HIGH RESOLUTION NUCLEAR MAGNETIC RESONANCE INVESTIGATIONS OF POLYMETHYLENIC PLANT BIOPOLYMERS: STRUCTURAL DETERMINATIONS AND POST-DEPOSITIONAL AMMONIA NITROGEN INCORPORATION

DISSERTATION

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By

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* * * * *

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ABSTRACT

Higher order plants have protective barriers on all of their exterior surfaces to control nutrient flow, prevent dehydration and protect against infection. These protective barriers contain long chain polymethylenic polyesters known as cuticular and suberinic biopolymers. Soil organic matter (SOM) and sedimentary organic matter (SDOM) contain a high proportion of polymethylenic carbon and many researchers have attributed the source of persistent SOM and SDOM to cuticular and suberinic biopolymers. Understanding the chemical composition of these materials is essential to understanding the soil and sedimentary processes which lead to creation of recalcitrant SOM and SDOM. A wide variety of analytical techniques are utilized within this study to characterize polymethylenic plant biopolymers with a focus upon advanced solid-state high resolution magic angle spinning (HRMAS) nuclear magnetic resonance (NMR) spectroscopy. Because the polymethylenic cuticular and suberinic biopolymers are significant SOM and SDOM precursors, they are also utilized within this study as proxies to examine chemical transformations which occur in these settings.

Suberan, a non-saponifiable suberinic biopolyester, is a likely source of persistent paraffinic SOM and SDOM. Previous structural investigation of suberan is limited and the cause for the chemical stability of this material has been unclear. In this study suberan isolated from River Birch (*Betula nigra*) bark is characterized using a wide variety of
analytical techniques including Fourier transform infrared (FTIR) spectroscopy, flash pyrolysis gas chromatography mass spectrometry (py-GCMS), tetramethyl ammonium hydroxide thermochemolysis (TMAH) GCMS, scanning electron microscopy (SEM), cross polarization magic angle spinning (CPMAS) $^{13}$C NMR spectroscopy, one-dimensional HRMAS $^{1}$H NMR spectroscopy and the two-dimensional HRMAS NMR spectroscopic techniques; $^{1}$H-$^{1}$H total correlation spectroscopy (TOCSY) and $^{1}$H-$^{13}$C heteronuclear single quantum coherence (HSQC). The FTIR, and GCMS analyses show suberan to be structurally similar to the closely related suberinic biopolymer, suberin. The SEM images depict a flat, ordered and crystalline physical character. The CPMAS $^{13}$C and HRMAS NMR analyses show suberan to contain a highly crystalline polymethylenic character and a lack of mid chain functionality, and these structural features set this biopolymer apart from the saponifiable suberin. We believe these features provide physical protection from saponification and lead to increased recalcitrance within depositional settings.

Solid-state HRMAS NMR spectroscopic techniques are used to analyze soil organic structures in two distinct pristine forest settings within Clear Creek Metro Park (Lancaster, Ohio). These techniques are also applied to the leaves and bark of Canadian Hemlock (Tsuga canadensis) and Yellow Birch (Betula alleghaniensis) trees which are the predominant plants at these two settings. By comparing the structures contained within soils and plant source materials we seek to determine whether structures indicative of cuticular and suberinic biopolymers persist within the SOM. Structural features associated with cuticular and suberinic biopolymers are observed within the soils and we attribute these SOM features to plant biopolymer inputs. Ester-linkages bind these plant
materials into macromolecules and some of the cross-peaks associated with these functional groups are not observed within the SOM NMR spectra. We propose that cuticular and suberinic biopolymers are significant contributors to the polymethylenic structures in SOM, but these macromolecules are partially depolymerized upon deposition to monomeric alkyl chains. It is also shown that some organic structures within both soils are not attributable to bark or leaf inputs.

Cutin is a polymethylenic cuticular biopolyester which is found within the leaves and fruit skins of higher plants. The abundance of cutin within plants and its polymethylenic structure indicate that it is likely a persistent SOM and SDOM precursor. Upon deposition this biopolymer undergoes chemical alterations. We use CPMAS $^{13}$C and HRMAS NMR spectroscopic techniques to provide evidence of aldehyde functional groups within the cutin structure. We propose that this highly reactive functional group is an important site where post-depositional nucleophilic substitutions may occur.

Nitrogen is incorporated into SOM and SDOM within depositional environments by biotic and abiotic processes, yet the mechanisms of this incorporation are still unclear. Amide nitrogen is the most abundant form of organic nitrogen within soils and sediments. Peptide nitrogen is a likely source of amides, yet peptides are labile leading to questions about the nature of persistent amides. We hypothesize that recalcitrant plant biopolymers can incorporate ammonia nitrogen into persistent structures leading to formation of persistent soil organic nitrogen structures. To explore abiotic nitrogen incorporation pathways, we reacted cuticular biopolymers, isolated from tomato (*Lycopersicon esculentum*) fruit skins and *Agave* (*Agave americana*) leaves, with isotopically labeled ammonium chloride ($^{15}$NH$_4$Cl) at a variety of environmentally relevant pH levels.
Elemental analysis and HRMAS $^{15}$N NMR spectroscopic techniques were utilized to show covalent ammonia nitrogen incorporation into cuticular biopolymers. We show the formation of both amines and amides which we attribute to nucleophilic substitution reactions occurring at carbonyl and carboxyl sites, respectively. We conclude that aldehydes, ketones and epoxides react with ammonia by nucleophilic substitution to form amines. We propose that the formation of amides occurs by ammonolysis of esters. We further propose that post-depositional abiotic ammonia nitrogen incorporation by cuticular biopolymers is a significant source of the persistent nitrogen structures found in SOM and SDOM.
Dedicated to Carrie, Kevin and Kyle
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CHAPTER 1

INTRODUCTION

1.1 Polymethylenic plant biopolymers

Plant biopolymers constitute the largest pool of living organic matter in the world. While plants are comprised of a large variety of biopolymers, almost all of their biomass can be attributed to four distinct classes of organic compounds; lignin, cellulose, hemicellulose and polymethylenic biopolymers. Polymethylenic biopolymers include cuticular and suberinic biopolymers which are found in the external surfaces of all higher order plants. These biopolymers enable higher plants to regulate nutrient flow, prevent dehydration and protect themselves from infection. In this study we focus on providing a greater understanding of the chemical nature and post-depositional fate of macromolecular polymethylenic cuticular and suberinic biopolymers as they become part of the soil organic matter. Cuticular biopolymers, cutin and cutan, provide structural support to the extracellular waxy lipid layers found within the shiny protective cuticles of leaves, conifer needles and fruit skins. The suberinic biopolymers, suberin and suberan, are contained within the cell walls of the outer layers of bark and roots. Both cuticular and suberinic biopolymers are thought to be composed of long-chain fatty acids that are
linked in varying fashions by ester bonds (Deshmukh et al., 2005, Graça and Santos, 2007). As such, these biopolymers could be readily saponified and depolymerized, but some of these biopolymers are not. Those that are completely saponifiable, cutin and suberin, are found within all higher plant surfaces, while non-saponifiable cutan and suberan are not as ubiquitous.

Estimations have been made that 180-1500 kg/hectare of cuticle material is found in the living plants within temperate forests and agricultural settings (Riederer, 1990). Within plant cuticles, the saponifiable biopolyester cutin comprises 40-80% of the cuticle weight: the remainder being extractable lipids and carbohydrates (Heredia et al., 2003). Because cutin is so abundant, it has been the focus of much research. Depolymerization gas chromatographic mass spectroscopic (GCMS) studies have shown cutin to be mainly comprised of ester cross-linked C_{16} and C_{18} \omega-hydroxy fatty acid monomers (Baker and Holloway, 1970; Walton and Kollatukudy, 1972; Kolattukudy, 1980; Holloway, 1982; del Rio and Hatcher, 1998). Many polymeric structures for cutin have been proposed (Zlotnik-Mazori and Stark, 1988; Jeffree, 1996; Ray et al., 1998; Fang et al., 2001); all based on the above monomer units as primary linkage points. Recently, Deshmukh et al. (2003) utilized high resolution magic angle spinning (HRMAS) nuclear magnetic resonance (NMR) spectroscopic techniques to show that primary and secondary alcohol functionality as well as \alpha-branched fatty acids are also important constituents within the polyester cutin. In the quest to understand the structural composition of cutin and its fate in soils using high resolution magic angle spinning (HRMAS) nuclear magnetic resonance (NMR) techniques, some new and previously unidentified reactive functional groups are described (Chapter 4).
The other cuticular biopolyester, cutan, provides a stronger barrier against dehydration than cutin and has only been isolated from drought adapted plants (de Leeuw and Largeau, 1993). Characterization of cutan by depolymerization GCMS and NMR techniques has shown C_{15}–C_{31} alkyl chains which are ester-linked to an aromatic backbone (Tegelaar et al., 1989b; McKinney et al., 1996; Schouten et al., 1998), and Deshmukh et al. (2005) have proposed a polymeric structure shown in Figure 1.1. In Chapter 5, we utilize both cutin and cutan to explore reaction pathways capable of leading to ammonia nitrogen incorporation.

Figure 1.1: Polymeric structural model of the cuticular biopolymer cutan as proposed by Deshmukh et al., 2005. Capital letters (A-L) denote specific functional groups identified within NMR spectra. Small case n indicates chain lengths of 25-32 methylene units while m indicates chain lengths of at least 6 methylene units. m + n = 31 or less.
The saponifiable suberinic polyester, suberin, is a cell wall biopolymer found in the protective and wound healing barriers of bark and roots. Numerous suberin depolymerization GCMS and NMR studies have shown long C\textsubscript{16} – C\textsubscript{22} alkyl chains containing alcohol, olefin, and epoxide functional groups ester-linked to glycerol and aromatic components (Holloway, 1984; Pacchiano et al., 1993; Graça and Pereira, 2000). Polymeric suberin structures have been proposed and shown to be comprised of two distinct domains: a polymethylenic domain covalently bound to a polyphenolic domain (Bernards and Lewis, 1998; Lulai and Corsini, 1998; Graça and Santos, 2007). In Chapter 3 we examine the post-depositional fate of suberin functional groups within forest soils.

Suberan has a limited research history and has only been isolated from a few sources (Tegelaar et al., 1995). Tegelaar et al. (1995) found a non-saponifiable suberinic polymethylenic biopolymer isolated from Birch (Betula japonica and Betula alba) bark and proposed the name suberan [suber = cork (lat.); -an = derived from]. To date, the pyrolysis GCMS analysis of Tegelaar et al. (1995) remains the sole published structural analysis of this recalcitrant biopolymer. It has been one goal of this thesis to provide a detailed structural characterization of suberan (Chapter 2), because polymethylenic soil organic constituents that survive the intense degradative action called humification are likely to be similar to suberan (Nierop, 1998).

1.2 Soil and sedimentary organic matter precursors

Soil organic matter (SOM) and sedimentary organic matter (SDOM) are comprised of a complex mixture of partially degraded plant litter and microbial biomass. Organic matter inputs are transformed or humified within these settings, becoming a
much more diverse mixture of molecular structures (Schnitzer, 1985; Kögel-Knabner et al., 1992; Hedges and Oades, 1997). Labile components of plant and microbial remains, such as cellulose, hemicellulose and peptides contain abundant polar functional groups which lend them to rapid degradation. Within a few years most of these inputs are recycled to CO₂ or dissolved and leached away, while the remaining organic material becomes resistant to degradation for a relatively long time (Hedges and Oades, 1997). It is well established that polymethylenic organic material persists within soil and sedimentary systems (Augris et al., 1998; Nierop, 1998; Hu et al., 2000). Many studies suggest cuticular and suberinic polymethylenic biopolymers and even specifically suberan are significant precursors to the persistent polymethylenic materials found in SOM and S₂OM (Largeau et al., 1986; Nierop, 1998; Collinson et al., 1999; Bull et al., 2000; Quénéa et al., 2006a). Augris et al. (1998) concluded that pyrolysates of the resistant organic residue fraction of forest soil “comprises an abundant contribution of selectively preserved macromolecular materials, cutans and/or suberans, directly inherited from higher plants”. Naafs et al. (2005) utilized a variety of spectroscopic techniques to analyze changes with depth of the polymethylenic organic material in a forest soil and showed that suberinic structures in soils persist, with a predominant recalcitrance among C₁₆ chain lengths. Simpson et al. (2002) performed diffusion NMR experiments on humic substances from an Oak forest and showed that C₁₆ and C₁₈ alkyl units represent a major input into soil biomass. It is clear that polymethylenic materials persist in soil and sedimentary systems and polymethylenic plant biopolymers are a likely source of this material.
1.3 Soil and sedimentary organic matter

Although polymethylenic materials are consistently shown to persist in soils, chemical alterations occurring within post-depositional environments have precluded definitive identification of the relative contributions of organic inputs to the stable carbon pool contained within SOM and SDOM. It is known, however, that the persistent fractions of SOM and SDOM have a major impact on global cycling of carbon, nutrients and contaminants (Stevenson, 1982; Deshmukh, 2003; Lal, 2004). Estimates have been made that soil contains 3.3 times the amount of carbon in the atmosphere and 4.5 times the amount of biotic carbon (Lal, 2004). Understanding the organic chemical reactions occurring within these depositional environments is important part of understanding global environmental processes, yet the organic compounds found within SOM and SDOM have revealed an extremely high degree of heterogeneity and complexity (Stevenson, 1982).

This complexity provides a difficult backdrop upon which to observe specific molecular transformations. For this reason, studies of SOM and SDOM transformations within depositional environments have often observed bulk properties such as enrichment or depletion of chemical functional groups (Kögel-Knabner, 2000). In order to gain a clearer understanding of soil and sedimentary reactions at a molecular level, specific SOM fractions, precursors, or model systems have also been utilized (Hsu and Hatcher, 2006; Rontani et al., 2005; Amrani et al., 2007). The use of SOM precursor plant biopolymers allows for molecular level examination of chemical transformations that occur within well established structures shortly after deposition. The goal of Chapters 4
and 5 of this thesis is to identify reactive sites and observe chemical transformations of cuticular biopolymers which are likely to occur within depositional environments.

1.4 Transformations within soil and sedimentary organic matter

The transformations that occur within soil and sedimentary systems are not well understood and involve recombination of microbial and plant materials by a complex mixture of reaction pathways including biological, photochemical and chemical processes (Collinson et al., 1997; Hsu and Hatcher 2006; Rontani et al., 2005; Amrani et al., 2007). Recently, studies have more specifically shown how persistent cuticular and suberinic biopolymers are transformed within depositional environments. Laboratory simulations of biological processes by lipase enzymatic cleavage of esters within lime cutin have shown a resistance to degradation leading to the conclusion that cross-linkages within cutin create a physical barrier to enzymatic processes (Ray and Stark, 1998). Rontani et al. (2005) performed visible light photo-oxidation of cutin and found the unsaturated components within the polymethylenic plant material oxidize to hydroperoxy-hydroxenoic acids (adds a hydroxyl next to a double bond). Although biological and photochemical processes are involved in post-depositional transformation of biopolymers, studies have also shown abiotic substitutions by nitrogen species play an important role (Nommik and Vahtras, 1982; Schulten et al., 1997; Knicker et al., 1996; Amrani et al., 2007).

Inorganic nitrogen is known to be reactive within SOM and SDOM and can react with a wide variety of oxygen functional groups, such as esters, ketones, aldehydes, and epoxides. These functional groups are contained within cuticular and suberinic
biopolymers and may be active sites for abiotic nitrogen incorporation. Davidson *et al.* (2003) recently showed that significant immobilization of inorganic nitrogen occurs in forest soils, challenging previously widely held beliefs that microbial processes are the dominant pathways for nitrogen immobilization in soil. Although nitrogen is widely believed to play a significant role in soil processes, limited development of analytical techniques capable of characterizing organic nitrogen has led to a lack of understanding about how nitrogen incorporates into SOM and SDOM.

### 1.5 Objectives and outline of the dissertation

This original research is designed to enhance our knowledge of the chemical structures and post-depositional fate of polymethyleneic plant materials, particularly cuticular and suberinic biopolymers. This work is split into two major focus areas. Firstly, characterization of cuticular and suberinic biopolymers isolated from a variety of plant materials in Chapters 2, 3 and 4. Secondly, analyses of a few important post-depositional chemical transformations of these materials within soil and sedimentary systems in are examined in chapters 3 and 5.

The non-saponifiable suberinic biopolymer, suberan, has been proposed as a major source of persistent polymethyleneic material found within SOM and SDOM, yet limited previous structural characterization of this biopolymer has been conducted (Nierop, 1998; Tegelaar *et al.*, 1995). It is unclear why the polyester suberan is not saponifiable. We hypothesize that it is structurally different than the closely associated yet saponifiable suberin biopolyester. Chapter 2 seeks to determine structural differences between these two biopolymers and to provide an explanation for the chemical resistance of suberan. In
order to provide detailed structural characterization we isolated suberan from River Birch (\textit{Betula nigra}) bark and utilized a wide variety of analytical techniques. These include Fourier transform infrared (FTIR) spectroscopy, flash pyrolysis GCMS, tetramethyl ammonium hydroxide thermochemolysis (TMAH) GCMS, scanning electron microscopy (SEM), cross polarization magic angle spinning (CPMAS) $^{13}$C NMR, one-dimensional high resolution magic angle spinning (HRMAS) $^1$H NMR and the two-dimensional HRMAS NMR techniques; $^1$H-$^1$H total correlation spectroscopy (TOCSY) and $^1$H-$^{13}$C heteronuclear single quantum coherence (HSQC). The combination of these techniques allowed for an extensive characterization of this material and facilitated proposal of a structural model not previously presented in the literature. We also observe significant structural differences between suberan and suberin, which likely contribute to the chemical resistance associated with suberan.

We know that the bark and leaves of higher order plants contain suberin and cutin, respectively. We hypothesize that these polymethylenic suberinic and cuticular biopolymers are likely significant contributors to the organic matter contained within soils. Chapter 3 examines the persistence of the polymethylenic components contained within the bark and leaves of higher order plants. We collected soil, leaf and bark samples from two pristine forest sites within the Clear Creek Metro Park (Lancaster, Ohio). One site is predominantly Canadian Hemlock (\textit{Tsuga canadensis}) while the other is predominantly Yellow Birch (\textit{Betula alleghaniensis}).

Solid soil, bark and leaf samples provide a significant analytical challenge. Derivatization can depolymerize samples to make them amenable to powerful analytical techniques such as GCMS and liquid state NMR, but also always alters the material prior
to analysis. To minimize alterations, we utilize solid-state HRMAS NMR techniques to identify the carbon and hydrogen functional groups contained within whole solid soils and plant materials. We then compare the organic functional groups contained within the soils to those contained within bark and leaf samples obtained from the same sites. By comparing the chemical functional groups within the soil and plant samples, we seek to determine if functional groups known to be contained within suberin and cutin are persisting in these depositional environments. Both soil samples indicate a strong presence of polymethylenic material and display chemical functional groups associated with suberin and cutin, while other structures observed within the soils are clearly not of this origin.

The cuticular biopolymer cutin is a major source of polymethylenic material in SOM and S<sub>D</sub>OM. It has been characterized previously and shown to contain some reactive functional groups such as epoxides and olefins (Deshmukh et al., 2003; 2005). The presence of reactive functional groups within this ubiquitous and recalcitrant biopolymer likely plays a significant role in chemical alterations occurring within depositional environments. In chapter 4 we utilize CPMAS<sup>13</sup>C NMR, one-dimensional HRMAS<sup>1</sup>H NMR, two-dimensional HRMAS<sup>1</sup>H<sup>-1</sup>H TOCSY NMR and two-dimensional HRMAS<sup>1</sup>H<sup>-13</sup>C HSQC NMR spectroscopic techniques to provide evidence of previously unidentified aldehyde functionality within cutin isolated from tomato (<i>Lycopersicon esculentum</i>) fruit skin and <i>Agave</i> (<i>Agave americana</i>) leaf cuticles. Aldehydes are very reactive electrophiles and likely participate in nucleophilic substitution reactions during the early stages of diagenesis (humification). The observation and discussion of the presence of aldehydes within cutin helped lead to the studies conducted within Chapter 5.
The presence of reactive sites such as aldehydes and epoxides within cuticular biopolymers leads to a potential for nucleophilic substitution reactions with ammonia nitrogen. Soil humic materials are known to contain nitrogenous functional groups but their origin is somewhat of an enigma because source materials for humic substances, lignin and polymethylene biopolymers, are typically devoid of nitrogen as components of their structure. Solid-state $^{15}$N NMR studies (Knicker and Lüdemann, 1995; Knicker et al., 2001) suggest that peptides become incorporated into resistant plant biopolymers during humification, mainly because the predominant NMR signal observed is that of amides. Ammonia is ubiquitous in depositional environments and is capable of participating in nucleophilic substitution reactions with electrophilic oxygen functional groups. We hypothesize in chapter 5, as an alternative to the hypothesis that peptides are the source of stable soil nitrogen, that ammonia nitrogen will incorporate into recalcitrant cuticular biopolymers via nucleophilic substitution reactions. To test our hypothesis we conducted laboratory reactions at various pH between isotopically labeled ($^{15}$N) ammonium chloride ($^{15}$NH$_4$Cl) and cuticular biopolymers isolated from tomato ($Lycopersicon esculentum$) fruit skin and Agave ($Agave americana$) leaf cuticles. The isolated biopolymers and reaction products were analyzed by elemental analysis, CPMAS $^{13}$C NMR and HRMAS $^{1}$H-$^{13}$C HSQC NMR techniques. The data clearly indicate that covalent abiotic ammonia nitrogen incorporation by cuticular biopolymers is a viable reaction pathway. While evidence of reaction with aldehydes or epoxides is shown, it is also shown that ammonolysis of esters is a significant reaction mechanism. This reaction pathway is a likely source of recalcitrant amide nitrogen found within soils and sediments.
CHAPTER 2

STRUCTURAL CHARACTERIZATION OF SUBERAN, ISOLATED FROM
RIVER BIRCH (Betula nigra) BARK

2.1 Introduction

The complex chemical mixtures contained within soil organic matter (SOM) and sedimentary organic matter (S\text{p}OM) are comprised of decaying plant detritus and microbial biomass. Studies have shown a strong polymethylenic character in the persistant fraction of SOM and S\text{p}OM. Both cuticular and suberinic biopolymers have been proposed an important source of this polymethylenic material (van Bergen et al., 1997; Augris et al., 1998; Nierop, 1998; Bull et al., 2000; Poirier et al., 2003; Quénéa et al., 2006a). When buried in peats that become coal, these materials are known to survive for millions of years (Tegelaar et al., 1991; 1995; Ewbank et al., 1996; Hatcher and Clifford, 1997). Studies have been conducted to discern the chemical nature of these biopolymers, because they are major input sources of the recalcitrant polymethylenic material found in SOM an S\text{p}OM (Kögel-Knabner et al., 1992; Nierop, 1998; Bull et al., 2000; Naafs and van Bergen, 2002; Deshmukh et al., 2003; 2005; Heredia, 2003; Naafs et al., 2005; Quénéa et al., 2006b).
Higher order plants have peridermal layers on their exterior surfaces that act as barriers to retain water as well as to resist microbial and fungal attacks. These layers contain lipids, polysaccharides, lignin and cuticular or suberinic biopolymers. The cuticular and suberinic biopolymers have been classified as cutin, cutan, suberin and suberan and are all long-chain polymethyleneic polyesters. The cuticular biopolymers, cutin and sometimes cutan, are contained within the cuticular layers of leaves and fruit while roots and bark contain the suberinic biopolymers, suberin and sometimes suberan. Cutin and suberin are defined as saponifiable, while cutan and suberan are defined as non-saponifiable. Depolymerization studies have shown cutin to be comprised of mainly C_{16} and C_{18} monomeric alkyl units containing primary (1°) alcohol, secondary (2°) alcohol and acid functional groups with the 2° alcohol groups located on C_9, or C_{10} (Baker et al., 1970; Kolattukudy, 1980; 1984; del Rio and Hatcher, 1998). High resolution magic angle spinning (HRMAS) nuclear magnetic resonance (NMR) investigation of cutin has supported these findings, also showing evidence for ester cross-linking and structures have been proposed (Fang et al., 2001; Deshmukh et al., 2003). Cutan depolymerization has yielded long-chain C_7-C_{33} polymethyleneic domains with alcohol and acid functional groups (McKinney et al., 1996; Boom et al., 2005). Cutan has been found to be a crystalline polymethyleneic cross-linked biopolyester with alcohol, aromatic, epoxide and acid functional groups, and a structure has been proposed (Deshmukh et al., 2005).

Suberin is a biopolymer that is localized in cell walls of the endodermis of primary roots and the periderm of mature roots and bark (Tegelaar et al., 1995; Franke et al., 2005). Investigators have proposed that the structure for this hydrolyzable polyester is
composed of long-chain (C_{11}-C_{24}) polymethylenic domains with carboxylic acids held
together by aromatic rings and esters (Kollatukudy et al., 1976; Kolattukudy, 1980; 1981;
Holloway 1984). Depolymerization studies of suberin have found the polymethylenic
monomers to be mainly comprised of C_{16}, C_{18}, C_{22} and C_{24} \omega-hydroxy fatty acids and
dicarboxylic acids (del Rio and Hatcher, 1998; Graça and Pereira, 2000). Suberinic
material is comprised of heteropolymeric material that occurs within two structural
domains, one a polymethylenic domain and the other a separate associated polyphenolic
domain (Bernards and Lewis, 1998; Lulai and Corsini, 1998; Graça and Santos, 2007).
The polyphenolic domain has been found in trace amounts compared to the
polymethylenic domain and comprises a small fraction (<5%) of the suberinic material
(Schreiber et al., 2005). Stark et al. (1992) have concluded from NMR spin diffusion
experiments that suberin polymethylenic chains are associated and/or bound to the cell
wall matrix. Yan et al. (1998) propose that suberin polymethylenes interface with cell
wall polysaccharides via suberin polyphenolics based on spin diffusion occurring more
rapidly from cell wall polysaccharides to aromatic than to polymethylenic groups. The
recent discovery of glycerol groups in depolymerization studies indicate that these
glycerol units constitute an important ester-linkage site for the long-chain acids and
alcohols (Graça and Pereira, 2000).

Although cutin, cutan and suberin have all been extensively characterized and
structures have been proposed, detailed structural description of a suberan biopolymer
that is analogous to cutan in its method of isolation has not been previously made.
However, like cutan, the chemical nature of suberan may be of great importance when
attempting to understand diagenetic processes and humification in soils, because below
ground plant biomass and the bark layer of many trees are important contributors to SOM and SODM. The focus of the current paper is on what we call suberan from bark, specifically the bark of the River Birch (*Betula nigra*). We chose the bark of *Betula nigra* because of its availability and the fact that much has already been learned of the chemistry of Birch bark (Tegelaar *et al.*, 1995; Johnson *et al.*, 2005).

The insoluble components of Birch bark, including suberan, have been previously investigated by pyrolysis-gas chromatography mass spectrometry (py-GCMS) (Tegelaar *et al.*, 1995). Tegelaar *et al.* identified an insoluble, non-saponifiable polymethyleneic biomacromolecule which they named suberan (suber = cork (lat.); -an = derived from). This substance was separated from acidified saponification liquors of a previously lipid-extracted bark residue. When these liquors were extracted with dichloromethane (DCM), the white suberan residue formed an insoluble precipitate at the aqueous/DCM interface. Suberan showed a unique distribution of pyrolysis products leading the authors to believe this material was different than previously studied biopolymers such as suberin, cutin and cutan. Tegelaar *et al.* (1995) found suberan to be comprised of a homologous series of \( n \)-alkanes, \( n \)-alk-1-enes, and \( \alpha,\omega \)-alkadienes with carbon lengths dominated by \( C_{20} \) and \( C_{22} \) while spanning from \( C_6 \)-\( C_{37} \). The analysis was limited to the destructive technique, py-GCMS, and no overall structure for suberan was proposed. We define suberan differently from Tegelaar *et al.* (1995), however. As discussed below, the material we envision as suberan is a residue obtained from saponification of suberin that has been treated to remove lipids, lignin, and carbohydrates. This definition of suberan matches that used to differentiate cutan from cutin.
We employ a number of techniques (most of which have not been previously applied) to characterize suberan at a level of structural detail that was previously unavailable. These include Fourier transform infrared (FTIR) spectroscopy, flash pyrolysis gas chromatography mass spectrometry (py-GCMS), tetramethyl ammonium hydroxide thermochemolysis (TMAH) gas chromatography mass spectrometry, scanning electron microscopy (SEM), cross polarization magic angle spinning (CPMAS) $^{13}$C nuclear magnetic resonance (NMR), one-dimensional high resolution magic angle spinning (HRMAS) $^1$H NMR and the two-dimensional HRMAS NMR techniques; $^1$H-$^1$H total correlation spectroscopy (TOCSY) and $^1$H-$^{13}$C heteronuclear single bond quantum coherence (HSQC). FTIR spectroscopy provides information about the bonds contained within the biopolymer structure, while py-GCMS and TMAH GCMS give information about polymer fragments formed during thermal and thermochemical breakdown into GC amenable molecules. SEM provides visual evidence of the highly ordered crystalline nature of this biopolymer. The NMR techniques are non-invasive allowing for analysis of intact cross-linked polymeric structures. CPMAS is used to determine functional groups present in whole biopolymers, but it provides limited resolution of overlapping $^{13}$C peaks and limited information about the connectivities within the polymer. One-dimensional $^1$H HRMAS NMR provides greater resolution that CPMAS. The two-dimensional HRMAS NMR techniques HSQC and TOCSY can provide for resolution of overlapping $^1$H or $^{13}$C peaks and information about connectivities, respectively. In short, our approach is one of the most comprehensive analytical examinations of suberan to date, utilizing both destructive and non-destructive techniques to provide structural insights.
2.2 Materials and methods

River Birch (*Betula nigra*) is a rapid growth angiosperm which prefers fine textural acidic soils and is native to the eastern half of the United States, as well as parts of Asia and Europe. *Betula nigra* bark was obtained by peeling layers from a mature live tree in Hilliard, Ohio. The bark of this plant peels off in large pieces making manual removal easy. The bark was washed extensively with ultrapure UVQ (18 Ω) water, lyophilized and ground to a fine powder. Isolation of the cuticular biopolymers within the bark was performed using a similar approach as was described by Deshmukh *et al.*, (2005) for cutan. Briefly, gentle reflux solvent extraction with methanol and chloroform to remove lipids (Holloway, 1984) was followed by removal of cellulosic components with sodium paraperiodate (Zelibor *et al.*, 1988) and finally removal of aromatic components with sodium hypochlorite (Lewin, 1984). The residues were rinsed extensively with ultrapure UVQ (18 Ω) water between each isolation process, and each treatment was repeated several times.

Following these extensive treatments, this residue was considered to be a mixture of the isolated suberin/suberan biopolymer mixture. To further isolate suberan from suberin the residue was saponified to hydrolyze suberin to its monomeric components and rinsed extensively leaving behind a solid suberan residue. The suberin/suberan mixture was refluxed for two hours in a 3% (w/v) potassium hydroxide / methanol solution (Holloway, 1984). The solution was centrifuged and the supernatant separated. Rinsing, resuspending, centrifuging and separating were repeated several times to assure removal of suberin. The pH was adjusted to 5 with glacial acetic acid to further solubilize any remaining suberin monomeric components. This residue was also washed several times.
times with ultrapure UVQ (18 Ω) water to remove any residual suberin and to remove the acetic acid. The remaining residue was a low density, white powder considered to be suberan.

*Fourier Transform Infrared Spectroscopy*

An FTIR spectrum was obtained to provide information about bonds present in the biopolymer. The suberan biopolymer was encased in a potassium bromide (KBr) pellet and analyzed on a Perkin Elmer 16PC infrared spectrometer. For the suberan IR transmission spectrum, ten scans were averaged over the region from 300-4500 cm⁻¹.

*Pyrolysis-GCMS*

Py-GCMS analysis of suberan was performed to produce fragments generated by flash heating at 610 °C in the absence of oxygen. A Chemical Data Systems pyrolyser superheated the ~0.2 mg samples within quartz tubes prior to flushing the residue into a Carlo Erba Mega 500 GC and Kratos MS25 MS. The samples were flash pyrolyzed and held for a 15 second delay period prior to being flushed into the GC with 99.995% purity helium gas. The GC used a 30 meter Restek RTx-5 silica capillary column (0.25 mm ID with 1.0 µm film thickness) with a 20:1 split injection. The column temperature was held at 40 °C for 2 minutes followed by an 8 °C/minute temperature ramp up to 300 °C where it was held for 10 minutes. The MS used 50 eV electron impact ionization and scanned over a mass range of 50-490 m/z. Peaks were identified by comparison to National Institute of Standards and Technology (NIST) and Wiley mass spectral peak libraries.

* Tetramethyl Ammonium Hydroxide Thermochemolysis GCMS *

Isolated suberan was derivatized by tetramethyl ammonium hydroxide thermochemolysis in vacuum sealed ampoules in order to convert the macromolecular
biopolymer into GC amenable fragments as described previously (McKinney et al., 1995). Suberan (∼0.7 mg.) was placed in glass ampoules with 200 uL of TMAH (25% in methanol, Aldrich), vortexed for 1 minute, the methanol was evaporated with nitrogen gas, and the ampoules were vacuum sealed. The reaction ampoules were heated to 250°C for 30 minutes and the reaction products were extracted with ethyl acetate. The extract was dried and resuspended in 100 uL ethyl acetate containing n-eicosane as an internal standard. The products were analyzed by GCMS. A Hewlett Packard 6890 gas chromatograph with a DB-5 methylsilicone coated fused silica capillary column (30 meter, 0.25 mm i.d., 0.1 µm film thickness, Supelco) used helium carrier gas (1 ml/min, 99.995%) with the temperature being ramped from 50°C - 75 °C at 25 °C/minute, 75 °C - 200 °C at 60 °C/minute, 200 °C - 250 °C at 10 °C/minute, 250 °C - 300 °C at 5 °C/minute and held at 300 °C for 1 minute prior to recycling. A LECO Pegasus II time-of-flight mass spectrometer with electron impact ionization (70 eV) was used to analyze the eluent. Peaks were identified by comparison to the National Institute of Standards and Technology mass spectral library within the LECO Pegasus II version 1.33 data acquisition and analysis software.

*Scanning Electron Microscopy*

The scanning electron microscopy was preformed by the Ohio State University Microscopic and Chemical Analysis Research Center within the department of geology. The isolated ground suberan powder was coated with gold and 100x, 500x, 1,000x, 2,000x, 5,000x and 10,000x magnification images were taken with a JEOL JSM-820 SEM with an Oxford energy dispersive X-ray detector.
Solid-state CP/MAS $^{13}$C NMR Spectroscopy

Solid-state $^{13}$C NMR analyses were performed on a Bruker AVANCE 300 MHz NMR spectrometer using the cross polarization and magic angle spinning (CPMAS) technique. Lyophilized samples were packed into 4 mm zirconia rotors with Kel-F caps and spun at 13 kHz at the magic angle (54.7°). The pulse program was a modified cpvacpd (cross polarization variable amplitude cross polarization decoupled) based on previous work (Dria et al., 2002). The decoupling was two-pulse phase modulated (TPPM) and the cross-polarization (CP) was ramped with a contact time of 2 ms and a recycle delay of 1 s. The spectral width was 19 kHz (260 ppm) and no apodization was used. Calibration was performed with a glycine external standard to the carboxyl signal at 176.03 ppm. The chemical shifts were referenced to tetramethylsilane (TMS).

Solid-state HRMAS NMR Spectroscopy

HRMAS NMR analyses were performed on a Bruker AVANCE 400 NMR spectrometer. Lyophilized solid samples were packed as a mixture with 99.9 % deuterated chloroform (CDCl$_3$) as a swelling solvent into a 4mm zirconia rotor with a Kel-F insert and Kel-F cap and spun at the magic angle at 6 kHz. A dual channel ($^1$H, $^{13}$C) HRMAS probe was used to acquire one-dimensional $^1$H NMR spectra, two-dimensional $^1$H-$^{13}$C heteronuclear single quantum coherence (HSQC) NMR spectra, and two-dimensional $^1$H-$^1$H total correlation spectroscopy (TOCSY) NMR spectra. The one-dimensional $^1$H spectra were acquired with a zgcppr (Bruker Biospin) pulse sequence and an optimized $^1$H 90° pulse time of 7.2 µs. A spectral width of 5208 Hz (13 ppm) centered at 6 ppm was scanned 32 times. The $^1$H-$^{13}$C HSQC spectrum was acquired using the invietgpsi pulse program, $^1$J coupling set to 145 Hz, and echo-antinecho TPPI (time
proportional phase incrementation) gradient selection. Exactly 300 scans were acquired
with a $^1$H 90° pulse time of 7.2 µs, $^{13}$C 90° pulse time of 4.6 µs and a 1 s recycle delay.
The F2 ($^1$H) time dimension (TD) had 1024 data points and spectral width of 4807 Hz (12
ppm) centered at 5 ppm. The F1 ($^{13}$C) TD had 512 data points and a spectral width of
26,161 Hz (260 ppm) centered at 120 ppm. The two-dimensional $^1$H-$^1$H TOCSY
spectrum was acquired using the mlevprtp.2 pulse program, with a mixing power of 11.5
dB, a mixing time of 0.2 s and 1 s recycle delay. The 90° pulse time was optimized to 7.2
µs. A spectral width of 4807 Hz (12 ppm) centered at 5 ppm was scanned 16 times. The
F2 had 2048 data points and the F1 had 512. Decoupling was achieved with TPPM, and
the processing function was QSINE.

2.3 Results and discussion

The focus of this investigation is on the structural characteristics of suberan from
River Birch bark, but it is salient to that investigation to discuss changes in the chemistry
of the bark as it is subjected to significant chemical processing to remove well known
biopolymers like lignin and cellulosic materials. The resultant suberinic residue is further
subjected to base hydrolysis with the intent to isolate a substance we call suberan. The
changes experienced by the bark as it is subjected to a series of chemical treatments is
best visualized by solid-state $^{13}$C NMR where quantitative changes in structural features
can be evaluated. It has been previously suggested, based on spin counting and Bloch
decay experiments that, once spectral conditions are optimized, CPMAS $^{13}$C NMR
spectra of bark and wood samples provide an accurate quantitative representation of the
functional groups present in these types of samples (Johnson et al., 2005; Deshmukh et
Thus, we chose not to obtain Bloch decay NMR spectra in this study and rely principally on the CPMAS approach.

All NMR peak assignments in this study were based on reference databases and previous studies (ACD/Labs, 2006, Gil et al., 1999; Simpson et al., 2001; Deshmukh et al., 2003, 2005; Ralph et al., 2004). The chemical shifts of functional groups observed for samples in this study are listed in Table 2.3. This table of shifts is partly based on chemical shifts observed for closely related cuticular materials (Deshmukh et al., 2003; 2005) and also based on two-dimensional NMR spectroscopy discussed below. Peaks are labeled according to an alphanumeric code, where the lettering (A, B, C, etc.) is based on specific functionality and the numeric subscripts denote positions as shown in the various model structures listed in Table 2.3.

**Solid-State CPMAS $^{13}$C NMR Spectroscopy**

CPMAS $^{13}$C NMR spectra of dried whole Birch bark (Figure 2.1), the suberin/suberan mixture (Figure 2.2) and the isolated suberan (Figure 2.3) all show evidence of aliphatic (A), ester (B,C), alcohol (D,E), carboxylic acid (F), branched ester (G), epoxide (I) and methoxy (J) functional groups. The CPMAS spectrum of whole Birch bark (Figure 2.1) and relative peak areas listed in Table 2.1 show that the natural Birch bark contains a large fraction of aliphatic, aromatic and hydroxyl functional groups. The most obvious components of whole bark are carbohydrates which account for most of the signals in the hydroxyl region and are particularly distinguished by the sharp peak at 110 ppm assigned to anomeric carbons in polysaccharides. The aromatic components are probably related to lignin in the bark, showing classical peaks at 56 ppm (methoxyl), 115 ppm (H-substituted aromatic), 130 ppm (C-substituted aromatic), and 150 ppm.
Figure 2.1: CPMAS $^{13}$C NMR spectra of milled but chemically untreated River Birch bark.
(O-substituted aromatic). The aliphatic region is dominated by peaks at 33 ppm and 30 ppm. Gil et al. (1997) used CPMAS and single pulse excitation $^{13}$C NMR and observed the same two methylene regions (30 and 33 ppm) within suberin which they attributed to variations in mobility. They proposed methylenes closer to chain linkages have lower mobility than mid-chain methylenes and that this factor was responsible for the chemical shift differences of polymethylenes. We disagree with this conclusion of Gil et al. (1997) and assign the chemical shift differences to be attributable to the presence of two types of polymethylene chains, one exhibiting a high degree of stacking order, or

<table>
<thead>
<tr>
<th>Chemical shift (ppm)</th>
<th>Aliphatic</th>
<th>Methoxy</th>
<th>Hydroxyl</th>
<th>Aromatic</th>
<th>Carboxyl</th>
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<td>0-45</td>
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<td>60-110</td>
<td>110-160</td>
<td>160-190</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.1:** Percentage distribution of carbon atoms in the untreated River Birch (*Betula nigra*) bark, suberin/suberan mixture and suberan samples as determined by solid-state CPMAS $^{13}$C NMR spectroscopy.
crystalline (33 ppm), and the other being disordered (30 ppm). A similar set of polymethylenic peaks was observed in cutin and cutan (Deshmukh et al., 2005). Smaller peaks at 15 ppm and 22 ppm can be assigned to terminal methyl, and \( \alpha \)-methylene respectively, in these long-chain structures.

After the extensive chemical treatments (periodate and chlorite) to remove polysaccharides and lignin and to isolate as a residue the suberinic biopolymers (suberin/suberan mixture), the fraction of carbon from aromatic structures decreases from 11.0% to 3.6% and, correspondingly, hydroxyl structures diminish from 48.9% to 11.8%, as expected. The loss and diminution in the spectra of lignin and polysaccharide signals appears to be incomplete. This is supported by the TMAH thermochemolysis results discussed below, in that lignin-like compounds are observed in suberan. It is, perhaps, expected that lignin-like materials could either constitute part of the suberan structure or simply be residual lignin that has escaped the bleaching effects of the chlorite treatments. The effectiveness of the aqueous reagents used for removal of lignin and polysaccharides may be diminished in the polymethylenic suberinic biopolymers that are expected to be quite hydrophobic, posing a challenge to reagent accessibility. Alternatively, it has been shown that a polyphenolic, lignin-like material, comprises one of the two structural domains within suberin (Graça and Santos, 2007). The polymethylenic material making up the second type of polymeric structural domain of suberin is the dominant constituent and is believed to be associated in an unknown fashion with the polyphenolic domain material (Bernards and Lewis, 1998; Lulai and Corsini, 1998).
The CPMAS spectrum of the suberin/suberan mixture (Figure 2.2) shows a dominance of aliphatic carbon while also providing evidence for many other functional groups. The amorphous aliphatic carbon peak at 30 ppm is far more intense than the crystalline aliphatic peak at 33 ppm indicating a predominance of mobile methylenes within the suberin/suberan mixture. Ester, alcohol, acid, epoxide and aromatic functional groups are prevalent while in a much smaller proportion to the polymethyleneic carbons. The carboxyl region shows one major peak at 173 ppm and a smaller peak at 166 ppm, both assigned mainly to ester functional groups.

Figure 2.2: CPMAS $^{13}$C NMR spectra of suberin/suberan mixture with peak assignments labeled.
Figure 2.3: CPMAS $^{13}$C NMR spectra of suberan with peak assignments labeled.
Upon further isolation of suberan by saponification and removal of suberin, several significant alterations to the CPMAS spectrum are observed (Figure 2.3). A new carboxyl peak appears at 181 ppm in the spectrum of the suberan. This peak is clearly assignable to fatty acid functionality. Another change in the spectra involves loss of the peak at 65 ppm (B1-ester) shown in the suberin/suberan mixture and emergence of a new peak at 63 ppm (D1-alcohol) in the isolated suberan. These shifts are consistent with the possibility that the separation of suberan from suberin generates acid and primary alcohol functional groups from esters within the suberinic material which contains a mixture of suberin and suberan. Another interesting feature of both Figure 2.2 and 2.3 is that they show α-branched esters (G), but the peak is more intense in the suberin/suberan mixture, indicating that suberan is comprised of more straight chain material. The other major change observed is in the polymethyleneic region where two peaks at 30 and 33 ppm are observed. The polymethyleneic carbon peak in the suberin/suberan mixture is predominantly at 30 ppm indicating mainly amorphous aliphatic material. Before saponification, the peak for crystalline polymethylenes is a less intense peak (33 ppm) on the downfield side of the polymethylene region. For suberan, the polymethyleneic region is dominated by the peak at 33 ppm indicative of a highly crystalline structure (Deshmukh, 2005). This highly crystalline nature distinguishes suberan from suberin in an analogous fashion to a similar differentiation observed in cutin and cutan (Deshmukh, 2003; Deshmukh et al., 2005).

Scanning Electron Microscopy

Figure 2.4, Figure 2.5 and Figure 2.6 are scanning electron microscopy images of isolated suberan. Figure 2.4 depicts images which are magnified 100x and 500x that
clearly show flat sheets of material. This flat morphology persisted through extensive chemical treatments, and was not physically degraded into a more amorphous configuration even though significant chemical alteration is observed (see discussion of the NMR data above). Figure 2.5 shows images that are magnified 1,000x and 2,000 times and appear to show a rigid crystalline nature. Figure 2.6 depicts images magnified 5,000x and 10,000x and clearly show what appears to be a highly ordered and crystalline material.

Figure 2.4: SEM photographs of suberan 100x and 500x zoom (images courtesy of S. Bhattiprolu).
Figure 2.5: SEM photographs of suberan 1,000x and 2,000x zoom (images courtesy of S. Bhattiprolu).

Figure 2.6: SEM photographs of suberan 5,000x and 10,000x zoom (images courtesy of S. Bhattiprolu).
Fourier Transform Infrared Spectroscopy

The IR spectrum of suberan shown in Figure 2.7 indicates the types of bonds present within suberan. The largest band is the broad O-H stretching band from 3700-3200 cm\(^{-1}\), indicative of alcohol and acid functional groups. The two intense sharp bands at 2916.9 and 2849.1 cm\(^{-1}\) are from C-H asymmetric and symmetric stretches within polymethylenic chains. The shoulder located at ~2700 cm\(^{-1}\) could be attributed to aldehyde functionality, yet no evidence of aldehydes is found in the NMR portion of this study (see below). The small band at 2344.7 cm\(^{-1}\) has been previously attributed to a C-O stretch within cationic carboxylic acids (Watanabe et al., 1973). The intense band at 1707.6 cm\(^{-1}\) is attributed to C=O stretching in acid and ester functional groups. The very low intensity C=C alkene stretch at 1640 cm\(^{-1}\) indicates a small degree of aliphatic unsaturation. Although the low intensity band at 1508.1 cm\(^{-1}\) is indicative of the C-C bonds within an aromatic ring, no aromatic C-H stretching at > 3000 cm\(^{-1}\) and no aromatic overtones in the 2000-1660 cm\(^{-1}\) region are observed, leading to the conclusion that suberan contains only a small aromatic component. The band at 1471.8 cm\(^{-1}\) is attributed to C-H scissoring in the polymethylenic chains. At 1410.9 cm\(^{-1}\) is a band showing O-H bending in carboxylic acids or esters. The band at 1262.7 cm\(^{-1}\) is due to C-O stretching in carboxylic acids or esters while the band at 1052.3 cm\(^{-1}\) is C-O stretching in alcohols. Methyl rocking at the end of polymethylenic chains appears as the band at 719.2.
Alcohol, ester and carboxylic acid functional groups are the major functions found within the IR spectra. Evidence of minor contributions from aromatics and alkenes are also found at 1508 cm$^{-1}$ and 1660 cm$^{-1}$ respectively. No clear evidence of ether bands at 3000-2800, 1200-1050, and 620-500 cm$^{-1}$ were found in the IR spectrum, but these bands may be of low intensity and buried under more intense overlapping peaks. Epoxides would appear as moderate intensity bands at 870-780 and 1300-1180 cm$^{-1}$, but are not observed in the IR spectra.
Py-GCMS

Py-GCMS is an invasive technique that thermally deconstructs the biopolymer into smaller pieces to facilitate GC separation. This technique gives useful information about the types of functional groups present in the biopolymer as well as information about the lengths of polymethylenic domains contained within the structure. Specific ion mass chromatograms are used to observe specific compound classes and in this study we have chosen three specific ion chromatograms to analyze fragments containing alkane (85), alkene (83) and fatty acid (73) domains (Tegelaar et al., 1995).

In Figure 2.8, we observe pyrolysis products that are mainly long-chain alkyl structures (alkanes and fatty acids). Peaks denoted by the specific ion fragment m/z 85 characteristic of alkanes are identified showing alkanes and fatty acids each having chain lengths of C8 to C21. A homologous series of alkanes and fatty acids extends to a maximum of C21 with the C21 peak being the most intense in the series. This pattern is different than that observed previously for suberan from Birch bark which extended from C6 to C37 (Tegelaar, et al. 1995), although Tegelaar et al. did observe a dominance of C20 - and C22 chains. The isolation treatments used in this study are more exhaustive than those of Tegelaar et al. (1995) and may be isolating a different material. Suberin, which may be closely associated with suberan in natural materials is known to have chain lengths up to C32 (Kolattukudy et al., 1976), and may not have been completely removed in the Tegelaar et al. (1995) study. Alternatively, the samples may be naturally different.

Figure 2.9 shows the m/z 83 single ion pyrogram of isolated suberan. This fragment is specific for alkyl structures containing at least one site of unsaturation, such
Figure 2.8: Select ion (m/z = 85) pyrolysis gas chromatogram of suberan showing a homologous series of alkanes and fatty acids ranging in chain length from C8-C21 with a dominant C21 alkane.
Figure 2.9: Select ion (m/z = 83) pyrolysis gas chromatogram of suberan showing a homologous series of alkenes ranging in chain length from C₈-C₂₁ with a dominant C₂₁ alkene, along with alkadienes ranging in chain length from C₁₄-C₂₁.
as the $n$-alk-1-enes observed in many pyrograms of polymethylenic materials. Both $n$-alkanes and $n$-alk-1-enes are known to be produced during pyrolysis by the cracking of C-C bonds in alkyl chains (Buckhorn et al., 1999; Quénéa, et al., 2006a). Many radicals are created during pyrolysis by homolytic C-C cleavage and they participate in secondary chain fragmentation, H· transfer and radical addition. Radicals formed during this process can terminate by H· elimination leading to alk-1-ene fragments or terminate through radical addition forming alkane fragments. The alkene peaks in the m/z 83 pyrogram increase with chain length as the alkanes do in the m/z 85 trace and once again the pyrogram is dominated by the $n$-C$_{21}$ peak. Smaller peaks assigned to $n$-alkadienes are also observed with chain lengths from C$_{14}$ to C$_{21}$. Figure 2.10 shows the m/z 73 ion chromatogram of suberan pyrolysis products. This fragment is characteristic of fatty acids. The peak intensity pattern for these fatty acids varies from C$_{3}$ to C$_{22}$ with the dominant chain length being C$_{8}$. The mass spectra indicate that these fatty acids are $n$-fatty acids. The last large peak in all three pyrograms is identified as docosanolide, a cyclic C$_{22}$ ester which is likely formed by intermolecular condensation between the ends of a C$_{22}$ alkyl chains with acid and primary alcohol functionality. This peak provides further evidence of C$_{22}$ being the dominant chain length.

In the pyrograms there is no predominance of even over odd carbon chain lengths in either the alkane/alkene or fatty acid series as has been observed in chemically degraded polymethylenic biopolymers, cutan and algaenan (Schouten, et al, 1998). The pyrolysis process cleaves biopolymers by way of a complex radical mechanism which can obscure this trend, which is observed in the TMAH GCMS data discussed below. Also, the series in both the alkane and alkene cases abruptly terminate at $n$-C$_{21}$. Previous
pyrolysis studies of long-chain fatty acids and their salts (Hartgers et al., 1995) show that 
α-cleavage of the precursor molecules occurs readily and yields a pattern of peak 
intensities for \( n \)-alkanes/\( n \)-alkenes similar to that observed here (e.g., gradually increasing 
peak intensity with increasing carbon number and abruptly terminating at one carbon unit 
less than that of the precursor molecule). This suggests that the fragmentation results 
from pyrolysis cleavage of an \( n \)-C\(_{22} \) precursor molecule, possibly a fatty acid or ester. It 
has been proposed that the unsaturated aliphatics detected by flash py-GCMS may be 
generated by H• elimination radical termination (Quénéa et al., 2006a), consistent with 
the case that the precursor is likely an \( n \)-C\(_{22} \) molecule or structural fragment.

Figure 2.10: Select ion (m/z = 73) pyrolysis chromatogram for suberan.
The TMAH derivatization technique has been effective for analysis of samples containing polar functional groups such as esters (McKinney et al., 1996; del Rio and Hatcher, 1998; del Rio et al., 1998). This technique provides for complimentary information with pyrolysis because it cleaves and methylates macromolecules at polar functional groups rather than breaking up alkyl C-C bonds. By maintaining the integrity of alkyl chains, the TMAH thermochemolysis technique provides valuable information about the chain lengths contained within the biopolymer. This technique is also able to identify aromatic moieties such as those found in lignin (Clifford et al., 1995).

In the TMAH GCMS chromatogram (Figure 2.11), the retention times between 270 and 330 s have been expanded to identify low intensity peaks which contain lignin-like polyphenolic structures. The labeled peaks at retention times of 272, 291, 309, 313 and 328 s all have mass spectra indicative of compounds typically identified in the TMAH thermochemolysis of lignin (Clifford et al., 1995). Bernards and Lewis (1998) have shown that the polyphenolic domain of suberin is structurally distinct and unique from lignin in that it is comprised mainly of hydroxylcinnamates. The TMAH data show no structures of this type for suberan, instead only lignin structural units are observed leading to the conclusion that the sodium chlorite chemical treatment used to remove lignin did not remove all of the lignin-like structures associated with the River Birch bark and any hydroxylcinnamates that may have been associated with suberin are probably removed by saponification. The aromatic regions observed in the NMR spectra shown below are attributable to residual lignin-like structures.
Figure 2.11: TMAH chromatogram of suberan with peak assignments as listed in Table 2.2.

<table>
<thead>
<tr>
<th>Peak number</th>
<th>Scan number</th>
<th>Mass</th>
<th>Tentative peak identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>583</td>
<td>282</td>
<td>n-Eicosane ($C_{20}$) - Internal standard</td>
</tr>
<tr>
<td>2</td>
<td>611</td>
<td>342</td>
<td>Octadecanedioic acid, dimethyl ester ($C_{18}$)</td>
</tr>
<tr>
<td>3</td>
<td>662</td>
<td>324</td>
<td>Eicosenoic acid, methyl ester ($C_{20}$)</td>
</tr>
<tr>
<td>4</td>
<td>673</td>
<td>?</td>
<td>Unknown</td>
</tr>
<tr>
<td>5</td>
<td>716</td>
<td>370</td>
<td>Eicosanedioic acid, dimethyl ester ($C_{20}$)</td>
</tr>
<tr>
<td>6</td>
<td>775</td>
<td>352</td>
<td>Docosenoic acid, methyl ester ($C_{22}$)</td>
</tr>
<tr>
<td>7</td>
<td>837</td>
<td>398</td>
<td>Docosanedioic acid, dimethyl ester ($C_{22}$)</td>
</tr>
</tbody>
</table>

Table 2.2: Identification of major peaks within the TMAH chromatogram (Figure 2.11).
The suberan TMAH GCMS chromatogram (Figure 2.11) is dominated by seven intense peaks listed in Table 2.2 and clearly show the presence of long-chain alkyl sections within suberan. Peak 1 is the internal standard, \( n \)-eicosane. Peaks 2, 5, and 7 are identified as diacid, dimethyl esters of chain length \( \text{C}_{18} \), \( \text{C}_{20} \), and \( \text{C}_{22} \), respectively. Peaks 3 and 6 are identified as a \( \text{C}_{20} \) and \( \text{C}_{22} \) monounsaturated acid methyl esters, respectively. Peak 4 is unidentified. Peaks 6 and 7, the most intense peaks, are identified as \( \text{C}_{22} \) esters and their presence is consistent with the py-GCMS data which indicates a predominance of \( \text{C}_{22} \) alkyl chains in suberan. All of the TMAH peaks show a tendency towards long (\( \text{C}_{18}-\text{C}_{22} \)) and even numbered alkyl chains which is in agreement with the depolymerization studies of suberin that have found suberin monomers to be mainly comprised of \( \text{C}_{16} \), \( \text{C}_{18} \), \( \text{C}_{22} \) and \( \text{C}_{24} \) \( \omega \)-hydroxy fatty acids and dicarboxylic acids (del Rio and Hatcher, 1998; Graca and Pereira, 2000). These chains may be located between two ester functional groups which, upon TMAH thermochemolysis, are transformed to dimethyl esters. The monomethyl esters may be formed by TMAH thermochemolysis of alkyl chains with a terminal methyl on one end and an ester on the other. This evidence of the dominance of alkyl and ester functionality within suberan is supported by the NMR data.

**Solid-state HRMAS NMR Spectroscopy**

The HRMAS \(^1\)H NMR spectra for suberan (Figures 2.12 and 2.13) are dominated by mid-chain methylene carbons (A2) at 1.3 ppm. Figure 2.12 shows the entire spectral width and the intensity is normalized to the largest peak, which is assigned to A2. A small peak at 7.2 ppm is attributable to the deuterated chloroform (CDCl\(_3\)) swelling.
<table>
<thead>
<tr>
<th>Code</th>
<th>Structure Type</th>
<th>$^1$H shift</th>
<th>$^{13}$C shift</th>
</tr>
</thead>
<tbody>
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<td>A1</td>
<td>R-CH$_2$-CH$_2$-CH$_3$</td>
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<td>14</td>
</tr>
<tr>
<td>A2</td>
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<td>30</td>
</tr>
<tr>
<td>B1</td>
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<td>65</td>
</tr>
<tr>
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<td>1.6</td>
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</tr>
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<td>1.3</td>
<td>26</td>
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<td>165</td>
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<td>1.3</td>
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</tr>
<tr>
<td>E3</td>
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<td>26</td>
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<td>I</td>
<td>R-CH$_2$-CHOCH-CH$_2$-R</td>
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<td>57</td>
</tr>
</tbody>
</table>

Table 2.3: Alphanumeric codes and NMR chemical shifts of functional groups identified in suberan.
Figure 2.12: One-dimensional HRMAS $^1$H NMR spectrum of suberan.

Figure 2.13: Enlarged one-dimensional HRMAS $^1$H NMR spectrum of suberan.
solvent, and no additional peaks with chemical shifts greater than 4.0 ppm are observed.

Figure 2.13 is an expanded view of the spectrum showing other significant protons in the sample. The peak at 4.0 ppm represents hydrogen $\alpha$ to the O side of an ester (B1). A large primary alcohol peak is shown at 3.6 ppm. This structural entity probably derives from saponification of esters. Terminal methyl protons are observed at < 1.0 ppm, and it is readily apparent that there are multiple types of terminal methyl groups. The different terminal methyl groups are assigned to a variety of alkyl segments including long chains and propyl regions of lignin-like structures (See discussion below). The cluster of peaks at 2.2 ppm is from protons $\alpha$ to the C side of an ester (C2), $\alpha$ to an acid (F2), and branched esters (G), all overlapping. Another cluster of peaks is found at 1.4 - 1.6 ppm and is assigned to protons $\beta$ to the O side of an ester (B2), $\beta$ to the C side of an ester (C3), $\beta$ to a primary alcohol (D2) and $\beta$ to a carboxylic acid (F3). The overlapping peaks make determination of the relative contribution of these functional groups difficult to ascertain in one-dimensional $^1$H NMR spectra, so we have employed two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR to assist in peak assignment.

$^1$H-$^{13}$C HSQC is a two-dimensional NMR technique with cross-peaks for resolution in both the $^1$H and $^{13}$C dimensions. One limitation of this technique is that only $^1$H and $^{13}$C atoms directly bound to one another appear in the spectrum. For example, the hydrogen and carbon atoms within carboxylic acid functionality (C1) do not appear as cross-peaks. The suberan HSQC spectrum (Figure 2.14) has cross-peaks (Table 2.3) in the carbon dimension ranging from 14 - 78 ppm and in the hydrogen dimension ranging from 0.8 – 4.0 ppm. The most intense peak is the mid-chain methylene peak (A2) at (1.3, 30). Note that cross-peaks will be identified by their chemical shifts in a parenthetical
expression where the first number represents the $^1$H chemical shift in ppm and the second represents the $^{13}$C chemical shift in ppm. This mid-chain methylene peak appears at 30 ppm in the carbon dimension indicative of amorphous structures rather than crystalline structures (33 ppm), appearing to contradict the CPMAS data for suberan discussed above. This altered shift in HRMAS NMR is attributable to the swelling solvent which imparts mobility to the alkyl chains and eliminates their ability to order into crystallites. The intensity of this (A2) peak creates t1 noise from unsuppressed $^1$H-$^{12}$C signals, and this appears as vertical streaking observed at 1.3 ppm (Simpson, 2001). Signals for terminal methyl groups are observed as two doublets at around (0.8, 15) and indicate a variety of terminal methyls. Long alkyl chains have terminal methyls (A1) that appear at (0.8, 14) and are part of the polymethylene spin system of A2 (see TOCSY discussion below) but are of very low intensity, indicating that most long alkyl chains are not terminated with methyl groups. Propyl side-chains associated with lignin-like structures have been shown to have terminal methyls with $^1$H shifts of 0.7-1.0 ppm (Ralph et al., 2004). The terminal methyls that constitute the dominant signals for methyl groups are assigned to the residual polyphenolic material as discussed with the TOCSY data shown below.

Ester functional groups are clearly identifiable in the HSQC spectrum. The $\alpha$ (B1), $\beta$ (B2), and $\gamma$ (B3) units on the oxygen side of esters show peaks that are all clearly displayed at (4.0, 65), (1.6, 29) and (1.3, 26), respectively. B1 is a well resolved peak indicative of a significant amount of ester functionality in suberan. The carbons which are $\alpha$ (C2), and $\beta$ (C3) to the carbon side of esters appear at (2.3, 34) and (1.6, 24),
Figure 2.14: Two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR spectrum of suberan with peak assignments for structures listed in Table 2.3.
respectively. Although the C2 peak overlaps with a peak assigned to acids (F2) and it is difficult to determine their relative contributions to this peak, the C3 peak is well resolved giving another clear indication of the significant presence of ester groups. Esters are normally considered to be saponifiable, so their residual presence in suberan is indicative of the fact that they are probably protected from saponification by physical entrapment within the biopolymer matrix.

Cross-peaks assigned to $\alpha$ (D1), $\beta$ (D2), and $\gamma$ (D3) primary alcohol units are found at (3.6, 63), (1.6, 33) and (1.3, 26), respectively. D1 and D2 are well resolved and non-overlapping, while D3 overlaps with ester peak B3 and secondary alcohol peak E3. The strong intensity of D1 and D2 are clear evidence of a significant amount of primary alcohol functionality within suberan. The $\alpha$ (E1), $\beta$ (E2), and $\gamma$ (E3) structures associated with secondary alcohols are found at (3.2, 78), (1.4, 34) and (1.3, 26), respectively. E1 is well resolved and of low intensity while E2 is less well resolved and of very low intensity. E3 overlaps with the ester cross-peak B3 and the primary alcohol cross-peak D3. The lower intensity of the cross-peaks for secondary alcohols relative to those of primary alcohols indicates a predominance of terminal alcohol functional groups which is in agreement with the one-dimensional HRMAS $^1$H NMR spectra.

The carbons $\alpha$ (F2), and $\beta$ (F3) to carboxylic acids appear at (2.3, 34) and (1.5, 27), respectively. The intense F2 peak overlaps with the ester peak C2, while the F3 peak does not overlap other functional groups but is poorly resolved and is of low intensity. Although the cross-peaks associated with carboxylic acid groups are not intense, they do affirm the CPMAS evidence for the presence of this functionality within suberan.
The peak at (2.4, 43) is very low intensity and attributed to an ester which is α-branched (G). This α-branched ester (G) function has been previously observed in cutan (Deshmukh et al., 2005) and may be an important structural feature associated with cross-linking of such biopolymers. The observed low abundance of this function may be significant in that it allows for a more highly ordered and crystalline structure which is probably responsible for providing physical protection from ester hydrolysis. The presence of the low intensity, well resolved peak I at (2.9, 57) is a clear indication for an epoxide group within suberan. Ether and olefin functional groups do not appear in the HSQC. Thus it is unlikely that these units are important parts of the biopolymer structure. The aromatic region shows no cross-peaks in the HSQC spectrum, even though a significant amount of aromatic intensity is observed in the CPMAS spectrum (Figure 2.3). We surmise that cross-peaks for aromatic structures present in the suberan isolate are not observed because they are dispersed over a wide chemical shift range and buried within the noise.

The two-dimensional $^1$H-$^1$H TOCSY NMR spectrum of suberan (Figure 2.15) shows cross-peaks deriving from the interaction between $^1$Hs located within the same spin system (refers to protons connected through protonated carbon backbones) which can extend over several bonds. This technique allows for observation of connectivities within the biopolymer, because cross-peaks arise from bonded functional groups within close proximity to one another. As expected, the dominant cross-peaks are from long chain methylenes (A2) at 1.3 ppm. Terminal methyls (A1), esters (B, C), primary alcohols (D) and carboxylic acids (F) all cross-correlate with A2 and are assigned as peaks 1-6 (Table 2.4, Figure 2.15). From the low intensity of the methyl cross-peak
Figure 2.15: Two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of suberan with cross-peak assignments as listed in Table 2.4 (below).
<table>
<thead>
<tr>
<th>Cross peak</th>
<th>Chemical shifts</th>
<th>Functionality assignments (codes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(0.8, 1.3)</td>
<td>Terminal methyl (A1) / Methylene (A2)</td>
</tr>
<tr>
<td>2</td>
<td>(1.3, 1.5)</td>
<td>Methylene (A2) / Carboxylic acid (F3)</td>
</tr>
<tr>
<td>3</td>
<td>(1.3, 1.6)</td>
<td>Methylene (A2) / Ester, Primary alcohol (B2, C3, D2)</td>
</tr>
<tr>
<td>4</td>
<td>(1.3, 2.3)</td>
<td>Methylene (A2) / Ester, Carboxylic acid (C2, F2)</td>
</tr>
<tr>
<td>5</td>
<td>(1.3, 3.7)</td>
<td>Methylene (A2) / Primary alcohol (D1)</td>
</tr>
<tr>
<td>6</td>
<td>(1.3, 4.1)</td>
<td>Methylene (A2) / Ester (B1)</td>
</tr>
<tr>
<td>7</td>
<td>(1.6, 2.3)</td>
<td>Ester (C3) / Ester (C2)</td>
</tr>
<tr>
<td>8</td>
<td>(1.6, 3.3)</td>
<td>Secondary alcohol (E2) / Secondary alcohol (E1)</td>
</tr>
<tr>
<td>9</td>
<td>(1.6, 3.7)</td>
<td>Primary alcohol (D2) / Primary alcohol (D1)</td>
</tr>
<tr>
<td>10</td>
<td>(1.6, 4.1)</td>
<td>Ester (B2) / Ester (B1)</td>
</tr>
<tr>
<td>11</td>
<td>(0.8, 1.0)</td>
<td>Terminal methyl</td>
</tr>
<tr>
<td>12</td>
<td>(0.7, 1.4)</td>
<td>Terminal methyl</td>
</tr>
</tbody>
</table>

Table 2.4: HRMAS $^1$H-$^1$H TOCSY NMR cross-peak assignments as shown in Figure 2.15.
within this spin system it is clear that the long alkyl chains are not terminated by methyl groups but by esters, acids, and terminal alcohols. Peak 8 is of low intensity and is assigned as a secondary alcohol (E1, E2).

Some cross-peaks are clearly assigned to the protons associated with a specific functionality as they formulate part of the same spin system. For example, peak number 7, assigned to the protons on a carbon $\alpha$ (C2) to an ester, shows a correlation with protons $\beta$ (C3) to the ester carbon. Similarly, peak number 10 shows the correlation of protons $\alpha$ (B1) and $\beta$ (B2) to the oxygen side of the ester. Another example is peak 9 which shows protons $\alpha$ (D1) and $\beta$ (D2) to a primary alcohol. All three of these cross-peaks derived from one type of functional group (ester or alcohol) are very intense. Peaks number 11 (0.8, 1.0) and 12 (0.7, 1.4) do not correlate with any other shifts observed within the spectrum, including the dominant 1.3 ppm shift of the mid-chain methylenes. These peaks have shifts previously observed for terminal methyls within lignin-like structures and are assigned as terminal methyls found within the polyphenolic structures. The lack of intensity and resolution precluded observation of any aromatic cross-peaks within the TOCSY spectrum. A very broad and low intensity hump is observed within the aromatic region of the HRMAS $^1$H NMR spectrum (only observed when spectrum in Figure 2.12 is magnified vertically) indicating that the protons of aromatic groups are broadly dispersed over a large chemical shift range. Such an effect would cause any TOCSY cross-peaks to be of insufficient magnitude to be observed.

Conclusions

Suberan isolated from River Birch (Betula nigra) bark and characterized extensively with a wide variety of techniques is found to be structurally very similar to
another suberinic biopolymer with which it is associated-suberin. The significant difference in the isolation and definition of these two biopolymers lies in the fact that suberin is saponifiable and suberan is not. It is evident that suberan retains ester functionality even though saponification is known to hydrolyze ester bonds. This conundrum is easily explained by the fact that the long-chain polymethylenic nature of suberan impacts a hydrophobic character to the material and leads to partial protection from extensive alkaline hydrolysis.

Suberan from River Birch bark is a polymethylenic biopolymer with alkyl chain lengths of mainly C_{22} with some C_{18} and C_{20}, as observed in the py-GCMS and TMAH GCMS data. Evidence of a significant contribution from esters, acids and primary alcohols are found in the IR, and the NMR spectra. It is most probable that the acid and primary alcohol functional groups within suberan are partially created during saponification of the suberin. Thus, the acids and primary alcohols are at least partially an artifact of the base hydrolysis. It is likely that the high crystalline order observed for suberan is also related to this hydrolysis process, as long-chain structures are released from their confinement within a more cross-linked polymer structure and are free to form crystallites. For example, what is not solubilized and removed during saponification of the suberin, because of a residual attachment to the polymer backbone, can reorient into crystallites readily due to free rotations of the long-chain structures. For such crystallites to form, it is also necessary that mid-chain positions be free of functional groups which would disrupt both the chemical shift and the \textit{trans} alignment of the polymethylenic structures required for the establishment of crystallinity.
Functional groups such as aromatics and epoxides are observed only as minor signals in the spectra. Aromatic carbons are only observed as weak signals in the CPMAS, and the TMAH GCMS data show them to be lignin-like structures. Epoxides are clearly present as small, well resolved peaks in the CPMAS and HSQC NMR spectra and appear to be present in suberan as well as suberin. Thus, there appears to be residual functional groups which could be envisioned as mid-chain substituents (epoxides) but these are minor components of suberan. The aromatic signals are probably from lignin that has not been totally removed by chlorite oxidation, perhaps because the hydrophobic suberin and suberan biopolymers have restricted access of the aqueous reagent to lignin sites.

Aside from the crystallinity, it appears that suberan biopolymers structurally share many similarities with suberin whose structural nature has been described in numerous previous studies (Kolattukudy et al., 1976; Kolattukudy, 1980; 1981; Holloway, 1984; Tegelaar et al., 1995; del Rio and Hatcher, 1998; Bernards and Lewis; 1998). The data shown here for suberan indicates that the chain lengths, the dominant ester functionality and the presence of various other functional groups are all features which have previously been observed in suberin. There are some major differences which indicate that suberan is structurally distinct. There is a possibility that suberan is distinct because it is formed as an artifact of the chemical isolation procedure employed in this study. The saponification process, being incomplete, may produce a polymer that is insoluble and resists further saponification due to its ordered polymethylene character, a characteristic that would have important implications for its survival in soil systems. Alternatively, suberan may be a distinct polymer that co-exists with suberin, perhaps originating from
natural processes within the bark that would hydrolyze the esters in suberin. In this explanation, saponification removes the suberin and retains the suberan, or partially saponified suberan, as a residue.

Graça and Santos (2007) have proposed a structure for suberin which contains secondary alcohol, epoxide or olefin functionality located mid-chain on almost all of the alkyl chains. Yet, the TMAH data in our study indicate that the majority of alkyl regions within suberan do not contain mid chain functionality and the NMR data show no evidence of olefins within suberan. While minor contributions from epoxides are observed within the NMR spectra of suberan and their chemical shifts indicate that they are in alkyl regions of the biopolymer, the absence of these structures in the TMAH GCMS data indicates that the majority of alkyl regions contained within suberan do not contain oxygen functional groups except at terminal positions. As mentioned above, the lack of oxygen functional groups at mid-chain positions is likely to enhance ordering of the long alkyl chains and be partially responsible for the existence of crystalline polymethylene. Graça and Pereira (2000) have found glycerol monomeric units within suberin and have proposed that they are involved in holding together the alkyl regions through ester-linkages. The experimental procedures of the current study are able to neither confirm nor deny that these types of structures are present in suberan. It is reasonable to assert that glycerol units may also be present in suberan and may be involved in the connectivity between alkyl chains as shown in a proposed structure for suberin (Graça and Santos, 2007).

The data presented herein supports the suggestion above that suberan is a distinct polymer that co-exists with suberin. It is clear that polymethylene in suberan are nearly
all in crystalline structures which serve to define its structure. Because both crystalline
and amorphous polymethyleneic domains are observed within the residue we call
suberinic material, the former being minor components of the polymethylene region of
the spectrum, this observation suggests to us that suberan does co-exist with suberin
within suberinic isolates. It is also likely that suberan, within suberinic materials that
have not been subjected to saponification, is composed of ester-linked polymethylene
lacking mid-chain substituents bound into a structure that is like the one proposed by
Graça and Santos (2007). In that proposed structure, the long-chain polymethylene are
ester-linked at both ends of the chain, to themselves, to glyceryl units, and to ferulic acid
units that form part of the polyphenolic portion of suberin. We do not know how suberan
might co-exist within the framework of this structural model of suberin at this point of
our investigation.

Figure 2.16 shows a rendition of the type of structural entity comprising suberan
as it was produced in this study, a non-saponifiable residue. This proposed structure
serves only as a model that contains structural features observed for our isolated suberan.
We include the glyceryl structural entity only based on previous work for suberin (Graça
and Santos, 2007). We do not have independent evidence for its existence within suberan.
The chemical shifts of such groups would overlap with those of primary and secondary
alcohols and glyceryl monomers likely would elute undetected with the solvent front in
the GCMS data precluding conclusive identification. We have no evidence of linkage to a
polyphenolic domain within suberan and attribute the residual aromatic, methoxy and
aromatic terminal methyl groups to a residual lignin like material which is not covalently
bound to the polymethyleneic material. The TMAH GCMS and py-GCMS indicate a
structure dominated by C22 polymethylenic chains with terminal oxygen functional

groups. The CPMAS $^{13}$C NMR data suggest that the long polymethylenic chains have

ester, carboxylic acid and terminal alcohol functionality and that the aliphatic carbon is

almost all crystalline in nature, demonstrating highly ordered stacking of saturated alkyl

chains.

The observed crystallinity of suberan and its potential recalcitrance to further

saponification are of importance to the understanding of humification in soils. SOM

demonstrates the presence of polymethylenic structures showing a high level of

crystallinity (Hu et al., 2000) and it is likely that the materials responsible for this are

partly derived from suberan-like units. Nierop (1998) has shown that suberan is a

potential contributor to SOM, so it is likely that the crystallinity observed in SOM is

partly derived from suberan. Deshmukh et al. (2005) have shown that cutan also shows

crystallinity, so this property is not unique to suberan. Thus, non-saponifiable

polymethylenic plant biopolymers share this characteristic which persists in SOM. In

fact, the persistence of these polymethylenic structures may be due to their demonstrated

recalcitrance to hydrolytic processes, similar to that observed upon sapinification.
Figure 2.16: Proposed model structural unit of suberan isolated from River Birch (*Betula nigra*) bark. R refers to extended suberan structures and is not specifically defined. The boxed areas represent methylenes that are or may be crystalline structural units.
3.1 Introduction

Plant and microbial remains are the major sources of organic carbon contributing to soil organic matter (SOM). Within soil systems, plant and microbial biopolymers undergo a series of degradative and recombinatory transformations creating a complex mixture of diverse organic structures (Hatcher et al., 1983; Nip et al., 1986; Tegelaar et al., 1991; Kögel-Knabner et al., 1992; Collinson et al., 1999). It is known that SOM has a persistent polymethylenic character which increases with depth (Augris et al., 1998; Nierop, 1998; Hu et al., 2000). Because of this feature, it is widely accepted that polymethylenic plant biopolymers are recalcitrant and resist microbial, photochemical and chemical degradation. Polymethylenic cuticular (cutin, cutan) and suberinic (suberin, suberan) biopolymers are found in the epidermal surfaces of higher plants such as leaves and bark and they have been proposed as a source of stable organic matter to soil systems (Tegelaar, 1995; Kögel-Knabner, 1992; Rasse et al., 2005). These sources of organic
matter are important because the hydrolysable polyesters, cutin and suberin, are found in the leaves/fruit and bark/roots of almost all higher order plants, respectively.

In this study we seek to determine the organic structures contained within forest soils and to compare them with structures contained in polymethylenic plant inputs to test the hypothesis that cuticular or suberinic materials are important contributors to the SOM developed in monocultural forest environments of southern Ohio. For this study we have chosen to characterize soils sampled from two pristine forest sites within Clear Creek Metro Park (Lancaster, Ohio). One site solely contains the coniferous evergreen, Canadian Hemlock (*Tsuga canadensis*) and the other site predominantly contains the deciduous hardwood, Yellow Birch (*Betula alleghaniensis*). Because no other significant plant species growth was occurring at these sites, the soil samples obtained for this study are limited to mostly Hemlock or Birch plant organic matter inputs. We also analyzed whole leaves and bark from live trees at the same two sites because the polymethylenic biopolymers contained within these materials are likely to persist in SOM. The bark samples analyzed within this study are likely sources of suberin, while the leaves are likely sources of cutin. Both suberin and cutin have been extensively characterized and we compare the structural features observed within this study with those previously observed within these polymethylenic plant biopolymers (Stark *et al*., 2000; Deshmukh *et al*., 2003; 2005; Graça and Santos, 2007). The goal of the current study is to identify organic structural components contained within forest soils and to relate them to structural components contained within major polymethylenic SOM input sources.

A number of studies conducted to provide structural information of soil and plant materials have used chemical isolation, chemical derivatization and pyrolysis to facilitate
analyses by common analytical techniques such as gas chromatography mass spectrometry (Tegelaar et al., 1995; McKinney et al., 1996; Nierop, 1998; Ray et al., 1998; Naafs et al., 2005). Isolation and derivatization techniques are often useful, but always have a risk of inducing chemical alterations, creating a material which is somewhat different than the source material. In the current study, to avoid any chemical alteration to the samples, we employ solid-state high resolution magic angle spinning (HRMAS) NMR techniques to characterize whole dried plant materials as well as the underlying soils collected from the A horizon. The analytical advantage of solid-state HRMAS NMR techniques is that they provide well resolved cross-peaks in spectra of complex solid samples allowing for identification of specific structural features and bond connectivities without the need for chemical derivatization.

CPMAS $^{13}$C NMR provides a semiquantitative determination of bulk functional groups while HRMAS NMR provides well resolved qualitative analysis. One-dimensional HRMAS $^1$H NMR allows for higher resolution spectra than traditional solid-state CPMAS $^{13}$C NMR, but is still limited by overlapping peaks. The two-dimensional HRMAS NMR techniques, $^1$H-$^1^3$C heteronuclear single quantum coherence (HSQC) and $^1$H-$^1$H total correlation spectroscopy (TOCSY) have recently become applicable to solid samples due to technological advances. The use of deuterated swelling solvents, such as chloroform-$d$ (CDCl$_3$) and dimethyl sulfoxide-$d_6$ (DMSO-$d_6$) greatly enhances solid-state NMR resolution. This is achieved by increasing molecular mobility thereby reducing anisotropic broadening and also by providing a deuterated shimming lock signal thereby increasing magnetic field homogeneity. Locking onto the deuterium signal allows for continual correction of magnetic field drift during the course of an analysis which can

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take as many as 24 hours to complete. We take advantage of these technological advances by utilizing solid-state two-dimensional HRMAS $^{1}\text{H}-^{13}\text{C}$ HSQC NMR to determine organic structures and solid-state two-dimensional HRMAS $^{1}\text{H}-^{1}\text{H}$ TOCSY NMR to determine bond connectivities within soils and directly compare them to chemically untreated whole plant materials.

3.2 Materials and methods

Sample preparation

The soil samples utilized in this study were sampled from a heavily shaded valley forest with walls of conglomerate black sandstone. The forest soils are dark, moist and sandy. Soil samples were collected from the A horizon of two sites within the Hemlock forest just north of the Hemlock trail within the Clear Creek Metro Park (Lancaster, Ohio). Soil samples were lyophilized and any visible plant remains were physically removed. Hydrochloric acid (10%) treatment for twelve hours (3x) and extensive rinsing with ultrapure UVQ (18 $\Omega$) water was followed by hydrofluoric acid (10%) treatment for twelve hours (3x) and rinsing to remove minerals and provide soil samples which were amenable to NMR analysis (Skjemstad et al., 1994).

Mature live leaves and mature live bark from standing Canadian Hemlock and Yellow Birch trees were collected, extensively rinsed with ultrapure UVQ (18 $\Omega$) water, lyophilized and ground. No chemical treatment of plant materials was conducted prior to NMR analyses in order to preserve their chemical integrity.

Solid-state HRMAS $^{1}\text{H}$ NMR

All solid-state high resolution magic angle spinning (HRMAS) NMR analyses
were performed on a Bruker AVANCE 400 NMR spectrometer equipped with a digital quadrature detector (DQD). All lyophilized solid samples were packed along with 99.9% deuterated chloroform (CDCl₃) or 99.9% deuterated dimethyl sulfoxide (DMSO- d₆) as swelling solvents into a 4mm zirconia rotor with a Kel-F insert and Kel-F cap and spun at the magic angle at 6 kHz. The one-dimensional HRMAS ¹H NMR spectra of plant materials were acquired with a “zg” pulse sequence (Bruker Biospin) while the demineralized soil sample spectra were acquired with a “zgpr” presaturation pulse sequence (Bruker Biospin). The bark and leaf samples had optimized ¹H 90° pulse times of between 6.5 and 6.75 µs. The demineralized soil samples had optimized ¹H 90° pulse times of 5.6 µs and 5.3 µs for the Hemlock and Birch soils, respectively. For the plant samples, a sweep width of 5208 Hz (13 ppm) centered at 5.4 ppm was scanned 16 times, while the demineralized soil samples had sweep widths of 5208 Hz (13 ppm) centered at 3.4 ppm and scanned 32 times.

**Solid-state CPMAS ¹³C NMR Spectroscopy**

Solid-state ¹³C NMR analyses of bark and leaves were performed on a Bruker AVANCE 300 MHz NMR spectrometer using the cross polarization and magic angle spinning (CPMAS) technique. Lyophilized samples were packed into 4 mm zirconia rotors with Kel-F caps and spun at 13 kHz at the magic angle (54.7°). The pulse program was a modified cpvaczp (cross polarization variable amplitude cross polarization decoupled) based on previous work (Dria et al., 2002). The decoupling was two-pulse phase modulated (TPPM) and the cross-polarization (CP) was ramped with a contact time of 2 ms and a recycle delay of 1 s. The spectral width was 19 kHz (260 ppm) and no apodization was used. Calibration was performed with a glycine external standard to the
carboxyl signal at 176.03 ppm. The chemical shifts were referenced to tetramethylsilane (TMS).

**Solid-state HRMAS $^1$H-$^{13}$C HSQC NMR**

A dual channel ($^1$H, $^{13}$C) HRMAS probe was used to acquire solid-state two-dimensional HRMAS $^1$H-$^{13}$C heteronuclear single quantum coherence (HSQC) NMR spectra. The $^1$H-$^{13}$C HSQC NMR spectra of plant materials were acquired using the “invietgpsi” (Bruker Biospin) pulse program with $^1$J coupling set to 145 Hz and echo-antiecho TPPI (time proportional phase incrimination) gradient selection. Between 100 and 150 scans were acquired for the plant samples with optimized $^1$H 90° pulse times of between 6.5 and 6.75 µs, and a 1.0 s recycle delay. The F2 ($^1$H) TD had 2048 data points and sweep widths of 5208 Hz (13 ppm) centered at 6.0 ppm while the F1 ($^{13}$C) TD had 512 data points and sweep widths of 27,172 Hz (270 ppm) centered at 130 ppm. The demineralized soil $^1$H-$^{13}$C HSQC NMR spectra were acquired using the “hsqcgpph” (Bruker Biospin) pulse program with $^1$J coupling set to 145 and states-TPPI gradient selection. Exactly 128 scans were acquired with optimized $^1$H 90° pulse times of 5.3 µs for the Birch soil and 5.6 µs for the Hemlock soil and a recycle delay of 1.0 s.

**Solid-state HRMAS $^1$H-$^1$H TOCSY NMR**

Solid-state two-dimensional HRMAS $^1$H-$^1$H total correlation spectroscopy (TOCSY) NMR spectra of the plant samples were acquired using the “mlevprtp.2” (Bruker Biospin) pulse program with phase sensitive TPPI. For the plant samples, the optimized $^1$H 90° pulse times were between 6.5 and 6.75 µs. The F2 TD had 2048 data points and the F1 TD had 512 data points while both dimensions had a sweep width of 5208 Hz (13 ppm) centered at 6 ppm. The TOCSY mixing time was 0.2 s and the mixing
power was 11.5 dB. For plant samples 60 scans were acquired with a 1.2 s recycle delay and the processing function was QSINE. The two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectra of the demineralized soils were acquired using the “mlevphpr” (Bruker Biospin) pulse program with states-TPPI. The optimized $^1$H 90° pulse time was optimized to 5.3 µs for the Birch soil and 5.6 µs for the Hemlock soil. The F2 TD had 4096 data points and the F1 TD had 512 data points while both dimensions had a sweep width of 6002 Hz (15 ppm) centered at 3.4 ppm. The TOCSY mixing time was 0.06 s and the mixing power was 14.5 dB. Exactly 32 scans were acquired with a 1.0 s recycle delay and the processing function was QSINE.

3.3 Results and discussion

The HRMAS NMR analyses conducted in this study allow determination of the organic functional groups contained within soils and plant materials collected from Clear Creek Metro Park (Lancaster, Ohio) specifically for evaluating the origin of SOM at two sites within a forested area of southern Ohio. The Canadian Hemlock and Yellow Birch sites, representing gymnosperm-dominated and hardwood forest sites, respectively, are discussed independently within this section and comparisons are discussed in the conclusions section. The functional groups within the soil samples are compared to those within the bark and leaves of the dominant overlying plants. These NMR analyses are not quantitative and provide for identification of functional groups without indicating their relative abundances. To provide indication of the relative abundance of carbon functional groups within the plant materials, solid-state CPMAS $^{13}$C NMR spectra were also obtained. All peak assignments in this work are based upon previous work, NMR...
databases and NMR simulation software (Deshmukh et al., 2003; 2005; Saito et al., 2007; Ralph et al., 2004; ACD, 2006).

**Canadian Hemlock site**

The HRMAS NMR spectra of the A horizon soil, as well as bark and leaves from Canadian Hemlock (the predominant plant contributing to the SOM at the site), provide a multitude of NMR cross-peaks that can allow evaluation of contributions to SOM from Hemlock bark and leaves. The one-dimensional HRMAS $^1$H NMR spectra of the samples shown in Figure 3.1 are all dominated by the alkyl methylene peak (1.3 ppm) indicating that the three samples all contain significant aliphatic material. The soil sample has a large broad terminal methyl peak (< 1.0 ppm) that is more prominent than in the Hemlock bark and leaves. The presence of a large variety of terminal methyl groups in the soil is indicated by the broadness of the peak and is attributed to post-depositional transformations leading to a wide variety of structural combinations which may include alkyl branching. Likewise, the sharp peaks at 1.1 ppm and the large shoulder at 1.5 ppm are attributed to branched alkyl methylene groups and these peaks are not observed in the Hemlock bark and leaf $^1$H spectra.
Figure 3.1: One-dimensional HRMAS $^1$H NMR spectra of Canadian Hemlock forest samples. Hemlock bark and leaves were swollen in CDCl$_3$, while the soil sample was swollen in DMSO-$d_6$. No significant peaks were observed with chemical shifts greater than 4.5 ppm.
Signals for oxygen-containing structures, such as esters, carboxylic acids and alcohols (1.6 – 2.3 ppm) are less abundant in the soil spectrum in comparison to those observed within the Hemlock bark and leaf spectra. If the major source of organic matter to the soil is from Hemlock plant tissues, then this indicates that humification, a process that transforms fresh plant material to SOM, involves chemical alterations occurring at or adjacent to these functional groups. The low intensity broad peak observed within the soil spectrum between 3.5 and 4.5 ppm is attributed to lignin methoxy groups as well as carbohydrates or peptides which are likely of microbial origin. No significant peaks were observed in the soil \(^1\)H spectrum with chemical shifts greater than 4.5 ppm. This finding is significant from the standpoint that aromatic moieties derived from lignin are not shown by this technique as significant contributors to the soil, a sharp contrast with traditional soils developed in forested ecosystems where lignin-derived aromatic compounds are significant contributors to SOM (Stevenson, 1994).

We employ the solid-state two-dimensional HRMAS \(^1\)H-\(^{13}\)C HSQC NMR spectroscopic technique to enable enhanced peak resolution and to examine connectivities of protons to \(^{13}\)C. The cross-peaks displayed within the soil \(^1\)H-\(^{13}\)C HSQC spectrum (Figure 3.2) are well resolved and their assignments are displayed in Table 3.1. Cross-peaks 1-5 are assigned to terminal methyl groups and confirm the fact that structural diversity exists for methyl groups within the Hemlock soil. Cross-peaks 6 and 9 indicate the presence of alkyl methylene groups associated with branched structures while signals for methine groups are not observed within the spectra. Cross-peak number 8 is attributed to the long chain alkyl methylene groups and is the most intense signal. Cross-peaks number 7, 10, 11, 12 and 15 all indicate the presence of carbon units that are
Figure 3.2: Two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR spectrum of demineralized soil from the A horizon of a Canadian Hemlock forest. No peaks with chemical shifts greater than 4.5 ppm in the $^1$H dimension or 80 ppm in the $^{13}$C dimension were observed for this sample. Numbered cross-peaks are identified as listed in Table 3.1.
<table>
<thead>
<tr>
<th>Cross-peak</th>
<th>$^1$H-$^{13}$C Chemical Shifts (ppm)</th>
<th>Functional groups</th>
<th>Bark</th>
<th>Leaves</th>
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<tr>
<td>1</td>
<td>(0.9 , 15)</td>
<td>terminal methyl</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>(0.9 , 17)</td>
<td>branched terminal methyl</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>3</td>
<td>(0.9 , 20)</td>
<td>branched terminal methyl</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
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Table 3.1: HRMAS $^1$H-$^{13}$C HSQC NMR cross-peak assignments for the demineralized soil from the A horizon of a Canadian Hemlock forest. Presence of these peaks within Canadian Hemlock bark and leaves is indicated. The spectra displaying these peaks are shown in Figures 3.2, 3.3 and 3.4.
in close proximity to ester, alcohol and carboxylic acid functional groups. Lignin methoxy functionality is displayed as cross-peaks number 14 and 16. No significant cross-peaks were observed with chemical shifts greater than 4.5 ppm in the $^1$H dimension or 80 ppm in the $^{13}$C dimension. This observation presents a bit of a conundrum. The presence of lignin is indicated by methoxyl resonances but aromatic units are not apparent. Either the signal-to-noise level was not sufficient to identify the aromatic cross-peaks expected from lignin moieties, or the aromatic structures are fully substituted such that there are few protonated aromatic carbons. Due to logistical constraints, CPMAS $^{13}$C NMR spectra were not acquired for the soil samples and the lignin content within these soils is unclear.

A two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR spectrum of Hemlock bark (Figure 3.3) was acquired to determine if the structural features within this material persist in the underlying soil. The cross-peak assignments and correlations to the soil structures are listed in Table 3.1. The HRMAS $^1$H-$^{13}$C HSQC NMR spectra of plant materials displayed in this study have inset CPMAS $^{13}$C NMR spectra. It is apparent that some organic structures observable within CPMAS $^{13}$C NMR spectra are not prominent within HRMAS $^1$H NMR spectra. The Hemlock bark $^1$H-$^{13}$C HSQC spectrum has one dominant terminal methyl cross-peak (1) assigned to long alkyl chains. Cross-peaks 6 and 9 are assigned to branched alkyl methylene groups although methines and terminal methyl groups associated with these structures are not observed. Cross-peaks 7, 11 and 12 are assigned to structures associated with esters, carboxylic acids and alcohols. As in the soil $^1$H-$^{13}$C HSQC spectrum, the dominant signal (cross-peak 8) in the Hemlock bark $^1$H-$^{13}$C HSQC spectrum is from long chain alkyl methylene groups. Lastly, cross-peak 16
Figure 3.3: Two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR spectrum of Canadian Hemlock bark. Numbered cross-peaks are identified as listed in Table 3.1. Ester peaks previously found within suberin biopolymers but not found within the Hemlock soil are denoted. Inset is the CPMAS $^{13}$C NMR spectra of Canadian Hemlock bark.
is assigned to lignin methoxy functionality indicating the bark sample contains some ligninous material. Ester, carboxylic acid, alcohol and alkyl methylene signals indicative of suberin biopolymers (see Chapter 2) are observed within the Hemlock bark $^1$H-$^{13}$C HSQC spectrum. Two of the ester cross-peaks, denoted as suberic esters in Figure 3.3 are shown within the Hemlock bark $^1$H-$^{13}$C HSQC spectrum but are not present in the soil $^1$H-$^{13}$C HSQC spectrum. This suggests that esters within Hemlock bark are not contributing to the soil. Thus, if the bark is to be considered an important organic contributor to SOM at this site, the long-chain alkyl esters within bark are at least partially transformed by the humification process such that they are no longer esters. Alternatively, bark organic matter does not contribute to SOM.

Another important source of organic matter to soil is leaf material. The two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR spectrum of Hemlock leaf is displayed in Figure 3.4 with cross-peak assignments and correlations to the Hemlock soil $^1$H-$^{13}$C HSQC spectrum shown in Table 3.1. Three distinct terminal methyl cross-peaks (1, 3, and 4) are observed within the Hemlock leaf $^1$H-$^{13}$C HSQC spectrum and they are all three also observed within the Hemlock soil $^1$H-$^{13}$C HSQC spectrum. The polymethylenic biopolymer cutin has been shown to contain a single terminal methyl (cross-peak 1), but not branched alkyl terminal methyl groups. These methyl groups contained within Hemlock leaves are not attributable to cutin (Deshmukh, et al., 2003; 2005), unless the cutin of Hemlock leaves is unique. The seven other cross-peaks labeled in the Hemlock leaf $^1$H-$^{13}$C HSQC spectrum are also observed in the Hemlock bark $^1$H-$^{13}$C HSQC spectrum (Figure 3.3) and are assigned to methylene (cross-peaks 6 and 9), alcohol (cross-peaks 7 and 10), and carboxyl (cross-peaks 7, 11 and 12) functional groups. Two
Figure 3.4: Two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR spectrum of Canadian Hemlock leaves. Numbered cross-peaks are identified as listed in Table 3.1. Ester peaks previously found within cutin but not found within the Hemlock soil are denoted. Inset is the CPMAS $^{13}$C NMR spectra of Canadian Hemlock leaves.
ester cross-peaks (denoted as cutin esters in Figure 3.4) observed within cutin \(^1\text{H}\)\(^{13}\text{C}\) HSQC spectra (Deshmukh et al., 2003; 2005) are observed in the Hemlock leaf \(^1\text{H}\)\(^{13}\text{C}\) HSQC spectrum, but not observed in the Hemlock soil \(^1\text{H}\)\(^{13}\text{C}\) HSQC spectrum (Deshmukh et al., 2003; 2005). It is clear that some esters, contained within both leaf material and bark, do not survive the humification process to be incorporated within the soil. Leaf and bark are undoubtedly major sources of organic matter to the soil, so we expect that they would contribute chemical structures. One can readily rationalize that the alkyl methyl groups associated with leaf material are incorporated, based on the observed cross-peaks of leaf and soil material.

Another important structural comparison tool is two-dimensional TOCSY \(^1\text{H}\)\(^1\text{H}\) NMR. The two-dimensional HRMAS \(^1\text{H}\)\(^1\text{H}\) TOCSY NMR spectrum of Hemlock soil is displayed in Figures 3.5, 3.6 and 3.7 while the cross-peak assignments are listed in Table 3.2. Figure 3.5 shows the full spectral window with an inset expanded view of the aromatic region of the Hemlock soil \(^1\text{H}\)\(^1\text{H}\) TOCSY spectrum and four distinct aromatic cross-peaks are denoted (29-32). Figure 3.6 shows an expanded view of the region with chemical shifts less than 2.8 ppm in both \(^1\text{H}\) dimensions while Figure 3.7 shows an expanded view of the region with chemical shifts below 2.5 ppm in one \(^1\text{H}\) dimension and between 2.8 and 6.0 ppm in the other \(^1\text{H}\) dimension. Numbered cross-peaks within the Hemlock soil \(^1\text{H}\)\(^1\text{H}\) TOCSY spectra are discussed below with the \(^1\text{H}\)\(^1\text{H}\) TOCSY spectra of the Hemlock bark and leaves.

The two-dimensional HRMAS \(^1\text{H}\)\(^1\text{H}\) TOCSY NMR spectrum of the Hemlock bark is shown in Figures 3.8, 3.9 and 3.10 and assignments for cross-peaks which are also observed within the Hemlock soil \(^1\text{H}\)\(^1\text{H}\) TOCSY spectrum are listed in Table 3.2.
Figure 3.5: Full spectral view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of demineralized soil from the A horizon of a Canadian Hemlock forest. Expanded views of selected regions are displayed as an inset or within Figures 3.6 and 3.7. Peaks 29-32 are denoted and assigned as listed in Table 3.2.
Figure 3.6: Expanded view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of demineralized soil from the A horizon of a Canadian Hemlock forest. Numbered cross-peaks are identified as listed in Table 3.2.

Figure 3.7: Expanded view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of demineralized soil from the A horizon of a Canadian Hemlock forest. Numbered cross-peaks are identified as listed in Table 3.2.
Table 3.2: HRMAS $^1$H-$^1$H TOCSY NMR cross-peak assignments for the demineralized soil from the A horizon of a Canadian Hemlock forest. Presence of these peaks within Canadian Hemlock bark and leaves is indicated. The spectra displaying these peaks are shown in Figures 3.5 through 3.15.
Figure 3.8: Full spectral view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of Canadian Hemlock bark. Expanded views of selected regions are displayed in Figures 3.9 and 3.10. Peak 31 is denoted within the aromatic region and identified as listed in Table 3.2.
Figure 3.9: Expanded view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of Canadian Hemlock bark. Numbered cross-peaks are identified as listed in Table 3.2.

Figure 3.10: Expanded view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of Canadian Hemlock bark. Numbered cross-peaks are identified as listed in Table 3.2. Ester peaks previously found within suberinic biopolymers but not found within the Hemlock soil are denoted.
3.8 shows the entire spectral window for the Hemlock bark $^1$H-$^1$H TOCSY spectrum and the labeled cross-peak (31) indicates that lignin structures contained within the Hemlock bark persist in this soil. Figure 3.9 displays seven cross-peaks within the Hemlock soil $^1$H-$^1$H TOCSY spectrum and their assignments are listed in Table 3.2. Cross-peaks 3, 6 and 8 are assigned to terminal methyl groups with cross-peak 3 attributed to a long alkyl chains while cross-peaks 6 and 8 are attributed to terminal methyl groups correlating with carboxyls and olefins, respectively. Cross-peak 3 has been previously observed in a $^1$H-$^1$H TOCSY spectrum of suberinic biopolymers (Chapter 2) while cross-peaks 6 and 8 are not of this origin. Cross-peaks 9, 12 and 15 are attributed to alkyl methylene groups correlating to carboxyl groups, while cross-peak 10 is attributed to alkyl methylene groups correlating to olefins. Cross-peaks 9, 10, 12 and 15 are attributable to suberinic structures. All cross-peaks in this region of the $^1$H-$^1$H TOCSY spectrum of suberinic materials (Chapter 2), other than additional terminal methyl cross-peaks, are also observed within the Hemlock soil and Hemlock bark $^1$H-$^1$H TOCSY spectra. Figure 3.10 shows five labeled cross-peaks within the Hemlock bark $^1$H-$^1$H TOCSY spectrum which are also observed within the Hemlock soil $^1$H-$^1$H TOCSY spectrum. These cross-peaks are all attributed to methylene groups correlating with carboxyl groups and olefins as listed in Table 3.2. Two alcohol cross-peaks in the Hemlock bark $^1$H-$^1$H TOCSY spectrum which have been previously observed within the $^1$H-$^1$H TOCSY spectrum of suberin material (Chapter 2) are absent in the Hemlock soil $^1$H-$^1$H TOCSY spectrum and are denoted as suberinic in Figure 3.10. This feature also indicates that if suberinic materials persist in this soil, they are transformed.
Figure 3.11: Full spectral view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of Canadian Hemlock leaves. Expanded views of selected regions are displayed in Figures 3.12 and 3.13.
Figure 3.12: Expanded view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of Canadian Hemlock leaves. Numbered cross-peaks are identified as listed in Table 3.2.

Figure 3.13: Expanded view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of Canadian Hemlock leaves. Numbered cross-peaks are identified as listed in Table 3.2.
The Hemlock leaf two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum is shown in Figures 3.11, 3.12 and 3.13. Cross-peaks which are also observed within the Hemlock soil $^1$H-$^1$H TOCSY spectrum are identified and assigned as listed in Table 3.2. No cross-peaks observed within the aromatic region of this spectrum are also observed within the Hemlock soil $^1$H-$^1$H TOCSY spectrum indicating Hemlock leaves are not a significant source of aromatic structures to this soil. Figure 3.12 shows ten cross-peaks which are observed within both the Hemlock leaf and Hemlock soil $^1$H-$^1$H TOCSY spectra. Cross-peaks 1, 2, 3, 5 and 8 are all assigned to terminal methyl groups as denoted in Table 3.2. Cutin only contains long chain alkyl terminal methyl groups (Deshmukh et al., 2003; 2005) and is not a source of the other terminal methyl cross-peaks. The observation of these cross-peaks within the Hemlock leaf and Hemlock soil $^1$H-$^1$H TOCSY spectra provides evidence that other leaf components besides the polymethylenic biopolyester, cutin, are persisting in this soil. Alternatively, the cutin from Hemlock leaf is structurally different from other cutins described previously (Deshmukh et al., 2003, 2005). Cross-peaks 6, 9, 10, 12 and 15 are also observed in the Hemlock bark $^1$H-$^1$H TOCSY spectrum and are attributed to methylene groups, carboxyl groups and olefins. Figure 3.13 shows five cross-peaks which are observed in the Hemlock leaf and Hemlock soil $^1$H-$^1$H TOCSY spectra. These cross-peaks are also observed within the Hemlock bark $^1$H-$^1$H TOCSY spectrum and also attributed to methylene groups, carboxyl groups and olefins. The presence of these features within the soil and plant materials indicates polymethylenic cuticular and suberinic biopolymers persist in this soil. The olefins within the Hemlock $^1$H-$^1$H TOCSY spectra show a selective preservation of the olefins with a chemical shift of 5.4 ppm and this is discussed in the conclusion section below.
Yellow Birch site

The dominant vegetation at this site is Yellow Birch whose organic matter is expected to be the dominant organic contributor to the soil. The HRMAS $^1$H NMR spectra of soil, bark and leaves from a Yellow Birch forest are shown in Figure 3.14. All three $^1$H spectra display a large peak at 1.3 ppm attributed to long chain alkyl methylene groups indicating polymethylenic structures comprise a significant portion of all these samples. The peaks for protons associated with carboxyl functional groups observed between 1.6 and 2.4 ppm within the plant materials are greatly diminished within the soil $^1$H spectra and indicate that carboxyl functional groups are not recalcitrant within this soil. Conversely, the peaks observed within the soil $^1$H NMR spectrum between 3.4 and 3.8 ppm are not as pronounced in the Birch bark and leaf $^1$H NMR spectra. These peaks are attributed to protons of methoxy groups derived from lignin as well as hydroxyl-substituted carbons likely derived from carbohydrates and peptides of microbial origin. No significant peaks with chemical shifts greater than 4.5 ppm were observed in the Birch soil $^1$H spectrum.
Figure 3.14: One-dimensional HRMAS $^1$H NMR spectra of Yellow Birch forest samples. Birch bark and leaves were swollen in CDCl$_3$, while the soil sample was swollen in DMSO-$d_6$. No significant peaks were observed with chemical shifts greater than 4.5 ppm.
The $^1$H-$^{13}$C HSQC spectrum of Birch soil shown in Figure 3.15 shows thirteen well resolved cross-peaks and their assignments are given in Table 3.3. Even after HF treatment to remove mineral material, limited cross-peak intensity is observed. Cross-peaks 1, 2 and 3 indicate a variety of terminal methyl groups within the soil attributed to long chain alkyl groups and alkyl branching. Further evidence of alkyl branching is found in cross-peaks 4 and 7 attributed to branched alkyl methylene groups. Cross-peaks 5, 8, 9 and 11 are all attributed to structural units associated with carboxyl and alcohol functional groups. The most intense cross-peak (6) is attributed to long chain alkyl methylene groups. Lignin methoxy cross-peaks (11 and 12) are also observed within the Birch soil $^1$H-$^{13}$C HSQC spectrum. The Birch SOM is mainly comprised of alkyl methylene groups, structures substituted by carboxyl groups, and also those containing alcohol groups likely derived from polymethylenic biopolymers as well as aromatic methoxy functional groups likely derived from lignin.
Figure 3.15: Two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR spectrum of demineralized soil from the A horizon of a Yellow Birch forest. No peaks with chemical shifts greater than 4.5 ppm in the $^1$H dimension or 80 ppm in the $^{13}$C dimension were observed for this sample. Numbered cross-peaks are identified as listed in Table 3.3.
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<th>Bark</th>
<th>Leaves</th>
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<td>Yes</td>
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<td>(0.9 , 20)</td>
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<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>(0.9 , 24)</td>
<td>branched terminal methyl</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>(1.3 , 23)</td>
<td>branched alkane</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>(1.3 , 26)</td>
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<td>Yes</td>
<td>Yes</td>
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<td>methylene</td>
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<td>Yes</td>
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<td>(1.3 , 32)</td>
<td>branched alkane</td>
<td>Yes</td>
<td>Yes</td>
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<td>(3.8 , 57)</td>
<td>lignin methoxy</td>
<td>Yes</td>
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Table 3.3: HRMAS \(^{1}H\text{-}^{13}C\) HSQC NMR cross-peak assignments for the demineralized soil from the A horizon of a Yellow Birch forest. Presence of these peaks within Yellow Birch bark and leaves is indicated. The spectra displaying these peaks are shown in Figures 3.16, 3.17 and 3.18.
Displayed in Figure 3.16 is the Birch bark two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR spectrum with cross-peaks also found within the Birch soil $^1$H-$^{13}$C HSQC spectrum labeled. The labeled cross-peaks are assigned as listed in Table 3.3. Even though the Birch bark and Birch soil $^1$H-$^{13}$C HSQC spectra both display multiple terminal methyl cross-peaks; only cross-peak 1 is observed in both. This cross-peak is attributed to long chain alkyl structures and indicates that these structures persist while other terminal methyl groups within the Birch bark are not transferred to the soil. Cross-peaks 4 and 7, attributed to branched methylene groups, are observed within the Birch bark and Birch soil $^1$H-$^{13}$C HSQC spectra indicating these structures persist in this soil. Cross-peaks 5, 8 and 9 are attributed to structural units containing carboxyl functionality and suberinic material within the Birch bark likely is the source of this material. Cross-peak 6 is attributed to long chain alkyl methylene groups and is the strongest signal in the $^1$H-$^{13}$C HSQC spectrum. Although wood is likely the main source of lignatious material to forest soils, the lignin methoxy cross-peak (13) observed within the Birch bark $^1$H-$^{13}$C HSQC spectrum indicates that Birch bark is also a source of this persistent structure. Ester cross-peaks observed within the Birch bark $^1$H-$^{13}$C HSQC spectrum known to be present in suberinic biopolymers (Chapter 2) but absent from the Birch soil $^1$H-$^{13}$C HSQC spectrum are denoted as suberinic esters in Figure 3.16. Their absence from the Birch soil $^1$H-$^{13}$C HSQC spectrum indicates that esters previously identified within suberinic biopolymers (Chapter 2) are not significantly preserved as such in this soil as discussed in the conclusions section below. In contrast to the other three plant materials analyzed in this study, the Birch bark is comprised of mostly aliphatic materials as indicated by the CPMAS $^{13}$C NMR spectrum inset in Figure 3.16.
Figure 3.16: Two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR spectrum of Yellow Birch bark. Numbered cross-peaks are identified as listed in Table 3.3. Ester peaks previously found within suberinic biopolymers but not found within the Hemlock soil are denoted. Inset is the CPMAS $^{13}$C NMR spectra of Yellow Birch bark.
Figure 3.17: Two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR spectrum of Yellow Birch leaves. Numbered cross-peaks are identified as listed in Table 3.3. Ester peaks previously found within cutin but not found within the Birch soil are denoted. Inset is the CPMAS $^{13}$C NMR spectra of Yellow Birch leaves.
Cross-peaks 1-9 from the Birch soil two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR spectrum are also observed within the Birch leaf $^1$H-$^{13}$C HSQC spectrum as shown in Figure 3.17. Structural assignments of these cross-peaks are listed in Table 3.3. All of the terminal methyl cross-peaks (1-3) which are observed within the Birch soil $^1$H-$^{13}$C HSQC spectrum are attributable to Birch leaf inputs. Cross-peaks 4-9, which are observed in the Birch soil and Birch bark $^1$H-$^{13}$C HSQC spectra are also observed in the Birch leaf $^1$H-$^{13}$C HSQC spectrum and are attributed to alkyl, alcohol and carboxyl functional groups. Ester cross-peaks observed within the Birch leaf $^1$H-$^{13}$C HSQC spectrum known to be present in cutin (Deshmukh, 2003; 2005) but absent from the Birch soil $^1$H-$^{13}$C HSQC spectrum are denoted as cutin esters in Figure 3.17. No lignatious cross-peaks are observed within the Birch leaf $^1$H-$^{13}$C HSQC spectrum and this plant material is not a significant SOM input source of aromatic structures.

Figures 3.18 and 3.19 show the Birch soil two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum. Within Figure 3.18 cross-peaks 16-22 are labeled with cross-peaks 20-22 being displayed within the inset expanded view of the aromatic region. Cross-peaks 16 and 17 are attributed to alkyl methylene correlation with alcohol groups while cross-peaks 18 and 19 are attributed to alkyl methylene correlation with olefins. Figure 3.19 displays an expanded view of the Birch soil $^1$H-$^1$H TOCSY spectrum with cross-peaks 1-15 labeled. All cross-peaks observed within the Birch soil $^1$H-$^1$H TOCSY spectrum are assigned as listed in Table 3.4. The assignment of these cross-peaks and their correlations to Birch bark and leaves are discussed below.
Figure 3.18: Full spectral view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of demineralized soil from the A horizon of a Yellow Birch forest. Expanded views of the selected regions are inset or displayed in Figure 3.19. Peaks 16-22 are denoted and assigned as listed in Table 3.4.
Figure 3.19: Expanded view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of demineralized soil from the A horizon of a Yellow Birch forest. Numbered cross-peaks are identified as listed in Table 3.4.
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<th>Bark</th>
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<td>Yes</td>
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<tr>
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</tr>
<tr>
<td>22</td>
<td>(7.0 , 7.3)</td>
<td>aromatics</td>
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<td>No</td>
</tr>
</tbody>
</table>

Table 3.4: HRMAS $^1$H-$^1$H TOCSY NMR cross-peak assignments for the demineralized soil from the A horizon of a Yellow Birch forest. Presence of these peaks within Yellow Birch bark and leaves is indicated. The spectra displaying these peaks are shown in Figures 3.19 through 3.23.
Figure 3.20: Full spectral view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of Yellow Birch bark. An expanded view of the selected region is displayed in Figure 3.21.

Figure 3.21: Expanded view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of Yellow Birch bark. Numbered cross-peaks are identified as listed in Table 3.4. Terminal methyl peaks previously found within suberinic biopolymers but not found within the Hemlock soil are denoted.
Figures 3.20 and 3.21 show the Birch bark two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum. The only five cross-peaks which are observed within both the Birch bark and Birch soil $^1$H-$^1$H TOCSY spectra are numbered within the expanded region displayed in Figure 3.21 and their assignments are listed in Table 3.4. Cross-peak 3 is attributed to long chain alkyl methylene groups while cross-peaks 4, 7, 10 and 14 are all attributed to methylene and carboxyl functional groups. These are the main functional groups observed within $^1$H-$^1$H TOCSY spectra of suberin biopolymers and indicate that suberinic structures are the main bark inputs able to persist within this soil. Two terminal methyl cross-peaks are denoted as suberinic within Figure 3.21 because they have been previously observed within $^1$H-$^1$H TOCSY spectra of suberin biopolymers (Chapter 2) and are observed within the Birch bark $^1$H-$^1$H TOCSY spectrum while they are not observed within the Birch soil $^1$H-$^1$H TOCSY spectrum. These terminal methyl groups are not attributed to long chain alkyl structures and do not persist within this soil.

The Birch leaf HRMAS $^1$H-$^1$H TOCSY NMR spectrum is shown in Figures 3.22 and 3.23. Figure 3.22 shows the full view of this spectrum and cross-peaks number 18 and 19 are labeled. These two cross-peaks are attributed to olefin structures and do persist in the Birch soil. Of particular interest is the trend observed within olefin cross-peaks which have chemical shifts of 5.2 and 5.4 ppm. Olefin cross-peaks with chemical shifts of 5.4 ppm are observed within the Birch soil $^1$H-$^1$H TOCSY spectrum while olefin cross-peaks with a chemical shift of 5.2 ppm are not observed. See conclusion section below for further discussion. Labeled in Figure 3.23 are eight cross-peaks which occur in the Birch soil and Birch leaf $^1$H-$^1$H TOCSY spectra and their assignments are listed in Table 3.4. Cross-peaks 1, 3, and 6 are terminal methyl groups and indicate that leaf inputs
Figure 3.22: Full spectral view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of Yellow Birch leaves. Expanded view of selected region is displayed in Figures 3.23. Peaks 18 and 19 are denoted and assigned as listed in Table 3.4.

Figure 3.23: Expanded view of the two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of Canadian Hemlock leaves. Numbered cross-peaks are identified as listed in Table 3.4.
can contribute some of the terminal methyl variety observed within the Birch soil $^1$H-$^1$H TOCSY spectra. Cross-peaks 7, 10, 14 and 15 are attributed to long chain alkyl methylene groups and carboxyl functional groups, while cross-peak 8 is attributed to long chain methylene groups correlating to olefins. All of these features are observed within cutin $^1$H-$^1$H TOCSY spectra (Deshmukh et al., 2003; 2005) and indicate that cutin structures persist within this soil.

3.4 Conclusions

The functional groups identified in soils and plant materials within this study indicate that leaves and bark are likely significant inputs to the SOM at these sites. Comparison of the CPMAS $^{13}$C NMR and HRMAS $^1$H NMR spectra of the plant materials indicates HRMAS techniques are not representing some of the structural features observed within the CPMAS spectra. In this study, we compare the structures which are observed within HRMAS NMR spectra of forest soils and plant materials to determine which features observed by this technique persist in forest soils. While many structural entities identified within the plant materials persist in the A horizon soils, reactive functional groups associated with certain structures within the bark and leaves are transformed through humification. The one-dimensional HRMAS $^1$H NMR spectra all show an intense peak at 1.3 ppm indicating that long-chain paraffinic structures are present within the soils and plant materials. SOM spectra for the two soils indicate an increase in the proportion and variety of terminal methyl groups in comparison to bark and leaves. While the $^1$H spectrum of Hemlock soil indicates an increase in alkyl branching, the Birch soil $^1$H spectrum does not display this feature. We propose that
increases in alkyl branching and terminal methyl group variation are due to post-depositional transformations of organic matter inputs. Both soil $^1$H spectra show a smaller proportion of structural entities associated with carboxyl functional groups than the plant materials indicating that these groups are being transformed via humification processes after deposition. One possible transformation is the hydrolysis of ester functional groups, but another is simply the degradation and removal of these structural units by micro-organisms. The Birch soil $^1$H spectrum shows a higher proportion of hydroxyl-containing structures than the source plant materials, which is attributed to an input of labile biopolymers such as cellulose and peptides to the soil.

The enhanced resolution associated with the two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR technique enabled specific identification of functional groups contained within the soils. Both soil $^1$H-$^{13}$C HSQC spectra confirm the polymethylenic contribution, long alkyl chain methylene groups, branched alkyl methylene groups and a variety of terminal methyl groups, to the SOM. Other than peaks in the terminal methyl and lignin methoxy regions, the $^1$H-$^{13}$C HSQC spectra of these two soils are identical indicating that soil processes generate similar organic structures from varied plant species.

Comparing the two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR spectra of plant materials with those of the soils indicates that bark and leaves contribute significantly to the SOM. The same two alkyl branching cross-peaks, the long chain alkyl terminal methyl cross-peak and the high intensity long chain polymethylene cross-peak are shown within all the $^1$H-$^{13}$C HSQC spectra, and we propose bark and leaves are the sources of these structures in the soils. At both sites, only the terminal methyl cross-peaks associated with long chain alkyl groups are matches within the bark and soil $^1$H-$^{13}$C HSQC spectra
while the leaf and soil $^{1}$H-$^{13}$C HSQC spectra also contain matching branched alkyl terminal methyl cross-peaks. This indicates that recalcitrant leaf inputs are not limited to polymethylenic cuticular and suberinic biopolymers. Unmatched terminal methyl cross-peaks shown in the soil $^{1}$H-$^{13}$C HSQC spectra are attributed to other SOM inputs and products of post-depositional transformations. Lignin methoxy peaks observed within both soil $^{1}$H-$^{13}$C HSQC spectra are observed within the bark $^{1}$H-$^{13}$C HSQC spectra but not the leaf $^{1}$H-$^{13}$C HSQC spectra, indicating that lignitios SOM components are partially attributable to bark inputs.

The presence of some cross-peaks associated with carboxyl- and alcohol-containing structures in the bark and leaf $^{1}$H-$^{13}$C HSQC spectra indicates structures derived from cuticular and suberinic biopolymers are present. Yet, many of the carboxyl (ester) cross-peaks shown in the bark and leaf $^{1}$H-$^{13}$C HSQC spectra are not shown in either soil $^{1}$H-$^{13}$C HSQC spectra. This indicates that polyesters derived from cutin and suberinic biopolymers are depolymerized in soils by the humification process. Hydrolysis of esters is rapid in acidic or alkaline environments but can also occur slowly in natural settings of neutral pH. Even though the pH of the Hemlock soil is 6.6 and the pH of the birch soil is 7.4, most esters do not persist in these soils. While the esters may not persist as such in the soil, it is likely that the paraffinic structures from the polyesters of cutin and suberin persist, perhaps contributing to the long-chain polymethylenic moieties seen in the HRMAS NMR spectra of the soils.

The two-dimensional HRMAS $^{1}$H-$^{1}$H TOCSY NMR spectra show functional groups contained within the soils and plant materials and their bond connectivities. The soil $^{1}$H-$^{1}$H TOCSY spectra support the presence of long chain methylene, branched alkyl,
varied terminal methyl, alcohol, and carboxyl functional groups. Also shown in the soil
$^1$H-$^1$H TOCSY spectra are olefin, ether and aromatic functional groups. The cross-peaks
shown in both soil $^1$H-$^1$H TOCSY spectra support a significant contribution of
polymethylene groups and a significant contribution to terminal methyl groups is likely
from the leaves. Terminal methyl groups observed previously within suberan (Chapter 2)
and Birch bark $^1$H-$^1$H TOCSY spectra which are not associated with long chain alkyl
groups are not observed within the birch soil $^1$H-$^1$H TOCSY spectrum. This shows that
polymeric suberinic material does not comprise significant fraction of the SOM at the
birch site. Additionally, ester functional groups, previously observed within suberan and
shown in the Birch $^1$H-$^1$H TOCSY spectrum, are not shown in the Birch soil $^1$H-$^1$H
TOCSY spectrum.

The olefins observed within the $^1$H-$^1$H TOCSY spectra show a post-depositional
transformation pattern which is observed at both the Hemlock and Birch sites. In the
Hemlock bark, Hemlock leaf and Birch leaf $^1$H-$^1$H TOCSY spectra, olefin cross-peaks
are shown at 5.2 ppm correlating to alkyl methylene groups at 1.3 and 2.1 ppm as well as
olefin cross-peaks at 5.4 ppm correlating to cyclic and polymethylene groups at 1.6, 1.7
and 2.1 ppm. In the leaf $^1$H-$^1$H TOCSY spectra the olefin cross-peaks at 5.2 ppm are
more intense than the cross-peaks at 5.4 ppm, yet in both soil $^1$H-$^1$H TOCSY spectra only
the cross-peaks at 5.4 ppm are shown. Structural differences associated with these two
olefin types have a significant impact on their recalcitrance in these soils. Olefin cross-
peaks matching those observed at 5.4 ppm have been reported within the polymethylenic
cuticular biopolymer cutin (Deshmukh et al., 2003) and we propose that aliphatic olefins
persist within these soils. Olefins within alicyclic plant resins have been shown to
resonate at 5.4 ppm (Clifford et al., 1997) and would likely cross-correlate with both cyclic and aliphatic methylene groups. The cross-peaks observed at 5.2 ppm within the bark and leaf $^1$H-$^1$H TOCSY spectra are attributed to resin structures and do not persist in these soils.

The NMR cross-peaks previously identified within cuticular and suberinic biopolymers are observed within all the plant materials. All of the polymethylenic NMR cross-peaks observed within the bark and leaf spectra are also observed within the soil spectra indicating that these components do persist in these environments. Functional groups such as alcohols, carboxylic acids and olefins, known to comprise cuticular and suberinic biopolymers, are also observed within all the bark, leaf and soil spectra. Cuticular and suberinic biopolymers are ester cross-linked into macromolecular structures and while ester cross-peaks are observed in the plant materials, many ester peaks are not observed within the soils. Terminal methyl groups and alcohols previously identified within macromolecular suberan (Chapter 2) are also observed within the Birch bark spectra, but not in the Birch soil spectra, indicating macromolecular suberinic structures are not prevalent. We propose that cuticular and suberinic biopolymers are a significant source of the persistent polymethylenic material found within these soils, but the many of their ester-linkages do not persist. These macromolecular biopolymers depolymerize into smaller recalcitrant polymethylenic structures after deposition.
CHAPTER 4

IDENTIFICATION OF ALDEHYDE FUNCTIONALITY WITHIN THE CUTICULAR BIOPOLYESTER CUTIN

4.1 Introduction

Aerial parts of higher order plants have a continuous extracellular cuticular membrane to prevent dehydration and infection. Estimations have been made that 180-1500 kg/hectare of cuticular material is found in the living plants within temperate forests and agricultural settings (Riederer, 1990). This cuticular layer is comprised of lipids, the macromolecular biopolymer cutin, and, in some cases, another biopolymer named cutan. While the lipid fraction of cuticular material is soluble in organic solvents, the biopolymers cutin and cutan are not. Cutin and cutan are cross-linked esters and provide a structural framework which supports the waxy lipids that are contained within and layered upon the cuticle surface. Within plant cuticles, the saponifiable biopolyester cutin is ubiquitous and comprises 40-80% of the cuticle weight (Heredia et al., 2003). Cutan, a non-saponifiable polymer, is less ubiquitous, being identified in only a limited number of plants (de Leeuw and Largeau, 1993, Gupta et al., 2006).

As is common with other plant parts, cuticular material eventually becomes incorporated into soil organic matter (SOM) and sedimentary organic matter (SdOM). As
it does, labile components are quickly recycled to CO\textsubscript{2} or dissolved and leached away, while the remaining organic material remains resistant to degradation for a relatively long time (Hedges and Oades, 1997). The hydrophobicity and low reactivity of cutin provides recalcitrance in most depositional environments, and polymethylenic material is known to persist in SOM and S\textsubscript{D}OM (Augris et al., 1998; Nierop, 1998; Hu et al., 2000). Cutin and suberin (another polymethylenic plant biopolymer found in bark) have been proposed as the major source of polymethylenic carbon in soils (Kögel-Knabner, 1992; Rasse et al., 2005). However, very little is known concerning the specific molecular transformations that convert these polymethylenic biopolymers to the aliphatic portion of SOM and S\textsubscript{D}OM.

While selective preservation of intact and relatively unaltered recalcitrant plant materials has been proposed (Hatcher et al., 1983; Tegelaar et al., 1991; de Leeuw and Largeau, 1993), most evidence indicates post-depositional reactions stabilize portions of the polymethylenic SOM and S\textsubscript{D}OM inputs leading to the creation of persistent OM (Nip et al., 1986; Tegelaar et al., 1991; Ishiwatari et al., 1992; Kögel-Knabner et al., 1992; Collinson et al., 1999; Mösle et al., 2002). Collinson et al. (1999) suggest formation of a macromolecular matrix by within-cuticle stabilization of normally degradable aliphatic constituents. They propose replacement of esters with other carbonyl functional groups and aliphatic stabilization by creation of additional cross-linkages possibly incorporating lipids into cuticular biopolymers. Gupta et al. (2006) provide evidence for \textit{in situ} polymerization of labile organic compounds during the preservation of fossil leaves and show the components of fossilized leaves are indicative of lipids and cutin polymerizing during diagenetic processes leading to a material which is more recalcitrant and resistant
to hydrolysis or degradation. Another proposal for stabilization of cuticle material involves cross-linkages via nitrogen and sulfur heteroatoms (Schnitzer, 1985; Eglinton et al., 1993; Bates et al., 1995; Hartgers et al., 1997; Filley et al., 2002; Knicker and Hatcher, 2002). Peptidic nitrogen and ammonia from the degradation of peptidic material are prevalent in depositional environments (Nommik and Vahtras, 1982; Cooper and Evans, 1983; Knicker and Hatcher, 1997). Highly reactive reduced sulfur species are abundant within the anoxic zone of sedimentary systems and are continually replenished by sulfate reducing bacteria (Mossmann et al., 1991; Luther et al., 2001). Sulfur and nitrogen within SOM and SOOM incorporate into natural organic matter during early digenetic processes (Nommik and Vahtras, 1982; Knicker and Hatcher, 2002; Amrani et al., 2007) and create more highly cross-linked and refractory structures (Sinninghe Damsté and de Leeuw, 1990; Amrani et al., 2007).

Thus, in order to understand cutin transformations occurring within depositional environments it is important to ascertain the structural features of cutin within the live plant material which might participate in diagenetic reactions. We expect that reactive organic functional groups capable of participating in cross-linking reactions or nucleophilic substitution reactions would be of most interest in this regard.

Cutin has been isolated from many sources including apple fruit, cherry fruit, lime fruit, tomato fruit skin, and the leaves of *Agave americana* (Holloway, 1982; Ray et al., 1995; del Rio and Hatcher, 1998; Deshmukh et al., 2003; 2005; Peschel et al., 2007). Depolymerization studies of cutin have shown the presence of mainly C$_{16}$ and C$_{18}$ hydroxy fatty acid monomers (Baker and Holloway, 1970; Walton and Kolattukudy, 1972; Kolattukdy, 1980; Holloway, 1982; del Rio and Hatcher, 1998). These studies have
often found additional minor components which were unidentified (Ray et al., 1995; del Rio and Hatcher, 1998). Based on depolymerization analysis, a general scheme for cutin monomeric structural units was proposed by Walton and Kolattukudy (1972) and this scheme is the one currently accepted (Figure 4.1).

A significant amount of recent information on the structure of cutin has been obtained by non-destructive methods such as NMR (Zlotnik-Mazori and Stark, 1988; Ray et al., 1998; Stark et al., 2000; Fang et al., 2001; Deshmukh et al., 2003; 2005; Kelleher and Simpson, 2006) and infrared spectroscopy (Luque et al., 1995; Benítez et al., 2004; Douliez et al., 2005). These studies have generally confirmed the previous structural models presented and have provided additional information on the details of cross-links and molecular mobility of the polymer chains. Deshmukh et al. (2003) utilized high resolution magic angle spinning (HRMAS) NMR spectroscopic techniques to show

<table>
<thead>
<tr>
<th>C_{16} Family</th>
<th>C_{18} Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH_{2}-(CH_{2})_{14}-COOH</td>
<td>CH_{2}-(CH_{2})<em>{11}-CH=CH-(CH</em>{2})_{11}-COOH</td>
</tr>
<tr>
<td>HOCH_{2}-(CH_{2})_{11}-COOH</td>
<td>HOCH_{2}-(CH_{2})<em>{11}-CH=CH-(CH</em>{2})_{11}-COOH</td>
</tr>
<tr>
<td>HOOC-(CH_{2})_{11}-COOH</td>
<td>HOOC-(CH_{2})<em>{11}-CH=CH-(CH</em>{2})_{11}-COOH</td>
</tr>
<tr>
<td>HOCH_{2}-(CH_{2})<em>{11}-CH-(CH</em>{2})_{n}-COOH</td>
<td>HOH_{2}C-(CH_{2})<em>{11}-CH=CH-(CH</em>{2})_{11}-COOH</td>
</tr>
<tr>
<td>\text{OH}</td>
<td>\text{O}</td>
</tr>
</tbody>
</table>

\[ n = 5, 6, 7, 8 \]
\[ m + n = 13 \]

Figure 4.1: Monomeric structural units for the cuticular biopolyester cutin. Adapted from Walton and Kolattukudy, (1972).
polymethylenic, carboxylic acid, ester, primary and secondary alcohol functionality as well as α-branched fatty acids and esters in cutin isolated from tomato (*Lycopersicon esculentum*) fruit skins. Fang *et al.* (2001) also used HRMAS NMR techniques to show polymethylenic, carboxylic acid, ester, and alcohol functionality as well as a previously unidentified terminal ethyl ester functionality within cutin isolated from lime (*Citrus aurantofolia*) fruit and proposed a structure containing this functionality. Ketone functionality has been observed within cutin isolated from lime fruit with only brief mention (Zlotnik-Mazori and Stark, 1988). The presence of ketones is important because this reactive functional group can play a significant role in post-depositional transformations. Also of importance in this regard is the presence of aldehyde and epoxide functionality. Epoxides have been identified in cutin from *Agave americana* (Deshmukh *et al.*, 2005), but the presence of aldehydes in cutin is not well established.

Many studies have proposed more elaborate structures depicting the polymeric molecular architecture of cutin based on the monomers shown in Figure 4.1 (Zlotnik-Mazori and Stark, 1988; Jeffree, 1996; Ray *et al.*, 1998; Fang *et al.*, 2001). They all depict a cutin structure comprised of long-chain hydroxy acids connected by cross-esterification. The structures all contain both mid-chain hydroxyl and terminal hydroxyl groups esterified to carboxylic acids on adjacent chains. Some structures include mid-chain hydroxyls cross-linked via ester bonds and such cross-links have been proposed to add structural stability to the cutin polyester. Until recently it was believed that cutin was only comprised of ester-linkages between fatty acid monomers, but Graça *et al.* (2002) have identified glycerol units comprising 1-14% of cutin. They propose that cutin
contains ester-linkages between the fatty acid monomers and also a glycerol backbone which is ester-linked to the fatty acid monomers.

Mid-chain hydroxyls and epoxides have been considered as important sites for cross-linking within the biopolymer (Ray et al., 1998; Heredia, 2003), but ketones or aldehydes have not been considered to be important cross-linking functional groups. The potential presence of aldehyde functionality within this natural material has been proposed but no direct evidence has been presented (Nawrath, 2002). In a recent review of the biopolymers cutin and suberin, Nawrath (2002) proposes that minor contributions to the cutin structure may be from long-chain aldehydes and ketones. Some other studies have displayed NMR spectra which indicate the presence of aldehydes as small peaks, but no discussion of their presence was mentioned (Ray et al., 1998; Deshmukh, 2003).

Aldehydes are known to be electrophilic and to participate in nucleophilic substitution reactions by accepting electron pairs. Studies have indicated that such functional groups can enter into cross-linking reactions in sedimentary systems, thereby inducing polymer stabilization (Schouten et al., 1993; Wakeham et al., 1995; Adam et al., 2000; Lückge et al., 2002; Amrani et al., 2007). The presence of aldehyde functionality within cutin provides a viable site for nucleophilic substitution allowing for heteroatom incorporation into SOM and SdOM. In this study one-dimensional $^1$H, two-dimensional $^1$H-$^{13}$C hetero single quantum coherence (HSQC) and two-dimensional $^1$H-$^1$H total correlation spectroscopy (TOCSY) high resolution magic angle spinning (HRMAS) NMR, as well as solid-state cross polarization magic angle spinning (CPMAS) $^{13}$C NMR techniques are utilized to evaluate the presence of aldehydes within cutin isolated from tomato (Lycopersicon esculentum) fruit skin and Agave (Agave americana)
leaves. These cuticular substances will be utilized in experiments of Chapter 5 where attempts are made to induce covalent coupling with nucleophiles likely to be found in sedimentary systems.

4.2 Materials and methods

Chemical isolation

Cutin was isolated from tomato (*Lycopersicon esculentum*) fruit skins and a cutin/cutan mixture was isolated from *Agave* (*Agave americana*) leaves using previous methods (Deshmukh *et al.*, 2003; 2005). Briefly, the tomato fruit was lanced and the skins were separated from the remainder of the mass. The *Agave* leaves were cut into sections (approximately 8cm x 8cm), and soaked in an oxalic acid (0.4% w/v) and ammonium oxalate (1.6% w/v) solution at 40°C for 48 hours prior to peeling the cuticular layers from the fleshy bulk material. The tomato skins and *Agave* cuticles were washed extensively with ultrapure UVQ (18 Ω) water, lyophilized and ground to a fine powder, with these whole cuticular layers being referred to in this study as residues R0. These lyophilized residues were stored in sealed glass containers with a Teflon lined caps for 18 months prior to further treatment. Gentle reflux solvent extraction with methanol and chloroform was performed three times to remove lipids (Holloway, 1984). Removal of cellulosic components was performed with sodium paraperiodate (Zelibor *et al.*, 1988) and these residues, referred to as R2, are considered to be isolated cuticular biopolymers. The tomato R2 is considered to be cutin while the *Agave* R2 is a mixture of cutin and cutan. The *Agave* R2 was saponified (1% KOH in 96% methanol) for two hours to
remove cutin (Holloway, 1984) leaving a cutan residue (*Agave* R3). The residues were rinsed extensively with ultrapure UVQ (18 Ω) water between each isolation process.

**Solid-State CPMAS $^{13}C$ NMR Spectroscopy**

Solid-state cross polarization magic angle spinning (CPMAS) $^{13}C$ NMR analyses were performed on a Bruker AVANCE 300 MHz NMR spectrometer using the cross polarization and magic angle spinning (CPMAS) technique. Lyophilized samples were packed into 4 mm zirconia rotors with Kel-F caps and spun at 13 kHz at the magic angle (54.7°). The pulse program was a modified cross polarization technique with variable amplitude cross polarization, based on previous work (Dria *et al*., 2002). The decoupling was two-pulse phase modulated (TPPM), and the cross polarization contact time of 2 ms was used with a recycle delay of 1 s. The spectral width was 19 kHz (260 ppm) and no apodization was employed. Calibration was performed by referencing to a glycine external standard the carboxyl signal at 176.03 ppm. The chemical shifts were referenced to tetramethylsilane (TMS).

**Solid-state HRMAS NMR Spectroscopy**

High resolution magic angle spinning (HRMAS) NMR analyses were performed on a Bruker AVANCE 400 NMR spectrometer. Lyophilized solid samples were packed as a mixture with 99.9 % deuterated chloroform (CDCl$_3$) or 99.9% deuterated dimethyl sulfoxide (DMSO– $d_6$) as a swelling solvent into a 4mm zirconia rotor with a Kel-F insert and Kel-F cap and spun at the magic angle at 3 kHz. A dual channel ($^1$H, $^{13}$C) HRMAS probe was used to acquire one-dimensional $^1$H NMR, two-dimensional $^1$H-$^{13}$C heteronuclear single quantum coherence (HSQC) NMR and two-dimensional $^1$H-$^1$H total correlation spectroscopy (TOCSY) NMR spectra. The one-dimensional $^1$H spectra for
tomato samples were acquired with a standard 90° pulse sequence with an optimized 90° $^1$H pulse time of 7.0 µs. The one-dimensional $^1$H spectra for *Agave* samples were acquired with a “zgeppr” (Bruker Biospin) pulse sequence and an optimized $^1$H 90° pulse time of 7.2 µs. For tomato samples, a spectral width of 5580 Hz (14 ppm) centered at 6 ppm was scanned 16 times, and for *Agave* samples a spectral width of 4808 Hz (12 ppm) centered at 5.4 ppm was scanned 16 times. All $^1$H-$^13$C HSQC spectra were acquired using the “invietgpsi” pulse program, $^1$J coupling set to 145 Hz, and echo-antiecho TPPI (time proportional phase incrementation) gradient selection. For tomato samples, 300 scans were acquired with a $^1$H 90° pulse time of 7.0 µs, $^13$C 90° pulse time of 4.6 µs and a 1 s recycle delay. For *Agave* samples, 500 scans were acquired with a $^1$H 90° pulse time of 7.2 µs, $^13$C 90° pulse time of 4.6 µs and a 1 s recycle delay. For tomato samples, the F2 ($^1$H) time dimension (TD) had 1024 data points and spectral width of 6675 Hz (17 ppm) centered at 2.15 ppm while the F1 ($^13$C) TD had 256 data points and a spectral width of 32,200 Hz (320 ppm) centered at 150 ppm. For *Agave* samples, the F2 ($^1$H) time dimension (TD) had 1024 data points and spectral width of 5580 Hz (14 ppm) centered at 5 ppm while the F1 ($^13$C) TD had 256 data points and a spectral width of 26,262 Hz (260 ppm) centered at 120 ppm. The two-dimensional $^1$H-$^1$H TOCSY spectrum of *Agave* R2 was acquired using the “mlevrtp.2” pulse program (Bruker Biospin), with a mixing power of 11.5 dB, a mixing time of 0.2 s and 1 s recycle delay. The 90° pulse time was optimized to 7.2 µs. A spectral width of 6402 Hz (16 ppm) centered at 3.3 ppm was scanned 48 times. The F2 had 2048 data points and the F1 had 512. Decoupling was achieved with TPPM, and the processing function was QSINE.
4.3 Results and discussion

Solid-state CPMAS $^{13}$C NMR spectra of milled but chemically untreated tomato fruit skins (tomato R0), chemically isolated tomato cutin (tomato R2), untreated Agave leaves (Agave R0), chemically isolated Agave cutin/cutan biopolymer mixture (Agave R2) and isolated Agave cutan (Agave R3) are shown in Figure 4.2. These biopolymers have been examined by NMR in previous studies, and the results of the current analyses confirm all of the previously observed structural features (Deshmukh et al. 2003; 2005). Integration of peak areas within all the CPMAS $^{13}$C NMR spectra (Table 4.1) indicates the dominance of polymethylene groups (53.6 - 86.0%) in these materials with

<table>
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<th>Chemical Shift (ppm)</th>
<th>Aliphatic</th>
<th>Methoxy</th>
<th>Hydroxyl</th>
<th>Aromatic</th>
<th>Carboxyl</th>
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<td>10.0</td>
<td>1.8</td>
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Table 4.1: Percentage distribution of carbon atoms in the cuticular biopolymer samples isolated from tomato (*Lycopersicon esculentum*) fruit skins and Agave (*Agave Americana*) leaves as determined by the solid-state CPMAS $^{13}$C NMR spectra displayed in Figure 4.1.
Figure 4.2: CPMAS $^{13}$C NMR spectra of cuticular biopolymer samples isolated from tomato (*Lycopersicon esculentum*) fruit skins and *Agave* (*Agave americana*) leaves. (1) tomato R0, chemically untreated tomato fruit skin. (2) tomato R2, chemically isolated cutin. (3) *Agave* R0, chemically untreated *Agave* leaf cuticle. (4) *Agave* R2, Chemically isolated mixture of cutin and cutan. (5) *Agave* R3, Chemically isolated cutan. Percentage contributions of functional groups based on integrated peak areas are displayed in table 4.1.
increasing proportion throughout the chemical treatments. The hydroxyl region (60-110 ppm) decreases substantially (32.9 to 17.3% for tomato and 16.5 to 11.3% for Agave) upon organic solvent extraction of lipids and removal of cellulosic components with paraperiodate (conversion of R0 to R2).

Of particular interest is the CPMAS $^{13}$C NMR spectrum of tomato R2 that displays two low intensity peaks in the carbonyl region. The peak at shown in Figure 4.2 at 202 ppm, most likely assigned to aldehydes, and a broader peak at 197 ppm, which we assign to ketone carbons (see discussion below), are observable in the R2 fractions of both plant materials while these peaks are not readily observed in the CPMAS $^{13}$C NMR spectra of their R0 and R3 fractions. The entire carbonyl region (190-220 ppm), comprised of aldehydes and ketones, constitutes only 0.1% of the untreated tomato fruit skin (R0) while comprising 0.6% of the isolated cutin biopolymer (R2). This region also increases from 0.3% (R0) to 0.5% (R2) for the Agave sample. Upon saponification of the Agave R2 sample, cutin is removed and this results in the loss of the majority of the carbonyl component, leaving a cutan residue (Agave R3) with only 0.2% carbonyl character, which is significantly less carbonyl character than the cutin/cutan mixture.

This trend indicates that the carbonyls are associated with the cutin fraction of the cutin/cutan mixture and likely a larger than 0.5% component of cutin isolated from Agave. The presence of 0.6% carbonyl within tomato cutin and at least 0.5% carbonyl within Agave cutin represents approximately one carbonyl on every tenth $C_{16}/C_{18}$ monomer. If 10% of the monomeric structural units within cutin possess this substituent, this could have a significant impact upon the fate of this biopolymer in sedimentary systems.
Figure 4.3: One-dimensional HRMAS $^{1}$H NMR spectrum of tomato (*Lycopersicon esculentum*) cutin (R2).
Figure 4.4: One-dimensional HRMAS $^1$H NMR spectrum of *Agave* (*Agave americana*) cutin and cutan mixture (R2).
The ensuing discussion is focused on residue R2 or the cutin fraction of Agave and tomato because this fraction is the only one displaying evidence of significant ketone/aldehyde functionality. The one-dimensional HRMAS $^1$H NMR technique applied for studies of R2 provides for high resolution spectra of these materials swollen in organic solvents (in this study CDCl$_3$ or DMSO – $d_6$). The HRMAS $^1$H NMR spectrum of tomato cutin (Figure 4.3) clearly shows an aldehyde signal at 9.7 ppm while also confirming all previously identified components; aliphatic (0.8 -1.3 ppm), ester (1.3 - 1.8, 2.2, 2.3, 4.0, 4.8 ppm), carboxylic acid (1.3-1.8 ppm), alcohol (3.2, 3.5 ppm), and aromatic (6.7 ppm) (Deshmukh et al., 2003). Figure 4.4 displays the HRMAS $^1$H NMR spectrum of the Agave cutin/cutan mixture (Agave R2) and also shows an aldehyde (9.7 ppm) as well as all previously observed functional groups (Deshmukh et al., 2005). The peaks at 9.7 ppm are clear evidence of aldehyde functionality within these samples. After saponification of Agave R2 to remove cutin and generate a residue called cutan (Agave R3), the aldehyde peak (9.7 ppm) disappears (spectra not shown).

The two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR technique provides for additional resolution by displaying carbon and hydrogen atoms directly bound to one another as cross-peaks. Peaks that overlap within either $^1$H or $^{13}$C NMR but not in both dimensions can become resolved cross-peaks. One limitation of this technique is that only $^1$H and $^{13}$C atoms directly bound to one another appear in the spectrum. For example, the hydrogen and carbon atoms within carboxylic acid functionality do not appear as cross-peaks. One can employ heteronuclear multiple bond correlation (HMBC) experiments to identify carbonyl groups not possessing a directly attached proton, but these experiments are inherently less sensitive, requiring large amounts of spectrometer
Figure 4.5: Two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR spectrum of tomato (Lycopersicon esculentum) cutin (R2).
Figure 4.6: Two-dimensional HRMAS $^1$H-$^{13}$C HSQC NMR spectrum of *Agave (Agave americana)* cutin and cutan mixture (R2).
time for the materials at hand. The HRMAS \(^1\text{H}-^{13}\text{C}\) HSQC NMR spectra of tomato cutin (tomato R2) and Agave cutin/cutan mixture (Agave R2) shown in Figures 4.5 and 4.6 display cross-peaks that have been previously observed and assigned (Deshmukh et al., 2003; 2005). They also clearly show aldehyde as a cross-peak (9.7 ppm \(^1\text{H}, 202\) ppm \(^{13}\text{C}\)) indicating the presence of this functionality, even though the previous studies did not specifically observe and identify them. Another cross-peak which has previously not been identified within cutin is also observed at 2.3 ppm \(^1\text{H}, 30\) ppm \(^{13}\text{C}\) and is assigned to a methyl ketone. Ketones are not observed within \(^1\text{H}-^{13}\text{C}\) HSQC NMR spectra because the carbon is not protonated; however, the carbon/proton pairs adjacent to the ketone are influenced by the presence of this functional group and appear as a distinct cross-peak. This peak is not resolved in the CPMAS \(^{13}\text{C}\) NMR or the one-dimensional HRMAS \(^1\text{H}\) NMR spectrum due to an overlapping ester/acid peak (2.3 ppm) in the \(^1\text{H}\) dimension and an overlapping methylene peak (30 ppm) in the \(^{13}\text{C}\) dimension. This is exactly the type of peak which is only observable with resolution in both dimensions and with the HRMAS \(^1\text{H}-^{13}\text{C}\) HSQC NMR technique. Similarly the alkyl carbons on the other side of the methyl ketone overlap with signals for units adjacent to esters and are not observable as distinct peaks. This technique provides evidence of both aldehyde and methyl ketone functional groups within cutin which supports the observation of two distinct peaks within the CPMAS \(^{13}\text{C}\) NMR. The chemical shifts of these cross-peaks indicate methyl ketone and aldehyde units are at the terminal end of long-chain structures within cutin.

Many HRMAS NMR investigations including, Deshmukh et al. (2003), have utilized deuterated dimethyl sulfoxide (DMSO – \(d6\)) as a swelling solvent as part of their analyses. DMSO is commonly used as an oxidizing agent within industrial processes to
convert terminal alcohols to aldehydes by a chemical process known as Swern oxidation (Mancuso and Swern, 1981). Although the oxidation of terminal alcohols to aldehydes by Swern oxidation requires activation of DMSO by an electrophilic agent, we were cautious to preclude this potential artifact within the current study. The cutin samples isolated within the current study were analyzed swollen in DMSO, but were analyzed again swollen in CDCl₃ to preclude any potential artifacts that may have occurred within DMSO. Comparison of the two spectra (not shown) reveals that one need not be concerned about Swern oxidation.

Two-dimensional HRMAS ¹H-¹H TOCSY NMR allows for observation of cross-peaks deriving from the interaction between ¹Hs located within the same spin system (refers to protons connected through protonated carbon backbones) which can extend over several bonds. As discussed previously, ketones do not appear in proton NMR spectra but aldehydes do. In order to investigate the chemical structural environment associated with the aldehyde functionality, ¹H-¹H TOCSY NMR analysis of the cutin/cutan mixture isolated from Agave leaves (Agave R2) was performed. Figure 4.7 shows the ¹H-¹H TOCSY NMR spectra of Agave R2 with three cross-peaks observed for the aldehyde. Aldehyde peak number 1 indicates cross-correlation (1.3 ppm, 9.7 ppm) with mid chain methylenes (γ or further from the aldehyde) which provides evidence that the aldehyde is likely at the end of a long alkyl chain known to dominate the cutin structure. Peaks number 2 (1.6 ppm, 9.7 ppm) and 3 (2.3 ppm, 9.7 ppm) are likely cross-correlations with carbon atoms that are located β and α to the aldehyde carbon, respectively. It is clear that aldehydes contained within cutin are at the end of long chains, most likely the C₁₆ or C₁₈ cutin monomeric units.
Figure 4.7: Two-dimensional HRMAS $^1$H-$^1$H TOCSY NMR spectrum of Agave (Agave americana) cutin and cutan mixture (R2).
In summary, the cutin isolated from tomato fruit skin and the cutin/cutan mixture isolated from *Agave* leaves clearly display aldehyde and methyl ketone functionality, most likely located at terminal positions on long-chain structures. Although the biopolyester cutin has been studied extensively, these features have not been well established within the literature. The relative proportion of these features is less than one percent of the overall carbon and has likely been considered insignificant. Alternatively, being natural samples, it is possible the samples used in the current study are different than those previously studied. After separating the cuticle from the bulk of the fruit, rinsing with ultrapure UVQ (18Ω) water, grinding and lyophilizing, these samples were stored for 18 months in a sealed glass container prior to further isolation and analysis. This aging period creates the possibility that terminal alcohols in the samples were oxidized to aldehydes while being stored. Rontoni *et al.*, (2005) have shown evidence of cutin oxidation by visible light, but they show formation of 9-hydroperoxy-18-hydroxyoctadec-10(\textit{trans})-enoic and 10-hydroperoxy-18-hydroxyoctadec-8(\textit{trans})-enoic acids formed by radical induced hydroxylation of sites α to mid-chain unsaturation. They show no evidence of terminal aldehyde or ketone formation by photo-oxidation. In CPMAS $^{13}$C NMR data shown by Deshmukh (2003) and Ray *et al.* (1998) evidence of aldehyde functionality within tomato cutin and lime cutin are displayed, respectively, yet there is no discussion of the 202 ppm peak, likely because it is of low intensity. This indicates that our samples are not unique in harboring this feature. It is evident that tomato cutin and an *Agave* cutin/cutan mixture either have aldehyde functionality in the plant biopolymers or oxidize to form aldehydes and ketones over time while isolated in a sealed dry glass jar. Formation of this functionality during chemical treatment (periodate)
Figure 4.8: One-dimensional HRMAS $^1$H NMR spectrum of tomato (*Lycopersicon esculentum*) cutin (R0).
to isolate cuticular biopolymers is unlikely because the aldehyde functionality is clearly observed within the HRMAS $^1$H NMR spectra of untreated plant material as shown in Figure 4.8 (tomato R0).

The presence of aldehydes within cutin is important because the reactivity of this functional group likely has a significant impact on the fate of this recalcitrant SOM and $S_D$OM input within post-depositional environments. It is known that SOM and $S_D$OM contain nucleophilic nitrogen and sulfur species within soil and wetland environments and that these heteroatoms participate in condensation reactions during humification. If aldehydes and methyl ketones within cutin provide electrophilic sites for heteroatom incorporation processes, they likely play a significant role in cross-linking and stabilization of polymethylenic plant biopolymers.

The long-term stabilization of cutin is an important phenomenon that has bearing on the evolution of polymethylenic materials in sedimentary systems. The observation of fossil cuticles in coals (Nip et al., 1986) has led to the suggestion that cutan, a more stable polymethylenic biopolymer in certain plants is responsible for the existence of aliphatic materials in fossil leaves, being preserved both physically and chemically intact (Tegelaar et al., 1989a) more so than other plant biopolymers. Cutin is generally regarded as a biopolymer less likely to survive long-term diagenesis due to the existence of saponifiable esters. Recently, Gupta et al. (2006) have challenged this “selective preservation” concept because they find preserved aliphatic fossil leaf material for plants whose modern analogs do not contain the resistant cutan. They propose that the lack of persistent biopolymers (cutan) in the modern leaf analogs along with the presence of an insoluble aliphatic biopolymer in fossilized leaf material requires the polycondensation of
lipids to explain the existence of the aliphatic fossil leaf material in the rock record. However, the persistence of aliphatic material is more easily rationalized as a cross-linking and stabilization of polymethylenic cutin biopolymers brought about by nucleophilic attack of sulfur- or nitrogen-containing mineral matter on the aldehyde and methyl ketone functional groups as shown by Amrani et al. (2007) and discussed in Chapter 5 of this thesis.
CHAPTER 5

AMMONIA NITROGEN INCORPORATION BY CUTICULAR BIOPOLYMERS

5.1 Introduction

The organic matter contained within soil and sedimentary systems is mainly comprised of the partially degraded remnants of plant and microbial biopolymers. Upon deposition, these well defined organic materials undergo a series of diagenetic transformations creating diverse organic compounds known as soil organic matter (SOM) and sedimentary organic matter (SdOM). These organic matter transformations involve degradation of biopolymers to smaller molecules ultimately evolving CO₂ as well as recombination and condensation reactions leading to complicated recalcitrant macromolecular structures (Schnitzer, 1985; Eglinton et al., 1993; Hartgers et al., 1997; Hedges and Oades, 1997; Collinson et al., 1999). During the early stages of diagenesis, inorganic nitrogen species are believed to incorporate into SOM and SdOM (Nommik and Vahtras, 1982; Barrett and Burke, 2000; Thorn and Mikita, 2000; Compton and Boone, 2002). Although nitrogen is thought to participate in the creation of recalcitrant SOM and SdOM, the mechanisms of incorporation and the structures formed during these processes are still unclear (Nommik and Vahtras, 1982; Schulten et al., 1997; Knicker et al., 1996; Amrani et al., 2007).
Nitrogen is an essential nutrient for plant growth which predominantly enters soils as inorganic ammonia (NH₃) generated by microbial nitrogen fixation of gaseous N₂ or from decaying plant matter (Rosswall, 1981; Stevenson, 1982). Within soil and sedimentary systems nitrogen becomes associated with SOM, stabilizing the nitrogen pool and allowing for storage and release of nitrogen to microbes and plant roots (Rosswall, 1981; Nommik and Vahtras, 1982; Barrett and Burke, 2000). Organic nitrogen contained within soils and sediments has been a major focus of soil research (Schulten and Schnitzer, 1998; Zang et al., 2000; Knicker et al., 2001; Bird et al., 2003). Nitrogen becomes associated with SOM mainly through biotic processes as soil and sedimentary plants and organisms utilize the various available inorganic nitrogen forms to synthesize biomass (Stevenson, 1982; Schnitzer, 1978). Studies utilizing isotopically labeled ammonia mixed with sterilized soils have shown abiotic ammonia reactions play a significant role in formation of soil organic nitrogen (Johnson et al., 2000, Barrett and Burke, 2002), but how this occurs is unknown. Ammonia is known to participate in nucleophilic substitution reactions with electrophilic functional groups, such as carbonyls and carboxyls, which are present within SOM and SDOM.

The major forms of organic nitrogen in soils and sediments are peptides and amino acids (Sowden et al., 1977; Nommik and Vahtras, 1982; Knicker and Lüdemann, 1995; Knicker and Hatcher, 1997; Knicker, 2002). Other forms of organic nitrogen also are known to exist in soils, including heterocyclic N (including purines and pyrimidines) and amino sugars (Schulten and Schnitzer, 1998). Peptides are labile compounds (easily degraded by microbes) and their persistence within soils and sediments has been a source of debate. In particular, their dominance over other forms of organic nitrogen has been a
central theme of the debate. Chemical analyses of the protein content in a wide variety of soils and sediments has revealed a maximum of 40% (Sowden et al., 1977) while solid-state $^{15}$N NMR analysis of soils and humic acids consistently show a dominant ($70^\circ$ %) peptide-like amide character (Knicker et al., 1995b; Knicker and Hatcher, 1997; Thorn and Mikita, 2000; Kögel-Knabner, 2000), implying that the major form of organic nitrogen is peptide nitrogen. Several explanations have been given for this discrepancy by Schulten and Schnitzer (1998), including low $^{15}$N abundance leading to poor NMR sensitivity and widely varying structures leading to broad unobserved NMR signals. Knicker (2002) has also addressed this discrepancy when utilizing cross-coupling $^{13}$C-$^{15}$N NMR techniques to show that organic nitrogen incorporated within a humic material correlates with alkyl carbon. Knicker (2002) concluded that inorganic nitrogen likely has incorporated into partially decomposed plant biopolymers. Because polymethylenic organic material within soils and sediments is hydrophobic and tends to resists degradation (Augris et al., 1998; Nierop, 1998; Hu et al., 2000), it is reasonable to assert that soil organic nitrogen contained within aliphatic domains may also resist degradation. The presence of alkyl organic nitrogen has led to the proposal that the CPMAS $^{13}$C NMR carboxyl peak (160-220 ppm) observed in soil samples likely contains a significant contribution from amides associated with aliphatic structures (Knicker and Lüdemann, 1995), either associated with peptides or other alkyl N species.

Another indication of aliphatic nitrogen compounds is found within GCMS analyses of soils and sediments. Alkyl nitriles have been observed within pyrolysates of older S$_{OM}$ with alkyl chain lengths ranging from C$_3$ to C$_{19}$, and it has been suggested that these structures originate during pyrolysis of alkyl amides and amines (Regtop et al., 129
observed long chain nitriles during GCMS analyses of organic matter from a young sediment and concluded that nitrogen-linked long-methylene-chain structures analogous to fatty acid amines / fatty nitriles are a precursor to nitrogenous compounds in petroleum. Although the presence of alkyl nitriles within pyrolyzates has been attributed to structures derived directly from algal cell wall biomacromolecules (Derenne et al., 1993), other studies find alkyl nitriles to be soil specific (Schnitzer, 1985; Schulten et al., 1997). Schulten et al. (1997) suggest these alkyl nitriles are formed by pyrolytic thermal decomposition of amines and that their presence in soils indicates significant concentrations of aliphatic amines formed by transformations of organic nitrogen during decomposition of plant precursors. They further conclude that alkyl amines, along with heterocyclic nitrogen compounds comprise the “unknown” nitrogen fraction in soils. Due to a lack of adequate methods for analysis of resistant soil organic nitrogen species, a significant fraction of this material is still defined as unknown. It is evident that a wide variety of structures including peptides (Tremblay and Benner, 2006), alkyl amides/amines of unknown origin (Knicker et al., 2001), and heterocyclic nitrogen compounds (Maie et al., 2006) comprise a large portion of the chemically resistant organic nitrogen within soils and sediments.

While the origin of peptides is obvious, there has been little explanation for the origin of alkyl amides/amines and for heterocyclic nitrogenous species. We focus, in our current investigation, on the origin of alkyl amides/amines in soil and sedimentary systems and propose that they originate from the abiotic reaction of ammonia with recalcitrant polymethylenic contributors to SOM and SpOM that have the requisite
functional groups. It is well established that phenolic organic matter binds ammonia in soils in non-exchangeable forms (Nommik and Vahtras, 1982; Thorn and Mikita, 1992). Recently, ammonia has also been shown to covalently bind with model aldehydes, octanal and 2-octenal, providing evidence of a possible cross-linking mechanism (Amrani et al., 2007). Because the aliphatic components of SOM and S_{OM} have these requisite functional groups (carbonyl and carboxyl), they are likely to react with ammonia and subsequently incorporate nitrogen.

Two major sources of polymethylenic organic matter to soils and sediments are the cuticular biopolymers, cutin and cutan. In this study we have selected these natural SOM and S_{OM} precursors as model compounds to investigate their potential to incorporate ammonia. Estimations have been made that 180-1500 kg/hectare of cuticular material is found in the living plants within temperate forests and agricultural settings (Riederer, 1990). This cuticular material comprises an extracellular membrane on the outside of the leaves and fruit of all higher order plants. This membrane which prevents dehydration and microbial infection contains lipids and the cross-linked biopolymers cutin and sometimes cutan. Within plant cuticles, the saponifiable biopolyester cutin is ubiquitous and comprises 40-80% of the cuticle weight (Heredia et al., 2003). Cutan, a non-saponifiable polymer, is less ubiquitous, being identified in only a limited number of plants (de Leeuw and Largeau, 1993, Gupta et al., 2006). These cuticular biopolymers have been proposed as the major source of persistent aliphatic materials found within SOM and S_{OM} (Kögel-Knabner et al., 1992; Ishiwatari et al., 1992).

Cutin is a cross-linked polyester that has been isolated from many sources including apple fruit, cherry fruit, lime fruit, tomato fruit skin, and the leaves of *Agave* 131
americana (Holloway, 1982; Ray et al., 1995; del Rio and Hatcher, 1998; Deshmukh et al., 2003; 2005; Peschel et al., 2007). Depolymerization studies of cutin have shown the presence of mainly C₁₆ and C₁₈ hydroxy fatty acid monomers (Baker and Holloway, 1970; Walton and Kolattukudy, 1972; Kolattukudy, 1980; Holloway, 1982; del Rio and Hatcher, 1998). Although cutin, a recalcitrant plant polyester, has been shown to contain aldehyde, ketone, epoxide, olefin, ester and carboxylic acid functional groups, it is unknown whether it participates in the incorporation of nitrogen (Chapter 4; Deshmukh et al., 2003). Also unknown is the role of cutan, a biopolyester comprised of long aliphatic chains, with primary alcohol, ester, carboxylic acid, and aromatic functional groups (Deshmukh et al., 2005). Both of these polymethylenic natural biopolymers contain functional groups which may allow them to react with ammonia nitrogen.

In the current study, incorporation of ammonia into cuticular biopolymers is evaluated by solid-state cross polarization magic angle spinning (CPMAS) $^{15}$N NMR, two-dimensional high resolution magic angle spinning (HRMAS) $^1$H-$^{15}$N heteronuclear single quantum coherence (HSQC) NMR and elemental analysis techniques. Cuticular biopolymers present a significant analytical challenge due to insolubility and low volatility. A major limitation associated with these $^{15}$N NMR techniques is that the natural abundance of the $^{15}$N isotope is only 0.37% and the gyromagnetic ratio is low compared to $^1$H’s, leading to low sensitivity. We overcome this analytical challenge by using 99+% $^{15}$N labeled ammonium chloride ($^{15}$NH₄Cl) as a reactant and utilize a technique, $^1$H-$^{15}$N HSQC, that detects $^{15}$N signals via their attached and more abundant $^1$H’s. We hypothesize that the oxygen functional groups contained within cuticular biopolymers can activate carbon structures to participate in nucleophilic substitution
reactions with ammonia thereby generating organic nitrogen compounds (Amrani et al., 2007). In these experiments, we varied the pH from 6.3 to 9.3 to examine the extent of reaction under environmentally-relevant pH conditions in soils and sediments. Of course, we expect that the amounts of available free ammonia, vs ammonium ion (pKa of ammonia is 9.24) would change over the course of selected pH values.

5.2 Materials and methods

Cuticular biopolymers

Cutin was isolated from tomato (Lycopersicon esculentum) fruit skins, while a cutin/cutan mixture and cutan were isolated from Agave (Agave americana) leaves. Isolation methods for these biopolymers have previously been described (see Chapter 4).

Ammonium chloride ($^{15}$NH$_4$Cl) reactions

Aqueous isotopically labeled ($^{15}$N) ammonium chloride ($^{15}$NH$_4$Cl) solutions were prepared by adding 600 mg of 99+ % $^{15}$N ammonium chloride (Aldrich) to 60 mL of ultrapure UVQ (18 Ω) water. Initial pH of the solutions was 5.4 and adjusted with potassium hydroxide (KOH, Aldrich) to achieve pHs of 6.3, 8.0, 7.5, and 9.3 for the various sets of reactions. Aliquots of the pH adjusted reagent (15 mL) were stirred magnetically with 200 mg of cuticular biopolymer in 50 mL roundbottom flasks. After displacing the headspace air with nitrogen gas, the reactions were sealed and allowed to proceed for two weeks in the dark.

The reaction products were vacuum filtered with GFF glass fiber filters. The solid residues were placed in ultrapure UVQ (18 Ω) water, shaken for 10 minutes and refiltered. This process was repeated three times to remove any residual water soluble
reactant ($^{15}$NH$_4$Cl). The residues were then rinsed with acetone three times by the same process and freeze dried.

**Elemental analysis**

Cuticular biopolymers and residues produced by reactions between biopolymers and isotopically labeled ammonium chloride ($^{15}$NH$_4$Cl) were analyzed for carbon and nitrogen composition in the laboratory of Dr. Gregory Cutter at Old Dominion University, Norfolk, Virginia, as described previously (Cutter and Radford-Knoery, 1991). Simultaneous carbon and nitrogen determination was achieved with a Carlo Erba NA-1500 elemental analyzer. Solid samples (~0.2 mg) were placed in tin sample cups with a vanadium pentoxide ($\text{V}_2\text{O}_5$) catalyst. Flash combustion of samples was achieved at 1010 °C followed by gas chromatographic separation of carbon dioxide, molecular nitrogen and sulfur dioxide followed by photoionization detection. Sulfanilamide (Carlo Erba) and a NIST estuarine sediment were both used as external standards for calibration.

**Solid-state CPMAS $^{15}$N NMR spectroscopy**

Solid-state cross polarization magic angle spinning (CPMAS) $^{15}$N NMR spectra were obtained using the basic ramp cross polarization pulse program with two-pulse phase modulated (TPPM) decoupling. The spectrometer was a 400 MHz Bruker AVANCE II with $^1$H resonating at 400.13 MHz and $^{15}$N resonating at 40.54 MHz. The samples were spun with a frequency of 10 kHz at the magic angle (54.7°) in a 4 mm zirconia NMR rotor sealed with a Kel-F cap. Exactly 61,440 scans were obtained with a 1.0 sec recycle delay. All nitrogen chemical shifts were externally calibrated to the glycine standard (32.6 ppm) with the spectra referenced to ammonia (NH$_3$) at 0.0 ppm (ACD, 2006).
Two-dimensional (2D) high resolution magic angle spinning (HRMAS) $^1$H-$^{15}$N heteronuclear single quantum coherence (HSQC) NMR was performed with a Bruker AVANCE II 400 MHz NMR spectrometer. The $^1$H resonance was at 400.13 MHz and $^{15}$N resonance was at 40.54 MHz. Pulsed field gradient capabilities were utilized with same low pulse level and electronics as a liquids probe (RF sending/receiving hardware, deuterium lock, and direct polarization transfer). The HRMAS probe utilized a 4 mm zirconia rotor with a Kel-F insert and Kel-F cap spun at 7 kHz at the magic angle (54.7°). The samples were swollen in (99.9%) deuterated chloroform (CDCl$_3$) to increase molecular mobility and provide a deuterium shimming lock signal. Relaxation delay time was 1.0 sec. Nitrogen chemical shifts were calibrated using formamide as an external standard resonating at 116.3 ppm referenced to ammonia (NH$_3$) at 0.0 ppm (ACD, 2006). The pulse program was “hsqcetf3gp” (Bruker Biospin) utilizing the echo-antiecho-TPPI (time proportional phase incrementation) gradient selection. Exactly 144 scans were acquired with a spectral width of 6,410 Hz (16 ppm) centered at 4.75 ppm and 1,024 data points in the F2 ($^1$H) dimension as well as 16,626 Hz (410 ppm) centered at 195 ppm and 256 data points in the F1 ($^{15}$N) dimension. The spectrometer was equipped with a digital quadrature detector (DQD) and the processing function was QSINE.

5.3 Results and discussion

Elemental analysis was performed to determine the amount of carbon and nitrogen present in cuticular biopolymers isolated from tomato (*Lycopersicon esculentum*) fruit skins and *Agave* (*Agave americana*) leaves. The results of the tomato
cuticle analyses are shown in Table 5.1. The chemically untreated tomato cuticles (tomato R0) show 0.99 % nitrogen and 53.6 % carbon content. The chemically isolated cutin from tomato fruit skins (tomato R2) shows 0.79% nitrogen content and 58.8% carbon. The high percentage of carbon is expected for this aliphatic biopolyester. While depolymerization studies have not shown nitrogen to be a component of cutin (Holloway, 1982; del Rio and Hatcher, 1998), it has been previously proposed that amino acids can be associated with cuticles (Holloway, 1982), and the 0.79% nitrogen content observed within cutin likely suggests the presence of some residual peptidic material. After reaction of the tomato fruit skin biopolymer samples with ammonium chloride, the carbon content remained relatively constant or increased slightly. A more interesting finding is associated with the nitrogen content of the biopolymers. The nitrogen content of the reactions carried out at pH 6.3 and pH 8.0 with both the tomato R0 and R2 samples remains relatively unchanged indicating minor nitrogen association during the reaction. It appears that, at these two pH levels, ammonium does not react significantly with the tomato based cuticular biopolymers. When the pH is elevated to 9.3 the dominant species in the reaction is ammonia which is a nucleophile. The reaction at pH 9.3 leads to elevated nitrogen content within the two tomato biopolymers; 1.82% for the tomato R0 sample and 2.49% for the tomato R2 (cutin) sample. This increase in nitrogen content is significant and is an indication that these biopolymers are able to react with ammonia to form organic nitrogen. The C/N ratio (Table 5.1) decreases from values greater than 50 for other fractions to values of 25 and 29 for the two isolates treated with ammonia at pH 9.3. The greater incorporation of nitrogen for the tomato R2 compared to the tomato R0
Table 5.1: Elemental analysis of tomato (Lycopersicon esculentum) fruit skin samples. Tomato R0 is chemically untreated, while tomato R2 is chemically isolated cutin. Each of these cuticular biopolymer samples was reacted with labeled ammonium chloride ($^{15}$NH$_4$Cl) at various pH, and the resulting products were analyzed for carbon and nitrogen content.

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<td>Tomato R0</td>
<td>53.66</td>
<td>0.99</td>
<td>54</td>
</tr>
<tr>
<td>$^{15}$NH$_4$Cl products</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 6.3</td>
<td>55.09</td>
<td>0.78</td>
<td>71</td>
</tr>
<tr>
<td>pH 8.0</td>
<td>57.58</td>
<td>1.10</td>
<td>52</td>
</tr>
<tr>
<td>pH 9.3</td>
<td>53.36</td>
<td>1.82</td>
<td>29</td>
</tr>
<tr>
<td>Tomato R2</td>
<td>58.80</td>
<td>0.79</td>
<td>74</td>
</tr>
<tr>
<td>$^{15}$NH$_4$Cl products</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 6.3</td>
<td>57.98</td>
<td>0.80</td>
<td>72</td>
</tr>
<tr>
<td>pH 8.0</td>
<td>58.53</td>
<td>0.97</td>
<td>60</td>
</tr>
<tr>
<td>pH 9.3</td>
<td>62.73</td>
<td>2.49</td>
<td>25</td>
</tr>
</tbody>
</table>
sample indicates that cutin, when purified by separation from associated extractable biopolymers, becomes more reactive with ammonia.

The elemental analysis of the Agave leaf samples shows a similar trend to that of the tomato fruit skin cuticle samples and the results are shown in Table 5.2. The carbon content of the untreated Agave leaf samples increases during the chemical isolation of the cuticular biopolymers, from 60.86% for Agave R0 to 64.61% for Agave R2 (cutin/cutan mixture) and to 68.00% for Agave R3 (cutan). This increase is expected as cutin and cutan are both polymethylenic structures with cutan having longer aliphatic chains and less oxygen functionality (Deshmukh et al., 2005). Upon reaction with ammonium chloride, the carbon content within all the reaction products decreases slightly (3-9%) from the carbon content of the respective unreacted biopolymers. The explanation for this decrease is not apparent.

The nitrogen content within all three Agave leaf samples prior to reaction with ammonium chloride is very low (0.24% to 0.32%). Following reaction with ammonium chloride the nitrogen content of the Agave R0 sample increases slightly from 0.24% to 0.46% and 0.60% for pH 7.5 and pH 9.3, respectively. The incorporation of nitrogen within the Agave R0 fraction is minimal and may be limited due to physical encasement by the lipid layer which remains intact in the R0 sample. In contrast, Agave R2 (cutin/cutan mixture) is slightly enriched in nitrogen at pH 7.5 (0.23% to 0.67%) but shows significant enrichment at pH 9.3 (0.23% to 1.49%). A change in C/N ratio from 281 to values of 87 and 42 for the R2 samples reacted at pH 7.5 and 9.3, respectively. After removal of organic soluble lipids (fraction R2) the cuticular biopolymers are able to react with ammonia whose availability is determined by the pH. The Agave R3 (cutan)
<table>
<thead>
<tr>
<th>Sample</th>
<th>%C</th>
<th>%N</th>
<th>C/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agave R0</td>
<td>60.86</td>
<td>0.24</td>
<td>254</td>
</tr>
<tr>
<td>$^{15}$NH$_4$Cl products</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.5</td>
<td>55.64</td>
<td>0.46</td>
<td>121</td>
</tr>
<tr>
<td>pH 9.3</td>
<td>57.05</td>
<td>0.60</td>
<td>95</td>
</tr>
<tr>
<td>Agave R2</td>
<td>64.61</td>
<td>0.23</td>
<td>281</td>
</tr>
<tr>
<td>$^{15}$NH$_4$Cl products</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.5</td>
<td>58.22</td>
<td>0.67</td>
<td>87</td>
</tr>
<tr>
<td>pH 9.3</td>
<td>62.00</td>
<td>1.49</td>
<td>42</td>
</tr>
<tr>
<td>Agave R3</td>
<td>68.00</td>
<td>0.32</td>
<td>213</td>
</tr>
<tr>
<td>$^{15}$NH$_4$Cl products</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.5</td>
<td>59.12</td>
<td>1.41</td>
<td>42</td>
</tr>
<tr>
<td>pH 9.3</td>
<td>59.05</td>
<td>1.55</td>
<td>38</td>
</tr>
</tbody>
</table>

Table 5.2: Elemental analysis of Agave (Agave americana) leaf cuticle samples. Agave R0 is chemically untreated, Agave R2 is a chemically isolated cutin/cutan mixture, and Agave R3 is chemically isolated cutan. Each of these cuticular biopolymer samples was reacted with labeled ammonium chloride ($^{15}$NH$_4$Cl) at various pH, and the resulting products were analyzed for carbon and nitrogen content.
sample shows the greatest incorporation of nitrogen at both pH 7.5 (0.32% to 1.41%) and pH 9.3 (0.32% to 1.55%). The C/N ratio drops from 213 in the untreated biopolymer to values of 42 and 38 at pH 7.5 and 9.3, respectively.

It is evident from the elemental analysis that the cuticular biopolymers, cutin and cutan, isolated from tomato fruit skins and Agave leaves are able to incorporate nitrogen from ammonium chloride into the aliphatic structure of the biopolymer. At pH 9.3, all of the samples utilized within this study incorporated significant nitrogen except for the Agave R0 fraction which may be unreactive due to the presence of lipids. At pH levels below 9.3, the samples do not incorporate significant nitrogen except for the Agave R3 (cutan) fraction. The cutan biopolymer is chemically resistant and its ability to incorporate nitrogen at pH 7.5 may provide a route for incorporation of nitrogen into a highly recalcitrant aliphatic material in a neutral to slightly alkaline pH environment.

Ammonia is ubiquitous in soil and sedimentary systems and, while many of these settings have pH lower than the pK_a of ammonium (9.24), the persistent nature of these biopolymers provides ample opportunity to react with ammonia. While the elemental analysis provides evidence for nitrogen incorporation within cuticular biopolymers, it does not indicate whether this nitrogen is bound to these biopolymers because non-covalent associations are also possible.

In order to provide evidence of covalent binding of ammonia to cuticular biopolymers and essentially provide information on the speciation of N in our samples, we performed 15N NMR analyses. Due to the low natural abundance of the 15N isotope, we were not able to observe any NMR peaks within the natural biopolymers prior to reaction with ammonium chloride (spectra not shown). We attribute all 15N NMR signals
observed within the spectra of the reacted biopolymers to the $^{15}$N labeled ammonium chloride ($^{15}$NH$_4$Cl) reagent influencing the incorporation of N into the biopolymer. Ammonia is expected to have a chemical shift of ~0.0 ppm while any residual ammonium ion shows a chemical shift of ~22 ppm. If labeled ammonia has bound covalently to the biopolymers, we would observe $^{15}$N NMR peaks downfield of 22 ppm.

Due to logistical constraints combined with sample availability, we were only able to obtain a CPMAS $^{15}$N NMR spectrum of tomato R0 after reaction with $^{15}$NH$_4$Cl at pH 9.3 (Figure 5.1). The elemental data discussed above show significant (1.82%) incorporation of N in this sample, and the spectrum clearly displays several distinct peaks. Integration of peak areas was conducted to indicate the relative abundance of the various nitrogen species and the results are displayed in Table 5.3. A broad, poorly defined peak appears between -10 and 10 ppm and this is attributed to residual ammonia. A second well defined peak is observed within the spectrum at 22 ppm and is assigned to residual ammonium ion. The entire spectral range from -20 ppm through 26 ppm is assigned to inorganic nitrogen non-covalently associated with the biopolymers and comprises about 18% of the CPMAS $^{15}$N NMR peak area. These two peaks provide evidence that inorganic nitrogen is not completely rinsed away from the biopolymers after reaction with ammonium chloride.
Figure 5.1: Full view of the CPMAS $^{15}$N NMR spectrum of tomato (*Lycopersicon esculentum*) fruit skin (tomato R0) after reaction with isotopically labeled ammonium chloride ($^{15}$NH$_4$Cl). Nitrogen chemical shifts referenced to ammonia (0 ppm).

<table>
<thead>
<tr>
<th>Chemical Shift (ppm)</th>
<th>Inorganic</th>
<th>Amine</th>
<th>Amide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato fraction R0</td>
<td>(-20)-26</td>
<td>26-50</td>
<td>92-140</td>
</tr>
<tr>
<td>reacted with $^{15}$NH$_4$Cl at pH = 9.3</td>
<td>18.2</td>
<td>22.3</td>
<td>60.5</td>
</tr>
</tbody>
</table>

Table 5.3: Percentage distribution of nitrogen functional groups as determined by integration of peak areas within the CPMAS $^{15}$N NMR spectrum of tomato (*Lycopersicon esculentum*) fruit skin (tomato R0) after reaction with isotopically labeled ammonium chloride ($^{15}$NH$_4$Cl).
The large well defined peak at 28-50 ppm is assigned to amines and comprises 22.3% of the peak area displayed within the CPMAS $^{15}\text{N}$ NMR spectrum of reacted tomato R0 (Figure 5.1, Table 5.3). Alkyl primary amines have a chemical shift of ~31 ppm while alkyl secondary amines have a chemical shift of ~40 ppm (ACD, 2006). The broadness of this peak indicates a variety of amines and suggests contributions from both primary and secondary amines. Amines are likely formed by nucleophilic substitution reactions occurring at carbonyls (aldehydes and ketones) or at epoxides. All of these functional groups are known to exist within cutin (Chapter 4; Deshmukh et al., 2003; 2005). Secondary amines generated by reaction with ammonia indicate two nucleophilic substitutions involving the same nitrogen atom. The presence of secondary amines provides evidence of newly formed nitrogen cross-linkages as previously shown (Amrani et al., 2007). These types of cross-linkages are believed to create more recalcitrant compounds and may be an involved in creation of persistent organic nitrogen found within SOM and $\text{S}_{\text{DOM}}$ (Nommik and Vahtras, 1982; Amrani et al., 2007).

The dominant peak within the CPMAS $^{15}\text{N}$ NMR spectrum of tomato R0 after reaction with $^{15}\text{N}$ labeled ammonia is assigned to amide N and comprises 60.5% of the integrated peak area (Figure 5.1, Table 5.3). This functional group could be generated by reaction of ammonia with esters or carboxylic acids known to exist within cutin. Reaction of ammonia with carboxylic acids is unlikely because a proton transfer from acid groups to ammonia is known to transform these groups into a salt comprised of carboxylate and ammonium ions. A more likely reaction involves esters being converted to amines by a process known as ammonolysis whereby ammonia initiates a nucleophilic substitution reaction on ester carboxyls. Cutin is a biopolyester and contains an abundance of ester
functionality. Another interesting feature (Figure 5.1) is that no peaks with $^{15}$N chemical shifts greater than 150 ppm are observed. If any imine, nitrile or oxygen bound nitrogen species (i.e. nitrite, nitrate) were present in the cutin after reaction, they would resonate in this region. If any of these compounds are present, their concentration is too low to be detected by the CPMAS $^{15}$N NMR technique.

Two-dimensional HRMAS $^1$H-$^{15}$N heteronuclear single quantum coherence (HSQC) NMR spectroscopy is also used to provide evidence for new covalent bonds formed between an isotopically enriched $^{15}$N ammonium chloride ($^{15}$NH$_4$Cl) reagent and cutin isolated from tomato fruit skins (tomato R2). In this experiment we focused on the cutin alone rather than the tomato R0 isolate as was done above. The two-dimensional HRMAS $^1$H-$^{15}$N HSQC NMR spectroscopy technique shows protons directly bonded to nitrogen as cross-peaks and the chemical shifts observed reflect the nature of the covalent bonds adjacent to the $^1$H-$^{15}$N units. As was the case with CPMAS $^{15}$N NMR, the HRMAS $^1$H-$^{15}$N HSQC NMR spectra of unreacted cuticular biopolymers did not show any peaks above the noise (spectra not shown). Once again, although the elemental analysis shows a small percentage of nitrogen (<1.0%), the low natural abundance of the $^{15}$N isotope precludes observation of any NMR peaks within the cuticular biopolymers before reaction.

Our rationale for reacting ammonia with the cutin biopolymer was that it has been shown to contain electrophilic sites such as aldehydes, ketones, epoxides, and esters (Chapter 4; Deshmukh et al., 2003; 2005), all of which are expected to yield either amine functionality, or amides, as would be the case with esters. No amine signals are detected within the HRMAS $^1$H-$^{15}$N HSQC NMR spectrum (Figures 5.2 and 5.3) of tomato fruit
Figure 5.2: Full spectral view of the HRMAS $^1$H-$^{15}$N HSQC NMR spectrum of cutin isolated from tomato (Lycopersicon esculentum) fruit skin after reaction with isotopically labeled ammonium chloride ($^{15}$NH$_4$Cl). Nitrogen chemical shifts referenced to ammonia (0 ppm).
Figure 5.3: Expanded view of the HRMAS $^1$H-$^{15}$N HSQC NMR spectrum of cutin isolated from tomato (*Lycopersicon esculentum*) fruit skin after reaction with isotopically labeled ammonium chloride ($^{15}$NH$_4$Cl). Nitrogen chemical shifts referenced to ammonia (0 ppm).
skin cutin reacted with ammonium chloride. The only cross-peaks observed in this spectrum are clearly in the amide region indicating that the main reaction is with esters to form alkyl amides. Amides are prevalent within soil and sedimentary systems, yet it is unclear what the relative importance is of abiotic reactions in forming amides in these settings (Thorn and Mikita, 2000). The only nitrogen observable by HRMAS $^{15}$N HSQC NMR within this study was added to the reaction mixture in the form of $^{15}$N-labeled ammonium chloride. Unreacted ammonia or ammonium non-covalently associated with the reaction products would have a nitrogen chemical shift of 0.0 and 22 ppm, respectively. No such peaks are observed in the spectrum, indicating that unreacted ammonium or ammonia was mostly removed from this sample during the exhaustive post reaction rinsing procedure.

The HRMAS $^{1}$H-$^{15}$N HSQC NMR spectrum of tomato fruit skin cutin after reaction with $^{15}$N labeled ammonium chloride shows six distinct peaks all in the region assigned to amide functionality (Knicker et al., 1995b; Knicker and Hatcher, 1997; Thorn and Mikita, 2000; Kögel-Knabner, 2000, ACD, 2006). The presence of six distinct peaks indicates a variety of amides whose specific assignments are not possible at this time. They could be derived from amides produced from various ester structural units within cutin (Graça and Pereira, 2000). It is also possible that some of the cross-peaks with equivalent nitrogen chemical shifts and varied proton chemical shifts (Figure 5.3) may be J-coupled and display splittings in the $^{1}$H dimension. It is clear however that the major reaction products are amides and the labeled ammonia likely reacted mainly with esters. Due to the demonstrated presence of strong nucleophiles such as ketones, aldehydes, and epoxides within the cutin structure, we expected to observe the presence of alkyl amines.
It is possible that amines were formed within this reaction, but their low concentration and structural heterogeneity precluded observation by HRMAS $^1$H-$^{15}$N HSQC NMR, at least for this fraction of cuticular biopolymers.

5.4. Conclusions

The elemental analysis and NMR analyses have confirmed that cuticular biopolymers are capable of incorporating ammonia nitrogen. The formation of alkyl amine and alkyl amide structures within cuticular biopolymers during reaction with labeled ammonium chloride ($^{15}$NH$_4$Cl) provides direct evidence for a nitrogen incorporation and possible stabilization pathway which has not been previously described in the literature. Because polymethylenic structures are known to persist in soil and sedimentary environments (Augris et al., 1998; Nierop, 1998; Hu et al., 2000), formation of alkyl nitrogen within polymethylenic and biologically refractory cuticular biopolymers is likely an important process involved in the sequestration of organic nitrogen in SOM and S$_D$OM.

Ammonia is ubiquitous and is a nucleophile which participates in substitution reactions with electrophilic sites within organic compounds. Cuticular biopolymers are highly aliphatic, yet they also contain several types of reactive oxygen functional groups that include esters, ketones, aldehydes and epoxides. Nucleophilic substitution by ammonia at these sites provides an abiotic reaction mechanism capable of forming amides from esters, as well as amines from ketones, aldehydes and epoxides. The NMR data shows formation of both amines (CPMAS only) and amides, while the elemental analysis data display a correlation between pH and nitrogen incorporation. At a pH of
where ammonia is prevalent, significant increases in nitrogen incorporation are observed. Our experimental design involves biopolymer isolation with methanol and chloroform, lyophilization and a sealed anaerobic reaction system with high ammonia concentration and no light. Under these conditions, we believe biotic processes are unlikely and we attribute the observed formation of amines and amides to abiotic nucleophilic substitution of ammonia.

The reaction of ammonia with cuticular biopolymers is one of several abiotic pathways for nitrogen incorporation into SOM and S:\D. Aromatic organic compounds have been shown to covalently bind nitrite, hydroxylamine and peptide nitrogen (Thorn et al., 1992; Thorn and Mikita, 2000; Hsu and Hatcher, 2005; 2006). Furthermore, previous studies have shown ammonia nitrogen to participate in abiotic incorporation within sterilized soils at elevated pH (Johnson et al., 2000; Barrett and Burke, 2002) and these studies proposed that the processes involved phenolic soil components. The data presented herein indicate that polymethylene biopolymers are also good receptors for ammonia. Our findings support the conclusion of Knicker et al. (2001) that alkyl nitrogen compounds are likely a contributor to the unknown fraction of soil organic nitrogen in that ammonia nitrogen incorporation by polymethylene biopolymers provides an explanation for the existence of the long-chain alkyl amines and amides within soils and sediments.

Peptides are chemically labile and the persistence of amide nitrogen in soils and sediments has prompted several investigations. Previous $^{15}$N NMR studies consistently show a dominant (>70%) amide nitrogen component within soil (Knicker et al., 1995b; Knicker and Hatcher, 1997; Thorn and Mikita, 2000; Kögel-Knabner, 2000), while wet
chemical studies consistently show that less than half of the nitrogen in soils is contained within peptidic structures (Sowden et al., 1977). Persistence of peptidic structures does occur within depositional environments and has been attributed to physical encapsulation (Knicker and Hatcher, 1997; Zang et al., 2000; 2001), but sources of persistent non-peptide amides remain unclear. We propose that the amine and amide functional groups observed within $^{15}$N NMR spectra of SOM and S$_2$OM could be partially attributable to the reaction of ammonia with persistent polymethylenic biopolyesters. Because these polymethylenic biopolyesters are thought to resist degradation, it is likely that nitrogen functional groups within their structures would also persist. Further studies will be needed to determine if the alkyl amines and alkyl amides created are recalcitrant components of SOM and S$_2$OM.
CHAPTER 6

SUMMARY OF RESULTS AND DIRECTIONS FOR FUTURE RESEARCH

6.1 Summary of results

This work is comprised of a series of investigations designed to provide detailed structural characterization of polymethylenic cuticular and suberinic plant biopolymers, and to provide insights into the post-depositional transformations which they undergo within soil and sedimentary systems. These polymethylenic biopolymers are a significant source of the persistent organic materials found within soils and sediments. Because these materials persist in soil settings their structures and transformations have a significant impact upon soil processes. We have utilized a variety of advanced analytical techniques with a focus upon solid-state high resolution magic angle spinning (HRMAS) nuclear magnetic resonance (NMR) spectroscopy in order to identify chemical structural features contained within whole plant materials, isolated plant biopolymers, soils and reaction products.

The non-saponifiable suberinic polyester, suberan, has a highly crystalline methylene character and a lack of mid-chain reactive functional groups. These features set this biopolymer apart from the closely related saponifiable suberinic biopolyester, suberin, and provide for chemical resistance. Cross polarization magic angle spinning
(CPMAS) $^{13}$C NMR spectra show a dominant crystalline methylene peak which is not the dominant methylene peak observed within suberin. HRMAS NMR spectroscopic and gas chromatographic mass spectroscopic investigations indicate a lack of mid chain functional groups within suberan. These physical and chemical structural features are different from those observed for suberin and we propose that these variances lead to chemical resistance. We further propose that suberan is a distinct biopolymer that coexists with suberin within River Birch (*Betula nigra*) bark and we provide a structural model for suberan.

In order to investigate the persistence of polymethylenic biopolymers and structures contained therein, we analyze whole bark and leaves from two sites comprised mainly of Canadian Hemlock (*Tsuga Canadensis*) and Yellow Birch (*Betula alleghaniensis*) as well as the underlying A horizon soils. Solid-state HRMAS NMR techniques allow for characterization of functional groups within whole plant materials without any chemical pretreatments, while the soils are acid demineralized to facilitate NMR analysis. By determining the structural features contained within soil organic matter (SOM) and comparing these features to those of whole plant materials, we are able to identify recalcitrant structures within the plant materials. Structures previously identified within polymethylenic cuticular and suberinic biopolymers are also compared to the structures contained within the soils and plant materials. We conclude that bark and leaves substantially contribute to SOM and many structures originate from cuticular and suberinic biopolymers. While the polymethylenic structures persist, the ester cross-linkages contained within these biopolymers partially depolymerize.
The cuticular biopolymer cutin is believed to persist within soils and sediments and is likely transformed upon deposition. We utilize a variety of NMR techniques to show the presence of highly reactive aldehyde functional groups within the cutin structure. We propose that this functional group is a likely site for post-depositional transformations leading to the complex aliphatic structures found within SOM and sedimentary organic matter (SdOM). Sulfur and nitrogen incorporate into SOM and SdOM during diagenetic transformations and nucleophilic substitutions at reactive oxygen functional groups is proposed as a mechanism of incorporation.

We show that ammonia nitrogen can incorporate into cuticular biopolymers forming organic nitrogen structures. By reacting isotopically labeled ammonium chloride (\(^{15}\)NH\(_4\)Cl) with cuticular biopolymers at a variety of environmentally relevant pH levels we simulate reactions which are likely to occur within soils and sediments. Elemental analysis shows that a significant amount of nitrogen is becoming associated with the cuticular biopolymers while \(^{15}\)N NMR spectroscopy provides evidence of covalent binding, generating amine and amide structures. Persistent soil organic nitrogen exists predominantly as amide structures and the sources of amide soil nitrogen are not well understood. Peptides contain amides and have been proposed as a source of these soil structures yet peptides are thought to be labile, leading to questions about how amides persist. We propose that the dominant amide signal consistently observed within SOM and SdOM \(^{15}\)N NMR spectra is partially due to polymethylenic plant biopolymers which have reacted abiotically with ammonia. Both ammonia and polymethylenic biopolymers are ubiquitous within soils and sediments. The recalcitrant nature of polymethylenic biopolymers provides a viable precursor for recalcitrant organic nitrogen structures.
including the poorly understood recalcitrant amides. We propose that the persistent organic amide nitrogen in soils and sediments is partially attributable the ammonolysis of esters contained within cuticular biopolymers.

### 6.2 Directions for future research

In this work we have provided evidence of structural features within cuticular and suberinic biopolymers which likely have a significant impact on their fate within soils and sediments. We believe that the highly crystalline polymethylenic domains contained within suberan lead to an increased chemical stability imparting recalcitrance within soils and sediments. We isolated suberan from River Birch bark, and Birch bark is the only source where this biopolymer has been previously identified. This leads to the question “If recalcitrant suberan is not found within a wide variety of plant materials, is it a major source of old SOM and S\textsubscript{p}OM?” Future investigations of plant materials, especially thin flat barks are needed to identify additional sources of suberan.

The most significant finding within this work involves abiotic ammonia nitrogen incorporation by polymethylenic plant biopolymers. We propose that ammonolysis of esters is a process whereby recalcitrant organic amide structures are being formed within polymethylenic domains. Ammonia incorporation by polymethylenic biopolymers has not been previously shown and leads to a series of interesting questions to be explored by future investigations. Laboratory simulations involving ammonia and model ester compounds followed by characterization of reaction products could elucidate more clearly the types of organic nitrogen structures being formed. If cuticular polymers are forming amides and amines via ammonia nucleophilic substitution, then it is possible that
suberinic polyesters participate in a similar reaction. Also, if amines are formed by ammonia reaction with cuticular biopolymers, then do these amines further react to form secondary or tertiary amines. If so, then amines formed in this way could provide a mechanism for formation of cross-linkages between polymethylenic structures. Cross-linkages of this type provide a potential stabilizing process leading to creation of persistent organic structures.

If nitrogen nucleophiles are interacting with polymethylenic biopolymers to form recalcitrant nitrogen structures, then do sulfur nucleophiles participate in a similar process? Sulfur incorporates into sedimentary organic matter and the mechanisms of incorporation are unclear. Reduced sulfur species found within sediments are strong nucleophiles and may incorporate into polymethylenic biopolymers thereby leading to recalcitrant organic sulfur structures. Sulfur NMR techniques are not well developed and may not be a viable technique for analysis of sulfurized polymethylenic reaction products. Carbon and proton NMR techniques also have limitations associated with detection of sulfur within these products. The chemical shifts associated with sulfur functional groups often overlap with those of oxygen functional groups and sensitivity limitations preclude observation of a small amount of sulfur incorporation. Derivatization techniques for sulfur-carbon bonds are well developed and utilizing chemical treatments such as methyl lithium / methyl iodide, could facilitate GCMS analyses of these reaction products. Use of GC separation coupled to sulfur specific flame photometric detection could facilitate identification of chromatographic peaks containing sulfur and assist in interpretation of GCMS data.

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Uncovering the mechanisms of nitrogen and sulfur incorporation into organic matter as well as those leading to formation of persistent organic structures within soils and sediments is critical to understanding global processes at a molecular level. The processes associated with nutrient cycling, contaminant cycling, and fossil fuel formation are all greatly affected by stable soil and sedimentary organic carbon, especially when these structures involve sulfur and nitrogen.
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