*16*) or to local anisotropic magnetic susceptibility that broadens the aryl signal. This broadening cannot be removed by MAS, making this signal undetectable, even by DP (*33*). However, the high correlation between aryl C and TGI ($R = 0.86$), and the aryl C and atomic H/C ratio ($R = -0.90$) indicate that the NMR data are, at least, semiquantitative. In addition, TGI presented negative correlations with labile groups, such as: alkyl (−0.73); N-alkyl/methoxyl (−0.83); and carbohydrates (−0.89). That indicates that this index is a good tool to estimate the degree of humification.

The first principal component (PC1) (Figure 2), calculated by PCA, accounted for 61% of the total variance and is characterized by positive loadings for the pyrogenic carbon aryl signal (broad featureless signal at 130 ppm) and negative loadings for the signals of carbohydrates, methoxyl, N-alkyl, alkyl (0−100 ppm), and O-aryl (151 ppm). The loadings of the PCA are the correlations between the original variables (spectra) and the principal components generated. In the present case, the increase of pyrogenic carbon derived compounds corresponds to a decrease of the contribution of the other compounds (carbohydrates, peptides, lignin etc). Should the contents of some components vary in a set of samples, that variation could be detected by PCA. Thus the PCA approach could be used to separate the components in a mixture. In this way, the positive loadings at 169 ppm can be attributed to carboxyl groups attached to pyrogenic carbon aromatic rings, and the negative loadings at 174 ppm to amide from proteins/peptides (*34*).

Thus, the larger the scores for this PC, the larger will be the contribution of pyrogenic carbon structures to the compositions of the HAs, and these structures are characterized by recalcitrant (aryl) and reactive groups (carboxyl). On the other hand, smaller scores indicate larger contributions of labile structures, and/or structures of lower degrees of humification, such as carbohydrates, proteins, and lignin. This PC separated the control soils samples with smaller scores (Figure 2), and it had positive correlations with TGI (0.82), O/H (0.82), and C/N (0.88) atomic ratios, and a negative correlation with the H/C (−0.90) atomic ratio. This confirms, by independent methods, that HAs from anthropogenic soils can be characterized with regard to high stability in terms of both structural (NMR and elemental composition) and thermal properties.

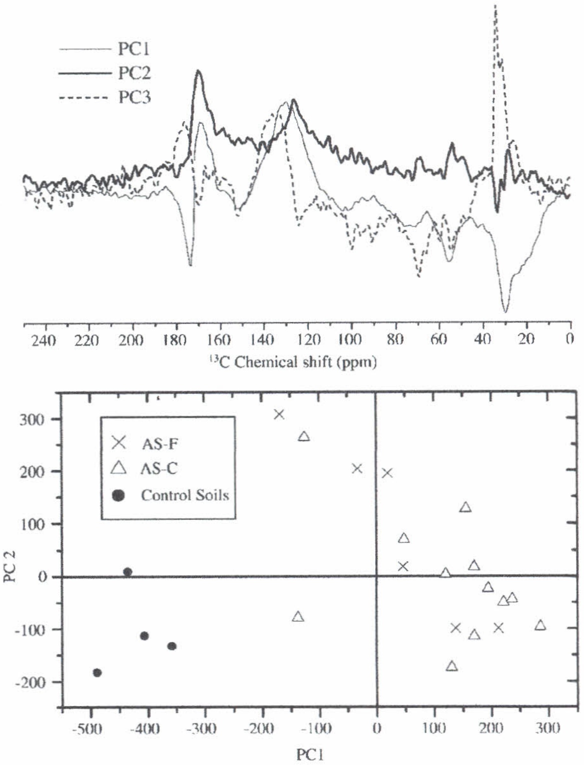

FIGURE 2. Upper: Loadings of PCA from full VACP spectra. Lower: Scores of PCA from full VACP spectra. PC1, 2, and 3: Principal components 1, 2, and 3; AS-F: anthropogenic soil under forest; AS-C: cultivated anthropogenic soil; control soils: non-anthropogenic soil.

The second principal component (PC2) accounted for 21% of total variance and is characterized mainly by a broad signal at 126 ppm and another at 170 ppm. The upfield shift of the aryl peak to 126 ppm is typical of charred residues attributable to polycyclic aromatic structures (*33, 37, 42*). The scores of this PC were smaller for the control soils, and varied within the anthropogenic soils group (Figure 2). The same tendency is observed for the first PC, indicating that the cultivation of AS does not alter significantly the quality of HAs and confirms the recalcitrance and resilience of HAs derived from pyrogenic carbon.

The third principal component (PC3) (Figure 2) accounted for 5% of total variance and is characterized mainly by sharp alkyl (33 ppm), aryl (136 ppm) and carboxyl/amide (177 ppm) signals. Due to negative loadings in the region of N-alkyl groups, this signal can be attributed to carboxyl. Thus these features indicate peripheral incorporation of fatty acids to the aromatic backbone (*7*). This PC discriminated just one sample (data not shown).

Despite the fact that there were not significant differences between the NMR spectra obtained by direct polarization and cross polarization (data not shown), and although there were not identifiable shifts of the aryl peaks at 126 ppm in the VACP spectra that would indicate the presence of polycyclic aromatic structures (*33, 37, 42*), there is convincing NMR evidence to corroborate the occurrence of polycondensed aromatic structures with carboxylic functionality in the ADE soil HAs. This evidence is provided by the identification, after PCA, of polycyclic aromatic structures with the typical aryl peak at 126 ppm. Further evidence is provided by the improved quality of the CSA spectra from applications of composite pulses. This indicates magnetic susceptibility heterogeneities, probably due to graphitic like structures (*33*).

From the loadings obtained by PCA, it was possible to separate different constituents of HAs, such as pyrogenic carbon-derived compounds (carboxylated aryl structures) and plant derived compounds (carbohydrates, proteins, and lignin). PCA of NMR data has permitted differentiation of functionalities from proteins and lignin from those of pyrogenic carbon. Our data indicate that HAs from anthropogenic soils have high contents of aryl and recalcitrant, ionisable, oxygenated functional groups (carboxyl and phenolic C), while the major functionalities in the HAs from adjacent non-anthropogenic soils are oxygenated functional groups, mainly from labile structures (carbohydrates, peptides, and lignin). That would suggest that the HAs from anthropogenic soils are more recalcitrant, and present more stable reactive groups. These differences can, at least in part, explain the higher and more sustainable fertility status of the anthropogenic soils, due the contribution of the OM to the soil CEC.

The data presented give evidence for the occurrence of pyrogenic carbon in the HA fraction of the SOM. The higher OM contents and the physical-chemical properties of the pyrogenic carbon probably have an important contribution to the higher CEC of these soils, and the ashes input contributed to the higher nutrients status. However, just the addition of nutrients would not guarantee the higher fertility, because these could be lost by leaching, for example, especially in the tropical regions. In this way, the combination of nutrient inputs (ashes) with better sorptive conditions (altered pyrogenic carbon) renders these soils more fertile than the similar surrounding soils without pyrogenic carbon inputs. That would indicate possibilities for a modern model of sustainable agriculture based in traditional knowledge.

The use of VACP NMR gave similar results to those obtained by direct polarization, and in a much shorter experimental time. The CSA filter technique, as proposed by Mao and Schmidt-Rohr (*28*), was not efficient for HA samples extracted from soils rich in pyrogenic carbon, probably as the result of their high magnetic susceptibility. However, the use of composite pulses for TOSS and the CSA filter was shown to be adequate for the study of samples rich in pyrogenic carbon.

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Supporting Information Available

Text giving further experimental details and additional information in the form of two tables and three figures, including a map displaying the sample sites, thermogravimetric characteristics, elemental compositions, and distribution of ^{13}C (%), determined by ^{13}C NMR, of humic acids extracted from Amazonian soils. This material is available free of charge via the Internet at http://pubs.acs.org.

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