DIVISION S-2—SOIL CHEMISTRY

Structural Components of Humic Acids as Determined by Chemical Modifications and Carbon-13 NMR, Pyrolysis-, and Thermochemolysis-Gas Chromatography/Mass Spectrometry

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ABSTRACT

The chemical structure of humic acids (HAs) extracted from a grassland surface soil and peat were studied using bleaching ($NaClO_2$ oxidation) and acid hydrolysis (6 *M* HCl) in combination with advanced analytical techniques: solid-state ^{13}C nuclear magnetic resonance (NMR), pyrolysis-gas chromatography/mass spectrometry (GC/MS), and tetramethylammonium hydroxide (TMAH) thermochemolysis-GC/MS. The purpose of the chemical treatments was to remove known structural fragments from the HA to study the building blocks and components of the macromolecule. Bleaching the peat HA resulted in an attack on the lignin structures leading to a significant reduction in the C-substituted and O-substituted aromatic C peaks (128 and 150 ppm) in the ^{13}C NMR spectrum. However, the bleached soil HA still contained residual aromatic C, suggesting that part of its aromaticity had originated from aromatic structures resistant to bleaching, possibly black C (charcoal). The pyrolysates of the bleached HAs contained mainly alkanes and alkenes (C_8 to C_{29}), whereas TMAH thermochemolysis yielded a homologous series of long-chain fatty acid methyl esters (FAMEs) (C_8 to C_{32}) and dicarboxylic acid dimethyl esters (DAMES). The data are comparable with those obtained from pyrolysis and thermochemolysis of plant cuticular materials, and therefore suggest that cuticular residues are an integral part of the HA macromolecule. The acid hydrolysis treatment removed esters, amides, carbohydrates, and some of the N-containing compounds from the HAs. This study demonstrates the effectiveness of bleaching and hydrolysis treatments together with advanced analytical techniques for characterization of aliphatic, lignin-derived (LG) and nonhydrolyzable N-containing structures associated with the HA macromolecule.

A major portion of soil organic matter (SOM) consists of humic substances (HS). Their structure, characteristics, and function have been studied extensively during the last century, particularly those of HAs. The traditional procedures used for studying HS structure include oxidation with $KMnO_4$, alkaline CuO, alkaline nitrobenzene, hypochlorite, HNO_3, peracetic acid, and H_2O_2; reduction using Zn-dust distillation and Na amalgam; base and acid hydrolysis; and enzymatic degradation (Christman et al., 1989; Griffith and Schnitzer, 1989; Stevenson, 1994). The primary objective of these treatments is to recover simple extractable monomers or fragments that can easily be analyzed and are believed to be representative of the HS's main macromolecular structure. However, the yield of some of these extractable components is relatively low. In this study, we have utilized a different approach whereby the residues remaining after chemical alteration are analyzed to obtain more structural information about the bulk macromolecule and about the alteration process itself.

Pyrolysis-GC/MS is a powerful analytical tool for obtaining molecular-level information about SOM and HS. In the past, the data from this technique have been used to design two- and three-dimensional model structures of HS (Schulten, 1995; Schulten and Leinweber, 1996). However, problems associated with pyrolysis (e.g., decarboxylation and formation of very polar products) have led to misinterpretations with respect to benzene carboxylic acids and fatty acids in HS (del Rio et al., 1994, 1998). A new technique, which has been recently developed to overcome this limitation, is thermochemolysis with TMAH. This method selectively cleaves ester and certain ether linkages (such as β-O-4 aliphatic-aryl bonds) in macromolecular organic matter (OM; Hatcher et al., 1995; Hatcher and Minard, 1996) through a chemolytic procedure that hydrolyzes and methylates ester and ether linkages, and assists in the depolymerization and methylation of lignin (Filley et al., 1999). This technique has been employed to characterize HAs, HS, composted OM, and SOM (del Rio et al., 1994, 1998; Hatcher and Clifford, 1994; Fabbri et al., 1996; Chefetz et al., 2000a,b), and is especially useful for detecting polar compounds such as long-chain fatty acids and benzenecarboxylic acids, as part of the HS structure. Another modern technique for the characterization of HS is solid-state NMR (Wilson, 1987; Preston, 1996; Mao et al., 2000). This technique offers a generic description of the major classes of C-containing groups in a sample and has contributed significantly to information regarding the chemical nature of HS.

Information gained from each analytical technique or chemical modification is important because the individual techniques complement each other. When used together, they provide awareness against biases that may evolve because of structural selectivity that each technique harbors. In this study, a combination of two chemical degradation techniques (oxidation using $NaClO_2$

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Abbreviations: AL, alkanes/alkenes; CPMAS, cross-polarization magic angle spinning; DAME, dicarboxylic acid dimethyl esters; FAME, fatty acid methyl esters; HA, humic acid; HS, humic substances; IHSS, International Humic Substance Society; LG, lignin-derived compounds; NMR, nuclear magnetic resonance; OM, organic matter; PR, protein-derived compounds; PS, polysaccharide-derived compounds; SOM, soil organic matter; TMAH, tetramethylammonium hydroxide.

and acid hydrolysis) together with advanced analytical characterization (^{13}C NMR, pyrolysis and TMAH thermochemolysis) were used to obtain data that contributes significantly to the knowledge and understanding of the building blocks in soil and peat Has.

MATERIALS AND METHODS

Samples, Humic Acid Extraction, and Purification

The peat sample was purchased from the International Humic Substances Society (IHSS). The soil sample was obtained from the Ellerslie Research Station, located south of the University of Alberta in Edmonton, Canada. Humic acids were extracted from the peat and soil samples according to the standard protocol of the IHSS (Swift, 1996).

Chemical Treatments

Subsamples from the isolated HAs were bleached and hydrolyzed. Bleaching was performed according to the method described by Wise et al. (1946) using 10 g of $NaClO_2$, 10 mL of acetic acid, and 100 mL of deionized water per gram of HA. The mixture was stirred overnight, then the supernatant was decanted and replaced by fresh solution. This procedure was repeated three times. Hydrolysis was carried out using 300 mL of 6 *M* HCl per gram of HA; the mixture was refluxed for 6 h. After each respective treatment, the residues were separated from the supernatant by centrifugation ($5000 \times g$, 15 min), dialyzed (Spectra/Por Membrane, MWCO 6000–8000; Fisher Scientific, Pittsburg, PA) against deionized water, and then freeze-dried.

Elemental Analysis

Elemental (C, H, and N) analysis was conducted in triplicates by Quantitative Technologies Inc. (Whitehouse, NJ).

Solid-State Cross Polarization Magic Angle Spinning Carbon-13 NMR Spectroscopy

Solid-state cross polarization magic angle spinning (CPMAS) ^{13}C NMR spectra, using the ramp-cross polarization technique (Cook and Langford, 1998) were obtained with a Bruker 300 MHz NMR-spectrometer (Bruker Analytic GmbH, Germany). To obtain spectra that would be quantitatively representative of the structural components of the sample, an optimal contact time was chosen to give representative intensities for all types of carbons. This was determined by analyzing the peak intensities at various contact times, ranging from 0.1 to 15 ms, with ramp-CPMAS (Wind et al., 1993). A contact time of 2 ms was chosen to be optimal. The spectrometer operates at a ^{1}H frequency of 300 MHz and a ^{13}C frequency of 75 MHz. The following optimized experimental parameters were used: ramp-cross polariztion contact time of 2 ms; recycle delay time of 1 s; sweep width of 27 kHz (368 ppm) and line broadening of 30 Hz. Freeze-dried samples were placed in a 4-mm rotor and spun at a frequency of 13 kHz at the magic angle (54.7° to the magnetic field). All samples were subjected to the same number of scans (25 200).

The ^{13}C NMR spectra were integrated into the following chemical shift regions: 0 to 50 ppm, paraffinic C; 50 to 112 ppm, alkyl-O or C-O, C-N bonds as in carbohydrates, alcohols, ethers, and amines; 112 to 163 ppm, aromatic and phenolic C; and 163 to 190 ppm, carboxyl, ester and amide; 190 to 215 ppm, carbonyl C (Hatcher et al., 1983).

Pyrolysis-Gas Chromatography and Mass Spectroscopy

Pyrolysis-GC/MS was performed using a Mega 500 series gas chromatograph (Carlo Erba, Milan, Italy), equipped with a CDS analytical pyroprobe-2000 controller, a CDS AS-2500 pyrolysis autosampler and a 30-m fused silica capillary column coated with chemically bound DB-1701 (0.25-mm i.d., film thickness 0.25 μm; Restek Corp., Bellefonte, PA). Humic Acid samples (~0.25 mg) were weighed and transferred to quartz tubes. Each tube was dropped into the pyrolysis chamber, which was subsequently heated to 615°C at a rate of 5°C ms^{-1} and was held at this temperature for 15 s. Detailed information regarding the pyrolysis procedure is described by Chefetz et al. (2000b).

Tetramethylammonium Hydroxide Thermochemolysis-Gas Chromatography and Mass Spectroscopy

Tetramethylammonium hydroxide thermochemolysis was conducted as described in earlier papers (Chefetz et al., 2000a,b). Briefly, HA samples (~1 mg) were placed in glass tubes with 200 μL of TMAH (25% [wt./wt.] in methanol). After the methanol was evaporated, the tubes were sealed under vacuum and subsequently placed in an oven at 250°C for 30 min. After cooling, the tubes were cracked open, an internal standard (*n*-eicosane) was added, and the tubes were extracted with ethyl acetate. The extracts were concentrated and injected (1 μL) into a 6890 series gas chromatograph (Hewlett Packard, Palo Alto, CA), equipped with a 15-m fused silica capillary column coated with chemically bound DB-5 (0.25-mm i.d., film thickness 0.1 mm; Supelco, Bellefonte, PA). The GC was directly coupled to a Pegasus II (Leco Corporation, St. Joseph, MI) time-of-flight mass spectrometer.

RESULTS

Hydrolysis and bleaching of HAs resulted in the recovery of varying amounts of residues. Elemental characterization of the HAs and their corresponding residues together with the yields for the treatments are presented in Table 1. Bleaching with $NaClO_2$ removed a large fraction (by weight) of the HA samples (92 and 78% for the peat and soil HAs, respectively). A significant loss in C content was recorded for the bleached soil HA (from 49 to 34%), whereas a minor increase in C content was recorded for the bleached peat HA. Both bleached HA samples exhibited higher H/C ratios than their corresponding untreated samples. Higher yields

Table 1. Treatment yields, elemental analysis, and atomic ratio characteristics of the studied samples. Elemental analysis values represent the mean of triplicate measurements with a coefficient of variation <5%.

Chemical treatment	Recovery	C	H	N	C/N	H/C
	g kg^{-1}	%				
Peat humic acid						
Bulk (untreated)		55.18	4.09	3.67	17.54	0.88
Bleached	80	56.48	6.54	4.23	15.58	1.38
Hydrolyzed	730	58.80	3.88	2.50	27.44	0.78
Soil humic acid						
Bulk (untreated)		49.10	3.84	4.26	13.45	0.93
Bleached	220	34.14	3.17	3.25	12.25	1.10
Hydrolyzed	610	60.33	3.33	2.60	27.07	0.66

(recovery) were obtained for the hydrolysis reactions (73 and 61%, for the peat and soil HAs, respectively).

Cross-Polarization Magic Angle Spinning Carbon-13 NMR

The CPMAS ^{13}C NMR spectra of the bulk and treated HAs are presented in Fig. 1. The CPMAS ^{13}C NMR spectra exhibited major peaks at: 30 and 32 ppm (methylene C), 56 ppm (methoxy C and a C of amino acids), 72 ppm (carbohydrates or aliphatic alcohols), 105 ppm (anomeric C of polysaccharides), 128 and 130 ppm (C-substituted aromatic C), 148 and 152 ppm (O-substituted aromatic C), and 172 ppm (carboxyl and amide C).

Distinct changes were observed between the spectra of the untreated soil HA and the untreated peat HA (Fig. 1U). The peaks at 130 ppm (aromatic C), and 72 and 105 ppm (polysaccharides) were more pronounced in the soil HA spectrum than in that of the peat HA. The methylene C peak (32 ppm) was sharp in the peat HA spectrum, whereas in the soil HA spectrum it was split into two peaks at 30 and 32 ppm. The O-substituted aromatic C peak (152 ppm) was seen only in the peat HA spectrum. The integrated areas of the peaks (Table 2) indicate that the peat HA contains 7% more alkyl-type carbons and that the soil HA is 5% richer in aromatic C structures. Both spectra exhibit a major carboxyl and amide C peak at 172 ppm, and a poorly resolved carbonyl peak at 190 ppm.

Bleaching of the peat HA resulted in extensive reduction of the aromatic C signal in the CPMAS ^{13}C NMR spectrum (Fig. 1B). According to the distribution of C functional groups we note that after $NaClO_2$ oxidation, the relative amount of aromatic carbons was reduced from 33 to 10% in this HA. However, the bleached soil HA still contained 22% of residual aromatic C. The peak at 56 ppm displayed the same trend as the aromatic C peak and was significantly reduced by bleaching. As a result of the significant reduction of the aromatic and methoxyl C peaks, the relative intensity of paraffinic carbons was doubled in the bleached peat HA (Table 2).

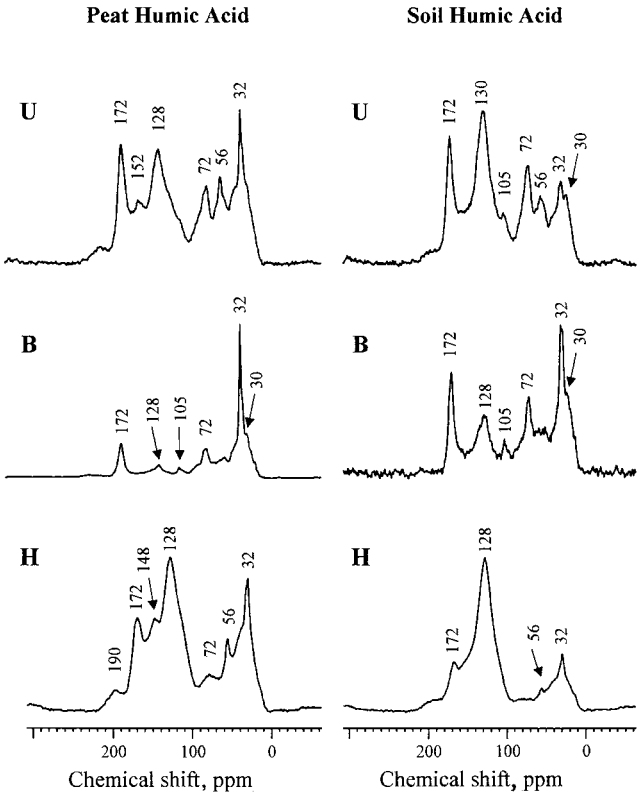

Fig. 1. The ^{13}C NMR spectra of the peat (left) and soil (right) humic acids (U, untreated; B, bleached; H, hydrolyzed).

Table 2. Distribution of soil and peat HA functional groups as determined by cross-polarization magic angle spinning (CPMAS) ^{13}C nuclear magnetic resonance (NMR).

Treatment C-containing group	Chemical shift, ppm	Bulk	Bleaching	Hydrolysis
		Percent of total C		
Peat humic acid				
Paraffinic C, and C bonded to other C	0–50	24.2	50.1	21.8
C-O and C-N in carbohydrates, alcohols, esters, and amines	50–112	27.5	27.4	20.7
Aromatic and phenolic C	112–163	32.8	10.4	43.0
Carboxyl, ester, and amide C	163–190	13.2	10.7	11.7
Carbonyl C	190–215	2.2	1.4	2.8
Soil humic acid				
Paraffinic C, and C bonded to other C	0–50	16.8	35.3	15.8
C-O and C-N in carbohydrates, alcohols, esters, and amines	50–112	28.9	30.0	14.1
Aromatic and phenolic C	112–163	38.0	21.8	58.5
Carboxyl, ester, and amide C	163–190	14.4	12.8	9.6
Carbonyl C	190–215	1.8	0.2	2.1

The absolute amount (weight units) of the aromatic and paraffinic C functionalities remaining after each treatment was calculated by multiplying the percentage of C functionality, as determined by CPMAS ^{13}C NMR spectra, by the yield of the treatment (the bulk sample containing 100 units of weight). The amount of aromatic C in the peat HA was reduced from 32.8 to 0.83 units after bleaching. Therefore, only 2.5% of the peat HA aromatic structures were resistant to bleaching. The aromatic units of soil HA contained compounds, which were more resistant to the bleaching treatment. Bleaching resulted in a reduction in the aromatic C, from 38 to 4.8 units, i.e., 12.6% of the original weight of the aromatic structure of soil HA. The total amount of paraffinic carbons left after bleaching was 7.7 weight units in the soil HA as compared with 16.8 units in the bulk sample (i.e., 54% of the paraffinic carbons were removed by the procedure). Fewer paraffinic carbons were left after bleaching of the peat HA (4 units), this amount representing only 16.5% of the original paraffinic C content in peat HA.

Acid hydrolysis resulted in a significant reduction of the carbohydrate and amine region (50–112 ppm). The peaks at 72 and 105 ppm were poorly resolved in the two hydrolyzed HA spectra (Fig. 1H), and this is consistent with other observation made from applications of this treatment to HS (Almendros, 1994; Stevenson, 1994). The total amount of polysaccharides (as calcu-

lated from the 60–112 ppm region in the CPMAS ^{13}C NMR spectra) left after this treatment was 11.5 and 7 weight units for the peat and soil HAs, respectively. In contrast with the trends observed by bleaching, fewer structural units were removed from the peat HA than the soil HA by the hydrolysis treatment. The nonhydrolyzed polysaccharides represented 55 and 30% of the original amount of polysaccharides for peat and soil HAs, respectively. Of course, it is likely that the peaks in the polysaccharide region of the spectra of hydrolyzed HAs are not polysaccharides at all but other types of alkyl-O carbons (e.g., lignin side-chains, alcohols, esters, etc.). The hydrolysis treatment also resulted in a significant reduction of the carboxyl and amide C peak, and therefore increased the relative intensity of the aromatic C peak in the ^{13}C NMR spectrum. The carboxyl and amide C content of the soil HA was reduced by 33% (from 14.4 to 9.6%). A lesser reduction of the carboxyl and amide peak was exhibited for the peat HA. The CPMAS ^{13}C NMR spectrum of the hydrolyzed peat HA exhibited a shift of the O-substituted aromatic C peak from 152 to 148 ppm. In addition to the reduction of carbohydrate signals in the hydrolyzed soil HA, the methylene C peak was significantly decreased as compared with the 128 ppm peak.

Pyrolysis-Gas Chromatography and Mass Spectroscopy

Total ion current chromatograms obtained by pyrolysis-GC/MS analysis for the bulk and treated soil and peat HAs are presented in Fig. 2 and 3, respectively. The main compounds identified in the chromatograms were grouped into six major classes: polysaccharide-derived (PS), protein-derived (PR), lignin-derived (LG), alkanes/alkenes (AL), fatty acids, and compounds that could not be assigned to a specific biological source. Peaks are identified in Table 3.

The major peaks in the bulk (untreated) soil HA chromatogram (Fig. 2U) were phenol (LG1), 4-methyl phenol (LG9), acetic acid (PS1), 2-furancarboxaldehyde (PS3), 5-methyl-2-furancarboxaldehyde (PS4), toluene (1), and acetamide (PR11). The bleaching treatment (Fig. 2B) resulted in a significant reduction of the LG peaks and corresponding increase in the peak intensities of alkanes and 1-alkenes (C_8 to C_{28}). However, intensities of these AL peaks are lower than those in the chromatogram of bleached peat HA (Fig. 3B). In addition to the presence of alkane and alkene pairs, the major peaks in the bleached soil HA chromatogram were pyridine (PR1), acetic acid (PS1), 2-furancarboxaldehyde (PS3), and toluene (1). The only LG peak identified in the bleached chromatogram was phenol (LG1), the relative intensity of which was significantly reduced compared with its original intensity in the bulk sample. Hydrolysis (Fig. 2H) reduced the number of polysaccharide- and protein-derived products, resulting in a relative increase of several alkane and alkene pairs, consistent with the observed increase in the paraffinic structures in the NMR spectra.

The major peaks in the untreated peat HA pyrolysis-

Table 3. Peak identification of pyrolysis-GC/MS products.

Lignin-derived peaks	
phenol	LG 1
2-methoxy phenol	LG 2
2-methoxy-4-methylphenol	LG 3
4-ethyl phenol	LG 4
2,6-dimethoxy phenol	LG 5
1,2-benzenediol	LG 6
1-(4-hydroxy-3-methoxyphenyl)-ethanone	LG 7
1-(4-hydroxyphenyl)-ethanone	LG 8
4-methyl phenol	LG 9
1-(4-hydroxy-3,5-dimethoxyphenyl)-ethanone	LG 10
Protein-derived peaks	
pyridine	PR 1
2-methyl pyrrole	PR 2
1-ethyl pyrrole	PR 3
2,5-pyrrolidinedione	PR 4
isoindole-1,3-dione	PR 5
3-hydroxybenzonitrile	PR 6
4-hydroxybenzonitrile	PR 7
1,3-isoquino linedione	PR 8
benzeneacetonitrile	PR 9
4-hydroxybenzonitrile	PR 10
acetamide	PR 11
3-hydroxypyridine	PR 12
indole	PR 13
2,3-dihydro-4-methyl indole	PR 14
3,9-diazatricycloy dodecan-2,8-dione	PR 15
benzonitrile	PR 16
pyrrole	PR 17
3-acetoxypyridine	PR 18
benzenepropanenitrile	PR 19
acetyloxy methyl propanenitrile	PR 20
Polysaccharide-derived peaks	
acetic acid	PS 1
propanoic acid	PS 2
2-furancarboxaldehyde	PS 3
5-methyl-2-furancarboxaldehyde	PS 4
2,3-dihydrobenzofuran	PS 5
3-phenyl furan	PS 6
6,10,14-trimethyl-2-pentadecanone	PS 7
1,3-isobenzofurandione	PS 8
4-methyl-1,3-isobenzofurandione	PS 9
benzofuran	PS 10
2-propanol	PS 11
1-hexadecanol	PS 12
2-methyl propanoic acid	PS 13
cyclopent-2-en-1,4-dione	PS 14
2-furanone	PS 15
3-methyl-5-methylindenfuranone	PS 16
2-furanmethanol	PS 17
2-octanol	PS 18
2-octylfuran	PS 19
2-hydroxy-3-methyl-2-cyclopenten-1-one	PS 20
Unassigned peaks	
toluene	1
1-ethyl-3-methyl benzene	3
1-ethyl-2-methyl benzene	4
1,3,5-trimethyl benzene	5
1,2,3-trimethylbenzene	6
butyl benzene	7
1,4-dimethoxybenzene	8
xylene	9
styrene	10
2-methyl phenol	11
3-methyl phenol	12
2,4-dimethyl phenol	13
3-ethyl phenol	14
1,3-benzenediol	15
benzaldehyde	16
indene	17
naphthalene	18
benzothiazole	19
benzoic acid	20
1,2-benzenedicarbosylic acid, bis(2-ethylhexyl) ester	21
2-2′-bipyridine	22
1-isocyanato-4-methoxy benzene	24
1,3-benzenediol	24
pyridatriazole	25
4-methylbenzaldehyde	26
1,4-dihydro-1,4-dimethyl-5H-tetrazol-5-one	27
2-phenoxyphenol	28

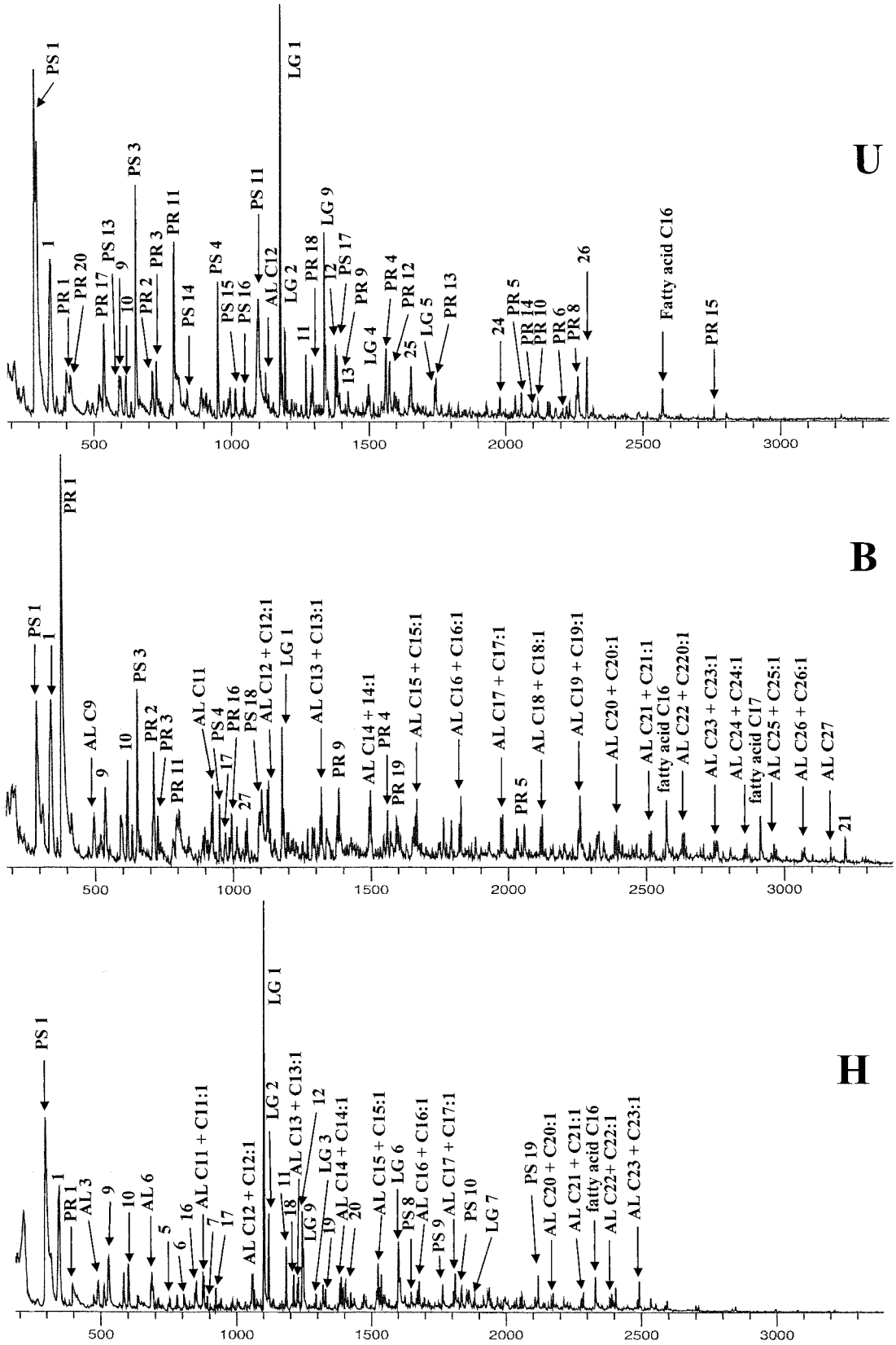

Fig. 2. Pyrolysis-GC/MS total ion current chromatograms of untreated (U), bleached (B), and hydrolyzed (H) soil humic acid (AL Cn: alkane/alkene; br: branched).

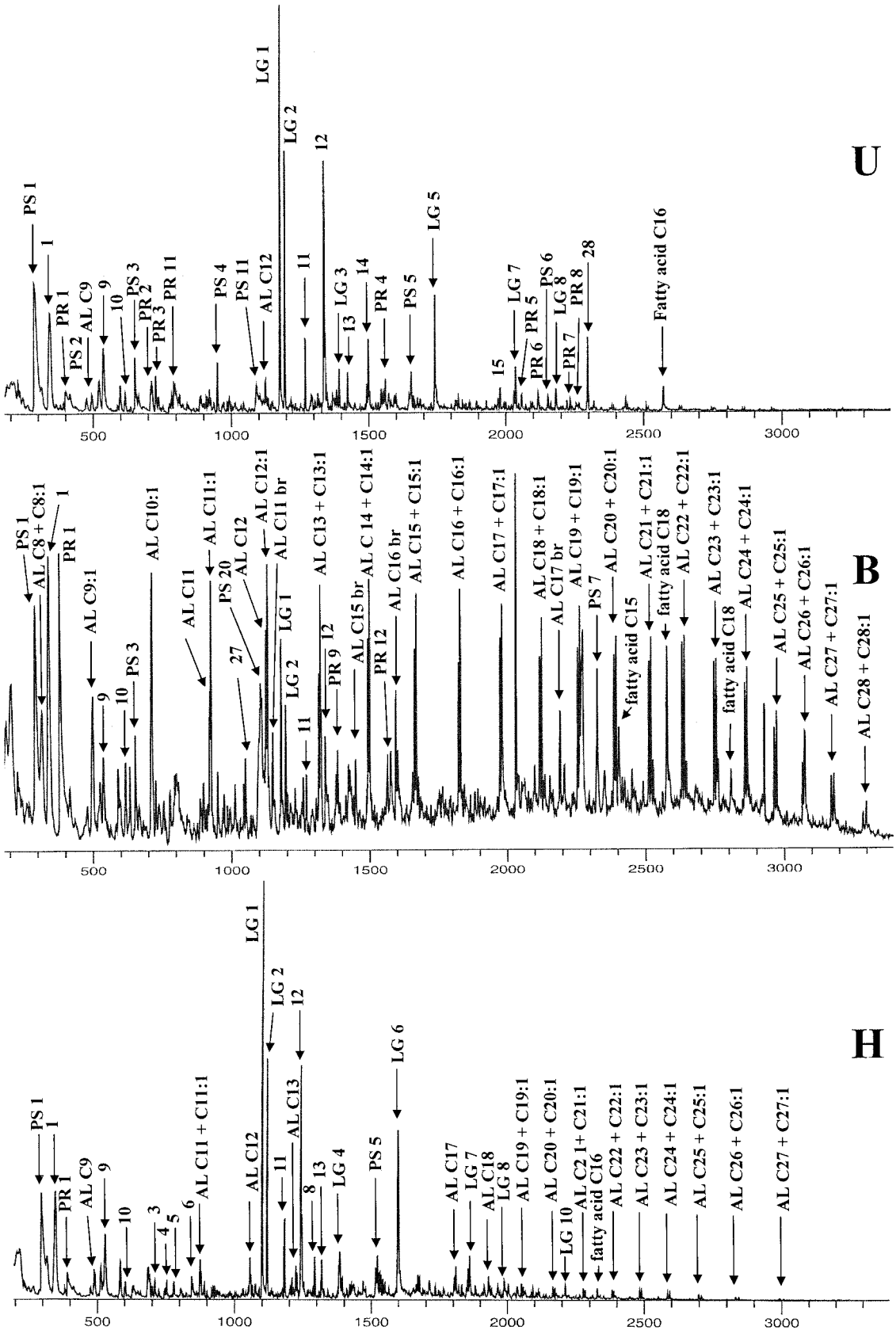

Fig. 3. Pyrolysis-GC/MS total ion current chromatograms of untreated (U), bleached (B), and hydrolyzed (H) peat humic acid (AL Cn: alkane/alkene; br: branched).

GC/MS chromatogram (Fig. 3U) were phenol derivatives: phenol (LG1), guaiacol (LG2) and 2,6-dimethoxy phenol (LG5), and 3-methyl phenol (12). The bleaching treatment, which selectively removes O-substituted aromatic components (lignin), resulted in a relative increase in the peak area of alkanes and 1-alkenes, and all homologs from C_9 to C_{27} were observed (Fig. 3B). In addition, several branched alkenes were identified. However, phenol and 2-methoxy phenol peaks were still present in the bleached peat HA chromatogram, suggestive of an incomplete bleaching and removal of lignin. Hydrolysis of the peat HA (Fig. 3H) resulted in little change, mainly a relative enhancement of the LG peaks (LG1, LG2, LG4, and LG6) and AL pairs, because of the removal of polysaccharides and carbonyl groups (such as in esters and amides).

Tetramethylammonium Hydroxide Thermochemolysis-Gas Chromatography and Mass Spectroscopy

Tetramethylammonium hydroxide thermochemolysis of the soil and peat HA samples (Fig. 4 and 5, respectively) yielded LG compounds (methylated p-hydroxyphenyl, guaiacyl, and syringyl compounds), nonlignin-derived aromatic compounds, heterocyclic N compounds, FAMEs, and DAMEs. Peaks are identified in Table 4 and labeled in Fig. 4 and 5.

The major peaks in the untreated soil HA chromatogram (Fig. 4U) were: C_4 and C_5 DAMEs; 1-methyl-2, 5-pyrrolidinedione; nonlignin-derived aromatic compounds such as methoxymethyl benzene, 2-propenoic acid 3-phenyl-methyl ester, 1,3,5-trimethoxybenzene, and 1,2,4-trimethoxybenzene; and several LG compounds, of which G6 and P5 were the most intense. Bleaching efficiently removed all LG units from the soil HA structure and the TMAH thermochemolysis products lacked the LG structures, except for P1. Consequently, the bleached soil HA (Fig. 4B) chromatogram was dominated by a series of FAMEs (C_{12} to C_{28}) and DAMEs (C_4, C_5, C_6, and C_9), and a distinct peak for 1,3,5-trimethoxybenzene (peak no. 16). These products are probably derived from the resistant plant biopolymer cutan as reported by McKinney et al. (1996). The chromatogram of the hydrolyzed soil HA (Fig. 4H) exhibited the same series of LG compounds and FAMEs as in the bulk HA, but the intensity of the 1-methyl 2,5-pyrrolidinedione peak was significantly reduced as was Peak No. 9.

Unlike the soil HA, the peat HA treated with TMAH thermochemolysis yielded more LG compounds from syringyl structures (S1, S5, and S6; Fig. 5U). The only LG compound identified in the bleached peat HA chromatogram was G6 and at a significantly reduced intensity (Fig. 5B). The bleached peat HA chromatogram exhibited a well-pronounced series of FAMEs (C_8 to C_{32}) with a strong predominance of even over odd C units. In addition to the series of FAMEs, DAMEs (C_4 to C_{10}) were also identified. The hydrolysis treatment enhanced the intensity of the LG peaks and FAMEs as compared with the chromatogram of the untreated HA.

Table 4. Peak identification of tetramethylammonium hydroxide (TMAH) thermochemolysis-CG/MS products.

Compounds derived from p-hydroxyphenyl structures	
P1	methoxybenzene
P3	(4-methoxyphenyl)-ethene
P4	4-methoxybenzaldehyde
P5	4-methoxyacetophenone
P6	4-methoxybenzoic acid, methyl ester
P18	3-(4-methoxyphenyl)-2-propenoic acid, methyl ester
Compounds derived from guaiacyl structures	
G1	1,2-dimethoxybenzene
G4	3,4-dimethoxygenzaldehyde
G5	3,4-dimethoxyacetophenone
G6	3,4-dimethoxybenzoic acid, methyl ester
G24	3,4-dimethoxyphenylacetic acid, methyl ester
Compounds derived from syringyl structures	
S1	1,2,3-trimethoxybenzene
S2	3,4,5-trimethoxytoluene
S5	3,4,5-trimethoxyacetophenone
S6	3,4,5-trimethoxybenzoic acid methyl ester
Other compounds	
1	3-methyl propanoic acid, methyl ester
2	4-methyl butanoic acid, methyl ester
3	benzaldehyde
4	benzene (methoxymethyl)
5	4-oxo-pentanoic acid, dimethyl ester
6	methyl-butanedioic acid, dimethyl ester
7	benzoic acid methyl ester
8	1-methyl 2,5-pyrrolidinedione
9	unidentified N-containing compound
10	2-methyl pentanedioic acid, dimethyl ester
11	2-methylene pentanedioic acid, dimethyl ester
12	2,5-dimethyl-1-propylpyrrole
13	benzenepropanoic acid, methyl ester
14	3,5-dimethyl benzoic acid, methyl ester
15	3-phenyl 2-propenoic acid, methyl ester
16	1,3,5-trimethoxybenzene
17	3,5-dimethoxybenzoic acid methyl ester
18	N,N-dimethyl-3-methoxypropylamide
19	4-methyl pentanoic acid, methyl ester
20	ethane, 1, 1′oxybis[2-methoxy]
21	N-n-propylmaleimide
22	isothiocyanato cyclohexane
23	1-(1-methyl-1H-pyrrol-2yl)-ethanone
24	3-methoxy-4-methylbenzoic acid, methyl ester
25	5-methoxy-2-methyl, 1H-indole
26	1-isocyano naphthalene
27	3,5-dimethoxybenzoic acid, methyl ester
28	2-(methylthio)-benzothiazone
29	dibutyl phthalate
30	phthalic acid, diisooctyl ester
31	methoxy acetic acid, methyl ester
32	3-methoxypropanoic acid, methyl ester
33	4-methyl pentanoic acid, methyl ester
34	1-cyclohexyl ethanone
35	1,4-dimethoxybenzene
36	1-(1-oxobutyl)pyrrolidine
37	2-ethyl-3-methoxy, 2-cyclopentenone
38	3-methoxy benzoic acid, methyl ester
39	1,2,4-trimethoxybenzene
40	4-methyl benzoic acid, methyl ester
41	2-ethyl-3-methoxy, 2-cyclopentenone
42	benzeneacetic acid, methyl ester

DISCUSSION

In the past, a valuable approach for characterizing HS was based on degradative methods that reduced macromolecular OM to smaller structural fragments, which were assumed to represent the main structural groups in the macromolecule (Stevenson, 1994). In this study, the opposite approach was used and CPMAS ^{13}C NMR, pyrolysis-GC/MS, and TMAH thermochemolysis-GC/MS were applied to examine the HA residue left after removal of specific structural components by acid hydrolysis and $NaClO_2$ oxidation.

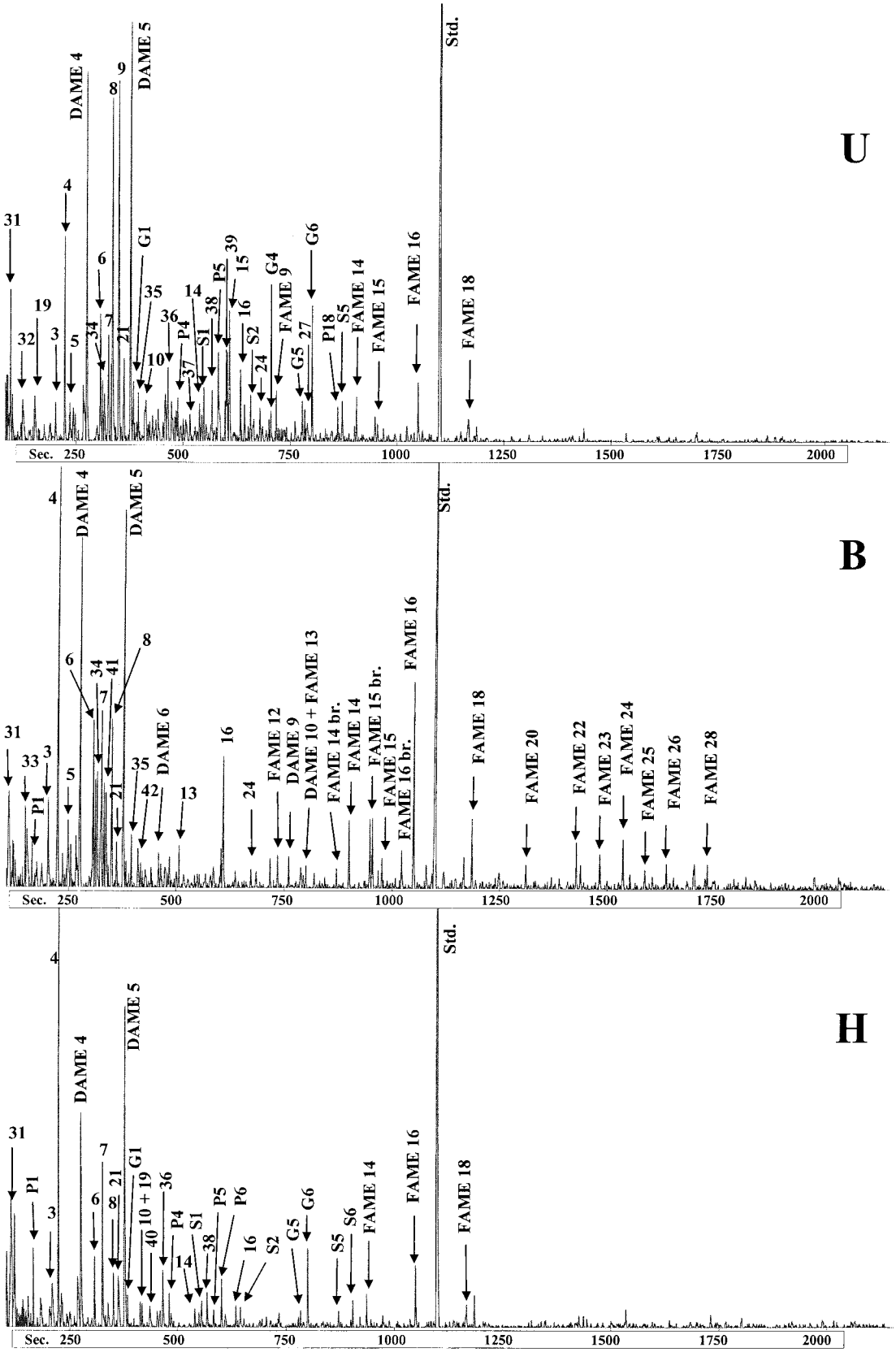

Fig. 4. Tetramethylammonium hydroxide (TMAH) thermochemolysis-GC/MS total ion current chromatograms of untreated (U), bleached (B), and hydrolyzed (H) soil humic acid (FAME, fatty acid methyl esters C no.; DAME, dicarboxylic acid dimethyl esters C no.; br., branched).

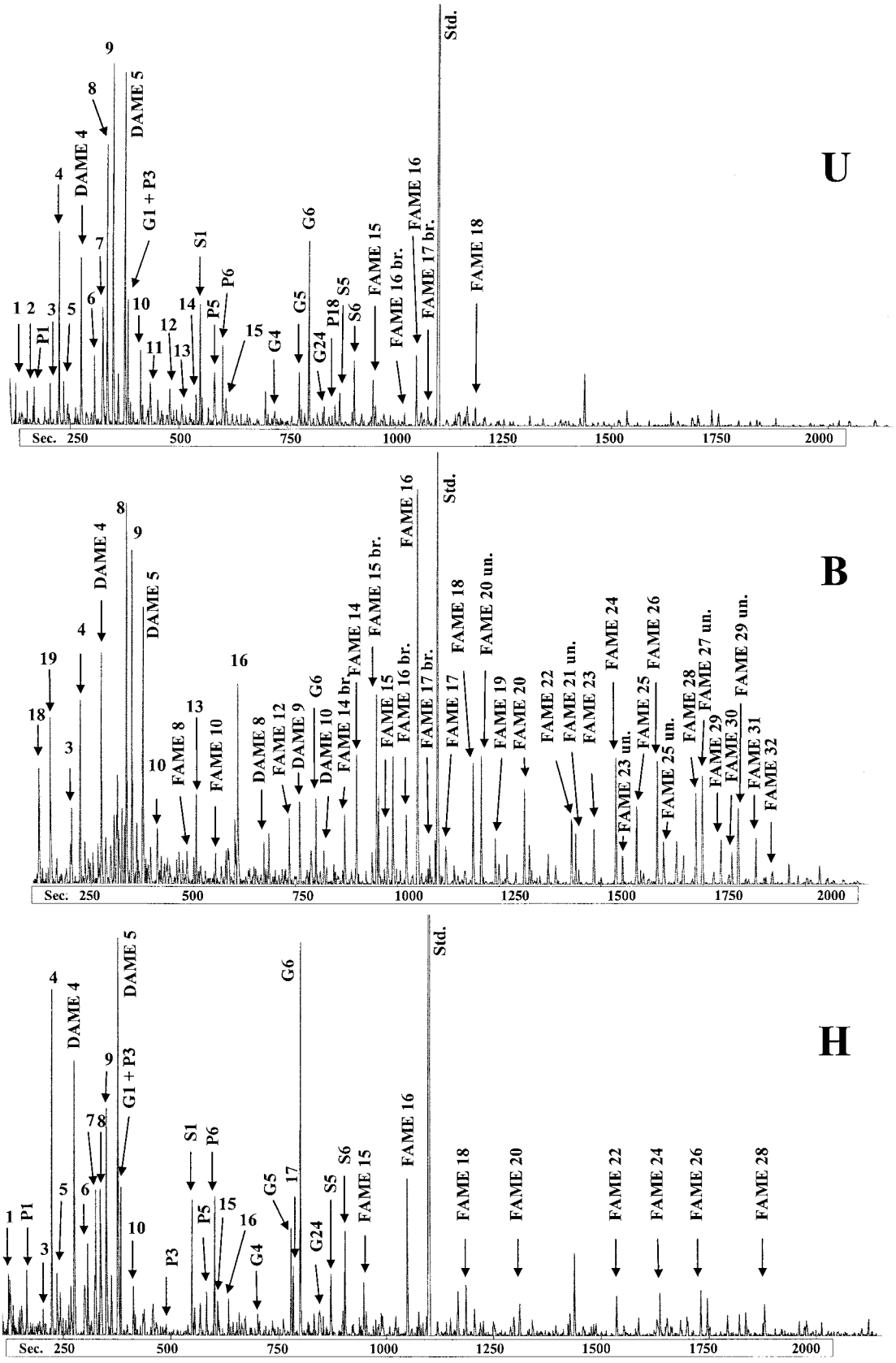

Fig. 5. Tetramethylammonium hydroxide (TMAH) thermochemolysis-GC/MS total ion current chromatograms of untreated (U), bleached (B), and hydrolyzed (H) peat humic acid (FAME, fatty acid methyl esters C no.; DAME, dicarboxylic acid dimethyl esters C no.; br., branched; un., unsaturated).

Bulk Humic Acid Samples

The soil HA was extracted from a surface layer of soil rich in OM, that was developed under grassland vegetation over glacial till. Although both soil and peat samples are considered well humified (Lyon, 1995; Salloum, 1999), the soil HA exhibited a higher level of C-substituted aromatic C and a lower content of phenolic C than the peat HA, as revealed by the ^{13}C NMR spectra. This is consistent with the background and nature of the source samples, as the peat contains more lignin-like matter than the soil. The substantial amount of alkyl-C in both HA samples is consistent with previous reports (Malcolm, 1989; del Rio et al., 1994; Almendros et al., 1998). The peat HA exhibited a sharp peak at 32 ppm which is assigned to carbons in long crystalline (rigid) polymethylenic $(CH_2)_n$ chains (Kögel-Knabner et al., 1992; Hu et al., 2000). The amorphous (mobile) polymethylenic C peak at 30 ppm was exhibited only as a small shoulder in the peat HA spectrum. However, the mobile alkyl C peak was more intense in the soil HA spectrum. The crystalline polymethylenic domains are expected to be more resistant to microbial degradation and therefore have longer residence times in soils than the amorphous (mobile) region (Hu et al., 2000). The relationship between the chemical nature of the polymethylenic domains in HAs and the degree of humification is not yet well understood and should be further investigated.

Pyrolysis-GC/MS analysis revealed high levels of compounds derived from polysaccharides. The PS peaks were more pronounced in the soil HA than in the peat HA, and this is consistent with the relative intensities of the polysaccharide regions exhibited in the ^{13}C NMR spectra. The pyrolysis-GC/MS data clearly indicate a higher contribution of LG residues in the peat HA structure than in the soil HA. Moreover, the peat HA pyrolysate chromatogram exhibited a relatively higher intensity for syringyl lignin units (such as 2,6-dimethoxy phenol) as compared with its relative intensity in the bulk soil HA chromatogram. This suggests that in the soil HA, the lignin is either derived mainly from gymnosperms, or the syringyl structures are selectively degraded during the humification process. The major LG compound identified in both pyrolysis chromatograms was phenol (Fig. 2 and 3). Phenol, in addition to being a pyrolysis product of lignin, has been reported to be generated as a pyrolysis product of polyphenolic compounds such as melanoidin- and polyphenolic-type components and amino acids, such as Tyr (van Heemst et al., 1999; Peulve et al., 1996).

In addition to the LG compounds, the pyrolysis-GC/MS chromatograms contained N-derived compounds such as pyrroles, indoles, pyridines, amides, and nitriles. The indoles may have originated from tryptophan-containing peptides, whereas the pyrroles can be derived from proline-containing peptides. The soil HA chromatogram exhibited a much higher content of these protein-derived compounds, suggesting a higher content of proteinaceous materials as part of the humic structure. The origin of the alkylated phenols (2- and 3-methyl phenols, and 3-ethyl phenol) that were present as dominant peaks in the peat HA samples, is unknown, and these could be derived from Tyr.

A higher proportion of LG peaks was produced by TMAH thermochemolysis of the bulk peat HA than that of the soil HA. Moreover, the peat HA yielded mainly the more oxidized acid-containing derivatives (such as P6, G6, and S6). The benzenecarboxylic acids in TMAH products are predominantly derived from lignin units where the α carbon of the side chain has been oxidized to a carboxyl group (Hatcher et al., 1995). This data support the pyrolysis finding, suggesting that both HAs contain lignin at an advanced stage of oxidation. Similar patterns of LG compounds have been reported in peat HA (del Rio and Hatcher, 1998), however a larger abundance of guaiacyl-like structures has been reported for forest soil HAs (Nierop et al., 1999). These data suggest that the sources of lignin (grass vs. wood) and the chemical nature of the HA are strongly related.

Under conditions of TMAH thermochemolysis, both untreated HA samples yielded saturated, unsaturated, and branched FAMEs of varying C chain lengths (from C_6 to C_{32}), with the most intense peaks being from C_{16} FAMEs. In addition, a series of DAMEs (C_4 to C_{28}) were also detected. A strong even-over-odd predominance was observed for this series, without a significant difference between the two HA samples. The presence of even-numbered long-chain fatty acids and dicarboxylic acids in both HA samples may be primarily because of an input of aboveground plant aliphatic biopolymers such as cutin and cutan (McKinney et al., 1996; del Rio et al., 1998), and belowground aliphatic plant biopolymers such as suberin and suberan (Nierop, 1998). Short-chain fatty acids and dicarboxylic acids (C_4-C_9), and the odd-C numbered and branched-chain fatty acids are commonly used as bacterial biomarkers. This set of data suggests that part of the HA macromolecular structure originates from refractory, highly aliphatic plant biopolymers (i.e., cuticular materials) and from microbial remains.

Bleaching

Oxidative degradation of HAs by hypochlorite has been used in the past to analyze soil HAs, coals, and HS from water (Christman et al., 1989). This reaction, in the case of aromatic substrates, involves electrophilic substitution of chlorine, followed by loss of aromatic character and oxidative cleavage of the ring. Chakrabartty and Kretschmer (1974) concluded that chlorine oxidation depends on the presence and reactivity of electronegative ring substituents. Aromatic rings activated by electron-withdrawing substituents such as nitro, hydroxy, methoxy, cyano, and carboxyl substituents can be oxidized, whereas condensed aromatic rings cannot. Therefore, this method is effective for decomposing noncondensed aromatic structures such as lignin-like and polyphenol units found in HAs. In this study, bleaching was conducted under acidic conditions and, therefore, chlorine dioxide or molecular O_2 were the bleaching agents (Hebeish et al., 1997).

The ^{13}C NMR spectrum of the bleached peat HA was

similar to that of insoluble and nonhydrolyzable organic residue from a forest soil (Augris et al., 1998). Both spectra are characterized by a sharp peak of long polymethylenic chains. These ^{13}C NMR spectra are similar to the spectra of cuticles from spruce needles (Kögel-Knabner et al., 1994). The highly aliphatic nature of the bleached HAs revealed by the ^{13}C NMR spectra supports the conclusion that highly refractory aliphatic plant biopolymers are an important and recalcitrant part of the HA structure. In a similar study by Hatcher et al. (1986) on peat samples from The Everglades, FL, paraffinic structures were found to be the dominant compounds of bleached whole peat. According to the ^{13}C NMR data, the contents of aliphatic bleach-resistant plant biopolymer-like compounds in the soil and peat HAs are 8 and 4% (by weight), respectively. The pyrolyzates of the bleached peat HA exhibited a series of alkane and alkene pairs similar to the pyrolysates of cutan from *Agave americana* (Kögel-Knabner et al., 1992; McKinney et al., 1996) and nonhydrolyzable organic residue from a forest soil (Augris et al., 1998). Tetramethylammonium hydroxide thermochemolysis of the bleached peat HA yielded methyl derivatives of long-chain fatty acids and dicarboxylic acids. The major FAMEs produced had 16 C atoms, which is the chain length of biopolymers typically found in cutin (del Rio and Hatcher, 1998).

The residual aromatic structures (after bleaching) in the ^{13}C NMR spectra are likely to be from condensed aromatic moieties that are not susceptible to bleaching via $NaClO_2$ or NaClO oxidation. The source of these compounds cannot be lignin or tannins because both are known to have a high degree of O-substituted aromatic carbons. We suspect that charcoal, charred plant materials, or coal fractions could be the origin of these structures, as these have been proposed to be an important pool of C in soils. Charring of plant materials was suggested to be one of the mechanisms of HA formation in volcanic ash soils (Shindo et al., 1986). Skjemstad et al. (1996) used photo-oxidation and NMR analyses to obtain quantitative measures of black C in soils and found that up to 30% of the total soil C can be estimated to be in the form of charcoal. Our data from the $NaClO_2$ oxidation and NMR suggest that materials whose origin may be black C are less likely to contribute to HA structure in our samples. Only 5% (by weight) of the soil HA is likely to originate from condensed aromatic structures. The data for the peat HA suggests less than 1% contribution of condensed aromatic structures to the humic macromolecular structure. These differences could be because of the differences between samples and the different oxidation methods used.

The presence of phenol and methoxybenzene peaks in the bleached chromatograms (pyrolysis and TMAH, respectively) indicates that phenol is not solely a LG compound as already discussed. Nonlignin-derived aromatic compounds, such as methoxymethyl benzene, benzaldehyde, and benzoic acid methyl ester probably represent highly humified lignin structures.

Acid Hydrolysis

The nonhydrolyzable soil HA constituted ~60% by weight of the original bulk sample, which is in the typical range for such residues of soil HAs (Orlov, 1995). However in the case of the peat HA, the hydrolysis residue constituted a much higher fraction of the bulk sample (73%), and this is more typical for HAs extracted from brown coals (Orlov, 1995). It was suggested that the degree of hydrolyzability of HS can be used as an indication of their degree of humification. As such, fulvic acid and fresh SOM are subject to much greater hydrolysis than highly humified substances. However, in the current study both HAs can be considered to be well humified, and the relative hydrolyzability does not reflect the maturity of the HA, but rather their constituent building blocks (i.e., origin). It is clear from the ^{13}C NMR spectra (Fig. 1) that the hydrolyzed peat HA contains more lignin-type structures (peaks at 56 and 148 ppm). The disappearance of the peak at 56 ppm in the hydrolyzed soil HA spectrum suggests that it was not from methoxyl C, but was, instead, contributed by other resonances, probably α carbons of amino acids (such as Arg, Glu, and Lys). The sharp peak at 128 ppm in the hydrolyzed soil HA spectrum suggests that most of the aromatic carbons in the HA structure are unsubstituted carbons or C-substituted (such as alkylbenzenes). The peak at 128 ppm in the hydrolyzed peat HA can be assigned to C_1, C_2, and C_6 carbons in *p*-hydroxyphenyl units of lignin (Hatcher, 1987). These units were the main peaks in the hydrolyzed peat HA pyrolysis chromatogram (phenol and 3-methyl phenol).

The hydrolysis procedure resolved a new peak at 148 ppm in the ^{13}C NMR spectrum of the peat HA. The peaks at 153 and 148 ppm can be used to distinguish hardwood from softwood lignin (Hatcher, 1987). The 153 ppm peak is assigned to C_3 and C_5 of syringyl units (hardwood), whereas the 148 ppm peak is assigned to C_3 and C_4 of guaiacyl units (softwood). The contribution of softwood lignin units to the humic structures is also supported by the TMAH thermochemolysis analysis. However, this cannot explain the shift of the O-substituted aromatic C peak from 152 ppm in the bulk sample to 148 ppm after hydrolysis. An explanation for this might be that the lignin-related peak in the bulk peat HA spectrum was poorly resolved and the resolution was improved only after removal of nonlignin components by acid hydrolysis. Alternately, cleavage of the β-O-4 bonds in the lignin structure resulted in changing the resonance of the ring carbons and enhancing the signal at 147 to 148 ppm.

Although acid hydrolysis has been reported to remove most amino acids and carbohydrates from soil HAs (Preston and Schnitzer, 1984), non-hydrolyzable N-containing compounds have been reported to contain 20 to 35% of the total N in soils (Stevenson, 1994). In this study, both TMAH thermochemolysis and pyrolysis of the hydrolyzed HAs yielded N-containing compounds such as 1-methyl-2,5-pyrrolidinedione and pyridine. These compounds could be derived from amino acids bound to phenolic or quinone groups (Stevenson,

1986), proteinaceous materials encapsulated in the humic structures (Knicker and Lüdemann, 1995; Knicker and Hatcher, 1997) or alkyl-substituted heterocyclic N-containing compounds which are structural constituents of the humic structure (Leinweber and Schulten, 1998). Schulten and Schnitzer (1998) reported that ~35% of the N in pyrolysis products of HS are heterocyclic N-containing compounds. However, ^{15}N NMR study carried out by Knicker et al. (2000) suggests that most of the N compounds in HS are in the form of proteins that are trapped in the HS macromolecule. The origin of these N-containing compounds is still unknown and should be further investigated.

CONCLUSIONS

This study demonstrates the effectiveness of bleaching as a tool for isolating aliphatic, polymethylenic, and condensed aromatic structures within the HA macromolecule. Further chemical analysis of the bleached samples can indicate the possible origin and mechanisms of formation of humic materials. The hydrolysis treatment was shown to be an important tool for studying the nature of LG and nonhydrolyzable N-containing compounds. Thus, our understanding of humification and HA structure can be further improved by applying chemical alteration methods in tandem with advanced analytical techniques such as CPMAS ^{13}C NMR, pyrolysis-GC/MS and TMAH thermochemolysis-GC/MS.

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REFERENCES

Almendros, G. 1994. Effects of different chemical modifications on peat humic acid and their bearing on some agrobiological characteristics of soils. Commun. Soil Sci. Plan. Anal. 25:2711–2736.

Almendros, G., M.E. Guadalix, F.J. Gonzalez-Vila, and F. Martin. 1998. Distribution of structural units in humic substances as revealed by multi-step selective degradations and ^{13}C NMR of successive residues. Soil Biol. Biochem. 30:755–765.

Augris, N., J. Balesdent, A. Mariotti, S. Derenne, and C. Largeau. 1998. Structure and origin of insoluble and non-hydrolysable, aliphatic organic matter in a forest soil. Org. Geochem. 28:119–124.

Chakrabartty, S.K., and H.O. Kretschmer. 1974. Sodium hypochlorite as a selective oxidant for organic compounds. J. Chem. Soc. Perkin Trans. 1:222–228.

Chefetz, B., Y. Chen, C.E. Clapp, and P.G. Hatcher. 2000a. Characterization of organic matter in soils by thermochemolysis using tetramethylammonium hydroxide (TMAH). Soil Sci. Soc. Am. J. 64: 583–589.

Chefetz, B., J.D.H. van Heemst, Y. Chen, C.P. Romaine, J. Chorover, R. Rosario, M. Guo, and P.G. Hatcher. 2000b. Organic matter transformation during the weathering process of spent mushroom substrate. J. Environ. Qual. 29:592–602.

Christman, R.F., D.L. Norwood, Y. Seo, and F.H. Frimmel. 1989. Oxidative degradation of humic substances from freshwater environments. p. 33–68. *In* M.H.B. Hayes et al. (ed.) Humic substances II. In search of structure. John Wiley & Sons, New York.

Cook, R., and C.H. Langford. 1998. Structural characterization of fulvic acid and humic acid using solid-state ramp-CP-MAS ^{13}C nuclear magnetic resonance. Environ. Sci. Technol. 32:719–725.

del Rio, J.C., and P.G. Hatcher. 1998. Analysis of aliphatic biopolymers using thermochemolysis with tetramethylammonium hydroxide (TMAH) and gas chromatography-mass spectrometry. Org. Geochem. 29:1441–1445.

del Rio, J.C., F.J. Gonzalez-Vila, F. Martin, and T. Verdejo. 1994. Characterization of humic acids from low-rank coals by ^{13}C-NMR and pyrolysis-methylation. Formation of benzenecarboxylic acid moieties during the coalification process. Org. Geochem. 22:885–891.

del Rio, J.C., D.E. McKinney, H. Knicker, M.A. Nanny, R.D. Minard, and P.G. Hatcher. 1998. Structural characterization of bio- and geo-macromolecules by off-line thermochemolysis with tetramethylammonium hydroxide. J. Chromatogr. A 823:433–448.

Fabbri, D., G. Chiavari, and G.C. Galletti. 1996. Characterization of soil humin by pyrolysis(/methylation)–gas chromatography/mass spectrometry; structural relationships with humic acids. J. Anal. Appl. Pyrolysis 37:161–172.

Filley, T.R., R.D. Minard, and P.G. Hatcher. 1999. Tetramethylammonium hydroxide (TMAH) thermochemolysis: proposed mechanisms based upon the application of 13C-labeled TMAH to synthetic model lignin dimer. Org. Geochem. 30:607–621.

Griffith, S.M., and M. Schnitzer. 1989. Oxidative degradation of soil humic substances. p. 69–98. *In* M.H.B. Hayes et al. (ed.) Humic substances II. In search of structure. John Wiley & Sons, New York.

Hatcher, P.G. 1987. Chemical structural studies of natural lignin by dipolar dephasing solid-state 13C NMR. Org. Geochem. 11:31–39.

Hatcher, P.G., and R.D. Minard. 1996. Comparison of dehydrogenase polymer (DHP) lignin with native lignin from gymnosperm wood by thermochemolysis using tetramethylammonium hydroxide (TMAH). Org. Geochem. 24:593–600.

Hatcher, P.G., and D.J. Clifford. 1994. Flash pyrolysis and in situ methylation of humic acids from soil. Org. Geochem. 21:1081–1092.

Hatcher, P.G., M.A. Nanny, R.D. Minard, S.C. Dible, and D.M. Carson. 1995. Comparison of two thermochemolytic methods for the analysis of lignin in decomposing wood: the CuO oxidation method and the method of thermochemolysis with TMAH. Org. Geochem. 23:881–888.

Hatcher, P.G., M. Schnitzer, L.W. Dennis, and G.E. Maciel. 1983. Solid state ^{13}C-NMR of sedimentary humic substances: new revelation on their chemical composition. p. 37–81. *In* R.F. Christman and E.T. Gjessing (ed.) Aquatic and terrestrial humic materials. Ann Arbor Science Publishers, MI.

Hatcher, P.G., E.C. Spiker, and W.H. Orem. 1986. Organic geochemical studies of the humification process in lowmoor peat. p. 195–213. *In* C.H. Fuchsman (ed.) The relationship of water with peat and its constituents. Elsevier Applied Science Publishers, Amsterdam.

Hebeish, A., A.A. Ragheb, K. Haggag, and A.A. Abd El-Rahman. 1997. Oxidation of moghat mucilage with sodium chlorite. Polym. Degrad. Stabil. 58:33–44.

Hu, W.G., M. Jingdong, B. Xing, and K. Schmidt-Rohr. 2000. Poly (methylene) crystallites in humic substances detected by nuclear magnetic resonance. Environ. Sci. Technol. 34:530–534.

Knicker, H., and H.-D. Lüdemann. 1995. N-15 and C-13 CPMAS and solution NMR studies of N-15 enriched plant material during 600 days of microbial degradation. Org. Geochem. 23:329–341.

Knicker, H., and P.G. Hatcher. 1997. Survival of protein in an organic-rich sediment. Possible protection by encapsulation in organic matter. Naturwissenschaften 84:231–234.

Knicker, H., M.W.I. Schmidt, and I. Kögel-Knabner. 2000. Nature of organic nitrogen in fine particle separates of sandy soils of highly industrialized areas as revealed by NMR spectroscopy. Soil Biol. Biochem. 32:241–252.

Kögel-Knabner, I., P.G. Hatcher, E.W. Tegekaar, and J.W. de Leeuw. 1992. Aliphatic components of forest soil organic matter as determined by solid-state ^{13}C NMR and analytical pyrolysis. Sci. Total Environ. 113:89–106.

Kögel-Knabner, I., J.W. de Leeuw, E.W. Tegekaar, P.G. Hatcher, and H. Kerp. 1994. A lignin-like polymer in the cuticle of spruce needles: Implications for humification of spruce litter. Org. Geochem. 21:1219–1228.

Leinweber, P., and H.-R. Schulten. 1998. Nonhydrolyzable organic nitrogen in soil size separates from long-term agricultural experiments. Soil Sci. Soc. Am. J. 62:383–393.

Lyon, W.G. 1995. Swelling of peats in liquid methyl, tetramethylene and propyl sulfoxides and in liquid propyl sulfone. Environ. Toxicol. Chem. 14:229–236.

Malcolm, R.L. 1989. Application of solid-state ^{13}C NMR spectroscopy to geochemical studies of humic substances. p. 339–372. *In* M.H.B. Hayes et al. (ed.) Humic substances II. In search of structure. John Wiley & Sons, New York.

Mao, J-D., W-G. Hu, K. Schmidt-Rohr, G. Davies, E.A. Ghabbour, and B. Xing. 2000. Quantitative characterization of humic substances by solid-state carbon-13 nuclear magnetic resonance. Soil Sci. Soc. Am. J. 64:873–884.

McKinney, D.E., J.M. Bortiatynski, D.M. Carson, D.J. Clifford, J.W. De Leeuw, and P.G. Hatcher. 1996. Tetramethylammonium hydroxide (TMAH) thermochemolysis of the aliphatic biopolymer cutan: insights into the chemical structure. Org. Geochem. 24: 641–650.

Nierop, K.G.J. 1998. Origin of aliphatic compounds in a forest soil. Org. Geochem. 29:1009–1016.

Nierop, K.G.J., P. Buurman, and J.W. de Leeuw. 1999. Effect of vegetation on chemical composition of H horizons in incipient podzol as characterized by 13C NMR and pyrolysis-CG/MS. Geoderma 90:111–129.

Orlov, D.S. 1995. Humic substances of soils and general theory of humification. A.A. Balkema Publishers, Rotterdam, the Netherlands.

Peulve, S., J.W. de Leeuw, M.A. Sicre, M. Baas, and A. Saliot. 1996. Characterization of macromolecular organic matter in sediment traps from the northwestern Mediterranean Sea. Geochim. Cosmochim. Acta 60:1239–1259.

Preston, C.M. 1996. Application of NMR to soil organic matter analysis: History and prospects. Soil Sci. 161:144–166.

Preston, C.M., and M. Schnitzer. 1984. Effects of chemical modifications and extractants on the carbon-13 NMR spectra of humic materials. Soil Sci. Soc. Am. J. 48:305–311.

Salloum, M.J. 1999. Sorption of organic compounds to soil and geologic samples with that vary in mineralogy and diagenetic properties. Ph.D. diss. Univ. of Alberta, Canada.

Schulten, H.-R. 1995. The three-dimentional structure of soil agromineral complexes studied by analytical pyrolysis. J. Anal. Appl. Pyrolysis 32:111–126.

Schulten, H.-R., and P. Leinweber. 1996. Characterization of humic and soil particles by analytical pyrolysis and computer modeling. J. Anal. Appl. Pyrolysis. 38:1–53.

Schulten, H.-R., and M. Schnitzer. 1998. The chemistry of soil organic nitrogen: A review. Biol. Fertil. Soils 26:1–15.

Shindo, H., Y. Matsui, and T. Higashi. 1986. Humus composition of charred plant residues. Soil Sci. Plant Nutr. 32:475–478.

Skjemstad, J.O., P. Clarke, J.A. Taylor, J.M. Oades, and S.G. McClure. 1996. The chemistry and nature of protected carbon in soil. Aust. J. Soil Res. 34:251–271.

Stevenson, F.J. 1986. Cycles of soil. John Wiley & Sons, New York.

Stevenson, F.J. 1994. Humus chemistry: Genesis, composition, reactions, 2nd ed. John Wiley & Sons, New York.

Swift, R.S. 1996. Organic matter characterization. p. 1011–1069. *In* D.L. Sparks (ed.) Methods of soil analysis. Part 3. SSSA Book Series no. 5. SSSA, Madison, WI.

van Heemst, J.D.H., P.F. van Burgen, B.A. Stankiewicz, and J.W. de Leeuw. 1999. Multiple sources of alkylphenols produced upon pyrolysis of DOM, POM and recent sediments. J. Anal. Appl. Pyrolysis 52:239–256.

Wilson, M.A. 1987. NMR techniques and applications in geochemistry and soil chemistry. Pergamon Press, Oxford, UK.

Wind, R.A., G.E. Maciel, and R.E. Botto. 1993. Quantitation in ^{13}C NMR spectroscopy of carbonaceous solids. p. 3–26. *In* R.E. Botto and Y. Sanada (ed.) Magnetic resonance of carbonaceous solids. American Chemical Society, Washington, DC.

Wise, L.E., M. Murphy, and A.A. D'Addicco. 1946. Chlorite holocellulose, its fractionation and bearing on summative wood analysis and on studies on the hemicelluloses. TAPPI 22:11–19.