

# The Identification of Plant Derived Structures in Humic Materials Using Three-Dimensional NMR Spectroscopy

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Here we demonstrate the application of 3-D NMR spectroscopy to structural studies of humic substances, the most abundant of organic compounds on earth. The increased spectral dispersion provided by the additional dimension is proven to be highly advantageous in separating the overlapping signals observed in 2-D spectra. Assignments of the major aliphatic structures and selected aromatic moieties are given as examples. We find that in a forest soil fulvic acid the major aliphatic materials are likely derived from leaf cuticles and further demonstrate that lignin signatures can be identified among the aromatic species. Once identified from the 3-D spectra, these structures can be assigned using the partial information available in 2-D, and in some cases, in the 1-D spectra. These signals are demonstrated to be characteristic to given samples of natural organic matter, and the case is made for their use as indicators of terrestrial biomarkers in mixtures of compounds with unknown origins.

## Introduction

Humic substances (HS) are formed by the chemical and biological transformations of plant and animal matter and are widely distributed in soils, waters, and sediments. These organic materials form major sources and sinks in global biogeochemical cycles (1) and can immobilize anthropogenic organic chemicals and heavy metals (2, 3); retain and release water and plant nutrients (4); and have potential uses in medicines (5). Knowledge of their molecular structures is essential to an adequate understanding of their varied roles in such environmental processes. Numerous wet chemical and spectroscopic methods have been applied in their study over the years, but as yet no single method or combination of methods has yielded a comprehensive structural framework of these materials. A number of recent multidimensional, solution-state NMR techniques have shown great potential in the study of humics. Buddrus et al. (6) first applied a 2-D experiment to study humic materials, which has recently been followed by a number of applications (7–17).

Although several structural fragments were identified from these results, the crowded spectra make interpretation of exact structures and conformations very difficult. Therefore, the spectral dispersion provided in a third dimension should prove highly advantageous.

This paper discusses the application of a 3-D HMQC-TOCSY experiment to a fulvic acid (FA) isolated from the surface of a pine forest soil and considers the implications of the results with reference to a range of natural organic materials.

## Experimental Section

Fulvic acid was isolated from the A<sub>h</sub> (surface) horizon of a pine forest site in the Harvard Forest at Petersham, Massachusetts. The FA was obtained by exhaustively extracting soil residue with NaOH at pH 12.6. The residue was that which remained after previous sequential, exhaustive extractions using first 0.1 M sodium pyrophosphate (adjusted to pH 7.0 with HCl) and then 0.1 M sodium pyrophosphate at pH 10.6. The humic acids and FAs were separated by precipitation with HCl, and FAs were then isolated using XAD-8 and XAD-4 resins in tandem as described elsewhere (18). The FA studied here represents ~5 wt % of the total extractable organic material. The whole soil used in <sup>1</sup>H-high resolution-magic angle spinning (HR-MAS) spectroscopic experiments was collected from the A<sub>h</sub> horizon of an oak forest soil located in Uragh Wood at Lough Inchiquin, Kenmare County, Kerry, Ireland. Prior to HR-MAS the soil was air-dried and passed through a 1-mm sieve. In addition, HS from the oak forest soil (Co. Kerry, Ireland) and from the surface (0–10 cm) of a marine sediment from Bayou Grande, Pensacola, Florida, were isolated for solution-state NMR studies. These were obtained by extracting soil or sediment with 0.1 M NaOH and filtering the extract (0.2-μm filter), followed by treatment with IR-1200H<sup>+</sup> cation-exchange resin (5 times). The HS were then freeze-dried.

Cutin was isolated from organically grown tomatoes. Tomato fruit (free of pulp and seeds) was suspended in a solution of oxalic acid (0.4% w/v) and ammonium oxalate (1.6% w/v) at 40 °C for 2 days, which loosened the cuticles for separation and collection (19). They were washed, freeze-dried, and ground in liquid nitrogen. The ground cuticles (less than 80 mesh) were successively extracted with chloroform, methanol, and 1:1 chloroform/methanol (v/v) with gentle reflux for 12 h for removal of soluble lipids (19). The extraction was repeated until dried extracts indicated absence of any dissolved material. The extracted residue was washed, freeze-dried, and treated with a 4.5% sodium periodate solution (pH adjusted to 4.1 with acetic acid) for 12 h (20). The filtered residue was resuspended in H<sub>2</sub>O and refluxed for 3 h to remove any polysaccharides associated with the cuticles. The residue, or cutin, was washed and freeze-dried.

Solution-state NMR data were acquired using a Bruker Avance 400 MHz spectrometer fitted with a QNP <sup>1</sup>H, <sup>13</sup>C, <sup>15</sup>N, <sup>31</sup>P probe. Samples (75 mg mL<sup>-1</sup>) were dissolved in DMSO-*d*<sub>6</sub>. One-dimensional <sup>1</sup>H spectra were collected (16 384 data points, 128 scans) and processed with an exponential function with a line broadening of 1 and zero-filled by a factor of 2. Two-dimensional Total Correlation Spectroscopy (TOCSY) (64 scans, TD (F1) 1024, TD (F2) 512) was done using an 80-ms mixing time, with Time-Proportional Phase Incrementation (TPPI). Processing was carried out using a sine-squared function with a phase shift of 90° in both dimensions and a zero-filling factor of 2. Heteronuclear Multiple Quantum Coherence (HMQC) spectra (128 scans, TD (F1) 1024, TD (F2) 512, J1 (<sup>1</sup>H-<sup>13</sup>C) 145 Hz) were acquired using a BIRD

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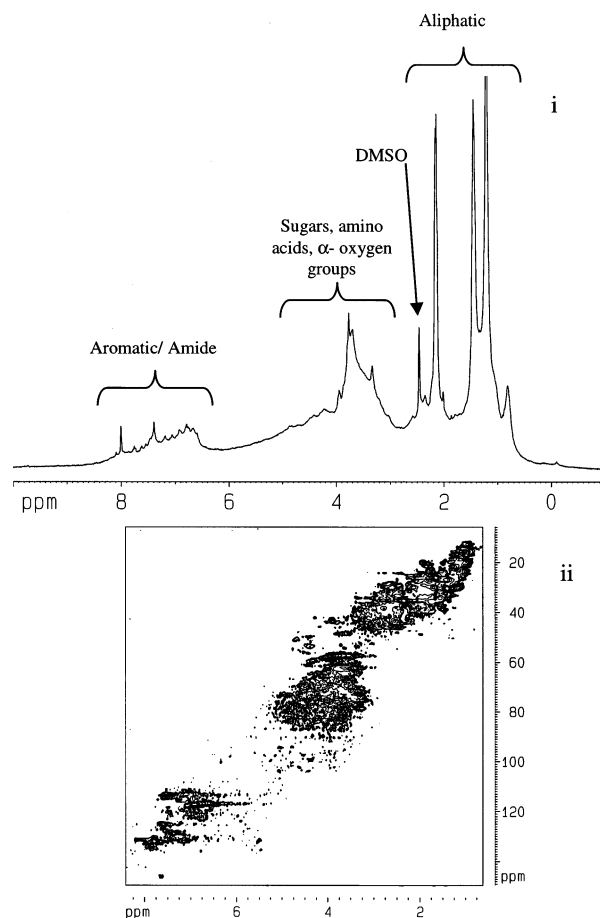


FIGURE 1.  $^1\text{H}$ -spectrum of FA isolated from the surface soil horizon of a pine forest (i). Bottom:  $^1\text{H}$ - $^{13}\text{C}$  HMQC-spectrum of the same sample (ii).

pulse train and TPPI (21). F1 was processed with a sine-squared function with a phase shift of  $90^\circ$ , while F2 was processed with a Gaussian broadening of 0.01 and line broadening of  $-1$ . Three-dimensional HMQC-TOCSY spectra (64 scans, TD (F1) 1024, TD (F2) 128, TD (F3) 128, J1 ( $^1\text{H}$ - $^{13}\text{C}$ ) 145 Hz) were acquired using a BIRD pulse train a mixing time of 80 ms and TPPI in F2 and F1. The 3-D cubic spectrum was processed with sine-squared functions, phase shifts of  $90^\circ$ , and a zero-filling factor of 2 in all dimensions.

The  $^1\text{H}$  HR-MAS NMR spectrum of the whole soil was acquired with a Bruker 600 MHz DMX spectrometer fitted with a 4-mm  $^1\text{H}$ - $^{13}\text{C}$ - $^{15}\text{N}$  HR-MAS probe; 1024 scans (TD 32 768) were collected, and the data set was processed with an exponential multiplication (line broadening of 4 Hz) and a zero-filling factor of 2.

## Results and Discussion

Studies were carried out on a FA, fractionated on the basis of charge density using XAD-8 resin (see Experimental Section). Even after fractionation the resulting mixture is complex, and both the 1- and 2-D NMR spectra display a high degree of signal overlap (see Figure 1). Identification of specific structures from the 1-D  $^1\text{H}$  spectrum is not possible, and although the introduction of a second dimension is certainly very beneficial (6–10, 16, 17), in such studies detailed interpretation from crowded regions of 2-D spectra is still extremely difficult and not always conclusive. Thus, the introduction of a third dimension, which provides additional connectivity or chemical shift information and increased spectral dispersion, is highly desirable. Figure 2 shows the entire 3-D cube from the HMQC-TOCSY experiment; the

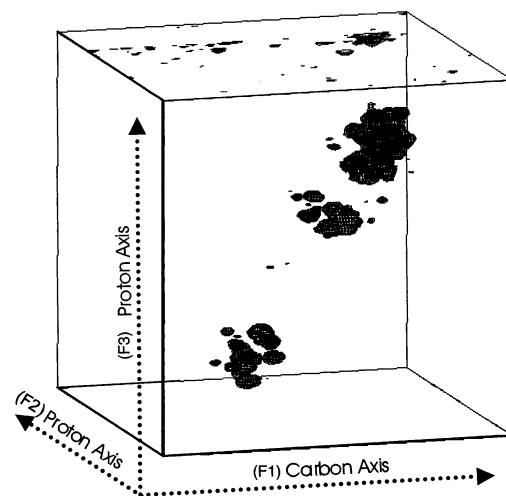


FIGURE 2. A 3-D HMQC-TOCSY spectrum of the pine forest FA. The F2–F3 plane contains TOCSY information.

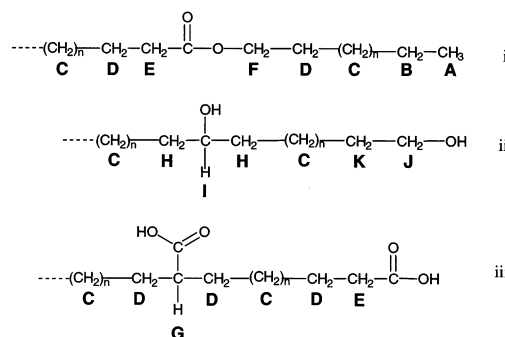
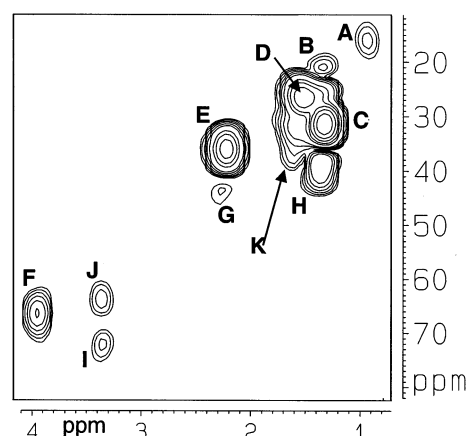


FIGURE 3. Two-dimensional spectrum produced from an F1–F2 slice through the 3-D HMQC-TOCSY spectrum of a pine forest soil FA at 1.3 ppm on the F3 (proton)-axis (Figure 2). Labels on cross-peaks correspond to the C–H structures in aliphatic chains (I–III).

$x$ -axis is assigned to the carbon frequency (F1), while the  $y$ -axis and  $z$ -axis are to proton frequencies (F2 and F3). TOCSY information is contained in the  $y$ – $z$  plane (F2–F3) and HMQC data in the  $x$ – $y$  (F1–F3) plane. The 3-D (F1–F2) plane contains both TOCSY and HMQC information. By taking a slice through the cube, 2-D spectra can be created that contain detailed coupling and chemical shift information for individual resonances. Taking slices through the F1–F2 plane is most productive. To create a slice, a point on the F3-dimension (proton) is selected. In Figure 3 is shown an F1–F2 slice through the largest peak in the proton spectrum, which results from main chain  $\text{CH}_2$  units (9) and occurs at 1.3 ppm (see Figure 1i). The resulting F1–F2 slice produces a spectrum

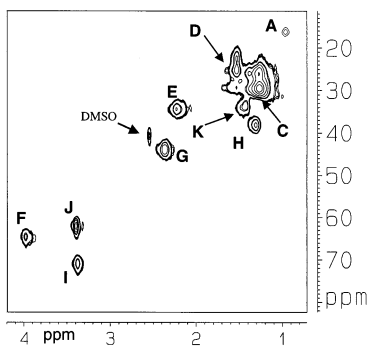


FIGURE 4. An  $^1\text{H}$ -HR-MAS HMQC spectrum of cutin from a tomato plant. For structural assignments see Figure 3.

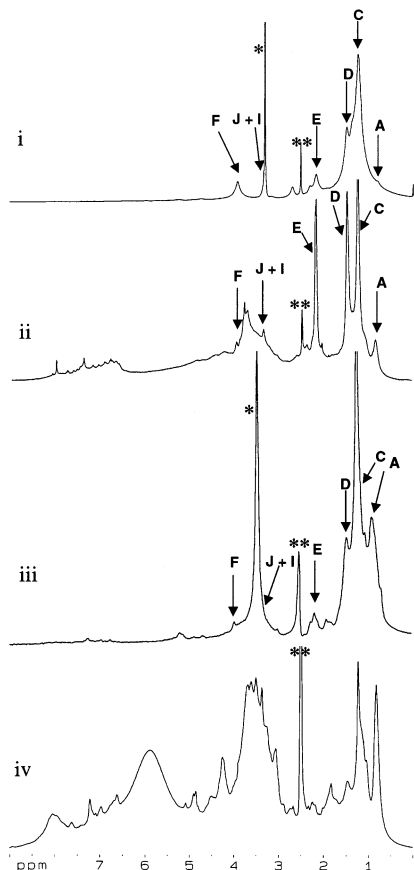


FIGURE 5. One-dimensional  $^1\text{H}$  NMR spectra of natural materials, letters point to resonances from NMR (see Figure 3): (i)  $^1\text{H}$  HR-MAS of a tomato cuticle; (ii)  $^1\text{H}$ -solution state spectrum of a pine soil FA; (iii)  $^1\text{H}$  HR-MAS spectrum of a whole soil from an oak forest (note the top of the methylene peak has been truncated in the diagram, to allow easier comparison of smaller resonances); (iv)  $^1\text{H}$ -solution state spectrum of an organic matter extracted from marine sediment. \* water signal in DMSO, \*\* DMSO solvent.

in which the cross-peaks describe the carbon and proton chemical shifts of the  $\text{CH}_2$  units themselves and all other units with which they couple. Standard interpretations become much easier as the slice contains only information from structures that contain a long aliphatic chain, i.e., there is much less spectral overlap than the 2-D HMQC data for this region (see Figure 11i). By combining chemical shift information, from various standard compounds, with the through-bond connectivity information, it is possible to conclude that the cross-peaks shown in Figure 3, describe 6 structural fragments. These can be summarized as aliphatic chains substituted with, mid chain carboxylic acids, mid chain

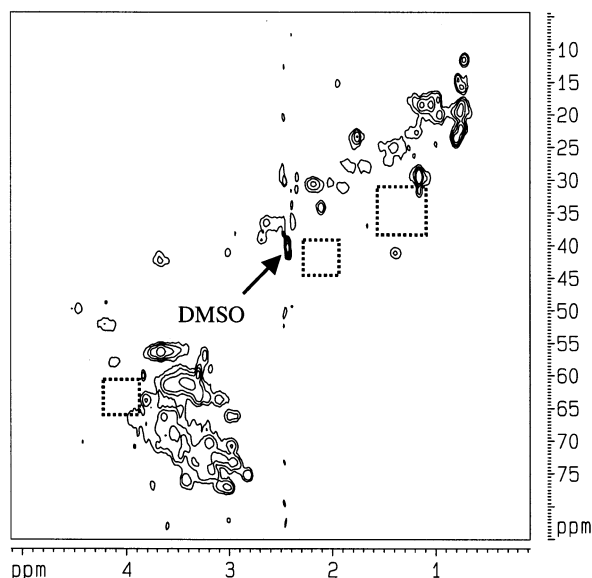


FIGURE 6. HMQC spectrum of a marine sediment extract from Pensacola, Florida. If cuticular components were dominant in the sample peaks, they would be expected to appear in the regions highlighted with boxes. The absence of these cross-peaks indicates the sample is not rich in cuticular material, and the aliphatic resonances result from other compounds, such as amino acids, fatty acids, etc.

hydroxyl groups, mid chain esters, terminal carboxylic acids, terminal hydroxyl groups, and terminal methyl groups. Note: the exact structures shown in Figure 3 are only as examples which highlight the six functionalities present. In reality the units will likely occur in different combinations and can generally be categorized as "esterified aliphatic chains which are substituted with carboxyl and hydroxyl functionalities". Such esterified structures indeed form a major biopolymer, cutin, which in plant leaves forms the water-repellant protective barrier known as the cuticle. NMR studies with cuticles from different sources indicate a basic generic structure for this biopolymer.

The HR-MAS HSQC spectrum of tomato cuticle is shown in Figure 4 for comparison. The close similarities between the aliphatic components in soil organic matter and the cuticular material in this and other studies (22, 23) are clear. All the structural components found in the cuticle can be seen to be present in the soil organic matter, and additional structural moieties are not present in any abundance. These similarities strongly suggest that the aliphatic structures in the soil organic matter are derived from cuticular materials. Considering the recalcitrant nature of this material, and its abundance in the forest canopy, it is not surprising that soil organic matter is enriched in cuticular materials. Indeed previous studies of forest soils have observed the accumulation of cuticular derived materials in the soil biota (24–26). It is interesting to note that a ratio of the main-chain methylene resonance (at 1.3 ppm) to other cuticular peaks is less in the soil organic matter extract than in the plant biopolymer itself. The relative abundance of terminal methyl and carboxyl groups in the soil organic matter can be explained by considering the selectivity of the soil extractant employed. The alkaline aqueous extract used to isolate FA will preferentially solubilize the shorter and more highly functionalized chains and leave behind the larger molecular weight plant material, which by its nature is very hydrophobic (it is the covering that protects plant leaves from water). These smaller more hydrophilic aliphatic components are likely to be produced in two ways: 1) primarily from the microbial and chemical breakdown of the plant cuticle in

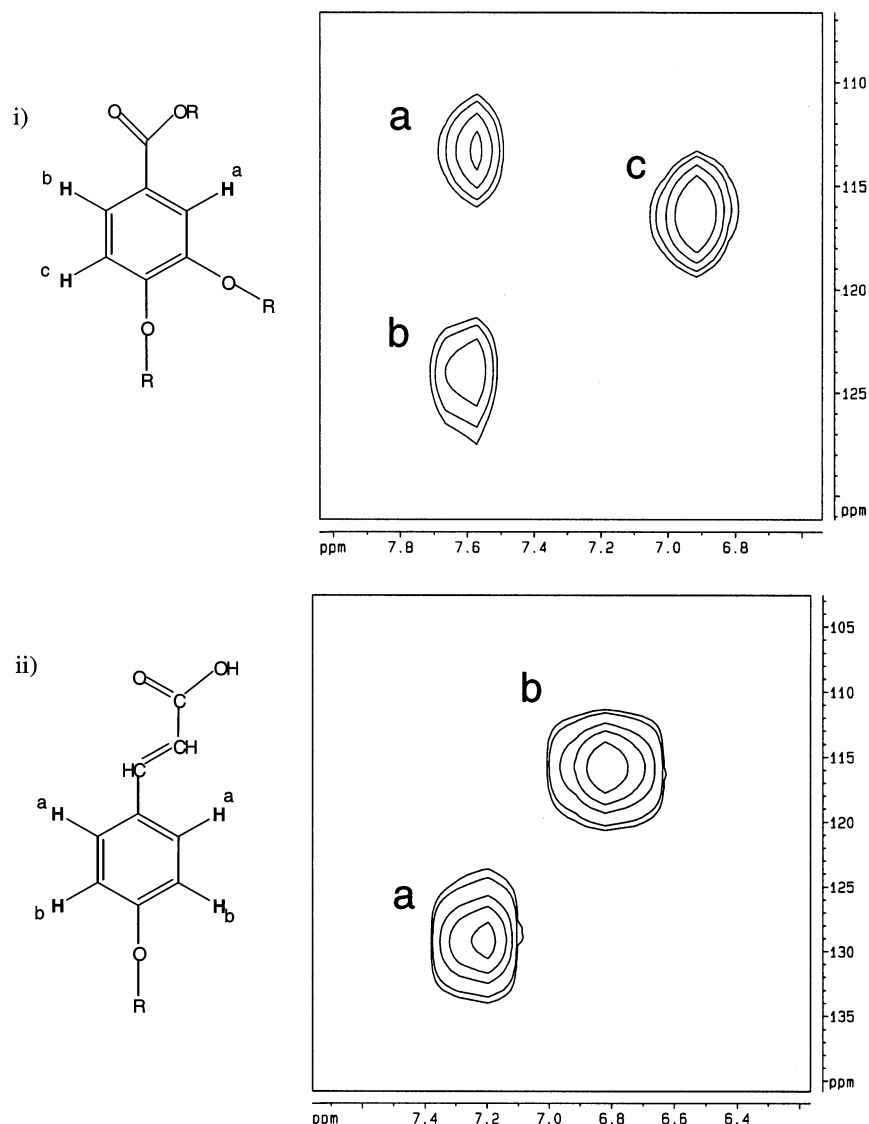


FIGURE 7. Assignment of pine forest soil FA aromatic structures from the F1–F2  $^1\text{H}$  slice at 6.9 ppm of the 3-D HMQC-TOCSY spectrum in Figure 2.

the natural environment and 2) from the cleavage of ester linkages brought about by the use of 0.1 M NaOH, which has become the standard solvent for the extraction of soil organic matter (27–32). It is unlikely, however, that the sodium hydroxide is responsible for breakdown and subsequent solubilization of all these aliphatic components. The abundance of intact esters in the NaOH extract is clear evidence that the extraction does not cleave all these functionalities. Furthermore, similar cuticular signals are seen to be prominent in the 1-D and 2-D spectra of isolates that have been extracted under mild conditions using pyrophosphate or water (33). This suggests, therefore, that a large majority of the extractable cuticular components have been released by chemical or microbial oxidation in the forest soil environment.

After specific signals have been assigned from the 3-D data, it is possible to identify the same cross-peaks in the 2-D HMQC data with greater confidence. In samples with sufficiently resolved signals, it is even possible to identify the resonances in the 1-D spectrum (see Figure 5). Consequently, by using 3-D NMR to analyze structures and their origins, previously unidentified signals in the 2-D and 1-D NMR can then be used as indicators of specific biomarkers in samples where the constituents have mixed origins, such as ocean sediments. Data in Figure 5 demonstrate that, once identified

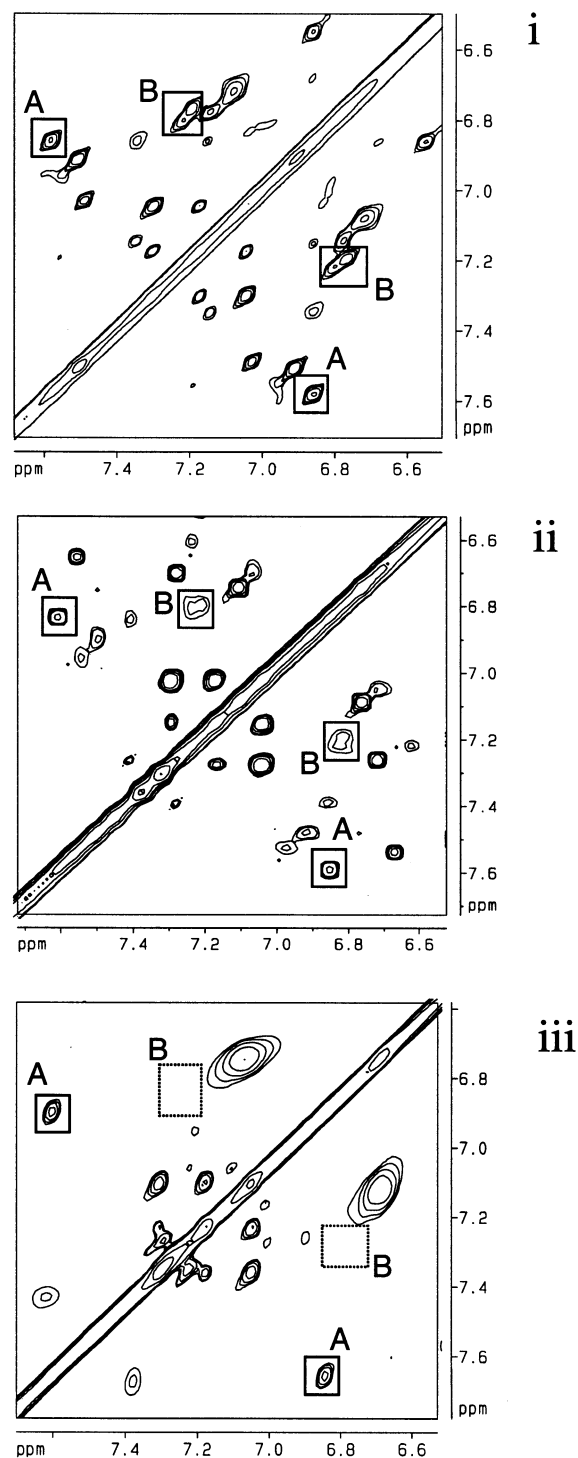
in 3-D, the signals from cuticular-derived material can be seen in the 1-D spectra of various natural organic materials. Comparison of the  $^1\text{H}$ -spectra from the pine forest FA (Figure 5ii) and oak forest whole soil using HR-MAS (Figure 5iii) shows similar aliphatic structures, which are further confirmed by 2-D HR-MAS NMR data for the whole soil sample (34). It is important to note that HR-MAS NMR will only detect the swellable constituents of a sample (34–37). DMSO is a highly penetrating solvent that can break hydrogen bonds; when employed as a solvent the soil became a gel, and thus under these circumstances we believe the vast majority of soil organic matter (which will swell to some extent) will be NMR visible. However this said, true solid domains persist after the addition of DMSO; these will be underestimated in the whole soil HR-MAS data. Although the  $^1\text{H}$ -spectra from the pine forest FA (Figure 5ii) and oak forest whole soil using HR-MAS (Figure 5iii) shows similar aliphatic structures, the ratios of the peaks show variations. The whole-soil spectrum displays a more intense peak at 1.3 ppm (representative of main chain  $\text{CH}_2$  groups), which suggests the abundance of long and relatively unfunctionalized chains, whereas the extract has less contribution from mid chain methylene, indicating that it contains shorter and more highly functionalized chains. This observation is consistent with the selective nature of the extraction and suggests that intact



cuticle materials are abundant in the soil but are not extracted by NaOH.

Figure 5iv shows the solution-state  $^1\text{H}$  NMR spectrum of a organic extract from a marine sediment sampled from Pensacola Bay in Florida. Such a sample has special significance as the Mississippi River drains ~60% of the North American land mass and contributes vast quantities of terrestrial carbon into the Gulf of Mexico. From the 1-D spectrum alone it is not clear whether cuticular signals can be detected or not. Although a peak at ~1.3 ppm is visible, suggesting a contribution from methylene, it is not possible to determine that these aliphatic signals are of cuticular origin. Thus, in this case, it is necessary to perform 2-D NMR experiments. In the 2-D HMQC spectrum it is clear that numerous signals characteristic of cuticle are missing (Figure 6). The implication is that either cuticular derived materials are not a significant input into the environment at this location or that the materials have been drastically transformed so that they are no longer recognizable as of cuticular origin. Aliphatic residues in these near coastal sediments are probably derived from other sources such as algae and microbial products, which may include fatty acids, alkanes, and peptides. However, it is important to note that this organic matter may be the result of a wide range of inputs. Some components, such as algaenan (a major structural component of some aquatic algae), may also contain aliphatic esters, that could, if due care is not taken, be mistaken for cuticle. Thus, for unambiguous identification of terrestrial inputs, like cuticles, it is important to consider all of the cross-peaks in the 2-D spectra. Using such an approach, the distinction can be made between structures of algaenan, which are characteristically unsaturated polyaldehydes, with some ester cross-linking (38–40) and cuticles that are predominantly saturated and hydroxylated polyesters (25, 41, 42).

In addition to aliphatic components, lignin, which is a major structural component of most terrestrial plants, is a good biomarker for terrestrial origins (43–46). Using the 3-D spectrum in the same manner as with the aliphatic structures, it is possible to make F1–F2 slices in the aromatic region and to identify aromatic units present in the pine forest FA. Figure 7i shows an example slice taken through the F3 proton axis at 6.9 ppm in the spectrum shown in Figure 2. From the F1–F2 slice we can see that two other protons couple with the proton at 6.9 ppm, thus indicating that the structure is trisubstituted and has three protonated positions on the ring. The carbon and proton chemical shifts of these proton positions can reveal the identity of the entire ring structure. Changes in substitution patterns, and the nature of the substituents themselves, considerably alter the chemical shifts at nonsubstituted positions. Comparison of the known chemical shifts of common lignin components (47) coupled with simulation of expected chemical shifts in various structural fragments indicate that an acid/ester functionality at the 1-position and methoxy/ether/hydroxyl substituents at 3- and 4-positions uniquely match the observed chemical shifts and couplings in the F1–F2 slice (see Figure 7i). This substitution pattern is very common to components in lignin, which is found in abundance in the forest soil organic matter. Another example of a lignin aromatic structure is given in Figure 7ii. Slicing through the proton axis at 6.8 ppm of the 3-D spectrum (Figure 2) yields an F1–F2 slice consistent with cinnamic acid/ester structures with a methoxy ether in the 4-position. Such cinnamyl structures are reported to be highly abundant in nonwoody sources (48) and therefore are likely derived from the forest floor vegetation. As with the aliphatic materials, once the aromatic signals have been identified from the 3-D experiment it is possible to make assignments in 2-D spectra. Due to severe overlap in the aromatic region of the 1-D spectrum and the relatively weak



**FIGURE 8.** TOCSY spectra highlighting the aromatic regions of various samples. Top: pine forest soil FA; Middle: oak forest soil FA; Bottom: marine sediment extract. A indicates 1,3,4-trisubstituted lignin structures, B para-substituted cinnamic moieties.

nature of the individual signals, identification of these signals is not feasible. However, in 2-D the added spectral dispersion and connectivity information make identification possible. Figure 8i shows the aromatic region of a TOCSY spectrum of the pine forest FA on which the identified signals are labeled (see Figure 7). Similar signals can be seen in the nonfractionated extract from an Irish forest soil (Figure 8). These findings are as per expectation, considering the similar nature of the lignin-rich canopies at both sites. In contrast, however, for the coastal sediment extract, the 1,3,4-substituted aro-

matic units can be identified, while signals from the cinammic units are missing (Figure 8iii). The presence of 1,3,4-substituted lignin units is a clear indication of the terrestrial origin of some of the carbon components in the coastal sediments. This is supported by studies employing copper oxide degradation in which numerous lignin biomarkers are identified in marine environments of which 1,3,4-substituted vanillyl structures are found to be the most recalcitrant (49). The absence of the cinnamic structures likely reflects the lability of these units in the environment due to rapid oxidation. This notion is also supported by observations of significant decreases in cinnamic units with respect to other lignin residues with increasing depth in forest profiles (33).

The full interpretation of 3-D experiment results is very time-consuming and difficult. The 2-D and 3-D spectra indicate that, in addition to aromatic and aliphatic structures, complex mixtures of polysaccharides and polypeptides are also in abundance. To ascertain the precise array of structures present in these complex mixtures would not be trivial and would likely require a detailed combination of wet chemical and spectroscopic methods. However, 3-D NMR spectroscopy does provide a powerful tool, that can be practically employed to identify, and indeed search for specific units in an intact mixture. Once identified from the 3-D experiments, many specific compounds will display characteristic resonances that are identifiable in 1-D and 2-D spectra.

The success of the 3-D technique with natural organic matter is highly encouraging. Although the time involved in collecting the data set is considerable, this will become less of an issue as cryogenically cooled probes and gradient amplifiers become commonplace. These technologies will lead to drastic improvement in the sensitivity and help to reduce artifacts, such as "T1 noise", in the experiments. Even without these amenities, we show that 3-D NMR of natural organic matter is feasible using standard hardware and can provide excellent results from which unambiguous interpretation is possible for numerous structures.

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