

Analyzing Water Soluble Soil Organics as Trifluoroacetyl Derivatives by Liquid State Proton Nuclear Magnetic Resonance

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Abstract: In forested ecosystems, water soluble organics play an important role in soil processes including carbon and nutrient turnover, microbial activity and pedogenesis. The quantity and quality (i.e., chemistry) of these materials is sensitive to land management practices. Monitoring alterations in the chemistry of water soluble organics resulting from land management practices is difficult because of the complexity and low concentration of these compounds. A procedure is described in which the water soluble organics are quantitatively derivatized with trifluoroacetic anhydride and then analyzed by liquid state proton nuclear magnetic resonance (^1H NMR). The procedure was applied to sample amounts as low as 0.03 mg from the forest floor and root exudates. The root exudate samples were dominated by aliphatic compounds with relatively few O-alkyl, olefinic and aromatic compounds. The chemistry of the samples originating from the forest floor differed dramatically with soil texture and treatment combinations.

Keywords: Water soluble organics, ^1H NMR, root exudates, acetylation

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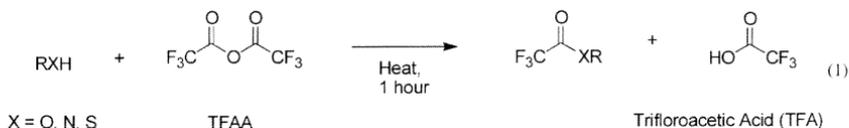
INTRODUCTION

Nuclear magnetic resonance (NMR) has been used extensively to study forest floor and soil organic matter (Baldoek and Preston, 1995; Hedges and Oades, 1997; Kögel-Knabner, 1997; Knicker, 2000; Mathers et al., 2000; Preston, 2001). Generally, solid state ^{13}C NMR has been used for these analyses and is useful in detecting the carbon chemical composition of the sample. The procedure is limited by the amount of carbon present in the sample since the ^{13}C isotope represents only 1.1% of natural carbon. Long analysis times are required to detect the ^{13}C signal in samples with low organic content. This is troublesome in samples with very low concentrations of organics such as soil solution samples. An alternative is to analyze the samples by liquid-state, high resolution proton (^1H) NMR. Since protons are abundant in organic samples, much lower detection limits and more rapid analyses are possible.

The primary disadvantage of ^1H NMR for the analysis of water soluble organics is that even trace amounts of water will produce a signal in the spectra. The water signal is typically the dominant signal in the spectra and overwhelms any signals in the general vicinity. In ^1H NMR, water will show a peak at 4.75 ppm and will overwhelm adjacent peaks such as those originating from amino acids and carbohydrates (approximately 3.0–5.0 ppm) (Sunghwan et al., 2003). Considering that water-soluble organics often contain amino acids and carbohydrates (Curl and Truelove, 1986; Gransee and Wittenmayer, 2000), the large water peak limits the utility of this procedure for these samples. Careful drying prior to dissolving the sample in a non-aqueous polar solvent will result in a smaller, but still interfering, water peak (Kaiser et al., 2003). Analysis of humic acids (Francioso et al., 2000) and dissolved organic matter (Francioso et al., 2000; Kaiser et al., 2002) has been accomplished by solution state ^1H NMR by selective irradiation of the water signal, thereby suppressing the signal. Unfortunately, selective irradiation does not completely remove the water signal, and adjacent peaks are also suppressed. Additionally, exchangeable protons, such as those attached to a heteroatom (i.e., amines, alcohols, carboxylic acids, etc.), will be suppressed. In solutions with very low sample concentrations, this is an unacceptable concession.

Conducting a ^1H NMR analysis using a non-polar solvent would eliminate any interference from water since water is not appreciably soluble in non-polar solvents. The challenge is to quantitatively transfer all the polar, water soluble material into a non-polar solvent. To accomplish this, the polar components in the sample would have to be converted to non-polar material by using a derivatizing agent. In this experiment, trifluoroacetic anhydride (TFAA) was selected as the derivatizing agent. Acetylation has been demonstrated to effectively and non-selectively react with a variety of polar compounds (Forbus et al., 1987; Wuts and Bergh, 1986). By selecting

TFAA, the trifluoroacetyl moiety is introduced on polar sites in the samples making them non-polar [Eq. (1)].



The introduction of the trifluoroacetyl moiety does not alter the general ^1H NMR spectra since the moiety does not have any protons and thus is invisible in the ^1H NMR spectra.

This study describe the TFAA derivatization and ^1H NMR analyses of root exudates and dissolved organic carbon (DOC). We define DOC as the material in the soil solution originating from the forest floor. Water-soluble organics, such as root exudates and DOC, influence carbon turnover, nutrient cycling, microbial activity, and transport of metals (Bowen and Rovira, 1991; Marschner, 1995; Gressel et al., 1995a; Guo and Chorover, 2003). The chemistry of these compounds can be sensitive to land management practices and may impact soil sustainability (Gressel et al., 1995b). Understanding the effects of land management practices on water-soluble organic matter and the resulting soil sustainability could provide the basis for selecting appropriate management practices. Changes in the chemistry of these compounds could be used as an early indicator of future trends in soil organic matter transformations.

Monitoring changes in the chemistry of water-soluble organics has been difficult because of the very low concentration and high complexity of the substrate. The objectives of this study are to (1) demonstrate the effectiveness of the derivatization technique on water soluble organic compounds and (2) demonstrate the effectiveness of liquid state ^1H NMR for the analysis of samples at very low concentrations.

MATERIALS AND METHODS

Site Description

The root exudates and DOC samples were obtained from larger ongoing studies examining the effects of intensive management on various measures of ecosystem productivity. The root exudates were collected from loblolly pine trees (*Pinus taeda* L.) at the Southeastern Tree Research and Education Site (SETRES) in the Sand Hills region of North Carolina (35°N latitude, 79°W longitude). Mean annual temperature and precipitation at the site are 17°C and 1210mm. The Wakulla series soil (sandy, siliceous, thermic Psammentic Hapludult) has a low water holding capacity, and is nutrient deficient for loblolly pine. The study is a 2 × 2 factorial with two levels of irrigation and fertilization, replicated on four blocks. Treatment combinations

were: (1) control (no irrigation or fertilization), (2) irrigation, (3) fertilization, and (4) irrigation plus fertilization. A complete description of the site and experimental design is provided by Albaugh et al. (1998).

Root exudates were only collected from the irrigated plots to eliminate any potential effects from fertilization. The irrigated plots were chosen over the control plots because the root exudates collection method, described next, requires the adsorbing media to be maintained in a moist condition. Typically, this is done by applying a set volume of water at the surface above the buried resin capsules once weekly. However, this action produces an artificial moisture column that is not uniform throughout the control plots. On sandy sites, such as SETRES, this artificial moisture column could be expected to increase microbial populations and activity resulting in pockets of microbial activity that are not typical of the plot. In the irrigated plots, this is not an issue since the irrigation is done uniformly across the plot.

Dissolved organic carbon samples were collected from two sites within the East Gulf Coastal Plain. The sites were located in Escambia County, Alabama (31°10'N 87°20'W) and Greene County, Mississippi (31°25'N 88°27'W). The climate was similar at both sites with an average growing season temperature of 24°C. The site in Alabama is a well-drained clayey soil of the Greenville series (Fine, kaolinitic, thermic Rhodic Kandudults) that was formed in clayey marine sediments. The site in Mississippi is a well-drained sandy soil of the Eustis series (Siliceous, thermic Psammentic Paleudults) that was formed in coarse-textured marine or fluvial sediments. The experiment was installed as a randomized complete block design replicated on four blocks. Two fertilization treatments were randomly assigned to the plots in each block: no fertilization (control) vs. fertilization at planting with 45 kg N ha⁻¹ and 50 kg P ha⁻¹. The fertilizer was applied as a broadcast application of 250 kg ha⁻¹ of diammonium phosphate.

Root Exudates

Root exudates were captured *in situ* onto XAD-7 resin capsules as described by Morse et al. (2000). The XAD-7 resins are a class of non-ionic carbonaceous resins that have been effectively used in capturing soluble organics in the soil (Morse et al., 2000; Johns et al., 1993; Skogley and Dobermann, 1996). In October 2003, XAD-7 resin capsules (UniBest, Inc., Bozeman, MT¹) were placed within the rooting zone approximately 10 cm below the mineral soil surface and 1 m from the tree stem. Although resin capsules can also trap microbial exudates and soil organic matter decomposition products, the placement of the capsules within the rooting zone results in the

¹The use of trade or firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture nor North Carolina State University of any product or service.

majority of the material captured to be root exudates. To minimize root disturbance adjacent to the resin capsules, a small spade was inserted into the soil at a 45° angle prior to placing the resin capsule in the soil (Fig. 1). The spade was then removed and the soil lightly tamped. After 7 days in the field, the resin capsules were excavated, placed in plastic bags, and returned to the laboratory for extraction and analysis.

Dissolved Organic Carbon

Zero tension lysimeters made with PVC materials were installed at the interface of the bottom of the forest floor (specifically Oa horizon/fragmented layer) and the surface mineral soil within each plot. Forest floor leachate was collected in amber glass bottles buried (45 cm below soil surface) adjacent to the lysimeters. Volume of leachate solution was measured at time of collection using a graduated cylinder. The volume data for the three lysimeters were averaged to obtain one volume value per plot per month. Leachate samples were collected once a month for 1 year (October 2001–October 2002). Samples were filtered through glass fiber filters (0.22 microns) within at least 48 h of collection and stored at 4°C until further analyses. The samples selected for this study were collected from the sandy site and clay site in July and August 2002, respectively. Dissolved organic carbon samples were collected from different soils and treatment conditions. The soil textures and treatments examined were sandy/control, clay/control and clay/fertilized.

Derivatization

The DOC samples were placed on a hotplate set at 50°C and evaporated down to approximately 5 mL. The samples were then placed in 10 mL test tubes and evaporated to dryness, in a SVC 100H Speed Vac Concentrator (Savant Instruments, Holbrook, NY), prior to derivatization. The root exudates were

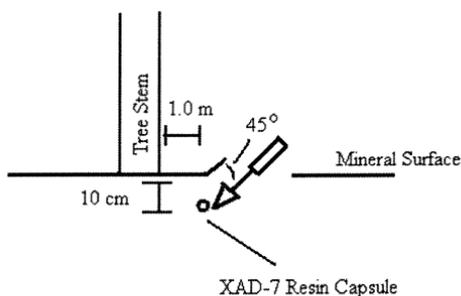


Figure 1. Schematic for burying the XAD-7 resin capsules.

derivatized while still absorbed onto the XAD-7 resin capsules. The resin is a chemically inert polyvinyl polymer which is neither altered nor extracted by this procedure. The derivatization involved adding 5 mL of neat TFAA to the samples and heating the samples at 50°C for 1h. In the case of the root exudates, the derivatization step also resulted in the extraction of the exudates from the resin capsules. Further extraction of the resin capsules did not result in any additional material being removed. The samples were allowed to come to room temperature, at which time 5 mL of chloroform (CHCl_3) was added. The derivatization was complete for all samples as the entire sample went into solution. For the root exudate samples, the resin capsules were then filtered through Whatman[®] WTP PTFE 1.0 μm membrane pore filters. The solutions were washed three times with 10 mL of 1% aqueous sodium bicarbonate solution. Any TFA produced in the reaction [Eq. (1)] was contained in the aqueous phase and was discarded. To remove any residual water, the organic phase was passed through a glass funnel fitted with a glass wool plug and approximately 200 mg of anhydrous sodium sulfate. The funnel was then rinsed with 2 mL of CHCl_3 and the wash was combined with the organic phase to completely transfer the derivatized material. The anhydrous organic solvent was evaporated off in the Speed Vac Concentrator. The residue was then brought back into solution in 0.75 mL of deuterated chloroform (CDCl_3) and analyzed by ^1H NMR and ^{19}F NMR.

A standard mixture of commercially available materials was made to test the reproducibility of the derivatization procedure. The standard mixture was made by thoroughly mixing 50 mg of lignin (Sigma-Aldrich, St. Louis, MO) and 50 mg of potato starch (Sigma-Aldrich, St. Louis, MO). Four 20 mg sub-samples of the standard mixture were placed in 10 mL test tubes, derivatized, and prepared for ^1H NMR analysis as described previously.

NMR Analyses

The ^1H NMR experiments were performed on a Mercury VX 400 Spectrometer (Varian, Inc., Palo Alto, CA) at an operating ^1H frequency of 400.137 MHz by means of a 5 mm 4NUC probe equipped with additional coils for z-gradients. Sixty-four scans were collected with a spectral width of 5200 Hz using a pulse width of 7 μs and a pulse delay of 1 s. The free induction decays (FIDs) were multiplied by an exponential line broadening function of 0.5 Hz prior to Fourier transformation to improve the signal/noise ratio of the spectra. All chemical shifts are reported relative to trimethylsilane which was used as an external standard. Compound class designations were based on the signal intensities in the following regions: alkyl (0–2.5 ppm); O-alkyl (2.5–5.0 ppm); olefinic (5.0–6.5 ppm); and aromatic and phenolic (6.5–9 ppm). Compound classes were designated based on the computerized integrals of the signal intensity in each chemical shift region and were calculated as percentages of the total area.

The ^{19}F NMR experiments were also performed on a Mercury VX 400 Spectrometer with 32 scans being collected for each sample. The spectral width was 51000 Hz using a pulse width of $5\ \mu\text{s}$ and a pulse delay of 4 s. The FIDs were multiplied by a 10 Hz exponential line broadening function prior to Fourier transformation. The NMR instrument was not calibrated to a standard and may have given erroneous chemical shift information. In this study this is not of great concern since the ^{19}F NMR experiments were only done to verify sample derivatization. Precise information on chemical shifts for ^{19}F NMR experiments could be obtained by calibrating the instrument with an accepted external standard such as trifluorotoulene.

Total Carbon Determination

To determine total carbon concentration in the root exudate samples, duplicate samples were prepared as described previously except that after the CHCl_3 wash, the solution was absorbed onto 75 mg of Chromosorb W in a test tube. The CHCl_3 was evaporated off in the Speed Vac Concentrator and, to ensure no residual CHCl_3 was still absorbed on the Chromosorb W, the samples were placed in a 50°C oven overnight. The absorbed material was then analyzed for total carbon and nitrogen on a Carlo Erba C/N/S analyzer (Fisons Instruments, Danvers, MA). The total carbon concentrations of the DOC samples were determined with a Shimadzu TOC Analyzer[©] Model 9300C (Shimadzu Instruments, Inc., Columbia, MD).

RESULTS AND DISCUSSION

Soluble organic matter in soils can be made up of several compounds varying in polarity. Among the more polar constituents of soluble organic matter are carbohydrates, particularly the simple sugars. Since these compounds are highly polar, they typically do not go into solution with a non-polar solvent. To test the applicability of the derivatization method, 50 mg of glucose was derivatized and prepared for ^1H NMR analysis as described previously. The derivatized glucose showed strong signals in the ^1H NMR spectrum (Fig. 2A). The major peaks were in the O-alkyl region (2.5–5.0 ppm) with some peaks being detected in the olefinic region (5.0–6.5 ppm). The presence of the electronegative fluorine atoms can shift the spectra slightly resulting in some signals from O-alkyl compounds being detected in the olefinic region. The derivatization of glucose was quantitative as all the material went into solution with CHCl_3 and produced strong signals in the ^{19}F NMR spectrum (Fig. 2B). The multiplets found in both the ^1H and ^{19}F NMR spectra are probably a result of coupling between the ^{19}F and ^1H atoms. This hypothesis is supported by the fact that earlier experiments in which there was residual TFA (no coupling possible) showed only a singlet in the ^{19}F NMR spectra (data not shown). The amount of glucose used in

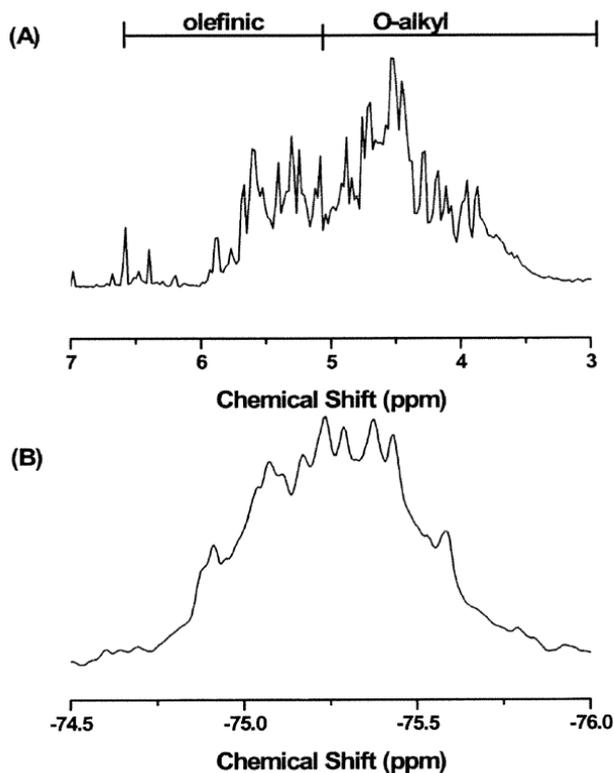


Figure 2. (A) ^1H NMR spectrum and (B) ^{19}F NMR spectrum of glucose derivatized with trifluoroacetic anhydride.

this experiment is considerably greater than the mean amount of DOC or root exudates collected (Table 1). Given the complete derivatization of the large amount of the polar glucose molecule, it is reasonable to expect that the derivatization would be quantitative for the DOC and root exudate samples. This

Table 1. Amount of root exudates and dissolved organic carbon (DOC) collected

Sample	Soil type	Treatment	Volume (mL)	Mean mass (mg)	N
Root exudate	Sand	Irrigated	N/A ^a	0.03 (0.01) ^b	8
DOC	Sand	Control	263	22.27	1
DOC	Clay	Control	107	15.49	1
DOC	Clay	Fertilized	57	25.52	1

^aNot applicable.

^bStandard error is shown in parentheses.

expectation is supported by the fact that the derivatized DOC and root exudates samples were completely dissolved by CHCl_3 .

The amount and composition of water soluble materials in forested ecosystems can be highly variable even when the samples are taken adjacent to each other. Consequently, a standard mixture of commercially available lignin and starch was used to test the reproducibility of the derivatization procedure. This mixture was selected because it provides a spectrum that covers most of the compound classes in soil (Fig. 3). There were no discernable differences in the spectra of the standard mixture replicates as all the spectrum could be superimposed on each other. The variability of the area percentages for each compound class was also very low (Table 2) demonstrating that the derivatization procedure was highly reproducible. Note that the

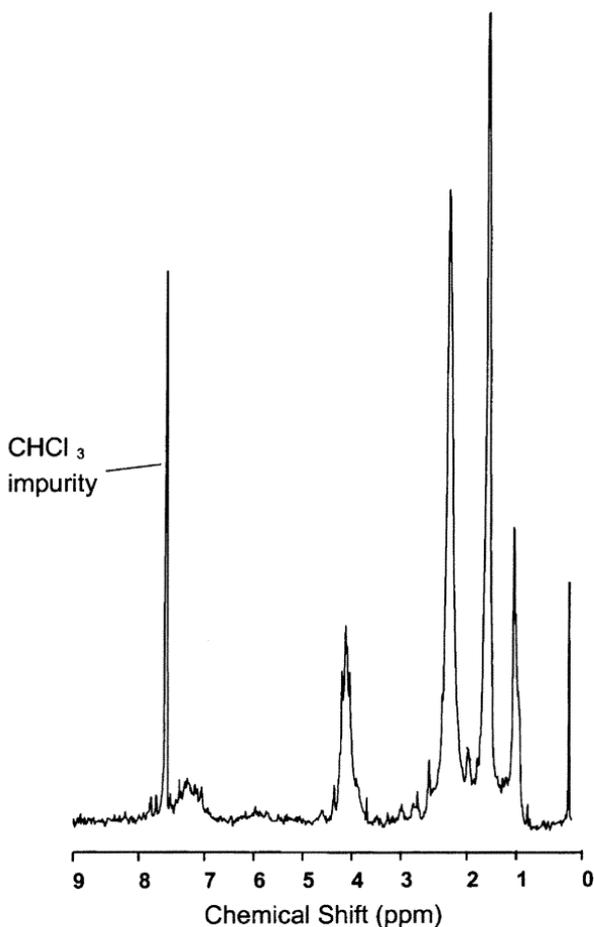


Figure 3. ^1H NMR spectrum of a 1:1 mixture of lignin and starch derivatized with trifluoroacetic anhydride.

Table 2. Mean area percentages for different compound classes in a 1:1 lignin:starch mixture ($n = 4$)

Compound class	Mean area percent ^a	Standard Error	Variance
Alkyl	72.77	0.52	1.08
O-alkyl	16.65	0.46	0.86
Olefinic	2.32	0.17	0.12
Aromatic/phenolic	8.24	0.49	0.08

^aValues exclude contribution of CHCl_3 signal.

O-alkyl signal originating from starch covered the chemical shift range of 3.5–4.0 ppm (Fig. 3); whereas the glucose signal covered a chemical shift range of approximately 3.5–6.0 ppm (Fig. 2A). The larger steric congestion found in polymeric compounds (i.e. starch) as compared with monomers (i.e., glucose) can result in a compression of the signal and a move to lower chemical shifts (Silverstein et al., 1981) and is probably the cause of the observed differences in signal position.

There is some overlap in the chemical shift regions for different compound classes and more investigation, such as mass spectrometry, would be required to conclusively determine compound identity. However, the ^1H NMR spectrum provides useful information on the overall chemistry of the sample. The spectrum of root exudates (Fig. 4A) is dominated by aliphatic compounds (0–2.5 ppm) but there are instances of O-alkyl (2.5–5.0 ppm), olefinic (5.0–6.5 ppm), and aromatic and phenolic (6.5–9 ppm) compounds. The sharpness of the peaks minimizes confusion as to the type of material present due to signal overlap. Since the derivatization procedure was demonstrated to be effective and quantitative for highly polar compounds (i.e., glucose), the dominance of aliphatics (non-polar compounds) in the spectrum is due to the sample composition and not to selective dissolution of non-polar compounds. The relative simplicity of the ^1H NMR spectrum of the root exudates is probably due to the fact that the root exudate collections were done over a short period of time (1 week) and under a controlled set of field conditions (i.e., irrigated plots). It is probable that longer collection times and different site treatments could result in dramatically different spectra that may reveal insight into treatment impacts on belowground carbon input from roots.

Dramatically different ^1H NMR spectra were observed for the DOC collected from the different soil texture and treatment combinations. The ^1H NMR spectrum of the DOC samples from the sandy/control sites (Fig. 4B) was dominated by aliphatic compounds (0–2.5 ppm) with small inclusions of O-alkyl (2.5–5.0 ppm), and aromatic and phenolic (6.5–9 ppm) compounds. In sharp contrast, the DOC samples from the clay/control sites (Fig. 4C) had very little aliphatic presence and

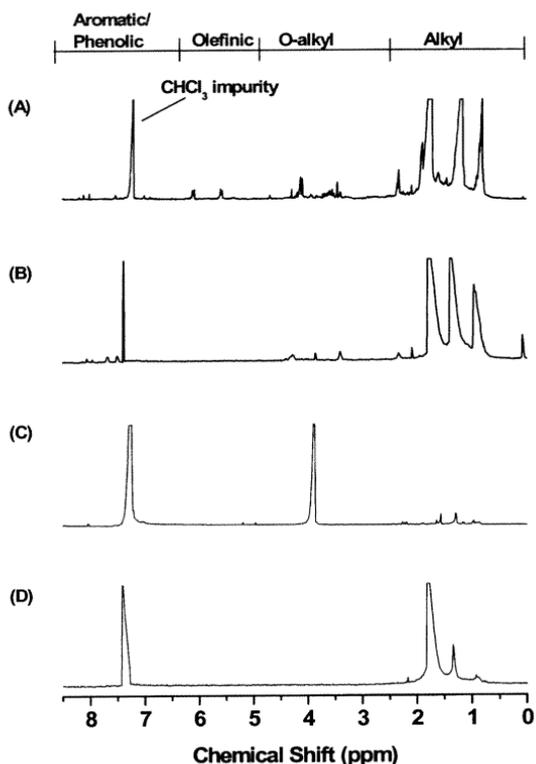


Figure 4. ^1H NMR spectra of trifluoroacetic anhydride derivatized (A) root exudates, (B) DOC samples from sandy/control sites, (C) DOC samples from clay/control sites, and (D) DOC samples from clay/fertilized sites.

were dominated by O-alkyl compounds with trace amounts of olefinic (5.0–6.5 ppm) and aromatic compounds. The sharp single peak at 3.9 ppm suggests that this signal is probably not from carbohydrates. Carbohydrates would be expected to produce a multiplet in this region as we saw for glucose (Fig. 2A), starch (Fig. 3) and in the root exudates (Fig. 4A). Since the CHCl_3 impurity is present at the expected shift (7.26 ppm), the possibility that there was a shift in the residual water peak from the expected signal position of 4.75 ppm to 3.9 ppm is ruled out. Finally, the DOC samples from the clay/fertilized sites (Fig. 4D) contained only aliphatic compounds. The significance of these vastly different spectra is beyond the scope of this manuscript; however, they do demonstrate the utility of the derivatization method coupled with high resolution ^1H NMR to detect compositional differences in DOC samples collected from different sources.

CONCLUSIONS

The derivatization procedure in which the trifluoroacetyl moiety is introduced on polar sites provides a means to determine the organic chemical composition of water soluble organics by ^1H NMR. This is particularly attractive because sample with very low concentrations of organics could be analyzed. The trifluoroacetyl moiety does not complicate the ^1H NMR spectra since it does not contain any protons. The utility of the procedure was demonstrated with DOC and root exudate samples. The complete spectra of compounds that could potentially be in a sample were detected without interferences. The signal intensities were generally sharp with little signal overlap; thus, simplifying the interpretation of the spectra. Very different spectra were observed for each sample (root exudates and each of the DOC samples) indicating that the chemistry of the soil solution can vary dependent on the source. This could be a very important observation in studies investigating soil processes including carbon sequestration, microbial activity, and soil absorption/desorption processes. Additionally, this research could provide a means of detecting changes in the chemistry (i.e., quality) of water soluble organics resulting from different land management practices.

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