PEDOSPHERE（土壤圈英文版）

Pedosphere ISSN 1002-0160/CN 32-1315/P doi:10.1016/S1002-0160(20)60019-4

Running Title: CHEMICAL AND SPECTROSCOPIC CHARACTERISTICS OF HA

Chemical and Spectroscopic Characteristics of Humic Acid from a Clay Loam Soil in Ontario after 52 Years of Consistent Fertilization and Crop Rotation

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ABSTRACT

Long-term fertilization and crop rotation can influence both organic carbon sequestration as well as the carbon composition of soils and the more resistant organic carbon compounds contained in humic acid (HA). This study examined the effects of fertilization (with/without) and crop rotation (continuous corn (CC), Kentucky bluegrass sod (SOD) and a corn-oats-alfalfa-alfalfa rotation (RC)) on the HA composition of soils from a 52-year field study in southern Ontario. HA samples were extracted from soils and elemental analysis, infrared spectroscopy, solid state C-13 nuclear magnetic resonance spectra and electron paramagnetic resonance methods were used to determine the influence of the crop management treatments on the characteristics of HA. Both fertilization and crop rotation affected the chemical characteristics of HA. Fertilization led to 5.9% increase in carbon, 7.6% decrease in oxygen and lower O/C and (N+O)/C ratios in HA as compared to the corresponding non-fertilized treatments. Rotation resulted in a lower proportion of C (48.1%) and a greater (N+O)/C ratio (0.7) relative to continuous cropping. Infrared spectroscopy analysis showed that HA contained more C-O groups in fertilized than non-fertilized CC and SOD treatments. Fertilization increased the O-alkyl-, phenolic-C and free radical contents of HA relative to non-fertilization

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treatments. Rotation decreased the aliphatic and carboxyl groups, and increased the O-alkyl, carbohydrate, aryl, phenolic groups and free radicals, relative to continuous corn and sod. Both long-term crop rotation and fertilization dramatically modified the soil HA composition of these soils. Significant relationships existed between the molecular composition of HA and SOC. Hence, humic acid characterization could be used as an indicator of the long-term sustainability of a crop management practice.

Key Words: rotation corn, fertilized treatment, bluegrass sod, continuous corn, structure characteristic

INTRODUCTION

Humic acid (HA) is the primary component of soil humic substances (HS) and plays a vital role in soil fertility and plant nutrition (Chen and Aviad, 1990; Schulten and Schnitzer, 1995). Small quantities of HA can enhance plant growth by improving the availability of micronutrients or lateral root initiation (Brady and Weil, 2008; Vaughan and Malcolm, 1985). The quantity and quality of soil HA may vary with agricultural practices. Fertilizer is an essential agricultural input around the world and the use of fertilizers have been found to increase soil organic matter (SOM) over time (Haynes and Naidu, 1998). Using a C-13 stable isotope technique, Zhang *et al.* (2012) found that long-term fertilizer addition to monoculture corn significantly increased the amount of newly formed soil organic C (SOC) but had little impact on the amount of native SOC in a previous study on this field site. Fertilizer addition could also affect the molecular structural characteristics of HA and this effect varies with fertilizer types. Chemical fertilizers could increase the contents of hydrogen in HA at the expense of the oxygen content (Zav'yalova, 2011). The combined application of chemical fertilizer and compost increased the O-alkyl- and aromatic-C contents relative to the application of chemical fertilizer only (Yanagi and Shindo, 2016). Soil amended with farmyard manure and chemical fertilizer increased the proportion of C in HA in a long-term field trial (Simonetti *et al.*, 2011). The use of farmyard manure can also increase the proportions of lignin, amino acids, peptides and proteins in soil HS (Ferrari *et al.*, 2011). Humification processes are very similar to geopolymerization by which the products of humification (HA) became more stable with advancing humification degree.

Crop rotation is a popular agronomic practice used to maintain crop health, improve soil quality, increase C inputs, the reactivity of SOM (Karlen *et al.*, 1997; Reeves, 1997; Wander and Traina, 1996) and decrease environmental impacts such as N_2O emissions (Drury *et al.*, 2008). Crop residues added to soil strongly influenced SOM quality and quantity (Samahadthai *et al.*, 2010). Changing crop types in rotation not only differentiates the amount of residues added but also the types of materials that decompose in soils. In a rice-wheat rotation agroecosystem, Zhang *et al.* (2011) found that applying maize residues increased the O-alkyl-C and aromatic-C contents of HA. In western Canada, the quality of SOM was found to be greatly affected by rotation in a barley system which was under-seeded with/without alfalfa (Campbell *et al.*, 1992).

Both rotation and N fertilizer rate significantly affected total soil C and N concentrations (Varvel *et al.*, 1994). The well-fertilized continuous-type rotations also had a 13% greater C content for soil than continuous wheat receiving only P fertilizer (Biederbeck *et al*., 1984). Long-term effects of crop rotation and fertilization were mostly observed with respect to the quantity of SOC (Kaiser *et al*., 2007; Omay *et al*., 1997). However, there are few studies examining the effects of fertilizer, rotation and their combination on the quality of SOC fractions such as HA. A 52-year field experiment in Woodslee, Ontario, Canada provides a unique opportunity to assess the impacts of long-term fertilization and crop rotation on the chemical nature of HA in these soils that resulted in dramatically differences in crop yields (Drury and Tan, 1995). Numerous studies have been conducted on this field trial to investigate the effects of long-term fertilization and crop rotation on soil structure and water desorption characteristics (McKeague *et al.*, 1987), denitrification and soil carbon (Drury *et al.*, 1998), C residue retention and chemical composition of SOM (monoculture corn only, Gregorich *et al.*, 2001), water and nitrate loss (Tan *et al.*, 2002), carbon retention (Zhang *et al.*, 2012), and water and nitrate partitioning (Woodley *et al.*, 2018), but this is the first time that the effects of these treatments on the compositions of HA has been investigated. The molecular structure of HA is a source of still unknown information (Baveye and Wander, 2019).

HA is one of the most structurally complex pools of the SOM. The chemical composition of SOM plays an important role in the storage of C (Jiménez-González *et al.*, 2019). Therefore, it is reasonable to hypothesis that the stabilization of SOC depending on the chemical composition of HA. And, the molecular composition of HA differs from fertilization and crop rotation. Therefore, the objectives of this paper are (1) to examine the influences of long-term consistent cropping rotation and fertilization on the characteristics of HA in the soil; (2) to test the relationship between SOC and the structural characteristic of HA. This work represents a more complete structural analysis of SOC, N, and the chemical characteristics for HA under different agricultural management practices which profoundly influence crop growth and yields.

MATERIALS AND METHODS

Experimental field and soil sample collection

Soil samples (Brookston clay loam soil: Orthic Humic Gleysol in the Canadian System of Soil Classification, poorly drained, fine-loamy, mixed, mesic Typic Argiaquoll in USDA Soil Taxonomy) used in this study were collected from a long-term cropping study on the Hon. Eugene F. Whelan Experimental Farm, Woodslee, Ontario (42 ° 13′ N, 82 ° 44′ W), 52-years after the experiment was initiated in 1959. This experimental site was converted to farmland from native grasses and mixed deciduous forest (C3-plants) in the middle to late 1800s. The land was used for corn (*Zea mays L.*), alfalfa (*Medicago sativa L.*), and red clover (*Trifolium pretense L.*) production from 1940 to 1954 and for continuous corn from 1956 to 1958. In 1959, 12 plots (each 76.2 m long by 12.2 m wide) were established and fertilization (with/without) and crop rotation were investigated (Fig. 1). The two fertilizer treatments were split into 6 plots with one plot seeded to continuous corn, one plot seeded to continuous Kentucky bluegrass (*Poa pratensis L.*) sod, and the remaining four plots seeded to corn-oat (*Avena sativa*)-alfalfa-alfalfa rotation with all four phases of the rotation grown every year (4 replicates). The fertilized treatments received 16.8 kg N ha^{-1} (NH_4NO_3), 29.3 kg P ha^{-1} (P_2O_5), and 27.9 kg K ha^{-1} (K_2O) per year at planting for all crops in rotation and an additional 112 kg N ha^{-1} (NH_4NO_3) per year for corn as a side-dress application. The non-fertilized treatments did not receive any mineral or organic fertilizer except for atmospheric deposition during the entire experimental period. Each plot was drained by a single tile (10 cm diameter, 70 cm average depth) running along the plot's longitudinal axis. All agronomic practices have remained the same during the entire period except for changes in plant varieties and populations. For more detailed soil and agronomic information on this site please see Stone *et al.* (1987), Drury and Tan (1995) or Woodley *et al.* (2018).

Fig. 1 The experimental design of cropping treatments in the field. Plot 1, corn phase of chemically fertilized (-F) corn–oat–alfalfa–alfalfa rotation; Plot 2, oat phase of fertilized rotation; Plot 3, chemically fertilized monoculture corn; Plot 4, first-year alfalfa of fertilized rotation; Plot 5, second-year alfalfa of fertilized rotation; Plot 6, chemically fertilized bluegrass sod; Plot 7, second-year alfalfa of unfertilized rotation; Plot 8, oat phase of unfertilized rotation; Plot 9, unfertilized monoculture corn; Plot 10, corn phase of unfertilized rotation; Plot 11, unfertilized monoculture sod; and Plot 12, first-year alfalfa of unfertilized rotation.

This study focuses on 6 treatments, including the fertilized continuous corn (CC-F), continuous Kentucky bluegrass sod (SOD-F) and rotation corn (RC-F, the corn phase) and non-fertilized continuous corn (CC-NF), rotation corn (RC-NF, the corn phase) and continuous Kentucky bluegrass sod (SOD-NF) treatments. The soil pH values of these 6 treatments are 6.98, 6.04, 6.17, 7.57, 6.69 and 6.55, respectively. The soil has an average texture of 280 g sand kg^{-1}, 350 g silt kg^{-1}, 370 g clay kg^{-1}. The mean 30-year annual air temperature is 8.9 ±0.4 ℃ and the annual precipitation is 832 ±257.11 mm.

From each plot, three replicate samples (0--20 cm) were collected along a transect midway between the tile (the center of the plot) and the plot edge to ensure that the samples were representative of the treatment and not unduly influenced by the tile or the plot edge. The samples were passed through a 2 mm sieve to make them homogeneous and then air-dried. Soil subsamples were finely ground (< 0.15 mm) and analyzed for total C and N content determination using a LECO-CN2000 analyzer (LECO Corporation, MI, USA). Because these soils are free of carbonates, the total C content was equivalent to the SOC content.

Extraction of HA and chemical analysis

HA was extracted from the bulk soils using the methodology published by the International Humic Substance Society (Swift, 1996). Briefly, the soil samples of 500 g (< 2 mm) were pre-treated with 250 mL of 1 mol/L HCl, extracted with 200 mL of 0.1 mol/L NaOH solution under a N_2 atmosphere, shaken for 24 h and centrifugated at 5000 × *g*. Both HCl (0.1 mol/L, 100 mL) and HF (0.3 mol/L, 50 mL) were used to mix with HA to remove silicate. The samples were then decanted and dialyzed to purify the HA. To determine if HCl and HF were removed during the dialysis procedure, subsamples of the HA mixture were tested by adding 0.1 mol/L $AgNO_3$. When Cl^- was still present, a precipitate formed. The dialysis was terminated when no precipitate formed which indicated all of the HCl was removed. The HA was freeze-dried and then ground to pass through a 0.25 mm sieve, and stored in airtight bottles. The quantity of the HA from these soil samples ranged from 1.5 to 3.9 g kg^{-1} soil. Elemental composition including

C, H, and N in the HA was measured using a Vario EL III elemental analyzer (Elementar, Germany). The O content was estimated as the mass difference, O%=100 - C% - H% - N%. Ash contents of the six treatments ranged from 0.01--1.83% and this was determined by ignition at a burning temperature of 750 ℃ in a muffle furnace for 3 hours.

Fourier Transform Infrared (FTIR) microspectroscopy of HA was performed in the range of 400--5000 cm^{-1} with 128 co-added scans at 8 cm^{-1} resolution using a Nicolet 750 micro-infrared instrument with KBr beam-splitter and a MCT/A detector (Thermo Scientific, Madison, WI). Precaution was taken to avoid moisture uptake.

Nuclear magnetic resonance spectra (C-13 NMR) of HA were obtained using a Bruker Avance III 400 instrument (Bruker, Switerland), equipped with a 4 mm probe at a spinning speed of 5 KHz and frequency of 100.37 MHz for C-13. The line broadening was 100 Hz. Acquisition conditions were 3000 μs contact time, 2 s recycle time and the scan number more than 10,000. The spectra were acquired by using C-13 cross-polarization/ total sideband suppression NMR spectroscopy (Mao *et al.*, 2012).

Electron paramagnetic resonance (EPR) measurements were performed using an A300 spectrometer (Bruker, America) with 9.519 GHz microwave frequency and 100 kHz modulation on 20 mg of HA enclosed in a quartz capillary.

Statistical analyses

A one-way ANOVA model was used to test whether differences in HA existed among fertilization and rotation treatments. Statistical analyses were performed using the SAS statistical software (SAS Institute, Cary, NC), and significance is reported at the 0.05 probability level. A least significant difference test (LSD) was conducted on the means when significant main effects occurred. The relationships between the SOC and the chemical compositions of the HA were examined using the Pearson correlation coefficient (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

SOC contents and elemental compositions of HA

There were significant differences in SOC concentrations among the treatments (Table I). After a half century of cropping practices, the soil under Kentucky bluegrass management contained 46.0 g kg^{-1} of SOC, which was 89% greater than the soils in rotation corn (24.3 g C kg^{-1}) and 133% greater than in continuous corn (19.7 g C kg^{-1}). Significantly greater amounts (about 20%) of SOC occurred in fertilized (32.74 g kg^{-1}) than in non-fertilized (27.30 g kg^{-1}) treatments over all three crop types. These results indicate that both long-term rotation and fertilization increased the SOC of the soils. More SOC in fertilized than in non-fertilized treatments may be due to higher crop biomass returns (stover and roots) which was a direct reflection of 2.7 times more corn grain yield (15.5% moist) in fertilized (8330 kg ha^{-1} yr^{-1} for RC and 5630 kg ha^{-1} yr^{-1} for CC) than in non-fertilized treatment (4160 kg ha^{-1} yr^{-1} for RC and 1040 kg ha^{-1} yr^{-1} for CC) at 15.5% moisture content based on the first 45 years of data. The soil total N concentrations responded to cropping practices in a similar manner as the SOC, with more N in the grass treatment (5.75 g N kg^{-1}) compared to rotation corn (2.98 g N kg^{-1}) and continuous corn treatments (2.78 g N kg^{-1}).

TABLE I

SOC and total nitrogen contents in the 0--20 cm depth and analysis of variance of fertilization (F) and rotation (R) effects

Treatments[a]	C			N		
	F	NF	Mean	F	NF	Mean
	g C kg^{-1} soil			g N kg^{-1} soil		
CC	21.37 ± 3.04	18.11 ± 1.66	19.74c[c]	3.02 ± 0.16	2.54 ± 0.45	2.78b
SOD	50.15 ± 6.53	41.84 ± 2.11	46.00a	6.94 ± 1.84	4.56 ± 0.04	5.75a
RC	26.70 ± 2.84	21.94 ± 3.28	24.32b	3.54 ± 0.71	2.42 ± 0.47	2.98b
Mean	32.74A[b]	27.30B		4.50A	3.18B	
ANOVA	*F value*	*Pr > F*		*F value*	*Pr > F*	
Fertilization (F)	91.66	<0.0001		23.01	<0.0001	
Rotation (R)	10.37	0.0074		10.99	0.0062	
F × R	0.78	0.4810		1.95	0.1849	

[a] CC: continuous corn; RC: rotation corn; SOD: monoculture Kentucky bluegrass sod; F: fertilization; NF: non-fertilization. Values are mean ± standard deviation;

[b] A-B: Means followed by different upper-case letter under the same soil property in the same row are significantly different from each other;

[c] a-c: Means followed by different lower-case letter in the same column are significantly different from each other;

Concentrations of C, N and O and the atomic ratios of HA were significantly affected by crop rotation, fertilizer application and their interactions (Table II, Fig. 2). The concentrations of C in HA were generally higher in fertilized than non-fertilized treatments, with 53.9% of C in CC-F versus 48.7% of C in CC-NF, 51.3% of C in SOD-F versus 49.9% of C in SOD-NF. The C concentrations of HA in rotation corn were similar for fertilized and non-fertilized treatments (48.7% -- 47.4%) (Fig. 2). These results are consistent with Zhang *et al.* (2011)'s findings that the crop biomass inputs in combination with fertilizer application can increase SOC contents in the bulk soil.

Fig. 2 Elemental composition and atomic ratios of HA extracted from soils under different fertilization and cropping management (ash-free). The vertical bar is the standard deviation (n=3). Means followed by the same lower-case letter are not significantly different from each other between the cropping types under the same fertilization treatment. Means followed by the same upper-case letter are not significantly different from each other between fertilization and non-fertilization under the same cropping.

TABLE II

Statistical analysis (*F* value and *P* level) of fertilization (F) and rotation (R) on the elements and the atomic ratios of soil humic acids

	C	H	N	O	H/C	O/C	C/N	(N+O)/C
R	$14.9^{***\ a)}$	0.070	0.487^{***}	13.9^{***}	0.008	0.011^{**}	7.17^{***}	0.011^{*}
F	37.2^{***}	0.228	0.092^{***}	49.2^{***}	0.000	0.022^{***}	0.920^{**}	0.022^{*}
R×F	10.5^{***}	0.093	0.184^{***}	13.2^{***}	0.004	0.010^{**}	0.913^{***}	0.011^{*}
Error	0.175	0.108	0.011	0.215	0.026	0.007	0.055	0.012

[a)] The data in the table represent F values; *, **,*** were the significant level at $P = 0.05$, 0.01, and 0.001, respectively.

For the fertilized treatments, the soil under rotation corn had a greater N concentration (4.8%) in HA relative to continuous corn (4.3%) and monoculture sod (4.5%), and there were significant differences in N concentrations of HA between corn in rotation and continuous corn. The N concentrations of HA in non-fertilized soils were greater in the SOD (4.8%) than in RC (4.5%) and CC (4.0%) treatments (Fig. 2). In contrast to the SOD which contained a greater N concentration in non-fertilized soil than the fertilized soil, the fertilized soils under CC and RC management had greater N concentrations than the corresponding non-fertilized soils under CC and RC. Higher N concentrations of HA in fertilized corn compared to non-fertilized corn treatments could be a direct reflection of fertilizer N inputs (Aleksandrova, 1980). However, we cannot explain why this N input did not show up in the SOD treatment and this is worthy of further study.

The C/N ratios of HA range from 11:1 to 15:1, with the highest for the fertilized continuous corn (14.6) and lowest for the fertilized rotation corn (11.8) in Fig. 2. According to Tan (2003), the HA with C/N ratios in these ranges are at the well-developed equilibrium stage of decomposition and accumulation of SOM which is not surprising since these treatments have been in place for 52 years. The lower C/N ratio associated with fertilized RC treatment could be attributed to N enrichment from both chemical fertilization and N_2 fixation by the alfalfa (Liu *et al.*, 2010). The ratio of H/C may illustrate the aliphaticity and sources of organic matter in soil (Zhao and Lv, 2012); however, the concentrations of H and the ratios of H/C were not significantly different among the treatments (Fig. 2). The highest C/N ratio and lowest H/C ratio (1.0) for CC-F suggested that the HA in this soil had the highest degree of condensation. Lower contents of O were found in the fertilized (37.1% and 41.8%) relative to non-fertilized CC and RC treatments (43.8% and 43.8%), which was consistent with the findings of other studies (Senesi and Brunetti, 1996; Zav’yalova, 2011). Since the O/C ratio may represent the degree of oxidation (Zav’yalova and Konchits, 2011), the smallest O/C ratio (0.5) for the HA in fertilized continuous corn treatment may indicate that the HA associated with this treatment contained more newly formed organic materials relative to the HA in other treatments (Dou *et al.*, 1992). Song *et al.* (2009) noted that the greater the ratio of (N+O)/C, the higher the polarity of HA. Given this knowledge, the HA had a higher polarity in non-fertilized than in fertilized soils.

Micro-infrared analysis

There were many similarities in the IR spectra among all HA samples (Fig. 3), the major bands were assigned as follows (Bellamy, 1975; MacCarthy *et al.*, 1985; Stevenson, 1982): a broad and rather intense band at 3366 cm^{-1} represented stretching vibrations of OH in phenols, acids and alcohols (Zhang *et al.*, 2011). It is worth of noting that water vapor in ambient air may have enhanced this peak. A band at 2934 cm^{-1} was attributed to the stretching vibration of the aliphatic asymmetric C-H group (Mao *et al.*, 2008). In addition, all HA had spectral characteristics in the wavenumber range of 2000 to 500 cm^{-1}, including: (1) COOH band with high intensities around 1711 cm^{-1} (carboxyl groups, Stevenson, 1972), (2) bands at 1658 cm^{-1}, ascribed to carbonyl C=O of quinone, (3) bands around 1453 cm^{-1}, attributed to C-H deformation of CH_3, and C-H bending of CH_2^-, and (4) the peaks near 1245 cm^{-1}, probably due to C-O stretch vibration in alcohols, phenols and carboxyl groups. Moreover, the peak at 1081 cm^{-1} was associated with the presence of carbohydrates (Zhang *et al.*, 2011). In general, differences in the IR spectra of HA between fertilizer and rotation treatments were observed and summarized in Table III, which showed changes in the ratios between the main absorbance peaks (Amir *et al.*, 2010). Higher ratio of 1658/1711 was obtained in RC-F treatment as compared to CC-F treatment ($P < 0.05$). And, the lower ratios of 1081/1245 in CC-F and SOD-F treatment were determined as compared to the non-fertilized treatments. The relative intensity of the IR absorbance peaks may indicate that rotation affects carboxylic groups (1658/1711), while fertilization has more impact on carbohydrates (1081/1245).

Fig. 3 Micro infrared spectra of HA extracted from soils under different fertilization and cropping treatments. F refers to treatments which are fertilized and NF to treatments that have not received fertilizers since 1958.

TABLE III

Ratios between main absorbance peaks of IR spectra of humic acids extracted from soils under different fertilization and crop management

		Ratios of IR peaks						
Treatments [a]		1658/3366[d]	1658/2934	1658/1711	1658/1453	1658/1245	1081/2934	1081/1245
CC	F	1.56A[c]a[b]	1.99 Aa	1.01Bb	1.81Aa	1.43Bb	0.57Ab	0.40Bc
	NF	1.48Aa	1.95 Aa	1.04Ac	1.89Ab	1.55Ab	0.64Aa	0.51Ab
SOD	F	1.54Aa	1.97 Aa	1.14Ba	1.79Ba	1.51Ba	0.64Ab	0.50Bb
	NF	1.55Aa	2.03 Aa	1.21Aa	2.03Aab	1.70Aa	0.71Aa	0.62Aa
RC	F	1.52Aa	2.12 Aa	1.14Aa	1.89Ba	1.56Ba	0.78Aa	0.57Ba
	NF	1.57Aa	2.13 Aa	1.16Ab	2.08Aa	1.70Aa	0.77Aa	0.62Aa

[a] CC: continuous corn; RC: rotation corn; SOD: monoculture Kentucky bluegrass sod; F: fertilization; NF: non-fertilization;

[b] Means followed by the same lower-case letter are not significantly different from each other between the cropping types under the same fertilization treatment;

[c] Means followed by the same upper-case letter are not significantly different from each other between fertilization and non-fertilization under the same cropping;

[d] The ratio refers to the IR intensity ratio of 1658 cm^{-1} and 3366 cm^{-1}.

C-13 NMR and EPR analysis

Fig. 4 and Table IV show solid-state C-13 NMR spectra of six HA samples examined with major signal intensities at 31, 57, 73, 103, 130, and 175 ppm, following in the pattern of six main chemical shift regions of the NMR spectra described by Marche *et al.* (2003). All spectra exhibited narrow and intense signals at 31--32 ppm, which were most likely due to unsubstituted saturated aliphatic-C from long-chain fatty acids, surface waxes, lipids, resins, protein-like structures or polymethylene in lipids, belonging to amorphous and crystalline CH_2 groups (Chen and Chiu, 2003; Tinoco *et al.*, 2004). Among all 6 treatments, the RC-F had the smallest amount of aliphatic structures (Table IV). The structures of HA tended to be more complicated under fertilization and rotation (Song *et al.*, 2018). The peaks at 56-58 ppm can be attributed to recalcitrant O-alkyl-C in lignin (Chen and Chiu, 2003), and the low signals for all samples may indicate low lignin content in HA (Oades *et al.*, 1987). The ring and anomeric-C of carbohydrates occur at 73 ppm and 103 ppm, respectively (González-Vila *et al.*, 1983). The peak at 103 ppm is also characteristic of non-protonated carbons from tannins (Preston *et al.*, 1997; Mao *et al.*, 2008), and in this regard there was more non-protonated aromatics occurring in the fertilized than in non-fertilized treatments in the current study. High signal of carbohydrates in the NMR spectra was also shown by relatively high signal peaks in IR spectra (Fig. 3). The peak at 175 ppm may come from quaternary carboxyl-C. HA with high carboxyl-C could have a positive effect on soil aggregation (Nègre *et al.*, 2002). The spectroscopic data indicate that aliphatic-C, carbohydrate-C, aryl-C and carboxyl-C were the major components of HA, and the HA have greater aliphaticity than aromaticity for the six treatments. This result was consistent with the finding of Zhao and Lv (2012) from an alluvial soil with 23 years' fertilization management.

Fig. 4 Solid-state C-13 NMR spectra of HA extracted from soils under different fertilization and cropping managements. F refers to treatments which are fertilized and NF to treatments that have not received fertilizers since 1958.

TABLE IV

Distribution of different C species in humic acids as determined using C-13 NMR spectroscopy

Type of C	Chemical shift	CC-F	CC-NF	SOD-F	SOD-NF	RC-F	RC-NF
aliphatic-C	δ0-40	22.19	28.58	24.81	22.09	19.48	21.07
O-alkyl-C	δ41-60	9.04	6.87	9.08	8.83	11.89	9.52
carbohydrate-C	δ61-105	17.31	15.68	16.66	18.48	19.18	18.73
aryl-C	δ106-150	28.16	25.35	26.36	26.43	30.77	26.83
phenolic-C	δ151-170	6.90	4.06	4.94	4.00	10.69	5.85
carboxyl-C	δ171-190	16.40	19.45	18.15	20.17	7.99	18.01
Aliphaticity a)	C%(δ0-105)/C%(δ0-170)	58.06	63.48	61.76	61.88	54.94	60.15
Aromaticity b)	C%(δ106-170/C%(δ0-170)	41.94	36.51	38.24	38.12	45.06	39.85

a) Aliphaticity= C% (δ0-105)/C% (δ0-170).

b) Aromaticity =C% (δ106-170)/C% (δ0-170).

The NMR analysis indicates that fertilization resulted in higher aromaticity of HA than non fertilization (Table IV). Since aryl-C is recalcitrant (Adani and Spagnol, 2008), the greater amount of aryl-C in the HA from the soil under RC-F management indicates that these HA samples are more stable relative to the HA extracted from other treatments. This suggested that RC-F management could be better than other treatments in improving soil structural stability. The stability of the HA in RC-F was also demonstrated by more phenolic-C and less carboxyl-C contents relative to the HA in other treatments. At the same time, more aryl-C in HA in fertilized rotation treatments than other treatments. Aryl-C belonged to hydrophobic component which had an essential role in controlling the processes of organic carbon sequestration (Song *et al.*, 2013).

Correlation analysis of properties among each other showed that the amounts of SOC in soils had a significantly ($P < 0.01$) negative correlation with the ratio of HA's IR intensities at 1658 cm^{-1}/3366 cm^{-1} (Fig. 5). This indicated that significant relationships existed between the molecular composition of HA and SOC. The increase of OH group or the decrease of C=O may promote the increase of SOC. Therefore, phenols, acids and alcohols were more characteristic of HA from soil with high SOC content. The band assigned to quinone was characteristic in HA from soils with low SOC content. Other factors such as the ratios of 1081/2934 and 1081/1245 were significantly ($P < 0.01$) negatively correlated with the content of C in HA. O, O/C, (N+O)/C and 1658/1711 also had a significantly ($P < 0.05$) negatively relationship with the content of C for HA. Therefore, the C content was affected by carbohydrates, aliphatic groups, alcohol, phenol, carboxyl groups and quinone.

Fig. 5 Correlation coefficient of the SOC and chemical composition of HA. Brown signifies positive correlation, and blue means negative correlation. This picture was drawn with matlab software.

The EPR spectra of HA contained single, symmetrical resonance line for different treatments (Fig 6). The g-factor in the 2.0064--2.0068 range was attributed to oxygen centered radicals, such as semiquinone radical anions (Zhao *et al*., 2018). Hence, it is a useful parameter for identifying the type of free radicals which are present. Relative to non-fertilized treatments, fertilization resulted in an obvious increase in the peak intensity (Fig. 6). The oxidization of phenols to quinones caused the higher content of free radicals (Song *et al*., 2017). This is consistent with higher aromaticity, phenolic-C and O-alkyl-C in Table IV, as will be expected for the less highly oxidized soil. (González Pérez *et al*., 2004; Song *et al*., 2017).

Fig. 6 EPR spectra of HA. F refers to treatments which are fertilized and NF to treatments that have not received fertilizers since 1958.

CONCLUSIONS

Long-term fertilization and rotation management changed the composition, structure and spectroscopic characteristics of soil HA. Fifty two years of continuous fertilization increased the contents of SOC and total N relative to non-fertilization. In addition to the impacts on C, N and O contents of HA, crop rotation (with/without fertilizer addition) strongly affected the C/N ratios of HA and fertilization significantly influenced O/C ratios. Relative to fertilization, non-fertilization treatment had higher O and lower C concentrations. Fertilization also led to higher aromaticity of HA with more O-alkyl-C and phenolic-C. Abundant aliphatic-C groups were associated with HA in continuous corn treatment, while higher O-alkyl-, carbohydrate-, aryl-, phenolic-C groups and free radicals, and less carboxyl-C were associated with rotation corn treatment. Hence, corn under a 4-year crop rotation with oats and alfalfa was found to have more stable C in the HA fraction compared to continuous corn. Crop rotation plus fertilization led to structurally complicated organic materials, which could favor the sequestration of organic C. And the increase of OH group or the decrease of C=O in HA may promote the increase of SOC. Consequently, crop rotation should be considered as an effective way to preserve humic substances in agricultural soil, especially when fertilizer is also applied. Therefore, HA composition could be used as an indicator for the relative C sequestration ability and soil quality of a soil and crop management practice.

ACKNOWLEDGMENTS

We would like to express our appreciation to Agriculture and Agri-Food Canada for maintaining the long-term plots. We also like to thank Mary-Anne Reeb for providing technical assistance in humic acids extraction. Other lab analyses were supported by the National Natural Science Foundation of China (No. 41571317, No. 41807113), Natural Science Foundation of Guangdong Province (No. 2018A030313940), and Twelfth five-year National Science and Technology support project for cycling of agricultural science and technology project (No. 2012BAD14B00).

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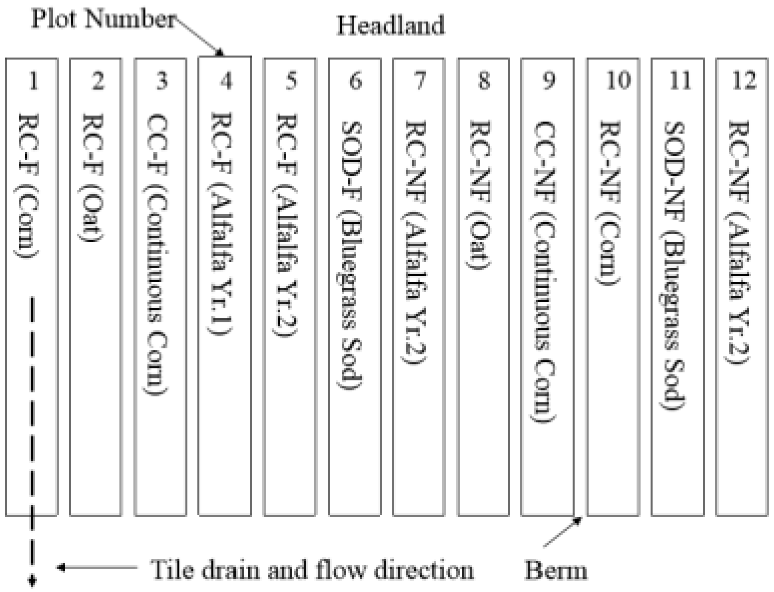
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2
RC-F (Oat)
3
CC-F (Continuous Corn)
4
RC-F (Alfalfa Yr.1)
5
RC-F (Alfalfa Yr.2)
6
SOD-F (Bluegrass Sod)
7
RC-NF (Alfalfa Yr.2)
8
RC-NF (Oat)
9
CC-NF (Continuous Corn)
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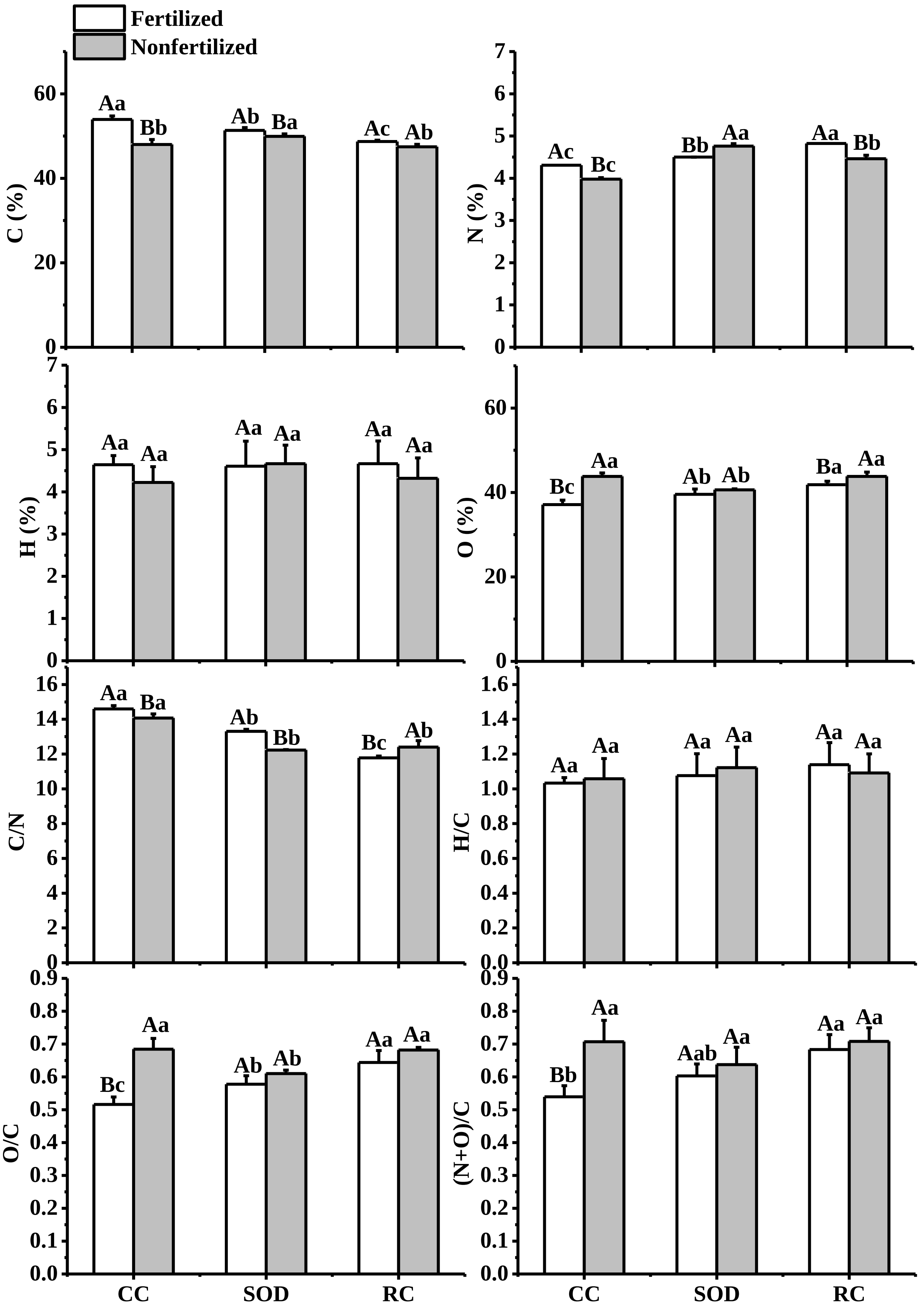
RC-NF (Corn)
11
SOD-NF (Bluegrass Sod)
12
RC-NF (Alfalfa Yr.2)
Tile drain and flow direction
Berm

Fig. 1


Fertilized
Nonfertilized
C (%)
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20
40
60
Aa
Bb
Ab
Ba
Ac
Ab
N (%)
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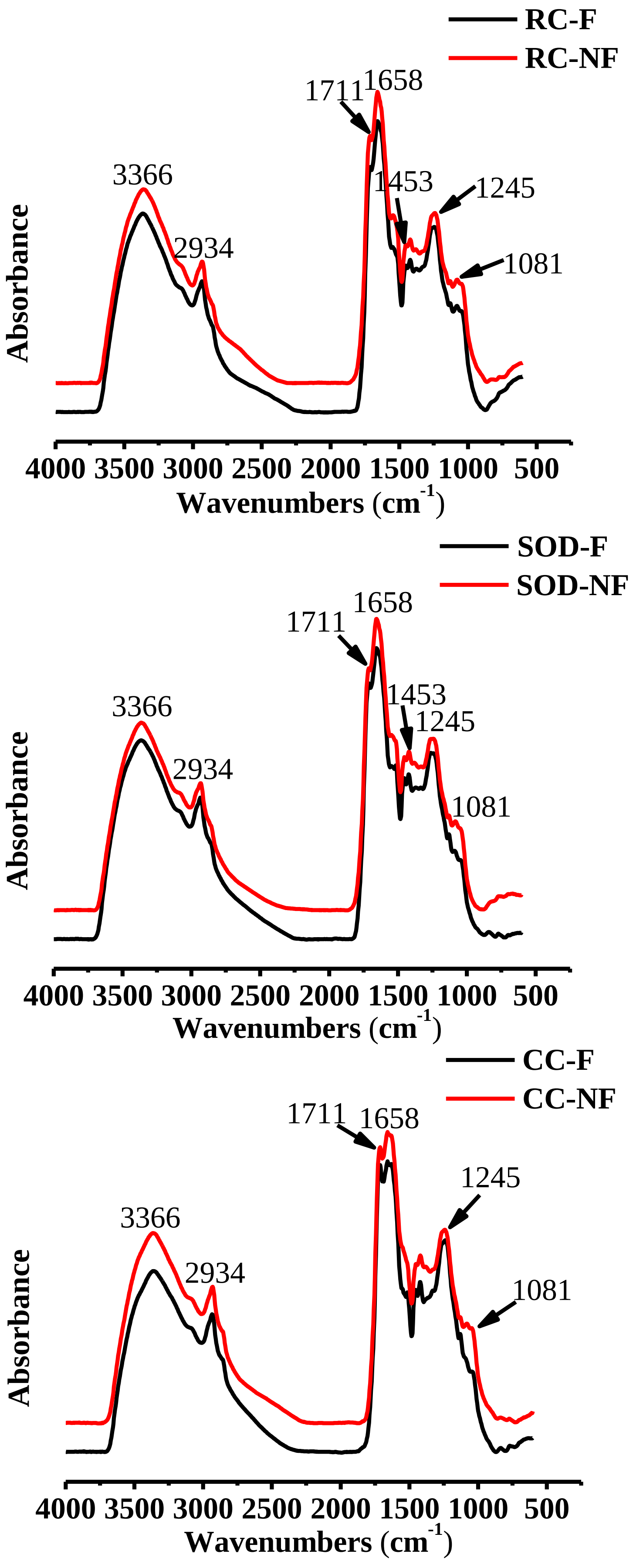
1
2
3
4
5
6
7
Ac
Bc
Bb
Aa
Aa
Bb
H (%)
0
1
2
3
4
5
6
7
Aa
Aa
Aa
Aa
Aa
Aa
O (%)
0
20
40
60
Bc
Aa
Ab
Ab
Ba
Aa
C/N
0
2
4
6
8
10
12
14
16
Aa
Ba
Ab
Bb
Bc
Ab
H/C
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Aa
Aa
Aa
Aa
Aa
Aa
O/C
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Bc
Aa
Ab
Ab
Aa
Aa
(N+O)/C
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Bb
Aa
Aab
Aa
Aa
Aa
CC
SOD
RC
CC
SOD
RC

Fig. 2


RC-F
RC-NF
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Absorbance
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Wavenumbers (cm^{-1})
SOD-F
SOD-NF
1711
1658
3366
1453
1245
2934
1081
Absorbance
4000 3500 3000 2500 2000 1500 1000 500
Wavenumbers (cm^{-1})
CC-F
CC-NF
1711
1658
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Absorbance
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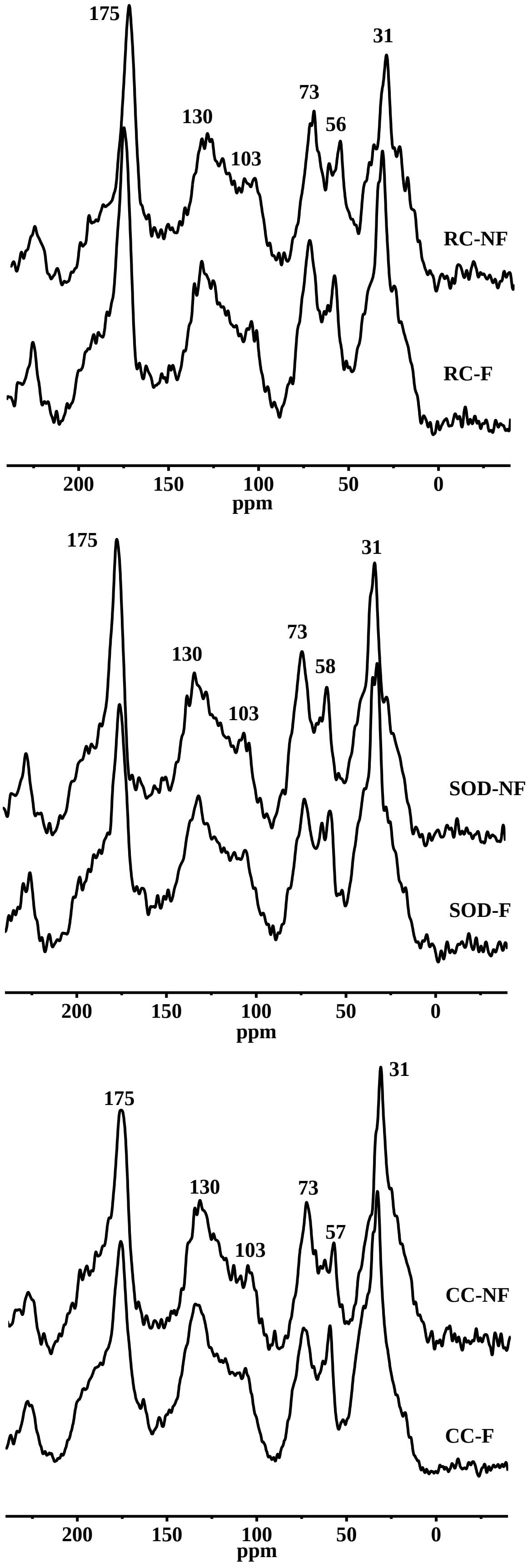
Wavenumbers (cm^{-1})

Fig. 3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RC-NF
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ppm
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73
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58
103
SOD-NF
SOD-F
200
150
100
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ppm
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CC-NF
CC-F
200
150
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50
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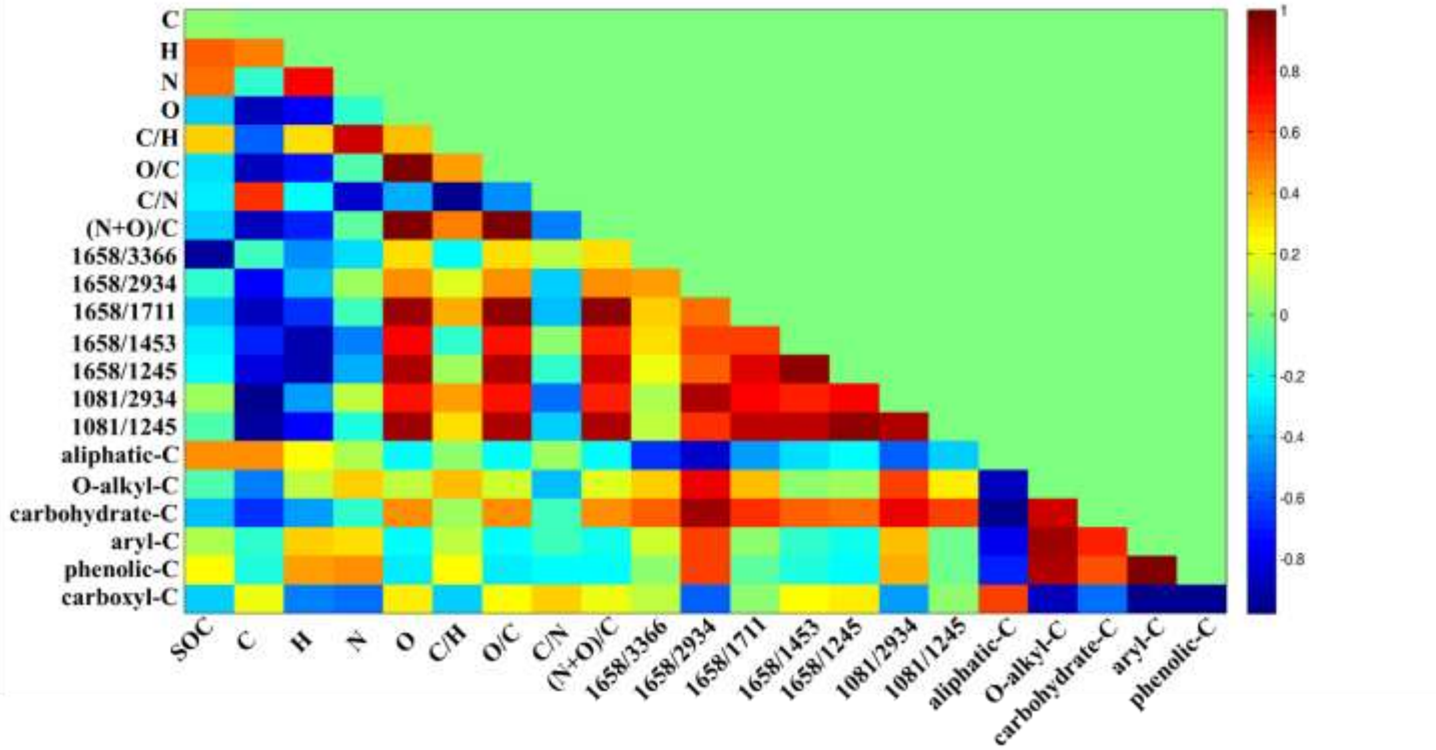
ppm

Fig. 4


C
H
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C/H
O/C
C/N
(N+O)/C
1658/3366
1658/2934
1658/1711
1658/1453
1658/1245
1081/2934
1081/1245
aliphatic-C
O-alkyl-C
carbohydrate-C
aryl-C
phenolic-C
carboxyl-C
SOC
C
H
N
O
C/H
O/C
C/N
(N+O)/C
1658/3366
1658/2934
1658/1711
1658/1453
1658/1245
1081/2934
1081/1245
aliphatic-C
O-alkyl-C
carbohydrate-C
aryl-C
phenolic-C
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-0.8

Fig. 5

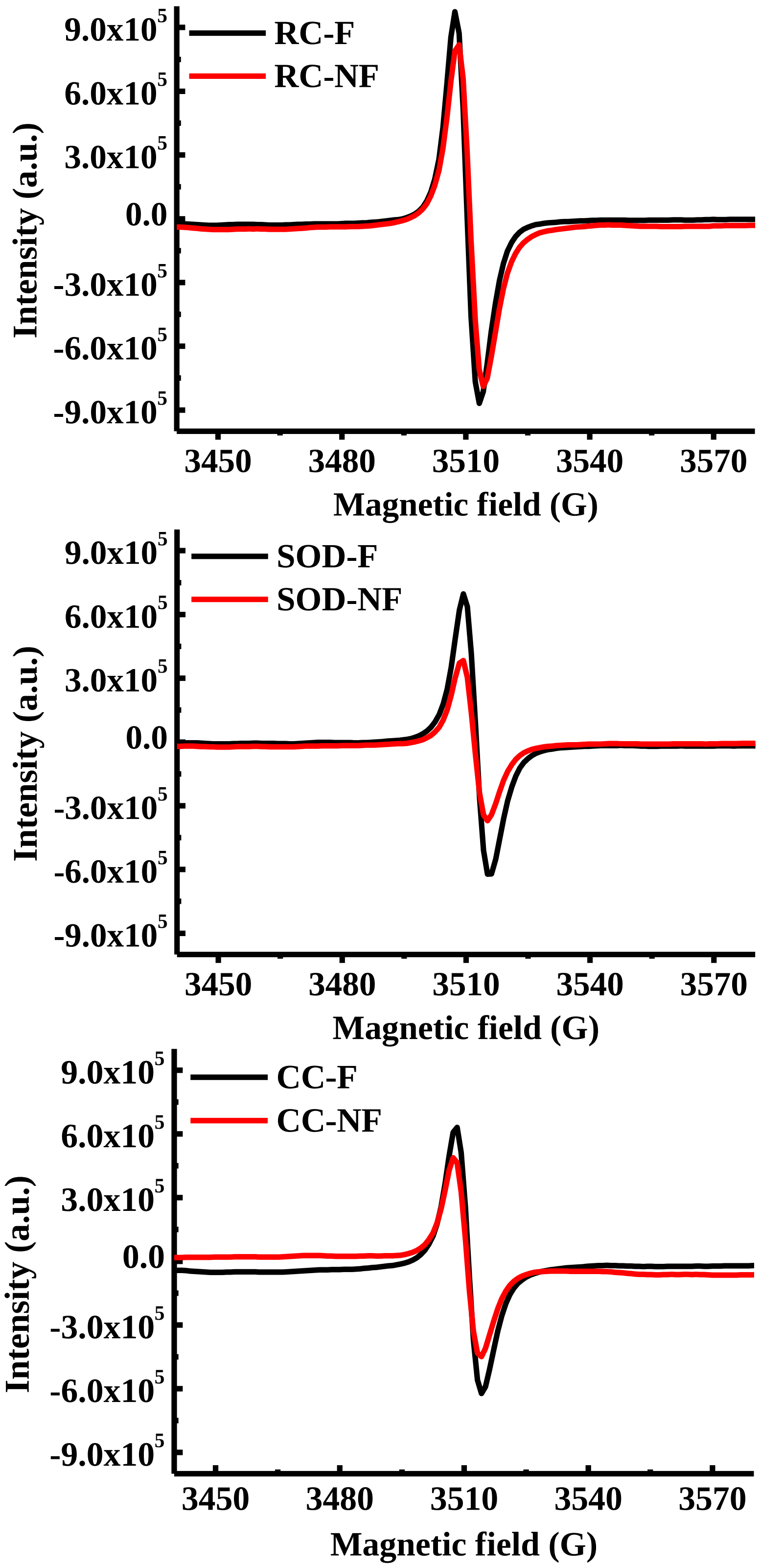
RC-F
RC-NF
SOD-F
SOD-NF
CC-F
CC-NF
Intensity (a.u.)
9.0x10^5
6.0x10^5
3.0x10^5
0.0
-3.0x10^5
-6.0x10^5
-9.0x10^5
3450
3480
3510
3540
3570
Magnetic field (G)

Fig. 6