Anal Bioanal Chem (2004) 378 : 1484–1503
DOI 10.1007/s00216-003-2422-z

REVIEW

**Robert L. Cook**

# Coupling NMR to NOM

Received: 1 August 2003 / Accepted: 13 November 2003 / Published online: 22 January 2004


**Abstract** This work itemizes and critically assesses several 1D and multi-dimensional nuclear magnetic resonance (NMR) techniques, in both the liquid (solvent suppression, APT, DEPT, INEPT, COSY, TOCSY, HSQC, HMQC, HMBC, NOESY, ROESY and others) and solid states (DP, SACP, RAMP-CP, CP-TOSS, MQ-DEPT, 2D $^{1}H$–$^{13}C$ HETCOR and others), which are relevant to the characterization of natural organic matter (NOM). The pros and cons of many of the discussed techniques are compared in an effort to provide guidance to the most beneficial utilization of these NMR instrumental techniques for researchers interested in gaining insight into various aspects of NOM.

**Keywords** Natural organic matter · Humic acid · Fulvic acid · Solid state · Liquid state · Multi-dimensional · Nuclear magnetic resonance

**Abbreviations** *1D* One dimensional · *2D* Two dimensional · *APT* Attached proton test · *BIRD* Bilinear rotation decoupling · *CP* Cross polarization · *COSY* Correlation spectroscopy · *CSA* Chemical shift anisotropy · *DEPT* Distortionless enhancement by polarization transfer · *DMSO* Dimethyl sulfoxide · *DOSY* Diffusion ordered spectroscopy · *DP* Direct polarization · *DQ* Double quantum · *FID* Free induced decay · *FT* Fourier transform · *FT-ICR-MS* Fourier transform-ion cyclotron resonance-mass spectroscopy · *HETCOR* Heteronuclear correlation · *HH* Hartmann–Hahn · *HMBC* Heteronuclear multiple bond correlation · *HMQC* Heteronuclear multiple quantum coherence · *HSQC* Heteronuclear single quantum coherence · *INEPT* Insensitive nuclei enhanced by polarization transfer · *LR-COSY* Long-range COSY · *MAS* Magic-angle spinning · *MQ* Multiple quantum · *MS* Mass spectroscopy · *NMR* Nuclear magnetic resonance · *NOE* Nuclear Overhauser enhancement · *NOESY* Nuclear Overhauser enhanced spectroscopy · *NOM* Natural organic matter · *PASS* Phase adjustment of spinning sidebands · *RAMP* Ramped amplitude · *RESTORE* Restoration of spectra via $T_{CH}$ and $T$ one rho ($T_{1\rho H}$) editing · *r.f.* Radio frequency · *ROESY* Rotating frame Overhauser enhancement spectroscopy · *SACP* Single amplitude cross polarization · *SOM* Soil organic matter · *SS* Spinning sideband · *TMS* Tetramethylsilane · *TOCSY* Total correlation spectroscopy · *TOSS* Total suppression of sidebands · *TPPM* Two-pulse phase modulation · *VCT* Variable contact time · *VSL* Variable spin lock · *WATERGATE* Water suppression by gradient tailored excitation

R. L. Cook (✉)
Department of Chemistry,
Louisiana State University and Southern University,
636 Choppin Hall, Baton Rouge, LA 70803, USA
e-mail: rlcook@lsu.edu

## Introduction

NOM plays a multitude of roles in the environment. These roles include soil fertility, pollutants' fate and transport including bioavailability, nutrient cycling, metal speciation, and carbon and nitrogen cycling, to name a few [1]. However, due to its complex nature, NOM has always posed challenges to analytical chemists. NOM appears to be more challenging to analyze than any biomolecule or family of biomolecules, such as proteins. In addition, there is no basis on which to isolate, separate, or purify all of the components of a NOM sample. In addition, even if one could separate NOM into individual components, one would not be able to model the NOM mixtures as one would have eliminated the properties which emerge via interactions. This reality is countered by the desire to derive molecular level models of NOM for modeling purposes. Thus, the ideal method for the analytical characterization of NOM interrogates the sample as a whole, but also allows one to obtain molecular level information. Although no analytical technique is perfect, as this would require one to monitor the sample without perturbing it at concentrations of environmental relevance, two analytical methods have

emerged as very promising [2]. The first is Fourier transform ion cyclotron resonance mass spectroscopy (FT-ICR-MS) as it allows one to use very small sample amounts and obtain mass data at a high enough resolution to assign exact empirical formulae to NOM samples. The other emerging technique is NMR. The combination of state-of-the-art FT-ICR-MS and NMR holds the promise of greatly advancing our molecular level understanding of NOM. While FT-ICR-MS is not the subject of this work, the reader is encouraged to read a recent review by Kujawinski [3] as well as the most recent papers on the subject by Cooper and co-workers [4, 5, 6], Hatcher and co-workers [2, 7] and others [8, 9, 10].

The focus of this work is the recent advancement in the application of NMR to NOM, discussed from a critical point of view. In putting this review together, a series of literature searches was carried out using both Sci-Finder® and Web of Science®, however, in all probability some work was missed, for which the author apologizes. Also, the number of papers in which NMR has been applied to the characterization of NOM is so vast that a complete survey is well beyond the scope of this review. Thus, only a select number of works will be presented here, with the author again apologizing in advance for any omissions.

There have been a large number of very good reviews written on the applications of NMR in the characterization of NOM [11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21]. One of the most encompassing, insightful, and referenced was written by Caroline Preston in 1996 [18]. However, the field of NMR has advanced greatly since that publication. These advances include new pulse sequences, two dimensional (2D) solid-state spectra, and 2D and 3D liquid-state studies of NOM (although the majority of the studies reviewed here have been on a class of NOM known as humic materials, NOM has been chosen as a universal descriptor). The aim of this review is to critically assess and provide some insight into these recent advances as well as the associated pros and cons of applying them to NOM samples.

## Setting the scene

The recent acceleration in the application of NMR to the study of NOM and the sophistication of the methods being used is derived from the great advances in NMR theory and hardware. The most obvious way in which these advances have helped in the study of NOM is through the introduction of high-field instruments, inverse probes, cryogenic probes, more sensitive solid-state probes, and high speed magic-angle spinning (MAS) probes, on the hardware side, as well as new pulse sequences and techniques such as shaped pulses and high resolution magic-angle spinning NMR. In addition, these advances have freed up NMR time on somewhat less cutting edge NMR equipment meaning that more NMR instrument time is available for NOM characterization and method development. Subsequently, the NMR characterization of NOM has advanced at a breathtaking pace, so much so that references written as little as two years ago are no longer current. In all probability, the same fate will befall this piece of work. As mentioned above, a survey of the literature was carried out from which it was determined that the current "state-of-the-art" in the characterization of NOM by NMR was in need of critical evaluation, especially in the area of solid-state NMR, but also in the area of liquid-state NMR.

This work concentrates on $^{1}H$ and $^{13}C$ NMR, and thus, it mentions $^{15}N$ or $^{31}P$ NMR only in passing. Also, the application of NMR in the study of pollutants and metal ions will not be covered as these areas deserve dedicated reviews.

## Solid-state NMR of NOM

The most used NMR method for the characterization of NOM is solid-state NMR, namely via cross polarization-magic-angle spinning (CP-MAS) $^{13}C$ NMR [18]. Solid-state NMR has many advantages over its liquid-state counterpart which include:

1. no concentration limit,
2. no solvent effects,
3. no concern in regards to heterogeneous sample dissolution,
4. minimal sample handling,
5. the capability of analyzing highly insoluble fractions such as humin and black carbon,
6. the stability of samples in the solid state, and
7. whole soils can be analyzed.

The CP-MAS NMR technique is deceivingly simple [22, 23, 24; the reader is referred to these monographs for more comprehensive explanations than are presented here] and consists of three parts. In the first part, known as cross polarization, the protons' (or, more correctly stated, the abundant spins, *I*) polarization is transferred to the carbons (or, more correctly stated, the rare spins, *S*). In the second part, the sample is physically spun at the magic-angle to the static magnetic field (it should be stated that the sample is spun at this angle for the entire experiment), while the third part involves decoupling the proton spins during acquisition. The cross polarization step can be expressed mathematically as $\gamma^{I}B_{1}{}^{I}=\gamma^{S}B_{1}{}^{S}$, where $\gamma$ is the gyromagnetic ratio of the nuclei and $B_1$ is the applied field. In the case of $^{1}H\rightarrow{}^{13}C$ cross polarization a theoretical enhancement of the $^{13}C$ signal by a factor of four can be achieved, due to the fact that the gyromagnetic ratio of protons is four times that of carbons. In addition, the recycling delay between pulses in the CP experiment is dictated by the spin–lattice relaxation ($T_1$) of the protons rather that the carbons. In extreme cases (e.g. crystalline polyethylene) this can translate into a thousand or more scans being collected in the same amount of time only one scan would take be if one was to excite the carbons with a 90° pulse to obtain a $^{13}C$ spectrum [21]. Thus, CP based techniques give a double benefit in terms of signal enhancement, and it is this increased signal that has made CP based methods standard for the NMR characterization

of NOM. The quest for signal in the characterization of NOM stems from the complex and heterogeneous nature of NOM. Typically, only very little signal arises from any given entity in the sample as such an entity is always very low in concentration. In addition, these entities are further diluted if one is analyzing a soil rather than an isolated fraction of NOM (e.g. humic acid).

MAS is used to alleviate line broadening to a large extent, if not entirely, due to chemical shift anisotropy (CSA), which arises from a non-spherical electron density distribution around the nucleus (this is especially important in $^{13}C$ NMR for aromatic, carboxyl, and alkene moieties) as well as, to some level, line broadening due to dipole–dipole interactions (Zeeman energy levels are shifted slightly by local fields around the nucleus, due to neighboring nuclei). In both cases these effects are removed in liquid-state NMR by the rapid and random tumbling of molecules induced by Brownian motions. In the solid state this rapid and random molecular tumbling does not occur. However, it can be shown that both of the abovementioned effects have a $(3\cos^2\theta-1)$ term in their mathematical descriptions. When this term is equal to zero one obtains the conditions found in the liquid state, that is *if* one can spin the sample at a high enough speed. The "magic" angle that this happens at is $\theta$=54.7°, and thus, the name: magic-angle spinning. Although commercially available probes allow sample spinning speeds ($\omega_r$) in excess of 30 kHz, and samples have been spun at speeds in the vicinity of 50 kHz, the proton pool still needs to be decoupled to remove dipole–dipole interactions to obtain high resolution $^{13}C$ solid-state spectra.

## Quantification and CP-MAS

### CP dynamics

The above discussion presents CP-MAS $^{13}C$ NMR to be a three-part technique, but it is, in fact, a four-part experiment, i.e.:

1. preparation of the protons,
2. polarization transfer,
3. acquisition (proton decoupling), and
4. MAS during the entire experiment.

Compared with most modern NMR experiments this corresponds to a simple pulse sequence, however, the simplicity of the pulse sequence is deceiving. The reason for this is that the dynamics of polarization transfer from protons to carbons is very complex, and can be expressed mathematically (with some assumptions) as follows [25]:

$$I(t) = I_0\left(1 - T_{IS}/T_{1\rho}^{I}\right)^{-1}\left[\exp\left(-t/T_{1\rho}^{I}\right) - \exp\left(-t/T_{IS}\right)\right] \quad (1)$$

where $I(t)$ is the observed intensity with a contact time during the CP process of $t$, $I_0$ is the ideal intensity, $T_{IS}$ is the CP time constant whose reciprocal is the rate at which CP takes place, $T_{1\rho}^{I}$ and $T_{1\rho}^{S}$ are spin–lattice relaxation rates in the rotating frame for the $I$ (abundant spins) and $S$ (rare spins), respectively. Finally, $t$ is the time allowed for the $I$ and $S$ spin pools to be in contact during the CP (only Hartman–Hahn CP is discussed here). One of the assumptions used to obtain the Eq. (1) is that $T_{IS}/T_{1\rho}^{S}\approx 0$. In addition, the efficiency of the cross polarization is related to the number of protons directly bound to the carbons in question or in their close spatial proximity [25], and for a complex sample such as NOM may require an even further expansion to take into account discretely fast and slow components. However, Eq. (1) demonstrates the double exponential behavior of the CP intensity, $I(t)$, versus contact time, $t$. This double exponential nature arises from the fact that it takes a finite amount of time for the polarization to be transferred from the protons to the carbons ($T_{IS}$) and increase the carbon signal, however, during this process protons are also relaxing (due to spin–lattice relaxation in the rotating frame $T_{1\rho}^{I}$). The exact time constants of $T_{IS}$ and $T_{1\rho}^{I}$ depend on the types of carbons and protons involved. If $T_{IS} \ll T_{1\rho}^{I}$, then the CP experiment can yield quantitative or very close to quantitative results. However, if $T_{IS} < T_{1\rho}^{I}$, or worse, $T_{IS} = T_{1\rho}^{I}$ or $T_{IS} > T_{1\rho}^{I}$ then CP experiment will yield non-quantitative results [26, 27, 28, 29].

### MAS and spinning sidebands

In addition to the dynamic concern addressed above, spinning the sample at the magic angle can interfere with the quantitation of a spectrum. To fully understand why this comes about one must first examine what is taking place when a sample is macroscopically spun at high speeds. This macroscopic spinning introduces a time dependence, which also leads to the strength of interactions under MAS being described by terms which oscillate at $\pm\omega_r$ and $\pm 2\omega_r$. For NOM by far the most important oscillating terms are those associated with CSA. These oscillating terms can be viewed as echoes, which arise from a refocusing of the magnetization due to the macroscopic spinning of the sample (a similar phenomenon can be seen in liquid-state NMR when a sample is poorly shimmed and spun, albeit at much lower speeds). When the free induced decay (FID) is transformed from the time domain to the frequency domain these oscillations manifest themselves in the spectrum as a series of spinning sidebands (SS) flanking the central isotropic line at intervals equal to $\omega_r$. For NOM samples SS can underlie unrelated isotropic lines resulting in an intensity distorted spectrum. In theory, if all species and CSA were known then these SS distortions could be eliminated and one would obtain a quantitative spectrum after making the appropriate corrections. However, in practice this is impossible for NOM as its exact molecular make-up is still unknown and sample dependent.

### MAS and the Hartmann–Hahn matching condition

In addition to SS, MAS also affects cross polarization. As a sample is spun at higher and higher speeds the profile of the Hartmann–Hahn (HH) match breaks down into a se-

ries of discrete peaks (fingers) in the frequency domain where the maxima are located at $0\omega_r$, $\pm\omega_r$, and $\pm2\omega_r$. The MAS speed at which the HH matching profile transitions from a continuum to a series of fingers is dictated by the strength of the local heteronuclear (*I–S*) and homonuclear (*I–I*) interactions and will be different for different chemical groups [30, 31]. It is also important to note that, as the sample spinning speed increases, the central ($0\omega_r$) match condition becomes less and less effective and succumbs to the first sideband ($\pm\omega_r$) match condition in terms of both efficiency and rate [31].

## Other considerations in terms of quantification

### Paramagnetic centers

The use of paramagnetic relaxation agents has a long history in the field of chemistry as these agents allow one to obtain a spectrum in a fraction of the time required without them. This time saving arises from the fact that one can perform many more scans in a set amount of time due the more rapid relaxation induced by the relaxing agent. The use of relaxation agents in NMR assumes a homogeneous effect across the whole sample, and such an assumption can be made for simple organic molecules or even a simple mixture of them. Unfortunately, in the field of NOM NMR the naturally occurring paramagnetic centers lead to non-quantitative spectra. This arises from the fact that the paramagnetic centers are not evenly distributed throughout the sample [32, 33, 34] and can lead to signal relaxation before a signal can be collected even in a simple one pulse experiment (vide infra). There are two categories of relaxation inducing centers in NOM. The first and most discussed category consists of metal ions, with iron being the most problematic. The second category contains organically stable radicals within the NOM organic matrix itself. The above issue has been addressed in the literature by Wilson et al. [35] and, more recently, in some very elegant and meticulous work by Smernik and Oades [26, 27, 28, 29, 34] as well as Keeler and Maciel [36]. In all cases, it has been shown by spin counting experiments, that the removal of inorganic paramagnetic centers leads to an increase in the total observable carbons. The best way to remove inorganic paramagnetics from solid samples such as soils and humin is a repeated treatment with a weak aqueous HF (2% *v*/*v*) using three or four cycles [26, 36]. However, questions have been raised in terms of possible chemical side reactions taking place within the NOM during the HF treatment. For soluble fractions cation exchange resins can be used, however, one should be concerned about losing a fraction of the sample to the column. At any rate, further investigation is needed to determine the importance of such side reactions or losses for the environmentally relevant chemistry of NOM. At the moment there is no simple solution to the problem of signal loss due to the organic radicals. Moreover, it was recently shown [36] that the removal of Fe can lead to the formation of organic radicals which is, in all probability, driven by the REDOX chemistry taking place within the NOM sample.

Paramagnetic ions have a much greater effect on CP based experiments than on single pulse experiments. This arises from the fact that they can reduce the $T_{1\rho}{}^I$ to such an extent that the $T_{1\rho}{}^I \leq T_{IS}$, thus cross polarization from the protons to the carbons does not take place since the protons will have relaxed before such a polarization transfer can take place.

### Background signals

Finally, background carbon signal from the end cap of the rotor, the probe's strator, and other probe components have been shown to be present in both DP and CP experiments [37, 38, 39, 40]. However, it has been shown that this background signal can be eliminated or subtracted and is only of great concern if DP experiments are being used and/or if the sample has a very low carbon content. Nevertheless, this effect should be checked for as its magnitude depends on the materials the probe is constructed from.

## How to acquire as quantitative solid-state $^{13}$C NMR spectra of NOM as possible (the same holds for any X nucleus)?

From the above discussion one would be led to believe that quantitation of all carbons within a NOM sample is an unachievable goal, and in all probability this is a correct statement. However, for those wishing to study NOM the information that NMR can provide is indispensable. Thus, there has been a great amount of effort made into obtaining as quantitative NMR spectra of NOM as possible. The major strategies put forward in the recent literature (since 1995) will be discussed below, both in terms of theory and applicability.

### The direct polarization (or Bloch decay or single excitation) method

If one can remove inorganic paramagnetic centers, and the concentration of organic paramagnetic centers (as can be determined by electron pair resonance otherwise known as electron spin resonance) is very small, then, in theory, one should be able to obtain a quantitative spectrum of the sample being analyzed under the appropriate conditions. These appropriate conditions are:

1. no SS interferences, and
2. the sample acquires full relaxation between excitation pulses.

Both of these conditions can be achieved. The elimination of SS interference can be accomplished either by using a low-field (2.3 to 4.6 Tesla) spectrometer or by spinning the sample at high speeds. The low-field approach is de-

rived from the fact that SS arise from CSA effects and these effects scale with the applied magnetic field. On the other hand, if one is not in the fortunate position of having a low-field NMR spectrometer, as is very often the case these days, then one must look for alternative methods of eliminating SS interferences. By far the most fundamental approach is to spin the sample at a higher spinning speed. In theory, the faster the better. The reason for this is that the faster the sample is spun the further away from the central isotropic line the SS move, to the point where they are out of the spectrum (the frequency window of interest). In addition to this benefit, the faster one spins the sample the more signal there is in the central isotropic line. The exact speed will depend on the rotor size and material as well as the size of the applied magnetic field. Thus, at first thought, from a simple NMR point of view, it would appear that one would wish to run the sample on the highest available field at a high enough spinning speed to eliminate SS interferences and take full advantage of the higher field in terms of the signal to noise ratio (*S*/*N*). However, this line of though is misleading as is does not consider the amount of sample one is analyzing. When this variable is taken into consideration the low-field option becomes very enticing. The reason for this reversal is that in order to spin a macroscopic sample at very high speeds one must place a great amount of stress on the vessel carrying the sample (rotor). One way of getting around the stress factor is to decrease the rotor's size, especially in terms of its radial dimension. In doing so one decreases the rotor's volume and hence the amount of sample it can hold. This can be illustrated by considering 14.00 mm, 7.00 mm, 4.00 mm, and 2.5 mm diameter rotors and assuming that all are 14.0 mm in length and that the diameter is the inner diameter (while, in reality, it is their outer diameter). Respectively, their volumes would be 2.16, 0.54, 0.18, and 0.07 mL, and if we consider that volume equals the amount of sample held, one quickly sees that there are advantages to large rotors. In reality for a full calculation one must consider the effect of field on *S*/*N* as well as fill factors and real rotor volume. When all of these factors are summed up the large rotor/small field route may have an advantage. However, presently and in the future low-field instrumentation may not be available to the large majority of scientists who wish to study NOM.

The relaxation condition requires that the $T_1$s for all the carbon types within the sample be measured, and that the delay between excitation pulses (or acquisitions) be set to five times the longest $T_1$. This can lead to cycle delays of a minute and a half (unless corrections are applied which, in turn, requires the time-consuming measurement of all the $T_1$s in the sample). If one considers that it can take thousands of scans to acquire a spectrum with sufficiently high *S*/*N*, due to the time averaging nature of NMR (*S*/*N*=[number of scans collected]$^{1/2}$, thus if one collects a spectrum with 128 scans and desires a twofold increase in *S*/*N* one must acquire a spectrum with 512 scans; similarly, if an increase in *S*/*N* by a factor of ten is desired then one needs to collect 12,800 scans) then the time required to collect a quantitative spectrum by DP-MAS can be very uneconomical. Thus, while the DP-MAS method is probably the most quantitative, it may not be the most appropriate method depending on the research group's circumstances.

## Quantitative CP methods

### CP/$T_1$-TOSS

This very elegant technique put forward first by Hu and Schmidt-Rohr [41] in the characterization of polymers and then applied to NOM by the same group along with other co-workers [42] addresses the quantification issues for DP-MAS data that have been acquired without full relaxation between acquisitions.

The technique can be rather time-consuming, as it requires one to obtain three spectra: one DP-MAS spectrum with too short a recycling delay and two CP/$T_1$-TOSS spectra with different delays between the CP and TOSS pulse sequences (in reality there are two additional 90° pulses between which this delay is sandwiched). The first spectrum is obtained with a very short delay between the CP and TOSS pulses on the order of 500 µs. The second spectrum is obtained with a delay that is equal to the recycling delay (delay between acquisitions) in the non-quantitative DP-MAS spectrum, typically in the order of 5 s. The relaxation dynamics are then analyzed and a correction factor is arrived at to correct the non-quantitative DP-MAS spectrum. However, this correction factor is flawed by the fact that is assumes a homogeneous $T_1$, which is definitely not the case for NOM samples. The longer this long delay is the closer the obtained DP-MAS spectrum will be to the quantitative DP-MAS spectrum and the smaller the correction as well as errors within the correction factor. It was found that a long enough delay had to be used so that the resulting second CP/$T_1$-TOSS spectrum was less than half the intensity of the original CP-TOSS spectrum. In some cases this required a delay longer than 25 s. If this half intensity condition was not reached then "large corrections errors" occurred. This suggests that the dynamics of every sample must be investigated which is a rather time-consuming proposition. In addition, this technique suffers from quantitative issues in regards to TOSS and CP discussed here. However, when applied correctly to NOM this technique has produced results that are in reasonable agreement with the chemical elemental analysis in an appreciably shorter time.

### RESTORE

The spin counting technique has been known for over two decades and was applied by Wilson et al. [35] early on in the study of humic materials by CP-MAS, however, it seems to have fallen out of favor. Recently Smernik and Oades along with co-workers [26, 27, 28, 29, 34] have done a series of very elegant experiments using spin counting to determine and quantify just how non-quantitative

CP-MAS methods are, especially when applied to highly complex and challenging soil samples. A most elegant use of spin counting has recently been presented by Keeler and Maciel [36] in which a whole soil and the classic fractions of SOM, namely humin, humic acid, and fulvic acid were characterized, and the spin counting technique was doubly calibrated to take into account the radial and longitudinal non-uniformities within a CP-MAS probe. The results from this study are in line with those found by Smernik and Oades along with co-workers [26, 27, 28, 29, 34]. However, Smernick and Oades [29] have developed a method known as RESTORE in which one can combine three CP-MAS spectra acquired under different conditions to yield, in theory, a quantitative spectrum. The first spectrum is a standard CP-MAS spectrum, followed by a $T_{1\rho}{}^{I}$ and a $T_{IS}$ adjusted spectra, respectively. A quantitative spectrum is arrived at by linearly combining the above three spectra. The mathematical relation used to combine them into one is derived from an intimate knowledge of the cross polarization taking place in the sample. Such a knowledge can only be gained by performing a series of time-consuming experiments in which $T_{1\rho}{}^{I}$ and $T_{IS}$ are precisely determined. The $T_{1\rho}{}^{I}$ value is obtained by applying a variable spin lock (VSL) pulse sequence in which the protons are spun locked for a variable time (delay, if you will) before CP is allowed to take place, and $T_{IS}$ is obtained by applying standard CP pulse sequences and varying the contact time, and using the $T_{1\rho}{}^{I}$ value previously determined by the VSL experiments. Both of these experiments are rather time-consuming as they require a series of spectra to be accumulated (the two measurement require 20 separate spectra to be collected, processed, and analyzed) after which the desired relaxation parameters are determined. The VSL technique is used in preference to the more traditional VCT technique to determine $T_{1\rho}{}^{I}$ as is removes slow $T_{IS}$ artifacts. The RESTORE technique, however, is very useful as it gives insight not only into the roles of the different relaxation rates, but also based on them allows one to obtain an edited spectrum from which chemical information can be derived. It may include information such as the presence of functional groups that may still have paramagnetic cations attached to them or are associated with stable radicals, moiety mobility, and the origin of the moiety (especially in terms of black carbon components such as charred wood).

When all is said and done, at this point, a rapid *quantitative* method to analyze carbon speciation of solid-state NOM samples by NMR is still beyond the realm of reality. In fact a DP-MAS spectrum collected under quantitative conditions (a high enough spinning speed to remove spinning sideband artifacts, a long enough delay between acquisition to allow for full relaxation, and a paramagnetic free sample [an impossibility for NOM]) still appears to be the only way of collecting a *quantitative* NMR spectrum on such samples. However, semi-quantitative data on the carbon speciation in solid-state NOM samples is highly desired and beneficial a large majority of the time, and at the moment the CP based methods appear to be best suited for this purpose.

## Semi-quantitative CP methods

### Single amplitude cross polarization (SACP)

This technique is by far the most used in the characterization of NOM of all types and from all sources. The application of SACP-MAS to NOM has been studied in great detail by Wilson [20], Snape [43], and Preston [18] and will not be fully analyzed in this work. In brief, the application of SACP-MAS to NOM samples is a fine balancing act between the rate of polarization build-up in unprotonated carbons (and even more remote unprotonated carbons) and $T_{1\rho}{}^{I}$ induced relaxation in highly protonated carbons. In reality it is a compromise between $T_{IS}$, $T_{1\rho}{}^{I}$ and the artificial amplification of proton rich carbons. Thus, it is required that one performs a series of experiments in which the cross polarization time (known as the contact time) is varied in order to characterize the cross polarization dynamics and then to determine the contact time which gives the most quantitative spectrum for all carbons. It should be noted that the optimal contact time for quantitative results does not necessarily deliver the most signal across the whole spectrum. In fact, for NOM one usually loses signal in order to obtain a more quantitative spectrum. In practice, one also usually compares the spectra obtained with different contact times to a spectrum which is considered quantitative, such as a DP-MAS spectrum collected under quantitative conditions. A quantitative liquid spectrum can also be used for the purpose of such a comparison, if the NOM being studied is fully soluble. In practice, it has been found that, as a general rule-of-thumb, 1 ms is the cross polarization time which appears to be the best compromise allowing for a quantitative, or more correctly stated, semi-quantitative $^{13}C$ NMR spectrum of NOM [18].

However, if one wishes to obtain as quantitative a spectrum of a NOM sample as possible with SACP, one must perform at least a series of experiments in which the contact time is varied. Subsequently, the natural logarithm of each peak's intensity is plotted against the contact time to yield a cross polarization build-up curve for each resolvable carbon species (usually ketonic [220–190 ppm], carboxyl [190–165 ppm], phenolic [165–150 ppm], aromatic [150–120 ppm], anomeric [120–90 ppm], carbohydrate [90–50 ppm], and aliphatic [50–0 ppm]; although more exact assignments are possible [36, 44, 45, 46, 47]). $T_{CH}$ and $T_{1\rho}{}^{I}$ are obtained by mathematically fitting the cross polarization to Equation 1 (where $T_{IS}$=$T_{CH}$ in this case) or to a more complex version of this equation. However, it should be noted that initial estimates of $T_{CH}$ and $T_{1\rho}{}^{I}$ can be obtained by fitting the early part and later part of the build-up curves, respectively. $T_{CH}$ and $T_{1\rho}{}^{I}$ can also be precisely measured by independent multi-spectra experiments [29]. In any case, the full characterization of the cross polarization dynamics of NOM is rather time-consuming and must be done for each sample if one is to get the most quantitative spectrum possible by this technique. This explains the popularity of the 1 ms rule-of-thumb as

it appears to give semi-quantitative results for the vast majority of samples, but the quantitation errors must be weighted against the inherent uncertainty of a solid-state NMR experiment on NOM, which is typically around 2 to 5%.

The quantitative abilities of SACP-MAS in the characterization of NOM have long been an area of concern as addressed by a large number of groups and studies (Ref. [36] and references therein), concluding that SACP-MAS spectra of NOM samples are the most quantitative when obtained at low fields (2.3T [100 MHz for protons or 25 MHz for carbons] to 4.6 T [200 MHz for protons or 50 MHz for carbons]) and at slow spinning speeds (approximately 4 to 5 kHz). The reason for this is that at low fields and slow spinning speeds SS interferences are of little concern and sample spinning has a minor, if any effect on the CP process. In addition, it has been argued that NOM spectra are so complex and consist of so many overlapping peaks that high fields deliver no improvement in resolution [36]. However, with recent high-field results this argument has been questioned [48, 49, 50, 51]. In addition, a large number of laboratories wishing to study NOM do not have a low-field solid-state NMR spectrometer at their disposal, and this problem will become more pronounced in the future as higher and higher magnetic fields become the norm in solid and liquid-state NMR. There are many reasons for this, but the most pressing appear to be:

1. most solid-state NMR instruments are multi-user instruments and the majority of the users correctly desire the highest field they can obtain funding for,
2. at the moment the lowest magnetic field commercial NMR vendors are delivering is 7 T and this will only go up (however, any of these magnets can be made into a 2.3 to 4.6 T magnet), and
3. funds for a purchase of a special low-field solid-state NMR instrument for the study of NOM as well as to keep it operational are also difficult to secure.

Thus, methods for obtaining semi-quantitative spectra at higher fields must be developed for the progression of the study of NOM by NMR by as many groups as possible. It has been realized that, for information gathered on high field (≥7 T) instruments, spinning sidebands are by far the most detrimental in obtaining semi-quantitative CP-MAS spectra of NOM. The two most theoretically and economically sound, as well as practical, approaches put forward to date are the CP-TOSS methods [42, 51] and fast sample spinning with RAMP-CP [33, 48, 49].

## Cross-polarization with total suppression of spinning sidebands (CP-TOSS)

Besides spinning the sample at very high speeds and interfering with the CP process, theoretically one can eliminate spinning sidebands in four other ways. The first is to synchronize the collection of the FID with the rotor period. This allows for a spinning sideband free spectrum as the anisotropic components average to zero over a complete rotor period, and thus, only the isotropic components remain [24]. However, this technique restricts the spectral width to such an extent that it is useless for the study of NOM. The second method is a two dimensional technique in which the sample "hops" in 120° increments while spinning [23]. This technique is technically difficult, and thus, very rarely used. The third method is known as phase adjustment of spinning sidebands (PASS) and involves separating sidebands according to their phase, simplifying the obtained spectrum [23]. The fourth, and by far the most utilized, is the CP-TOSS technique [22, 23, 24] and, as far as can be determined, it is the only one of the four techniques to be applied to NOM [42, 44, 45, 46, 47, 51]. This cross polarization technique has been around for a long time and has been used extensively. The initial development of the CP-TOSS technique was by Dixon and co-workers [52, 53] in which they showed that the technique eliminated spinning sidebands from CP-MAS spectra. The underlying principle of the technique is that $\pi$ (180°) pulses can change a precessing nucleus's phase. Dixon showed that by the astute choice of delays between $\pi$ pulses based on the rotor's period it is possible to cancel out the spinning sidebands due to the change in their phase by displacing the spin echoes in time. In theory, a series of $\pi$ pulses need to be used, but in practice only four $\pi$ pulses are generally used. The phases of the $\pi$ pulses are cycled to compensate for pulse imperfection which could lead to incomplete sideband suppression (which is the whole point of this pulse sequence). Also, it is highly preferable to use composite $\pi$ pulses as the finite length of a single $\pi$ pulse reduces the efficiency of this technique. In addition, in regards to the pulse sequence, it should be noted that the power level of the $\pi$ pulses applied to the $S$ nucleus must be set so as to prevent possible CP from taking place during their application. Thus, one can see that CP-TOSS pulse sequences are far more complicated than the standard SACP pulse sequence. However, with modern instrumentation the above precautions can be addressed, facilitating the application of the CP-TOSS technique.

However, in general CP-TOSS signals are not quantitative [21, 24, 54] and if there are many spinning sidebands (as is the case in NOM) CP-TOSS does not work well, and small residual spinning sidebands are observed. In fact, even under the most ideal conditions CP-TOSS is non-quantitative as the suppressed sideband intensity is not fully or equally added to the isotropic band [24]. In addition, relaxation (most importantly $T_2$) takes place during the evolution of the CP-TOSS pulse sequences, and thus, signal with a relatively rapid $T_2$ relaxation will be reduced in the final spectrum. Finally, for powdered samples the isotropic signal can be reduced due to destructive interferences (signals from different carousels, a carousel of crystallites can be viewed as being oriented with the same Euler $\alpha$ and $\beta$, but different $\gamma$ angle) [24]. The more complex a sample is the more sidebands there are to suppress. In reality, full suppression does not take place and small residual sidebands prevail. Thus, for complex samples such as NOM, CP-TOSS is inherently non-quantitative.

As pointed out above, in reality, only semi-quantitative spectra can be obtained by *any* method that uses CP. In the studies that have applied CP-TOSS to humic materials it has been found that semi-quantitative spectra are obtained [51, 55]. This is most probably due to the fact that the $T_2$ relaxation is not a major concern during the CP-TOSS sequence since $T_2$ of NOM appears to be on the millisecond time scale [49, 51] if the sample is low in paramagnetic centers. In addition, it appears that the non-quantitative aspects of CP-TOSS especially for NOM may be less significant than the 2–3% variation in reproducibility (vide supra). The CP-TOSS method has the advantage of being able to yield semi-quantitative spectra at low spinning speeds allowing one to use large sample volumes at high magnetic fields.

### Ramped amplitude cross polarization (RAMP-CP)

As discussed above, by spinning the sample at sufficiently high speeds one can remove the spinning sidebands from the spectral window of interest. Thus, one can add the spinning sideband intensities to the isotropic intensities to achieve as quantitative a result as possible (the same holds for DP-MAS). This approach has the added bonus of refolding more of the signal lost due to the spinning sidebands back into the isotropic band. Also, it has been shown that due to motional modulations one obtains a more quantitative spectrum of some NOM samples by spinning them at higher speeds [48]. Thus, it would be very beneficial to obtain a CP-MAS spectrum of NOM at high spinning speeds, especially as commercial solid-state NMR probes currently allow spinning speeds of up to 35 kHz. The problem of obtaining CP-MAS spectra at high sample spinning speed has been addressed by a number of groups. At the moment it appears that the solution put forward by Smith and co-workers [56, 57, 58] of varying the amplitude of one of the pulses (either on the $I$ or $S$ transmitter [channel]) during the HH condition, over a frequency range large enough to cover the sample spinning speed, is the most accepted and used. Soon after Smith and co-workers developed RAMP-CP Cook et al. [48, 49] applied it to NOM. RAMP-CP works on the principle that a HH match will always be achieved for some part of the contact time while SACP (single amplitude CP)-MAS may not if the HH matching condition becomes too narrow. This simple concept has large theoretical ramifications in terms on the amount of signal obtained and the quantitative nature of this signal. One of the major drawbacks of the RAMP-CP technique is that an exact HH match is not always achieved during the ramped pulse. However, this can be offset by placing the RAMP at the center of the HH match on the first order sideband match, and allowing for a longer contact time. This longer contact will enhance the effect of $T_{1\rho}{}^I$ relaxation and may render some carbons associated with proton with short $T_{1\rho}{}^I$ reduced in terms of intensity or invisible altogether. To minimize this effect RAMP-CP usually takes place on the first order sideband of the HH match, and thus takes advantage of the more rapid and efficient polarization transfer of this condition. This reduces the required contact time which, in turn, reduces the effect of $T_{1\rho}{}^I$. The fact that RAMP-CP does not hold the CP condition during the full contact time means that it may, in fact, reduce the effect of $T_{1\rho}{}^I$ relaxation as the $I$ and $S$ spins are not held in intimate contact. Also, the equations that have been developed [59] assume that the HH match condition is held during the whole contact time, which is not the case for RAMP-CP or any other variable amplitude CP pulse sequence. In fact RAMP-CP can be viewed as integrating the area under the HH matching condition in the frequency domain rather than sampling *a point* (ideally the maximum) along the HH matching condition as SACP does. Thus, the applications of these CP based equations must be done with caution. In fact, a difficult to accomplish modification involving a Lorentzian function to model the sweep through the CP condition which accounts for the width of the HH matching condition (which depends on the vicinity of the carbons to the proton pool, the number of protons in each pool, and the sample spinning speed) is warranted. However, it has been shown that one can model the cross polarization dynamics of the RAMP-CP at least at the semi-quantitative level, especially if both a fast and a slow components are included [50].

The fact that the RAMP-CP integrates the HH matching condition (by varying the $B_1$ condition of one of the transmitters) profile rather than samples a single point means that it corrects for some of the major non-quantitative limitations of the CP-MAS method. One of the major reasons for this is that it allows one to "spin" the spinning sidebands out of the spectral window of interest, and thus, remove the effect of spinning sideband interferences. In addition, it allows for more than just one HH match to be satisfied, thus allowing equal matching of HH conditions within a sample as complex as NOM, especially at high sample spinning speeds. It also allows for a HH match to be maintained for long-term experiments, where electronic drift may mean that the initial instrumental parameters set for an ideal HH matching condition do not remain the same over the time of the experiment due to the HH matching condition being extremely sensitive to the strength of the applied $B_1$ field. The drifting of either or both of the $B_1$ field strengths over time (particularly for experiments with long acquisition times as are sometimes needed for samples with low carbon content) can lead to the loss of signal, or even worse, to non-quantitative CP. This is primarily the case for remote carbons (such as carboxylic, ketonic, and aromatic) as they have much narrower HH matching conditions compared to the protonated carbons. Also, the $B_1$ fields in CP-MAS probes are rather inhomogeneous (more correctly stated, they are non-uniform) both radially and vertically, but especially vertically. These inhomogeneities are due to the way in which the single solenoid coil is designed. The effect is again a HH mismatch condition as one moves away from the center of the coil as the $B_1$ field decreases. The exact shape of the $B_1$ field profile can be calculated to a certain extent [60, 61] and thus, in theory, one can correct for this effect. Thus, $B_1$ variations are compensated for by RAMP-CP

[31, 57, 58] and, in doing so, RAMP-CP delivers more quantitative results. This is especially the case for carbons with a narrow HH matching condition, in particular at high sample spinning speeds. The exact speed at which this will become important will vary between carbon types.

Finally, it should be noted that, in theory, RAMP-CP allows for cross polarization via the more efficient adiabatic CP process as the RAMP-CP experiment enables a population inversion, and thus may eliminate some of the quantitative issues raised due to $T_{1\rho}{}^{I}$ relaxation during the long contact time [62].

## Comparison of semi-quantitative methods

From the discussion above it can be seen that any CP based technique can only, in theory, deliver semi-quantitative spectra when applied to NOM samples due to the interplay between the number of protons attached to a specific carbon and the interplay between $T_{CH}$ and $T_{1\rho}{}^{I}$ in the dynamic of the CP process. Nevertheless, CP methods are by far the most used to study NOM samples today and will be heavily used for the foreseeable future due to the time savings they afford. Thus, for consistency between published spectra it is desired that a standard method be developed. However, before this can be done some issues must be cleared up. The first is that RAMP-CP is more time-consuming to set up [55], and that the original RAMP-CP set-up for the study of NOM samples was done by simply choosing an arbitrary time based non-quantitative liquid data [42]. With modern instrumentation the set up and optimization of RAMP-CP is just as simple as SACP and simpler than CP-TOSS. However, the appropriate contact time has to be determined, as with any CP method. This was already done by Cook et al. (though not reported, as it should be standard operating procedure similar to calibrating a pH electrode) by doing a series of contact time experiments and then mathematically fitting the build-up curves in the standard manner. In addition, spectra obtained at different contact times were compared to a liquid-state spectrum obtained under quantitative condition [48]. It should be noted that the sample used by Cook et al. was the very soluble Laurentian fulvic acid. Consequently, RAMP-CP does not require any more time to set up correctly compared to a standard SACP-MAS experiment in order to obtain the most quantitative data possible.

Some studies have examined both CP-TOSS and RAMP-CP, along with low-field SACP [42, 55]. Both groups were in agreement that both SACP-MAS spectra acquired at low field and RAMP-CP spectra acquired at high field were much more quantitative than those obtained with SACP-MAS at high fields. However, in regards to the quantitative nature of the spectra acquired with the CP-TOSS method at high-field techniques, the two groups' results disagree. The work of Mao et al. shows that CP-TOSS delivers spectra that are quite quantitative [42]. However, the results of Peuravuori et al. show that CP-TOSS delivers results that are still not highly quantitative [55]. The results of Peuravuori are in line with the fundamental characteristic of sideband editing by the TOSS method [63]. This discrepancy between results from two independent groups is both interesting and concerning. Of particular concern is the large number of samples and experiments each group carried out, and may indicate that even more work needs to be performed with the CP-TOSS technique and different versions of the CP-TOSS pulse sequences to fully analyze how quantitative this technique is for the characterization of NOM. In the studies discussed above RAMP-CP was also examined and compared to SACP-MAS as in three other studies [48, 49, 50]. In all cases RAMP-CP has been able to deliver very good results in terms of quantitation. However, Mao et al. proposed that a carefully optimized SACP experiment using the first order sideband HH matching condition will yield similar results to those of a RAMP-CP, even though their results did not support the proposition (one would assume the use of an optimized CP condition for their study, especially as they used a 800 μs contact time rather than the 1 ms "rule-of-thumb", especially after making a point for the importance of such optimization) [42]. Cook et al. investigated this possibility and found it not to be the case [33]. There are two possible reasons for this. The first and most likely is due to the non-uniformity of the $B_1$ fields in CP-MAS probes. As the sample was spun at a sufficiently high speed, the HH matching conditions for some types of carbons may have been narrowed to such an extent that their maxima were not being sampled throughout the whole sample, as has been shown by Peersen et al. [31]. Thus, nuclei with broad HH profile, such as aliphatic carbons, will be less affected by this phenomenon then those with narrow HH profile, e.g. carboxyl carbons. This will lead to a decrease in the observed carboxyl carbon signals, and hence, to a non-quantitative spectrum. The same holds true for drifts in the $B_1$ fields in time over the period of a long acquisition. The second explanation is that due to transient oscillations in the SACP build-up curves (which are absent in RAMP-CP build-up curves) non-quantitative CP takes place as was put forward by Dria et al. [50]. However, it should be noted that the study by Dria et al. supports the proposition by Mao et al. [42]. Possible reasons for this are that the HH profiles for all sampled carbons were broad enough at the spinning speeds they used (higher than those reported by either Cook et al. or Mao et al.) and/or the $B_1$ fields produced in their instrumental setup were very stable over time and homogeneous throughout a large portion of their sampling volume. It has also been stated that the signal increase obtained at higher field is lost due to the smaller sample volume one must use in order to achieve the sample spinning speeds needed to remove spinning sideband interferences [36, 42]. However, this may not always be the case as shown by the work of Dria [50], as larger sample volume is not always better in terms of *S/N*, especially per unit volume. The reason for this is that the larger the sample the larger the coil must be and the further away from the majority of the sample the coil will be. This means that weaker $B_1$ fields will occur, resulting in weaker decoupling conditions, which

in turn leads to shorter $T_{1\rho}^{I}$ [64] and less efficient cross polarization. Dria et al. have shown that RAMP-CP gives twice as much signal as SACP with everything else equal and ten times as much, or more, compared to an older low-field instrument. Thus, it would appear that very little is lost by using a smaller sample volume, at least as far as going down to a 4 mm rotor and CP-MAS probe.

On aggregate it appears that RAMP-CP with high sample speeds is the method of choice for obtaining a semi-quantitative spectrum of NOM as it:

1. allows one to remove spinning sideband artifacts,
2. integrates the HH matches rather than samples a single point, and thus, removes non-quantitative effects induced by drifts in the $B_1$ strengths in time or across the sample volume,
3. delivers twice as much signal as standard SACP per unit time (thus spectra can be run in 1/4 the amount of time),
4. is easily set up on modern instruments, and
5. might enable adiabatic CP.

CP-TOSS appears to be the next method of choice as it also removes spinning sidebands, but further investigation is required due to the conflicting results obtained by this method to date. It should be noted that modern decoupling pulse sequences such as two-pulse phase modulation (TPPM) should be used whenever possible in the analysis of NOM, as done by Dria et al. [50].

## Spectral editing methods

At least some of the time it is highly desirable to have as quantitative results as possible. Other times it is more important to obtain structural information, as is the case in most NMR structural studies of large biomolecules such as proteins. In the study of NOM such structural information will allow for better models to be proposed and utilized in simulation studies which are proving to be so crucial in almost all areas of science, in general, and chemistry, in particular, thanks to the proliferation of fast computers.

### 1D methods

The longest used of all solid-state spectral editing techniques in the study of NOM is CP itself, for example, it allows one to edit carbons based on their proton multiplicity via a short contact time (view protonated carbons) or long contact time (remove protonated carbons). The next are relaxation methods which allow one to edit spectra based upon $T_1$ relaxation (the simplest version of this is to use a delay shorter than is needed for full relaxation of the spectrum, and mathematically fit the magnetization build-up) and $T_2$ relaxation (in these experiments there is a delay between excitation of the carbons via CP or DP and the time in which the spectrum is collected, with the protons being under the influence of dipolar decoupling; it is very desired to refocus by a 180° pulse on the carbons to generate a Hahn spin echo during the delay between excitation and acquisition [evolution period]). Dipolar dephasing is another old editing technique that is based on allowing the protons to diphase the carbon signal and is accomplished with a pulse sequence very similar to the $T_2$ pulse sequence discussed above, but without dipolar decoupling being applied to the protons. Thus, during the evolution period of the pulse sequence the protons diphase the carbon magnetization via heteronuclear couplings. With an evolution time in the range of 30 to 150 µs (usually 60 to 75 µs for NOM samples) one suppresses the protonated carbons and is left with the unprotonated carbons as well as protonated carbons with weak heteronuclear couplings to protons, e.g. $CH_3$ groups. Although the above techniques are useful, especially when combined [49], they give only limited information.

A highly beneficial editing technique would be one capable of distinguishing $CH_2$ from CH (and the less pressing $CH_3$, due to the dipolar dephasing experiment discussed above) groups. Such a technique exists and was introduced by Wu et al. [65]. It combines a series of $^{13}C$ CP-MAS spectra with appropriate CP periods (either short or long), depolarization periods, and polarization inversion periods. The technique allows one to obtain the full CP-MAS $^{13}C$ spectrum and three subspectra based on $CH_n$ multiplicity. These include: a spectrum consisting of $CH_3$ and unprotonated carbons, another one consisting of $CH_2$ carbons, and yet another one consisting of CH carbons (the technique, in fact, requires the collection of four spectra to be then linearly combined to produce the abovementioned subspectra). However, it should be noted that this technique requires one to empirically determine the coefficients used for the linear combinations via the use of model compounds. This technique has been applied to a humic acid by Keeler and Maciel [66] and yielded very informative subspectra that helped to address some of the assumptions made in the literature. Regrettably, the technique is very time-consuming when applied to NOM as indicated by the Keeler and Maciel study in which a 9.5 mm rotor holding 700 mg of humic material was used, and the set of three subspectra required more than 156 h to collect.

Recently, Schmidt-Rohr and Mao [46] have introduced a new method for the efficient selection of CH groups by the suppression of $CH_2$ and quaternary-carbon signals and applied it to a series of model compounds as well as humic acid. The method is based on the fact that the SI spin pair is invariant under spin-pair dipolar decoupling, while $SI_2$ due to its two *I*s is quickly dephased in terms of heteronuclear multiple-quantum (MQ) coherence. Thus, one can suppress $CH_2$ signals while allowing the CH signals to remain by using MQ coherence and distortionless enhancement by polarization transfer (DEPT). The quaternary carbons are removed from the spectrum by subtracting an equivalent dipolar dephased spectrum from the MQ spectrum mentioned above. This approach leads to the loss of the majority of the $CH_3$ signal, which can be further suppressed by using a simple $T_1{}^{13}C$ filter of 0.5 to 1.5 s [46]. This work shows that one can very effectively ob-

tain CH only spectra, and is superior to the method discussed above for non-crystalline samples. Also, despite a maximum efficiency of 14% a CH only spectrum of a humic acid sample could be obtained in just under 3.5 h. However, Schmidt-Rohr and Mao [46] also showed that a chemical filter could be incorporated into the pulse sequence which, in turn, allowed one to assign peaks to NCH or CCH(C,C) carbons using simple, and by far more accessible, double-resonance hardware rather than the triple resonance SPIDER NMR technique introduced shortly before by the same group [45]. In addition, the MQ-DEPT method is more quantitative as it is less prone to selective $T_2$ dephasing [46].

### 2D NMR

Another way of editing a spectrum is to disperse the data into a second or even third or fourth dimension. This allows one to overcome some of the peak overlap which plagues 1D NMR spectra of humic materials, but more importantly (as will be discussed in more detail in the liquid section of this work) it also allows for this dispersion to occur in a controlled manner. Solid-state 2D NMR has been sparingly applied to NOM [44, 45, 47, 49, 67]. In all cases rather broad lines are observed, however, this is much less severe in the cases where the 2D spectrum maps out carbon–proton correlations [44, 45, 47]. The carbon–proton 2D work (labeled this way as they are carbon detected experiments) has been based on the standard solid-state HETCOR principle [68, 69, 70], however, the pulse sequence has been elegantly modified. The following modifications have been included:

1. *Dipolar dephasing filter*. As discussed above, this filter suppresses all carbons except for the unprotonated carbons and protonated carbons with weak heteronuclear couplings and allows for a simpler 2D spectrum to be obtained [44].
2. *$T_2$ filter.* This edits from the spectrum carbons based on their mobility and proximity to paramagnetic centers, thus resulting in a simplified spectrum [44].
3. *Lee-Goldberg CP rather than Hartmann–Hahn CP*. Lee-Goldberg CP arranges the $^1$H spin-lock to be at the magic angle in the rotating frame [24, 71], which means that the heteronuclear *I–S* dipolar coupling is maintained, and thus, rapid transfer of magnetization can take place. This technique suppresses the $^1$H homonuclear dipolar coupling, and thus, only carbons in very close vicinity to protons are polarized. Consequently, a removal of the spin diffusion that typically takes place in Hartmann–Hahn polarization is accomplished. For NOM samples this means that a more precise, and thus simplified spectrum is possible [45, 47].
4. *Chemical shift anisotropy recoupling*. As discussed above [45].

By combining the above techniques Mao et al. [44, 47] have been able to start connecting the moieties within humic materials and testing proposed models. Also, their work has shown that for some humic materials it appears that lignin structures are present. Thus, from this very elegant work a large amount of new information on humic materials has been derived. The fact that these are solid-state methods means that they can be extended to whole soils and other insoluble samples which constitutes a definite advantage over the liquid-state experiments discussed below. However, more research needs to be done on these methods as they apply to NOM, and due to their theoretical complexity and novelty (in some cases within the NMR community as a whole) it will require some time for these techniques to become standard methods in the study of NOM.

## Liquid-state NMR

In the history of NMR the first "true" NMR publication by Purcell et al. [72] was on solids and was very quickly followed by Bloch et al. [73] work on liquids. It should be noted that the two groups independently developed and submitted their work for publication within a month. However, historically liquid-state NMR has progressed ahead of its solid-state sibling. The reason for this is that Brownian motions remove line broadening mechanisms. In addition, the relaxation processes within a liquid sample are much more favorable to producing spectra with narrow lines and allowing for more scans to be acquired. Consequently, the liquid state has become the predominant state in which NMR spectra are acquired and for which NMR techniques were and continue to be developed for. But, as can be seen from the discussion above, solid-state NMR is beginning to catch up mainly in the area of method development. However, liquid-state NMR is still ahead by some distance.

In the area of NOM characterization, until very recently the opposite has been true. The major reason for this is the long held idea that NOM was so complicated that the improved resolution offered by the liquid state did not offset its disadvantages in terms of concentration limit, solubility, solvent effect, and sample preparation, even though this has been argued to the contrary for a long time [18]. Recently, the resolution advantage of liquid-state NMR has started to be harnessed in the characterization of NOM, especially when data are dispersed into another dimension. This part of the review is set up quite differently compared to the solid-state section, as most of the cutting edge NOM liquid-state NMR work uses well established liquid-state techniques that have been used by biochemists and chemists for over two decades. Nevertheless, the overall aim as set out at the start of the paper is the same. For more detailed explanations of the methods discussed below the reader is referred to the monographs by Cavanagh et al. [74] and Levitt [75] as well as a very good and modern review by Renolds and Enrique [76]. For a guide on how to set these techniques up on an NMR spectrometer the reader is referred to the monograph by Braun et al. [77].

## 1D methods for the study of NOM

Traditionally, $^{1}H$ NMR has been the method of choice for the investigation of small molecules due the high natural abundance and receptivity (measured by gyromagnetic ratio) of protons, especially in the field of chemistry. However, for NOM this has not been the case for two reasons. Firstly, the solvent of choice for NOM work is water and thus, one has to deal with a very strong water signal in the $^{1}H$ NMR spectrum. Secondly, $^{1}H$ NMR does not deliver the resolution $^{13}C$ NMR can (this is even more pronounced in solid-state NMR). Thus, historically $^{1}H$ NMR has not been very informative in the study of NOM, and information provided by other nuclei such as $^{13}C$, but also $^{15}N$ and $^{31}P$, has been sought (although work on $^{15}N$ and $^{31}P$ is highly informative it is outside of the scope of this review).

### $^{1}H$ NMR

This would be the method of choice for rapid screening and characterization of dilute NOM samples. Traditionally water causes a major problem due to the dynamic range limitations of the electronics needed for the collection of water's large NMR signal which saturates the electronics at the gain needed to detect the NOM (due to digitization limitations of analog-to-digital conversion). Thus, the signal of water must be attenuated to allow for the detection of the signal of interest. This attenuation can be accomplished in a number of ways. The most popular is the suppression of the water signal by using a diffusion filter in combination with pulse sequences that manipulate the magnetization of the water. The best known of these is the WATERGATE (water suppression by gradient tailored excitation) [78, 79]. This technique has been successfully applied by Lee et al. [80] to NOM samples. By using this water suppression technique the authors were able to obtain $^{1}H$ spectra of NOM samples at concentrations as low as 2 mg/mL. Cook et al. [81] have also shown that an alternative method known as excitation sculpting performs equally as well as WATERGATE. However, in all cases any signal close to water will be affected, thus this limitation must be kept in mind. In addition, great care is required when optimizing these pulse sequences and each sample requires a separate optimization as the frequency center for the shaped pulse is crucial for the success of either of these two highly useful methods in the study of NOM. It is worth noting that the chemical shift of the water signal is very sensitive to temperature. Also, it should be remarked that either of these water suppression techniques can be incorporated into the 2D pulse sequences discussed below, and modern versions of these pulse sequences can suppress more than one solvent signal.

Another way around the intense solvent signal is to use a deuterated solvent. In the case of $D_2O$ one will still be left with a substantially large water signal (or more correctly HDO signal, which will shift with temperature) due to the exchangeable protons of NOM. This, in fact, will be a problem with any solvent with exchangeable protons. The next most popular, after water, solvent for NMR studies of NOM is DMSO (in reality DMSO-$d_6$; e.g. Refs. [81, 82]). DMSO is a very good solvent as can be determined by its lower dipole moment and is better at solvating groups, such as aromatics, than $H_2O$, as has been seen to be the case for NOM [83]. However, it is highly recommended that DMSO only be used from a freshly broken ampoule [81]. The use of DMSO allows one to see the exchangeable protons, such as in carboxylic groups, that are invisible in spectra in which water has been used as the solvent. In addition, one can see amide protons as well as their disappearance by the addition of small amounts of $D_2O$ [82].

Regardless of whether DMSO-$d_6$ or $D_2O$ (or $D_2O$ and NaOD) is used as the solvent, one obtains a very complex proton spectrum. It consists of broad lines due to broad envelopes of peaks (as derived from the 2D methods discussed below) as well as broad peaks which arise from macromolecules or macromolecular assemblies of molecules (the broad lines arise due to the long correlation times inherent to either of these cases) which are overlaid by a series of sharp lines that may arise form either small molecules or from highly mobile segments of large macromolecules or macromolecular assemblies of molecules. However, the obtained spectra are also extremely complex because of the chemical complexity of NOM, and due to the narrow spectral window being investigated brought about by the small chemical shift dispersion of protons. This, in turn, means that chemical group assignments in NOM samples can only be made at the macroscopic level when one only considers the proton spectrum. Nevertheless, quantitative spectra can be obtained in a much more time efficient manner compared to any of the solid-state NMR techniques discussed above or any of the liquid-state $^{13}C$ experiment discussed below (usually a quantitative $^{1}H$ spectrum can be obtained in a matter of 10 min).

It should be noted that high resolution spectra can be obtained for NOM by very thorough shimming of the sample, such that one can determine the effect of deuterium on the signal of ammonium ions and distinguish $NH_4^+$, $NH_3D^+$, and $NH_2D_2^+$ ([81], in this study the assignment of the ammonium ions was confirmed by $^{14}N$ NMR, $^{1}H$–$^{15}N$ HSQC, and $^{1}H$–$^{1}H$ TOCSY experiments). Thus, even at the high concentrations needed for the 2D spectra discussed below (30 to 150 mg of NOM per milliliter of solvent) viscosity is not a problem as further supported by the 2D results discussed below and the $^{14}N$ results obtained by Cook et al. [81].

### $^{13}C$ NMR

As in solids, $^{13}C$ NMR has been the nucleus of choice to monitor NOM in the liquid state, as $^{13}C$ has a larger chemical shift dispersion than $^{1}H$, and more of the sample is interrogated by $^{13}C$ compared to $^{15}N$ and $^{31}P$. However, care must be taken when one wishes to acquire a quantitative $^{13}C$ spectrum. As with the solid state, all paramagnetic impurities must be removed (in the real world paramagnetic

centers must be kept to minimum), full relaxation must take place, and the protons must be decoupled by inverse gated decoupling [18]. In this decoupling scheme the decoupler is switched on just before the excitation pulse, and then switched (gated) off during the recycling time after acquisition and before the next excitation pulse. This leads to decoupling free of population effects, which removes nuclear Overhauser enhancement (NOE), thus *yielding a quantitative spectrum* if the system under investigation is allowed to fully relax between excitation pulses. Usually a 45° pulse is used to excite allowing a shorter delay between acquisitions. However, it should be noted that to obtain a quantitative $^{13}C$ liquid-state spectrum of NOM with satisfactory *S/N* can take in excess of two days, and thus validating the need for CP-MAS (vide supra). If a NOM sample is fully soluble then quantitative liquid-state $^{13}C$ NMR is very comparable to quantitative solid state obtained using DP $^{13}C$ NMR in terms of instrument time and requires much more commonly available instrumentation.

The real power of liquid-state $^{13}C$ NMR in the recent past has been the fact that one can edit spectra based on carbon multiplicity. This can be done in many ways such as the attached proton test (APT), insensitive nuclei enhanced by polarization transfer (INEPT), and distortionless enhancement by polarization transfer (DEPT).

### APT

The APT pulse sequence consists of a spin-echo (90°–$\tau$–180°–$\tau$–acquire) pulse sequence. By gating the decoupler, one can distinguish the multiplicity of the carbons in the sample. The APT (also known as gated spin-echo or spin-echo Fourier transform) method relies on the fact that the magnetization vector components of the CH, $CH_2$, and $CH_3$ carbons do not rotate synchronously but are characteristically different. Thus, by choosing the appropriate $\tau$ period ($\tau$=1/*J* in seconds) and gating the decoupler off during one of the $\tau$ periods, *the quaternary and $CH_2$ carbons will have one phase which is 180° out of phase with the CH and $CH_3$ carbons*. This method has recently been applied to NOM by Cook et al. [81] in which it was shown that the majority of the aromatic carbons were protonated, and thus, could not be functionalized, while the majority of the functionality was on the carbohydrate moieties (they further proved this point with 2D NMR techniques which are discussed below).

### INEPT

In this experiment polarization is transferred from the proton to the carbons via a population exchange, due to the *S* nucleus (carbons in this case) and the protons sharing a common energy level. The delays (sandwiching the 180° pulse on the proton channel) in the pulse sequence are set to 1/(4*J*) in seconds. Thus, *only carbon atoms with directly bound hydrogens are observed*. In theory, the INEPT pulse sequence can also be used to edit spectra based on different carbon multiplicity, however, in practice it is the DEPT that is the preferred method for such editing. The INEPT pulse sequence has been used to interrogate a NOM sample and it was shown that a large proportion of the aromatic carbons in this sample were protonated, and hence, not functionalized, while the carbohydrate moieties appeared to be highly functionalized (these findings were also supported by APT and 2D NMR results) [81].

### DEPT

This experiment starts off in a very similar manner to the INEPT pulse sequence with a 90° pulse after which the magnetization evolves under the influence of the proton–carbon couplings for a period equal to 1/(2*J*), followed by a 180° pulse applied on the proton channel while, at the same time, a 90° pulse is applied on the carbon channel. This produces a state, known as heteronuclear multiple quantum coherence (HMQC), in which both the proton transverse and carbon magnetization evolve coherently. This new state is allowed to evolve for a period of 1/(2*J*) at which time a $\theta$° pulse is applied on the proton channel in concert with a 180° refocusing pulse on the carbon channel, after which a period of 1/(2*J*) is allowed to pass before the signal is acquired. The angle, $\theta$°, is varied between 45° (CH phased up, $CH_2$ phased up, and $CH_3$ phased up), 90° (CH phased up, $CH_2$ no signal, $CH_3$ no signal), and 135° (CH phased up, $CH_2$ phased down, $CH_3$ phased up). By combining the spectra produced with these three different pulse angles one can produce separate spectra for each carbon multiplicity. This method was used by Shin and Moon [84, 85] and more recently by Randall and co-workers [86]. Since the results can be made quantitative Shin and Moon developed an "average" structure for the NOM they were studying. These studies display the power of spectral editing techniques (as well as the solid-state editing presented above) and how elegant studies designed for NOM samples based on these techniques can be. However, it must be noted that peak overlap does not allow for a breakdown of the full carbon spectrum into absolute spectra of different carbon multiplicities, and thus caution must be exercised in interpreting any of these edited spectra. The same holds for spectra of NOM produced by *any* editing technique (including HSQC spectra, unpublished data of Cook).

## 2D methods for the study of NOM

Due to the complexity of samples, even in the liquid-state peak overlap is a major problem in the study of NOM by NMR. Thus, the dispersion of the data into a second, or third, and even forth dimension would be highly beneficial. The first application of liquid-state 2D NMR spectroscopy to NOM can be traced back to the work of Buddrus and co-workers [87] followed by a long period in which liquid-state 2D NMR methods were not used in the

characterization of NOM. However, in the last few years this has started to change, and the advantages of multi-dimensional NMR in the characterization of NOM have become apparent [81, 82, 86, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107]. The 2D NMR experiments used in the majority of the studies used in the characterization of NOM can be broken down based on through bond interactions (homonuclear and heteronuclear), through space interactions, and diffusion (not reviewed here). This subdivision shows the control one has over the data dispersion in 2D NMR, and why 2D NMR is such a powerful and useful technique for the study of complex systems such as biogeopolymers. For all the studies presented below the reader is highly encouraged to use the phase sensitive version of the pulse sequences and the gradient version of these experiments (if the available hardware allows for this).

## Through-bond connectivities

Homonuclear shift correlation

There are two different types of experiment within this class. Both have been used in the characterization of NOM.

### *Correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY)*

There are two major experiments in this subclass. The first is the well known COSY in which one correlates chemical shifts of coupled nuclei. This is accomplished by modulating the amplitude of each nucleus by the chemical shift frequency of the nuclei it is coupled to. In practice, however, *one can only detect protons that are coupled within one or two bonds*. There are special COSY pulse sequences such as the COSY-90 (in which both pulses are 90°), phase sensitive COSY (it removes the skewed lines of the standard COSY experiment which arise from the addition of both cosine and sine components within the same FID), COSY-45 (in this experiment the second pulse is 45°, allowing one to distinguish between $^{2}J$ [one bond separated] and $^{3}J$ [two bond separated] as well as it narrows the diagonal peak), Long-range COSY (LR-COSY, allows one to observe signals between protons which are connected by very small coupling constants), and Double quantum filtered COSY (DQ-COSY, in which one double quantum transition takes place leading to two advantages, the first being the suppression of water signals as water has no double quantum transition, and secondly, the diagonal peak is phased into adsorption allowing one to avoid having to deal with the tailing of the dispersion in the diagonal peaks as is normal in phase-sensitive COSY).

The TOCSY experiment can be viewed as a super COSY, except for the multiple-coherence transfer which is induced between all the spins of a coupled system, regardless of whether the protons in the system are coupled to one another or not. This is accomplished by inserting a spin lock pulse in the COSY pulse sequence needed for the coherence transfer in place of the second 90° pulse. The spin-lock converts weakly coupled spins into strongly coupled spin systems by a continuous r. f. irradiation in a direction perpendicular (e.g. the $x$ direction) to the main magnetic field ($B_0$), the $z$ direction, creating a $B_1$ field. This only works if the r.f. field strength exceeds the $z$ component corresponding to the chemical shifts of the spins. If this is the case, the magnetization components aligned along the $x$-axis are locked there, their differences vanish, and the strong coupling condition is fulfilled. Under this condition, the coherences of spins are coupled together. Thus, during the spinlock, a spin and its coupling partner(s) transfer their in-phase coherence directly into the other's in-phase coherence in an oscillating manner. This oscillation frequency is directly proportional to the coupling constant between the spins. The resulting 2D spectrum contains cross-peaks with respect to all of the coupled spins whether they are directly coupled or not. Thus, *the covalent connection between all protons in a coupled network can be mapped out via the cross-peaks in a TOCSY spectrum*, emphasizing the power of TOCSY over COSY for complex molecules such as biogeopolymers. In fact, very recently it has been shown by Cook et al. [81] that coupled networks of up to seven protons can be observed in NOM using the TOCSY method.

In the analysis of NOM samples COSY and TOCSY based methods have been used by a large number of investigators [81, 82, 92, 93, 95, 98, 99, 100, 101, 103, 104, 105, 106, 107]. While both techniques have their place, they appear to be most useful when used in combination. The choice of which COSY method to use is one to be given great thought. From the knowledge that NOM produces very complex spectra in which peak overlap will be of concern, the double-quantum COSY experiment looks very attractive as a good starting point, and it has been shown to give superior results in the analysis of NOM by Simpson et al. [104]. Though expected, it is very comforting that one can apply general finds from the area of biomolecular NMR to NOM NMR. But, it should be noted that DQ-COSY is significantly less sensitive than basic COSY, with the cross peaks having intensities of about 40% of those in a basic COSY experiment collected under comparable conditions [76]. Thus, if the basic COSY requires 1 h to collect, a DQ-COSY spectrum with the same *S/N*, will take more than 6 h [108]. It should be noted that homonuclear correlation spectra require a much shorter time to acquire than the heteronuclear experiments discussed bellow, thus in the big picture, the extra time cost of the DQ-COSY spectrum is not preventative in the analysis of NOM. It would be very informative to combine a series of COSY techniques along with the TOCSY technique in the study of NOM. A combination of COSY-45, Long-range COSY, TOCSY, and DQ-COSY (to resolve peaks near the diagonal that may not be resolved by the other techniques) can be envisioned for a thorough mapping of the proton coupled network. The closest work to this combination of homonuclear correlation peaks that has appeared in the literature to date is that of Hertkorn et

al. [95], in which both COSY and TOCSY spectra of two NOM samples (a fulvic acid and a humic acid) were analyzed in great detail and compared. From this work it was found that some cross peaks apparent in the TOCSY spectrum were not apparent in the COSY spectrum, and vice-versa, for both samples analyzed. This effect is due to the self cancellation occurring because of the overlap of antiphase coherence. When the published homonuclear correlation spectra of NOM are compared, especially the TOCSY results for NOM, unique but similar patterns emerge. These patterns have been assigned to coupled networks *within* the aliphatic (0–2 ppm), functionalized aliphatic (2–3 ppm), heteroatom substituted (3–5 ppm), and the aromatic/amine regions (6–9 ppm). Specific partial structures can be assigned, such as intra aliphatic chains (2.3–0.5 ppm [$F_2$]→2.3–0.5 ppm [$F_1$], presented as 2.3–0.5→2.3–0.5), deoxy sugars, ethers, and esters (4.4–3.0→1.4–1.0), functionalized aliphatic chains with a single heteroatom (4.5–3.2→3.0–1.4), intra carbohydrates with anomeric carbons (4.4–1.0→4.8–4.0), methylated alkenes (2.0–3.0→4.0–5.0), and alcohols (1.0–2.0→3.0–4.0). The above assignments have been made based on the observed cross peaks [81, 95]. However, other groups have stated that the observed cross peak patterns arise from peptide like structures consisting of amino acids [82, 97]. But, one must be careful making such assignments as close inspection and interpretation by Hertkorn et al. [95] of their own data compared to data in the biochemical literature showed that none of the cross peaks found for their samples arose from peptide structures or amino acids. Cook et al. [81] also showed that none of the nitrogen NMR data they collected ($^{14}$N and $^{1}$H–$^{15}$N HSQC spectra) showed any peptide like structures and, in all probability, the amino acid cross peaks observed in their spectra between the aromatic/amine region and substituted aliphatic region in the TOCSY spectrum arose because of ROESY break through. The peptide like signals in the data presented by Fan [98] and Simpson [82] are in all likelihood due to peptide like structures present and arise from the sample isolation method as well as the fact these samples (the exact source of the sample in Ref. [82] is not specified) are isolated from surface soils, and thus, may contain relatively young materials which are not present in the samples analyzed by Hertkorn et al. [95] or Cook et al. [81]. However, when comparing TOCSY results between different NOM studies the all important mixing time must be taken into account. In the studies which have examined NOM samples by TOCSY the mixing time appears to range from 37 ms to 70 ms, with 50 ms being the middle ground, though further investigation into this parameter is needed. The more NOM samples that are analyzed by COSY and/or TOCSY methods in combination with other methods (as discussed below) the more of a data base that will be formed to draw more concrete and general conclusions from. It is interesting to note that Simpson et al. [101, 105] have reported TOCSY spectra of NOM in a soil matrix and a diffusion edited DOSY spectrum of a NOM fraction, thus showing the diversity and importance of this pulse sequence for future environmental studies.

## Heteronuclear Shift Correlation

The coherence phenomenon discussed above is in no way restricted to homonuclear systems. It also takes place in heteronuclear systems. In fact, an even larger array of heteronuclear experiments is possible since pulses can be applied selectively to either species and broadband heteronuclear decoupling can be employed as seen fit. For most NOM samples $^{1}$H and $^{13}$C and/or $^{1}$H and $^{15}$N would be of the greatest interest, however, for some samples $^{1}$H and $^{31}$P may be of interest as well. These experiments are useful for NOM in order to:

1. unravel overlapping peaks in the proton spectrum by exploiting the chemical shift dispersion of the carbon (nitrogen or phosphorous) spins, and vice versa,
2. *correlate a proton spectrum with a carbon spectrum for assignment purposes*, and
3. increase sensitivity via indirect detection.

The classic heteronuclear correlation technique known as HETCOR uses carbon detection, though recently this technique has been displaced by proton detection (also known as "inverse detection") schemes. This new methodology requires inverse detection probes (in these probes the proton coil is the inner coil, i.e. closest to the sample, while in a "typical" probe the proton coil is on the outside) and new consoles (alternatively, an older type spectrometer console can be re-wired). However, the great gain in sensitivity more than compensates for this effort as is evidenced in the biochemical literature. The magnitude of this sensitivity gain for carbon is about 30, while for nitrogen it is about 300!

### Heteronuclear multiple quantum coherence (HMQC) and heteronuclear single quantum coherence (HSQC)

The HSQC and the HMQC methods are very closely related. However, while in the HSQC method uses single quantum coherence, the HMQC method uses multiple quantum coherence. This means the HMQC requires fewer pulses, and is thus less sensitive to pulse calibration errors than the HSQC method. However, the resolution of the HMQC method is limited in the $^{13}$C dimension due to the $^{1}$H multiplet pattern, while the resolution of the HSQC method depends only on the $^{13}$C linewidths. Consequently, if the sample gives a crowded $^{13}$C NMR spectrum, as almost all NOM do, then the HSQC method is the method of choice. The superior resolution of HSQC over HMQC was discussed by Bax et al. [109] over a decade ago while comparing the two methods (it should be noted that Bax developed the HMQC experiment). However, it is again comforting to know that this has also been recently confirmed to be the case for NOM [104], and thus it appears that the vast practical NMR experience of the biochemical and other chemical fields (such as carbohydrate chemistry) can also be applied to NOM samples. Thus, the HSQC experiment will be the focus of the discussion below.

From the discussion above it appears that inverse experiments are far superior to their non-inverse cousins, though not without some potential problems. The first problem is that, by making $^{13}C$ the indirect dimension, one trades the higher resolution of $^{13}C$ dimension for the lower proton resolution. However, as will be shown in the discussion section for NOM, this may not necessarily be a serious drawback relative to the severe overlap of signal in the $^{13}C$ dimension. The second, and more serious problem, is that by using proton rather that $^{13}C$ detection $^{12}C$ is not filtered out. In other words, all proton signals are detected regardless of whether they are attached to a $^{12}C$ or a $^{13}C$. This is of concern in the study of NOM since only unlabeled samples are available. For an unlabeled sample, based on the natural abundance of $^{13}C$ to $^{12}C$, only 1.1% of the signals observed will be due to $^{1}H$,$^{13}C$ pairs, not to mention the signal due to the protonated solvent. The solvent issue can be overcome to a large extent by using non-protonated solvents, while the non-$^{13}C$ associated protons can be eliminated in part by phase cycling. Because phase cycling takes place after digitization of the signal the dynamic range problem cannot be reduced, and the cancellation $^{1}H$,$^{12}C$ signal is not perfect. Thus, problematic $t_1$ noise ridges arise at all proton shift frequencies (it is called $t_1$ noise as this noise arises in the $t_1$ time domain of the 2D experiment as appears in the $F_1$ dimension of the final 2D spectrum).

The use of pulse field gradients can alleviate these problems. This is because pulse field gradients can be used to very selectively choose the desired coherence pathways. This selectivity is based on the speed at which different coherences dephase under the influence of a gradient pulse and the ratioing (usually gradient strength) of the two gradient pulses (the initial gradient to cause a phase twist and the second to refocus the phase twist caused by the initial gradient pulse). In practice, only the part of the proton magnetization that was a $^{13}C$ coherence during the initial gradient pulse will be refocused by the second gradient pulse. In addition to overcoming the $t_1$ noise problem, gradients can also be used to suppress the solvent signals.

The only problem with using gradients is that only the carbon $x$ or $y$ component is transferred back to the protons. This minor problem can be overcome by adding a double reverse INEPT step, one for the $x$ and one for the $y$ component. With these modifications the gradient experiment is theoretically as sensitive as the normal HSQC experiment *if* relaxation effects due to the longer time needed to execute this pulse sequence and the pulse imperfections (since more pulses are used) are ignored. However in practice, even though there are signal losses due to relaxation and pulse imperfections, the artifact suppression using the gradient selections more than makes up for the signal loss so caused.

A cousin of the HSQC method is the heteronuclear correlation (HETCOR) method which uses $^{13}C$ detection rather than $^{1}H$ detection. In theory, HETCOR can deliver higher resolution than HSQC due to the much larger chemical shift dispersion inherent in $^{13}C$ compared to $^{1}H$. In fact, using a bilinear rotational decoupling (BIRD) decoupled HETCOR pulse sequence one can resolve peaks differing by as little as 0.01 ppm in both $^{1}H$ and $^{13}C$ dimensions [110]. In fact, it has been argued that this resolution advantage of HETCOR over HSQC would be beneficial in the analysis of NOM samples [90], however, when one views the resolution of the obtained peaks, the resolution of the HSQC experiment is more than adequate. Thus, unless one must obtain data beyond the resolution capabilities of the HSQC experiment, the HSQC appears to be the best way of acquiring proton–carbon correlations for protonated carbons.

When one looks at the HSQC (and HMQC) spectra of NOM reported to date in the literature [81, 82, 88, 89, 90, 93, 94, 95, 98, 99, 100, 103, 104, 105, 106, 107], they are all very complex (with the exception of the highly fractionated NOM sample reported in Ref. [100]), but can be broken down into four general groups. These include: the aliphatic region which covers the $^{1}H$ region of 0.4 to 3.4 ppm $^{1}H$ chemical shift range as well as the $^{13}C$ region of 5 to 40 ppm (represented as (0.4–3.4→5–40) henceforth), the single heteroatom substituted aliphatic (2.6–5.3→40–85), the anomeric (4.2–5.6→85–105), and the aromatic (6.0–9.0→105–145) regions. Depending on the sample there may be more or less overlap, however, as a general trend, the aliphatic and substituted aliphatic regions are very crowded and consist of peaks overlying a continuum, while the anomeric region is usually well resolved. The aromatic region is usually crowded but not to the extent of the aliphatic regions. Hertkorn et al. [95] have shown how NOM HSQC spectra compare to HSQC spectra of eleven possible constituents of NOM. By using this method they once again concluded that peptide like structures were not present in their samples. One of the major issues that will need to be addressed in future studies of NOM samples by HSQC (and any other heteronuclear correlation technique) is what the optimum $J_{CH}$ is, or would a range of coupling constant be more useful. A quick survey of the literature shows that $J_{CH}$ values ranged from 140 to 150 Hz, when this all important parameter has been reported.

## Heteronuclear multiple bond correlation (HMBC)

The HSQC experiment has one major weakness when it is applied to NOM. *Only carbons with protons directly bonded to them are detected*. Consequently, carboxylic, phenolic and other functionalized carbons are not detected by this technique. However, the HSQC pulse sequence can be modified into the HMBC experiment via a lengthening of the heteronuclear coupling delay. The HMBC is a modified HSQC experiment which relies on the $^{2}J_{CH}$ and $^{3}J_{CH}$ coupling rather than $^{1}J_{CH}$ coupling. Thus, *non-protonated carbons will be observed*. An example of this pulse sequence is as follows: $90°X(^{1}H)–t_1–90°(^{13}C)–\tau_2–90°(^{13}C)–t_1/2–180°X(^{1}H)–t_1/2–acq.(^{1}H)$. The duration of $t_1$ is set to $1/(^{2}J_{CH})$ while $\tau_2$ is set to between 50 and 80 ms. In this pulse sequence the direct ($^{1}J_{CH}$) correlations are suppressed by a low-pass $J$ filter. The second pulse on the carbons creates the zero- and double-quantum coherence of interest. The

180° pulse on the proton channel subsequently causes the zero- and double-quantum components to interchange, removing the $^{1}H$ chemical shift from the $t_1$ modulation frequency. Following the final carbon pulse, the proton signals that arise from the multiple-quantum coherence are modulated by the homonuclear proton coupling and the $^{13}C$ chemical shifts. Due to the long $\tau_2$ delay used, the proton signal is phase twisted at the start of $\tau_2$, making phase-sensitive processing of the proton dimension undesirable. As discussed above for the HSQC experiment, gradient coherence selection is also the best option for the HMBC experiment, even if 50% of the signal is lost. The reason for this is that $t_1$ noise suppression more than makes up for the sensitivity loss in terms of *S*/*N*.

Although the HMBC is of great importance in the characterization of NOM, only two reports containing HMBC spectra could be found. The results from both of these reports show how important the HMBC experiment is in the study of NOM samples. One of the reasons why this experiment may not have received the attention it deserves is the amount of time it takes to acquire the spectra. Also, the initial HMBC reported in the literature [100] was relatively simple, and in some readers' view, may not have produced enough results to justify the expense in terms of NMR time. The abovementioned HMBC spectrum was for a NOM sample that was highly simplified due to a superb fractionation method used in its isolation. However, on a much more complex sample Cook et al. [81] have shown that HMBC spectra of NOM are extremely information rich and play a major role in the analysis of NOM. One of the weaknesses of the HMBC experiment for general application is the fact that it requires a single long-range coupling constant to be chosen. However, with the complexity of NOM samples this may, in fact, be a strength. One can alleviate this weakness by using methods such as the ACCORD-HMBC or by acquiring a series of HMBC spectra with different coupling constants. The results reported by Simpson et al. [100] appear to show that only aromatic moieties were found to be functionalized (this is derived from the fact that only the aromatic part of the HMBC spectra was shown). Conversely, Cook et al. [81] showed that the majority of the functionality on the sample they analyzed resides on the aliphatic and substituted aliphatic moieties with a very small amount of functionality on the aromatic moieties. The findings by Cook et al. [81] are consistent with data previously derived from solid-state NMR data collected on the same sample [49], providing very comforting validation of these two very different NMR approaches. Since both groups used a coupling constant of 5 Hz, the differences between the Simpson et al. [100] and Cook et al. [81] data must have arisen form differences in the samples themselves.

## Combining 2D methods to get a bigger picture

All the techniques discussed thus far are based on following a covalent bond network in a variety of ways. However, on closer inspection it can be seen that, in combination, they allow one to follow the covalent bond network in a way which is equivalent to walking down the molecule one atom at a time as long as one is able to see all the atoms. Two groups have attempted such a walk [81, 100]. The approach that Cook et al. [81] adopted was to use the HMBC data as a starting point and then connect it to the TOCSY data, and vice versa. This loop was followed until a full dead end, i.e. the end of the molecule, was reached. HSQC data were used to couple the $^{1}H$ assignment derived from the TOCSY data with $^{13}C$ assignments. The HSQC data also acted as a cross-check and verification of the TOCSY and HMBC data. Once the assignments and connectivities of the carbons and protons have been determined a "fingerprint" was established, which was then used to search data-bases. If more then one molecule was found to match-up with this "fingerprint" then chemical and NOM knowledge was used to eliminate unlikely candidates. In all such cases only one full match was found [81]. Thus, this allowed for an identification of molecules that can be used in the study on NOM by simulation methods such as molecular mechanics. Also, this allows one to put forward molecular level based models, rather than the moiety level models derived by previous studies. However, it should be noted that the Cook et al. method of analysis is extremely time-consuming and will need to be automated before it can be used as a standard method for the analysis of complete data sets obtained by multiple 2D NMR methods on a NOM sample.

A few words of caution in regards to the use and analysis of 2D liquid-state NMR data are important at this time. Due to relaxation processes one may *not* be seeing all the entities present in NOM. This is especially the case for large entities which may be invisible to the NMR experiments as they will have very short $T_2$ induced by slow tumbling rates in solution, and thus, experience a less homogenous magnetic field. This, in turn, leads to these entities producing broad NMR lines which will be lost in the baseline; likewise for samples which contain paramagnetic centers. Also, in the case of 2D experiments the situation becomes worse the longer the pulse sequence takes to evolve, and thus more signals are lost due to $T_2$ relaxation. This leads to a strong emphasis on small molecules. Similar biasing of data towards small molecules is also highly probable in FT-ICR-MS data due to the ionization step. Another area of concern when interpreting 2D data is the effect of conformation on chemical shift, especially when comparing the data to model compounds as done by Fan et al. [98], Hertkorn et al. [95], and Cook et al. [81]. A third and related concern is the effect of solvent on the observed chemical shifts. Again, this is more of a concern when comparing the obtained data to those of model compounds, and is the other reason why so few molecular models were put forward to date [81]. In addition, when critically comparing results, one must make sure that the conditions under which the NMR data were obtained are identical (or that the differences between them have been accounted for).

## Combining 2D techniques into one experiment

Although the 2D methods presented above are very valuable in their own right, even more information is possible by combining the techniques either in a time economic 2D method or into a full-blown 3D method. Although this is very tempting, $T_2$ relaxation becomes an even bigger concern. However, both Cook et al. [81] and Simpson et al. [100] have successfully interrogated NOM samples with a 2D combination of TOCSY and HSQC. The power of expanding into three dimensions is very apparent from the work of Simpson et al. [106]. However, it appears as if one loses signal due to the relaxation and the long evolution time of the pulse sequence in both the 3D case (signals apparent in the 2D HSQC spectrum are not apparent in the 3D HMQC-TOCSY spectrum [106]) and in the 2D case (signals in the HSQC spectrum were not apparent in the TOCSY-HSQC data [81]). Thus, while the data obtained from these combination experiments can be very informative it must be kept in mind that one is, in all probability, editing the data due to $T_2$ relaxation.

## Through-space connectivities

This class of NMR experiments is based on dipole–dipole interactions which are usually observed by nuclear Overhauser enhancement spectroscopy (NOESY). It is nearly always a proton–proton experiment, and thus will be considered as such throughout this paper. NOESY has proven itself to be the most important class of NMR experiments in the study of macromolecular structures, especially in the biological sciences. However, when applied to NOM the results have yielded very poorly resolved spectra [82, 91, 96, 98]. In fact, only in the work reported by Simpson [82] does one see much resolution at all. The major reason for this, as pointed out by Hertkorn [96], arises from the fact that contributions from chemical exchange "swamp" those from spatial relationships. Another concern is that compounds with molecular weights of between 750 to 2000 Da [76] may be invisible in a NOESY spectrum due to correlation time considerations, depending on the static magnetic field, the viscosity of the solution, internal mobility, and molecular shape and size [111]. However, there is a solution to both of these issues in the form of rotational frame Overhauser enhanced spectroscopy (ROESY). In this experiment the protons are spin locked in the rotating frame during the evolution period. This causes the correlation time to become a non-factor, and in addition, it phases peaks due to exchange and the diagonal peaks 180° to the true NOE peaks. Thus, in theory, one should be able to produce a spectrum that shows only NOE peaks but, if there is overlap with stronger exchange peaks then, once again, no information is obtained. It should be noted, however, that signals obtained in a ROESY spectrum are only about one fourth of the intensity of signals obtained in a comparable NOESY experiment. Hertkorn et al. [96] shown that, even in dry DMSO, exchange is a major problem which severely limits the amount of spatial relationship information one can obtain for NOM samples. Nevertheless, Simpson [82] has shown that informative NOESY and ROESY spectra can be obtained. Again, this difference is most probably due to the sample being analyzed and shows that further study is needed into the applicability of NOESY or ROESY spectral methods to NOM samples.

Although not discussed here, diffusion based techniques are also very useful in the study of NOM as shown by the work of Larive and co-workers [112] and more recently by Simpson [106].

## Conclusion

As can be seen from the discussion above, the characterization of NOM by NMR is a very exciting and rapidly growing area, especially in multi-dimensional NMR. Although the application of multi-dimensional methods to NOM samples still needs further study, already these techniques are being used to look at and derive information on some very difficult "real world" environmental issues as shown by the work of Mao et al. [47], Hertkorn et al. [94], and Kaiser et al. [107]. The advances are expected to continue, especially by combining analytical techniques such as NMR and mass spectroscopy with a method of separation at the front end, otherwise known as high-performance liquid chromatography–NMR–MS (or hyphenated techniques). The progress will be further accelerated as manufacturers realize there is a market for instruments dedicated to NOM characterization, as the work of Simpson with Bruker has demonstrated [101, 102]. However, we must be careful not to create a situation where only a handful of NOM research groups have access to the instrumental capabilities. Since the review by Preston in 1996 [18] great progress has been made and some of the challenges she put forward have been met, such as the application of multi-dimensional NMR methods. With progress being unstoppable, the methods that are now considered to be state-of-the-art will not be for long, promising further insight into the complex systems that are known as NOM.

## References


1. Stevenson FJ (1994) Humus chemistry; genesis, composition, reactions, 2nd edn. Wiley, New York
2. Hatcher PG, Dria KJ, Swunghwan K, Frazier SW (2001) Soil Sci 166:770–794
3. Kujawinski EB (2002) Environ Forensics 3:207–216
4. Stenson AC, Marshall AG, Cooper WT (2003) Anal Chem 75:1275–1284
5. Stenson AC, Langing WM, Marshall AG, Cooper WT (2003) Anal Chem 74:4397–4409
6. Fievre A, Solouki T, Marshall AG, Cooper WT (1997) 11: 554–560
7. Kujawinski EB, Hatcher PG, Freitas MA (2002) Anal Chem 74:413–419
8. Brown TL, Rice JA (2000) Anal Chem 72:384–390

9. Alomary A, Solouki T, Patterson HH, Cronan CS (2000) Environ Sci Technol 34:2830–2838
10. Solouki T, Freitas MA, Alomary A (1999) Anal Chem 71: 4719–4726
11. Simpson AJ (2001) Soil Sci 166:795–809
12. Zakrezewska J, Zujovic Z, Vucelic D (2000) New Adv Anal Chem:P1/291–P1/358
13. Mathers NJ, Mao XA, Xu ZH, Saffigna PG, Berners-Price SJ, Perera MCS (2000) Aust J Soil Res 38:769–787
14. Knicker H, Kogel-Knabner I (1998) ACS Symp Ser 707: 339–356
15. Dec J, Bollag J-M (1997) Soil Sci 162:858–874
16. Kogel-Knabner I (1997) Geoderma 80:243–270
17. Leenheer JA (1997) Characterization of natural organic matter by nuclear magnetic resonance spectroscopy. In: Nanny MA, Minear RA, Leenheer JA (eds) Nuclear magnetic resonance spectroscopy in environmental chemistry. Oxford University Press, New York, pp 213–220
18. Preston CM (1996) Soil Sci 161:144–166
19. Wershaw RL, Mikita MA (1987) (eds) NMR of humic substances and coal: techniques, problems, and solutions. Lewis, Chelsea
20. Wilson MA (1987) NMR techniques and applications in geochemistry and soil chemistry, Pergamon, New York
21. Axelson DE (1985) Solid-state nuclear magnetic resonance of fossil fuels. Multiscience, Minister of Supply and Services Canada (M39–16/1985E)
22. Schmidt-Rohr K, Spiess HW (1994) Multidimensional solid-state NMR and polymers. Academic Press, New York
23. Stejskal EO, Memory JD (1994) High resolution NMR in the solid state; fundamentals of CP/MAS. Oxford University Press, New York
24. Deur MJ (2002) (ed) Solid-state NMR spectroscopy; principles and applications. Blackwell, Malden
25. Kolodziejski W, Klinowski J (2002) Chem Rev 102:613–628
26. Smernik RJ, Oades JM (2000) Geoderma 96:159–171
27. Smernik RJ, Baldock JA, Oades MJ (2002) Solid State Nucl Magn Reson 22:71–82
28. Smernik RJ, Baldock JA, Oades MJ, Whittaker AK (2002) Solid State Nucl Magn Reson 22:50–70
29. Smernik RJ, Oades JM (2003) Euro J Soil Sci 54:103–116
30. Stejskal EO, Schaefer J, Waugh JS (1977) J Magn Reson 28: 105–112
31. Peersen OB, Wu X, Smith SO (1993) J Magn Reson Ser A 106:127–131
32. Cook RL, Langford CH (1999) A biogeopolymeric view of humic substances with application to paramagnetic metal effects on $^{13}C$ NMR. Special publication, Royal Society of Chemistry, 247 (Understanding Humic Substances) pp 31–48
33. Cook RL, Langford CH (1999) Polymer News 24:6–15
34. Smernik RJ, Oades JM (2000) Commun Soil Sci Plant Anal 31:3011–3026
35. Wilson MA, Vassallo AM, Perdue EM, Reuters JH (1987) Anal Chem 59:551–567
36. Keeler C, Maciel GE (2003) Anal Chem 75:2421–2432
37. Smernik RJ, Oades JM (2001) Solid State Nucl Magn Reson 20:74–84
38. Preston CM (2001) Can J Soil Sci 81:225–270
39. Preston CM (2000) Can J Soil Sci 80:227–229
40. McGill WB, Roy JL (2000) Can J Soil Sci 80:231–234
41. Hu W-G, Schmidt-Rohr K (2000) Polymer 41:2979–2987
42. Mao J-D, Hu W-G, Schmidt-Rohr K, Davies G, Davies EA, Xing B (2000) Soil Sci Soc Am J 64:873–884
43. Snape CE, Axelson DE, Botto RE, Delpuech JJ, Tekely P, Gerstein BC, Pruski M, Maciel GE, Wilson MA (1989) Fuel 68:547–560
44. Mao J-D, Xing B, Schmidt-Rohr K (2001) Environ Sci Technol 35:1928–1934
45. Schmidt-Rohr K, Mao J-D (2002) J Magn Res 157:210–217
46. Schmidt-Rohr K, Mao J-D (2002) J Am Chem Soc 124: 13938–13948
47. Mao J-D, Hundal LS, Schmidt-Rohr K, Thompson ML (2003) Environ Sci Technol 37:1751–1757
48. Cook RL, Langford CH, Yamdagni R, Preston CM (1996) Anal Chem 68:3979–3986
49. Cook RL, Langford CH (1998) Environ Sci Technol 32:719–725
50. Dria KJ, Sachleben JR, Hatcher PG (2002) J Environ Qual 31:393–401
51. Mao J-D, Hu WG, Ding G, Schmidt-Rohr K, Davies G, Ghabbour EA, Xing B (2002) Int J Environ Anal Chem 82:183–196
52. Dixon WT (1982) J Chem Phys 77:1800–1809
53. Dixon WT, Schaefer J, Sefcik MD, Stejskal EO, Mckay RA (1982) J Magn Reson 49:341–345
54. Axelson DE (1985) Fuel 66:195–199
55. Peuravuori J, Ingman P, Pihlaja K (2003) Talanta 59:177–189
56. Peerseen OB, Wu X, Kastanovich I, Smith SO (1993) J Magn Reson Ser A 104:334–339
57. Metz G, Ziliox M, Smith SO (1996) Solid State Nucl Magn Reson 7:155–160
58. Metz G, Wu X, Smith SO (1994) J Magn Res Ser A 110: 219–227
59. Mehring M (1983) Principles of high resolution NMR in solids. Springer, Berlin Heidelberg New York
60. Nielsen NC, Bildsoe H, Jakobsen HJ (1992) J Magn Res 98:665–673
61. Horne D, Kendrick RD, Yannoni CS (1983) J Magn Res 52:299–304
62. Marks D, Vega S (1996) J Magn Res 118:157–172
63. Randall EW, Mahieu N, Ivanova GI (1997) Geoderma 80: 307–325
64. Wind RA, Maciel GE, Botto RE (1993) Carbon-13 NMR spectroscopy of carbonaceous solids. In: Botto RE, Sanada Y (eds) Magnetic resonance of carbonaceous solids. Am Chem Soc, Washington, DC, pp 3–26
65. Wu X, Burns ST, Zilm KW (1994) J Magn Res Ser A 111: 29–36
66. Keeler C, Maciel GE (2000) J Mol Struct 550/551:297–305
67. Mao J, Ding G, Xing B (2002) Commun Soil Sci Plant Anal 33:1679–1688
68. Caravatti P, Braunschweiler L, Ernst RR (1983) Phys Lett 100:305–310
69. vanRossum BJ, Forster H, deGroot HJM (1997) J Magn Reson 124:516–519
70. Lesage A, Sakellariou D, Steuernagel S, Emsley L (1998) J Am Chem Soc 120:13194–13201
71. Lee M, Goldgerg WI (1965) Phys Rev 140:A1261–A1271
72. Purcell EM, Torrey HC, Pound RV (1946) Phys Rev 69:37–38
73. Bloch F, Hansen WW, Packard M (1946) Phys Rev 69:127–130
74. Cavanagh J, Fairbrother WJ, Palmer AG, Skelton NJ (1996) Protein NMR spectroscopy; principles and practice. Academic Press, New York
75. Levitt MH (2001) Spin dynamics: basics of nuclear magnetic resonance. Wiley, New York
76. Reynolds WF, Enriquez RG (2002) J Nat Prod 65:221–224
77. Braun S, Kalinowski H-O, Berger S (1998) 150 and more basic NMR experiments; a practical course. Wiley–VCH, New York
78. Piotto M, Saudek V, Sklenar V (1992) J Biomol NMR 2:661–665
79. Sklenar V, Piotto M, Leppik R, Saudek V (1993) J Magn Reson A 102:241–245
80. Lee GSH, Wilson MA, Young BR (1998) Org Geochem 28: 549–559
81. Cook RL, McIntyre DD, Langford CH, Vogel HJ (2003) Environ Sci Technol. 37:3935–3944
82. Simpson A (2001) Soil Sci 166:795–809
83. Wang K, Dickinson CL, Ghabbour EA, Davies G, Xing B (2003) Soil Sci 168:128–136
84. Shin H, Moon H (1996) Soil Sci 161:250–256

85. Shin HS, Rhee SW, Lee BH, Moon CH (1996) Org Geochem 24:523–529
86. Ivanova GI, Randall EW (2003) Cent Eur J Chem 1:10–26
87. Buddrus J, Burba P, Lambert J, Herzog H (1989) Anal Chem 61:628–631
88. Haiber S, Burba P, Herzog H, Lambert J (1999) Fresenius J Anal Chem 364:215–218
89. Haiber S, Herzog H, Burba P, Gosciniak B, Lambert J (2001) Fresenius J Anal Chem 369:457–460
90. Haiber S, Herzog H, Burba P, Gosciniak B, Lambert J (2001) Environ Sci Technol 35:4289–4294
91. Chien Y-Y, Bleam WF (1998) Environ Sci Technol 32: 3653–3658
92. Wang L, Mao X, Yang Y (1998) Bopuxue Zazhi 15:411–420
93. Schmitt-Kopplin P, Hertkorn N, Schulten H-R, Kettrup A (1998) Environ Sci Technol 32:2531–2541
94. Hertkorn N, Claus H, Schmitt-Kopplin P, Perdue EM, Filip Z (2002) Environ Sci Technol 36:4334–4345
95. Hertkorn N, Permin A, Perminova I, Kovalevskii D, Yudov M, Petrosyan V, Kettrup A (2002) J Environ Qual 31:375–387
96. Hertkorn N, Schmitt-Kopplin P, Perminova IV, Kovalevskii D, Kettrup A (2001) Two dimensional NMR spectroscopy of humic substances. In: Swift RS, Sparks KM (eds) Proc 9th Int Conf of the Int Humic Substances Soc, Understanding and Managing Organic Matter is Soils, Sediments, and Waters, University of Adelaide, Australia 21–25 September 1998. IHSS, St Paul, MN, pp 149–158
97. Morris KF, Cutak BJ, Dixon AM, Larive CK (1999) Anal Chem 71:5315–5321
98. Fan TW-M, Higashi RM, Lane AN (2000) Environ Sci Technol 34:1636–1646
99. Kingery WL, Simpson AJ, Hayes MHB, Locke MA, Hicks RP (2000) Soil Sci 165:483–494
100. Simpson AJ, Burdon J, Graham CL, Hayes MHB, Spencer N, Kingery WL (2001) Euro J Soil Sci 52:495–509
101. Simpson AJ, Kingery WL, Shaw DR, Spraul M, Humpfer E, Dvortsak P (2001) Environ Sci Technol 35:3321–3325
102. Simpson AJ, Kingery WL, Spraul M, Humpfer E, Dvortsak P, Kerssebaum R (2001) Environ Sci Technol 35:4421–4425
103. Simpson AJ, Kingery WL, Hayes MHB, Spraul M, Humpfer E, Dvortsak P, Kerssebaum R, Godejohann M, Hofmann M (2002) Naturwissenschaften 89:84–88
104. Simpson AJ, Salloum MJ, Kingery WL, Hatcher PG (2002) J Environ Qual 31:388–392
105. Simpson AJ (2002) Magn Reson Chem 40:S72–S82
106. Simpson AJ, Kingery WL, Hatcher PG (2003) Environ Sci Technol 37:337–342
107. Kaiser E, Simpson AJ, Dria KJ, Sulzberger B, Hatcher PG (2003) Environ Sci Technol 37:2929–2935
108. Hull WE (1994) In: Croasmun WR, Carlson RMK (eds) Two-dimensional NMR spectroscopy; applications for chemists and biochemists, 2nd edn. VCH, New York, p 307
109. Bax A, Ikura M, Kay LE, Torcia DA, Tschudin R (1990) J Magn Reson 86:304–318
110. McLean S, Reynolds WF, Wang JP, Jacobs H, Jean-Pierre LL (1994) Magn Reson Chem 32:422–428
111. Neurhaus D, Williamson MP (2000) The nuclear Overhauser effect in structure and conformation analysis, 2nd edn. Wiley–VCH, New York, p 37
112. Dixon AM, Larive CK (1999) Appl Spectrosc 53:426A–440A