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Does solid-state ^{15}N NMR spectroscopy detect all soil organic nitrogen?

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Abstract. Virtually all of the N detected by ^{15}N cross polarization (CP) NMR spectra of four HF-treated soil clay fractions is amide N. However, the intensity of this ^{15}N CP NMR signal (per unit N) is 27–57% lower than detected for a wheat protein, gliadin. There are two possible explanations – either the amide N in the soil clay fractions produces proportionately less NMR signal than does the amide N in gliadin, or part of the N in the soil clay fractions produces little or no NMR signal. The cross polarization dynamics of the gliadin amide resonance and amide resonances detected for the soil clay fractions are very similar and thus should produce similar amounts of signal, ruling out the first possibility. Therefore up to half or even more of the organic N in these soil clay fractions must be in a form that is insensitive to NMR detection. For a model compound (caffeine), non-protonated heterocyclic N produced less than 20% of the signal of an equivalent amount of amide N in gliadin. Results from several ^{13}C NMR techniques provide further evidence that much of the undetected N in the soil clay fractions may be heterocyclic.

Introduction

More than 90% of soil nitrogen (N) exists in an organic form (Schulten and Schnitzer 1998; Friedel and Scheller 2002). Organic N also accounts for a similarly large and important pool of N in sediments (Patience et al. 1992; Knicker et al. 1996a; Knicker and Hatcher 1997; Zang and Hatcher 2002) and ocean waters (McCarthy et al. 1997). In all of these environments (soils, sediments and ocean waters), the supply of N to plants and microorganisms is in large part controlled by the rate of organic N mineralization. Despite the importance of organic N in a range of settings, there remains considerable disagreement within the scientific community as to its chemical nature (Schulten and Schnitzer 1998). This disagreement arises from seemingly contradictory results from different analytical techniques, each of which either leaves a sizable fraction of N unaccounted for or is subject to potential biases, or both.

The most long-standing method to characterize organic N involves acid hydrolysis followed by chemical or chromatographic detection and quantification of small molecular products such as amino acids, amino sugars and

nucleotides (Schulten and Schnitzer 1998 and references therein; Greenfield 2001; Friedel and Scheller 2002). Based on numerous published studies on acid hydrolysis, Schulten and Schnitzer (1998) concluded the 'average' composition of soil organic N to be 40% amino acid, 5–6% amino sugar, 35% heterocyclic N and 19% ammonium. However, in this analysis, whilst the amino acid and amino sugar contents are based on their identification and quantification in the hydrolysates, the contribution of heterocyclic N is not based on direct detection, but is mostly made up of 'non-hydrolysable' and 'unidentified hydrolysable' fractions. Only around 3% of total soil N can be directly identified as heterocyclic N in nucleotides (Schulten and Schnitzer 1998). Another complication is that the ammonium content includes organic N that is hydrolysed to ammonium under the reaction conditions (Schulten and Schnitzer 1998).

By using variations on the standard conditions of hydrolysis, the proportion of soil N that can be hydrolysed may be increased. Leinweber and Schulten (2000) found that treatment of the residue of the standard hydrolysis procedure with a dithionite/citrate/bicarbonate (DCB) reagent that removes pedogenic oxides, released around one third of the N that had been considered non-hydrolysable. Hydrolysis of the DCB-treated residue released even more N, much of which was identified as amino acid N. Greenfield (2001) found that for many soils a combination of acid and alkaline hydrolysis released more N from soils than did acid hydrolysis alone. Both of these studies suggest that the figure of 35% heterocyclic N is high, even if all of the non-hydrolysable and unidentified hydrolysable N is indeed heterocyclic N.

The main rationale for assuming that the unidentified organic N is heterocyclic is that most other organic N forms should be hydrolysable in strong acid. Processes such as the Maillard reaction have been identified that combine amino acid and carbohydrate precursors into highly-coloured, nitrogen-rich, high molecular weight materials (Benzing-Purdie et al. 1983; Benzing-Purdie 1986; Skene et al. 1997; Schulten and Schnitzer 1998). Heterocyclic N has been identified in these products using ^{15}N NMR spectroscopy (Benzing-Purdie et al. 1983; Benzing-Purdie et al. 1986; Skene et al. 1997).

Analytical techniques based on pyrolysis followed by chromatographic identification of the organic fragments released provide some evidence for non-hydrolysable heterocyclic N in soils (Leinweber and Schulten 1998; Schulten and Schnitzer 1998 and references therein; Leinweber and Schulten 2000), although heterocyclic N can also be produced from protein under pyrolysis conditions (Zang and Hatcher 2002). Patience et al. (1992) reported that the majority of organic N in some marine surface sediments was heterocyclic, based primarily on results from X-ray photoelectron spectroscopy (XPS). However, a more recent XPS study (Keleman et al. 2002) casts doubt on the assignments of this earlier study.

Solid-state ^{15}N NMR spectroscopy provides an alternative technique for characterizing organic N. Being a solid-state technique, it avoids the biases and artifacts inherent with methods that involve a chemical or pyrolytic degradation step. In principal, solid-state ^{15}N NMR spectroscopy should enable the

detection of all types of organic N with equal efficiency. In almost all ^{15}N NMR spectroscopic studies of soils, the vast majority of detected N is found in a single resonance at a chemical shift characteristic of amide N (Knicker et al. 1993; Clinton et al. 1995; Knicker 2000a; Knicker and Skjemstad 2000; Knicker et al. 2000). The presence of heterocyclic N in soils is detected as a small, indistinct shoulder at best (Mahieu et al. 2000). In fact, the ^{15}N NMR resonance for amino N is always more prominent than that for heterocyclic N in soils (Knicker et al. 1993; Clinton et al. 1995; Knicker 2000a; Knicker and Skjemstad 2000; Knicker et al. 2000; Mahieu et al. 2000). Schmidt-Rohr et al. (2004) recently reported the detection of substantial amounts of amide N attached to aromatic rings in soils used for insensitive rice production, using a novel NMR technique.

Nitrogen-15 NMR spectra of organic N in sediments (Knicker et al. 1996a; Knicker and Hatcher 1997; Knicker and Hatcher 2001) and dissolved organic N (McCarthy et al. 1997; Dignac et al. 2000) are similarly dominated by amide signal. To improve signal-to-noise ratios, a number of researchers have utilized ^{15}N labeling in composting studies. In such studies utilizing ^{15}N-labeled plant material (Knicker and Lüdemann 1995; Hopkins et al. 1997; Knicker 2002), ^{15}N labeled algal material (Knicker 2000b; Knicker et al. 1996a; Zang et al. 2001) or ^{15}N labeled nitrate and ammonium (Knicker et al. 1997; Bedrock et al. 1998; Cheshire et al. 1999), the signal from amide N predominated, and very little heterocyclic N was detected. On the other hand, incubations of plant material with ^{15}N-labeled urea (Benzing-Purdie et al. 1992; Skene et al. 1997) and humic fractions with ^{15}N-labeled nitrite (Thorn and Mikita 2000) formed considerable quantities of non-amide N. Incubations of soil with ^{15}N-labeled trinitrotoluene (TNT) also resulted in the formation of non-amide TNT-breakdown products containing various forms of organic N (Knicker et al. 1999; Bruns-Nagel et al. 2000; Knicker et al. 2001). Two types of organic matter in which the signal for heterocyclic N does constitute most of the ^{15}N NMR signal detected are chars (Derenne et al. 1993; Knicker et al. 1996b; Keleman et al. 2002), including the char fraction of soil (Knicker and Skjemstad 2000), and fossil organic matter such as coal and kerogen (Knicker et al. 1995; Knicker et al. 1996a; Keleman et al. 2002; Knicker et al. 2002).

Importantly, even after acid hydrolysis of some sediments, Knicker and Hatcher (1997, 2001) found that ^{15}N NMR still detected mostly amide N, showing N that survives hydrolysis should not automatically be assumed to be non-amide N. A mechanism of encapsulation within other organic matter components was proposed as the reason why the amide was not hydrolysable in these organic-rich sediments.

There are a number of potential shortcomings of ^{15}N NMR for the analysis of organic N. Nuclear magnetic resonance spectroscopy is inherently insensitive, and ^{15}N NMR spectroscopy especially so, given the low natural abundance (0.37%) and gyromagnetic ratio of the ^{15}N nucleus. Natural abundance ^{15}N NMR spectra of organic matter are therefore usually 'noisy', hampering identification of minor components. It has been pointed out that there are

many different types of heterocyclic N that resonate at different frequencies, and that detection of signal spread across a number of low-intensity peaks would be difficult in low signal-to-noise ratio spectra (Schulten and Schnitzer 1998). Studies that utilize ^{15}N labels only cover the early stages of the humification process and cannot probe aromatization processes that may take hundreds or thousands of years (Schulten and Schnitzer 1998). A potential for overlap of amide and heterocyclic N resonances also exists, although a technique called double cross polarization has been used to show this is not a major problem, at least for some ^{15}N-labeled composted materials (Knicker 2000b; Zang et al. 2001; Knicker 2002).

All natural abundance ^{15}N NMR studies of organic matter, and most that used ^{15}N labels, have employed the cross polarization (CP) technique. For natural abundance studies, using the CP technique is unavoidable, as the alternative direct polarization (DP) technique, otherwise known as Bloch decay (BD), is too insensitive to produce a signal on a practical timescale. The limitations of CP in solid-state ^{13}C NMR spectroscopy are well documented (Kinchesh et al. 1995; Preston 1996; Preston 2001). The proportions of aromatic and carbonyl C are often underestimated in ^{13}C CP NMR analyses of organic matter (Fründ and Lüdemann 1989; Mao et al. 2000; Smernik and Oades 2000b; Mao et al. 2002), due at least in part to slow rates of polarization transfer to ^{13}C nuclei remote from nearest ^{1}H neighbours. This problem is expected to be more severe for ^{15}N CP NMR spectra, as the lower gyromagnetic ratio of the ^{15}N nucleus results in a weakening of the critical dipolar coupling responsible for polarization transfer. Furthermore, the short contact times of 1 ms or less that are usually employed to characterize organic N (see, for example, Knicker et al. 1997; Knicker, 2000a, b; Knicker and Hatcher 2001; Knicker et al. 2002) can bias the acquisition of signal intensity toward directly protonated nitrogen types such as amide, at the expense of that derived from non-protonated N. Keleman et al. (2002) showed using model compounds that long contact times were required to acquire signal from non-protonated N efficiently. Furthermore, Keleman et al. (2002) found that the detection of non-protonated N was sensitive to the magnetic field strength of the spectrometer. On a 400 MHz spectrometer, pyridine N (non-protonated) gave roughly half the signal of pyrrole N (protonated) at a contact time of 5 ms, whereas on a 200 MHz spectrometer both N types gave signal of equal intensity at this contact time. Most ^{15}N NMR studies of organic N have been conducted on 300 or 400 MHz spectrometers.

It is perhaps, then, not surprising that amide N is the dominant N form detected in solid-state ^{15}N studies of organic N, as other forms are likely to be under-represented, to varying degrees, depending on the acquisition conditions. What is therefore required is a method to determine the proportion of N that is actually detected by NMR spectroscopy. Spin counting (Smernik and Oades 2000a, b) is such a technique. Spin counting measures the 'NMR observability' of a nucleus in a sample, expressed as the amount of NMR signal per unit mass of the nucleus in the sample, divided by the amount of NMR

signal per unit mass of the nucleus in a reference material known to give a quantitative NMR signal. We have successfully applied spin counting to solid-state ^{13}C CP NMR spectroscopy of organic matter, and shown that the observability of C (C_{obs}) in charcoal-like structures in soils is around 30%, compared to an observability of over 70% for most other organic matter components (Smernik and Oades 2000a, b). We have also shown that some alkyl structures in organic matter are under-represented in ^{13}C CP NMR spectra of soils (Smernik and Oades 2000b) and sewage sludge (Smernik et al. 2003a).

In this paper we apply a spin counting approach to answer the question "does solid-state ^{15}N NMR spectroscopy detect all soil organic nitrogen?" Since amide is the predominant form of N detected in soil organic matter, gliadin, a wheat protein in which most of the N is also amide, is used as a reference. In addition, spin counting is carried out on two model compounds, glycine and caffeine, to determine whether the different types of N in these molecules (amine and heteroaromatic) produce less signal than amide N in ^{15}N CP NMR spectra.

Materials and methods

Sample collection and preparation

The collection and preparation of the soil clay fractions used in this study has been described in detail previously (Baldock et al. 1992). Briefly, soils were collected from the A horizon (0–10 cm), air-dried and sieved to less than 2 mm. Soils were fractionated quantitatively on the basis of particle size following ultrasonic disruption. The 0.2–2 μm fractions were chosen for this study on the basis that this size fraction was relatively enriched in organic matter, and organic N in particular. Carbon contents of the soil clay fractions were determined used a LECO CR12 combustion furnace. Some properties of the whole soils are presented in Table 1.

The soil clay fractions were de-ashed with HF using the procedure of Skjemstad et al. (1994) in order to isolate the organic matter for NMR analysis. Treatment with HF provides two advantages: (i) it removes most paramagnetic species that can reduce the sensitivity of NMR detection, and (ii) it concentrates the organic matter by removing most of the mineral fraction.

Table 1. Classification and properties of the soils (adapted from Baldock et al. 1992).

Soil	Soil type	Climate	pH	Organic C (mg g^{-1})
Millicent	Mollisol	temperate	8.5	37
Mt Schank	Andosol	temperate	6.1	48
Malanda	Oxisol	tropical	5.4	61
Urrbrae	Alfisol	mediterranean	5.6	23

A number of studies have indicated that HF-treatment has minimal effect on organic matter chemistry (Skjemstad et al. 1994; Schmidt et al. 1997; Mathers et al. 2002; Keeler and Maciel 2003). Carbon and nitrogen contents of the HF-treated 0.2–2 μm soil fractions were determined using a Carlo-Erba 1001 CHN analyzer (Hedges and Stern 1984), and are presented in Table 2. Given that HF-treatment efficiently removes both inorganic C and inorganic N, the C:N ratios presented in Table 2 reflect C:N ratios of the organic matter. Corresponding carbon and nitrogen recoveries were similar for each sample, indicating that HF-treatment had minimal effect on C:N ratios. Carbon recoveries ranged from 57% to 85%, whilst N recoveries ranged from 62% to 86% (Table 2).

Glycine and gliadin were used as purchased from Ajax Chemicals, and Fluka Chemicals, respectively. Caffeine (Sigma Chemicals) was doped with 1% copper in order to increase the rate of T_1H relaxation. Solutions of caffeine and copper(II) chloride were mixed and the resultant solution was freeze-dried. This concentration of copper has been shown previously not to affect the intensity of NMR signals (Smernik and Oades 2000c).

NMR spectroscopy

Solid-state ^{13}C NMR spectra were acquired with magic angle spinning (MAS) and high-power ^{1}H decoupling on a Varian Unity 200 spectrometer fitted with a Doty Scientific high-speed MAS probe at a ^{13}C frequency of 50.3 MHz. Samples were packed into 7 mm diameter cylindrical zirconia rotors with Kel-F end-caps and spun at 5 kHz at the magic angle. Free induction decays were acquired with a sweep width of 40 kHz. A total of 1216 data points were collected for all spectra, representing an acquisition time of 15 ms. All spectra were zero filled to 8192 data points and processed with a 50-Hz Lorentzian line broadening and a 0.010-s Gaussian broadening. Chemical shifts were externally referenced to the methyl resonance of hexamethylbenzene at 17.36 ppm.

Carbon-13 CP spectra represent the accumulation of 4000 scans and were acquired using a 1 ms contact time and a 1 s recycle delay. Carbon-13 DP spectra represent the accumulation of 1000 scans and were acquired using a 90 s recycle delay. All DP spectra were corrected for background signal

Table 2. Carbon and nitrogen contents of the HF-treated 0.2–2 μm soil fractions, and carbon and nitrogen recovery on HF-treatment.

Soil	C content (mg g^{-1})	C recovery (%)	N content (mg g^{-1})	N recovery (%)	C:N ratio
Millicent	242.5	85	25.9	86	9.4
Mt Schank	493.5	57	53.1	62	9.3
Malanda	343.2	61	33.1	66	10.4
Urrbrae	228.0	78	20.9	71	10.9

(Smernik and Oades 2001). Background signal for CP spectra was generally less than 1% of total signal and was not corrected for.

Solid-state ^{15}N NMR spectra were acquired with magic angle spinning (MAS) and high-power ^{1}H decoupling on a Varian Unity INOVA 400 spectrometer with a Doty Scientific supersonic MAS probe at a ^{15}N frequency of 40.5 MHz. Samples were packed into 7 mm diameter cylindrical zirconia rotors with Kel-F end-caps and spun at 5 kHz at the magic angle. Free induction decays were acquired with a sweep width of 25 kHz. A total of 5000 data points was collected for all spectra, representing an acquisition time of 100 ms. All spectra were zero filled to 131072 data points and processed with a 50-Hz Lorentzian line broadening and a 0.010-s Gaussian broadening. Chemical shifts were externally referenced to glycine at 347 ppm (Knicker and Hatcher 2001).

Nitrogen-15 CP NMR spectra of the soil clay fractions represent the accumulation of 145×10^3 to 245×10^3 scans and were acquired using a 1 ms contact time and a 1 s recycle delay. Nitrogen-15 spin counting experiments are described in detail below.

Carbon-13 spin counting experiments were performed using the method of Smernik and Oades (2000a, b). Glycine was used as an external intensity standard (i.e. the glycine spectrum was acquired separately to those of the samples). For CP spin counting experiments, differences in spin dynamics between the sample and the glycine standard were accounted for using the method of Smernik and Oades (2000a), except that a variable spin lock (VSL) rather than a variable contact time (VCT) experiment was used to determine $T_{1\ \rho}H$ (Smernik et al. 2002). Errors in C_{obs} are estimated to be $\pm10\%$ in CP and $\pm15\%$ in DP (Smernik and Oades 2000a). Carbon-13 RESTORE NMR subspectra were generated using the method of Smernik and Oades (2003). The RESTORE technique generates ^{13}C NMR subspectra of organic matter components characterized by different cross polarization dynamics, and provides a way to examine the causes of quantitation deficiencies in CP spectra.

Results and discussion

^{15}N CP NMR spectra of the soil clay fractions

The ^{15}N CP NMR spectra of the soil clay fractions are shown in Figure 1. The spectrum of each soil contains a large, broad resonance centred at -260 ppm, which can be assigned to amide N. For the Mt Schank soil, a small resonance at -347 ppm is clearly discernable above the noise. This can be assigned to amine N. For the Mt Schank soil, the intensity of the amine peak was approximately 5% of that of the amide peak, as determined by spectral integration. An amine resonance is barely discernable for the Millicent and Malanda, but not for the Urrbrae soil clay fractions (Figure 1). Spinning

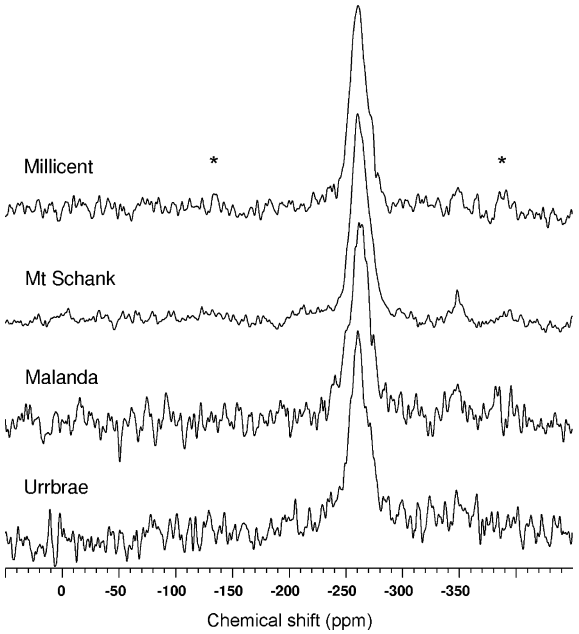

Figure 1. Solid-state ^{15}N CP NMR spectra of the HF-treated soil clay fractions. Number of scans acquired: Millicent, 156×10^3; Mt Schank, 245×10^3; Malanda, 145×10^3; Urrbrae 156×10^3.

side-bands of the amide peak, expected 125 ppm either side of the central band and denoted with asterisks in Figure 1, are also barely discernable for the Millicent and absent for the other soil clay fractions.

The spectra presented in Figure 1 are similar in appearance to previously published natural abundance ^{15}N CP NMR spectra of soils and soil clay fractions (Knicker et al. 1993; Knicker and Skjemstad 2000; Knicker et al. 2000; Mahieu et al. 2000). The spectra in Figure 1 result from the accumulation of 145×10^3 to 245×10^3 scans (see figure caption), over a period of 2 to 3 days. Previous studies have employed a shorter delay between consecutive scans (recycle delay) of 150 ms (Knicker and Skjemstad 2000) or 200 ms (Mahieu et al. 2000), enabling the accumulation of up to 3×10^6 scans (Knicker and Skjemstad 2000) in a similar time. The recycle delay of 1 s used in this study was chosen to avoid signal loss through saturation. This is a requirement for spin counting.

Comparison of ^{15}N CP NMR signal intensity – spin counting

The scarcity or absence of non-amide signal in ^{15}N CP NMR spectra of soil organic matter has often been taken as proof that N types other than amide are rare or absent. However, as discussed above, there are a number of reasons to suspect that other types of N, in particular those without directly attached ^{1}H

nuclei may be severely under-represented in ^{15}N CP NMR spectra. Therefore, it is important to gauge the quantity of NMR signal detected, as well as chemical shift distribution of the signal that is detected. Quantification of NMR signal intensity in this way is termed spin counting.

In its most simple form, spin counting involves calibration of signal intensity for the sample against that of a reference sample, taking into account the mass of N in each (Equation 1).

$$N_{\text{obs}}(\text{uncorrected}) = \frac{\left[\frac{\text{NMR signal intensity}}{\text{mass in rotor} \times \text{N content}}\right]_{\text{sample}}}{\left[\frac{\text{NMR signal intensity}}{\text{mass in rotor} \times \text{N content}}\right]_{\text{reference}}} \quad (1)$$

For this study, we have chosen gliadin, a commercially available wheat protein as our reference for ^{15}N NMR spin counting. The ^{15}N CP NMR spectrum of gliadin (Figure 2) is very similar to those of the soils (Figure 1), with an amide signal at −264 ppm by far the most intense, and indeed the only clearly discernable, resonance.

Values of N_{obs} (uncorrected), calculated for the soil clay fractions using Equation (1), with gliadin as a reference ranged from 43–73% (Table 3). In other words, the soil samples produced around one half to three quarters as much ^{15}N CP NMR signal as did the gliadin reference, even though amide N was the predominant signal in all cases. There are two possible explanations for this: (i) the amide N in soils produces less signal than the amide N in the gliadin reference or (ii) the soils contain non-amide N that is not detected. Both of these possibilities are discussed in detail in the following sections.

Cross polarization dynamics in ^{15}N CP NMR spectra of soil clay fractions and gliadin: Does the amide N in soils produce less signal than the amide N in gliadin?

The signal produced by a ^{15}N nucleus in a cross polarization experiment depends on the way ^{15}N and ^{1}H nuclei behave and interact in the presence of

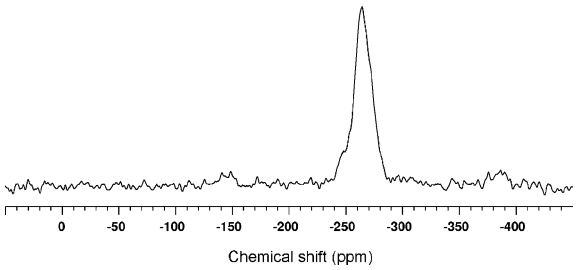

Figure 2. Solid-state ^{15}N CP NMR spectrum of gliadin (6592 scans, 1 ms contact time, 4 s recycle delay).

Table 3. Results of ^{15}N CP spin counting for HF-treated 0.2–2 μm soil fractions and model systems.

	N_{obs}(uncorrected) (%)	$T_{1\rho}H$ (ms)	N_{obs}(corrected) (%)
Gliadin	100[a]	3.45	100[a]
Millicent	73	3.22	74
Mt Schank	49	2.97	52
Malanda	43	3.25	44
Urrbrae	50	3.21	51
Glycine	101	26.45	78
Caffeine	22	105.77	16

[a]Gliadin N_{obs} values are 100% by definition.

spin locking radiofrequency pulses during the contact time. The dependence of signal intensity (I) on contact time (t) can be approximated by Equation (2).

$$I = \frac{1}{\alpha}[1 - \exp(-\frac{\alpha t}{T_{NH}})][\exp(-\frac{t}{T1\rho H})] \quad (2)$$

where I is the signal intensity, $\alpha = 1-(T_{NH}/T_{1\rho}H)$, the rate of cross polarization is $1/T_{NH}$, and the rate of ^1H relaxation is $1/\ T_{1\rho}H$. By varying the contact time, the effect of the key parameters controlling signal intensity (T_{NH} and $T_{1\rho}H$) can be examined.

Figure 3 shows the results of variable contact time (VCT) experiments on gliadin and one of the soil samples (Mt Schank). The VCT curves are very similar, indicating that rates of signal build-up (T_{NH}) and decay ($T_{1\rho}H$) during the contact time are also similar for the two materials. Therefore the low N_{obs}(uncorrected) value (49%) determined for this soil cannot be attributed to differences in the cross polarization dynamics between the amide signal

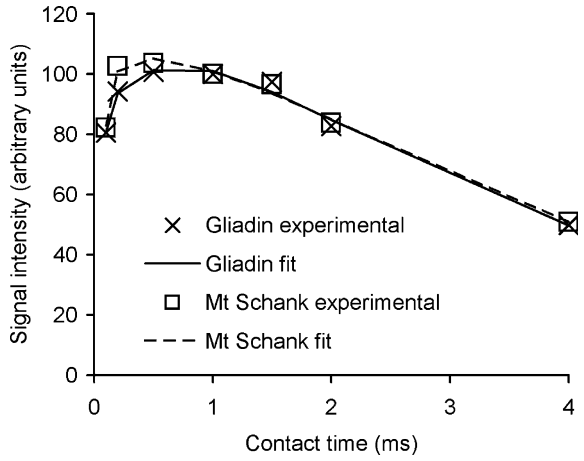

Figure 3. Plots of signal intensity versus contact time for ^{15}N variable contact time (VCT) NMR experiments on gliadin and Mt Schank soil. The curves provided are a guide only.

detected in the soil sample and the amide signal detected in the gliadin. In other words, it would appear that the reason that this soil clay fraction produced around half as much signal as gliadin was not because the detected nitrogen nuclei were only producing half as much signal, but rather half the N in the soil was producing virtually no detectable signal.

It is not feasible to routinely carry out ^{15}N VCT analysis for every soil sample. In order to obtain data for the Mt Schank soil sample with adequate signal-to-noise ratio, an acquisition time of over one week was required. However, one of the two key parameters, $T_{1\rho}$H, can be gauged more readily, via detection by ^{13}C, rather than ^{15}N ($T_{1\rho}$H is a property of the ^{1}H nuclei rather than the ^{15}N nuclei). This approach assumes that the ^{15}N nuclei are associated with the same ^{1}H population as are the ^{13}C nuclei. In one sense this is clearly true, as amide N must be associated with amide C at the very least. A process known as spin diffusion averages $T_{1\rho}$H at scales less than around 10 nm (Zumbulyadis 1983), a scale that represents tens of bond-lengths ($\sim$0.1–0.2 nm). It is true, however, that $T_{1\rho}$H is usually not uniform throughout samples of soil organic matter (Smernik and Oades 2003), and therefore it is possible that there exist N-rich domains of organic matter characterized by $T_{1\rho}$H relaxation rates more rapid than the average value for the organic matter, as detected via ^{13}C. The results of ^{13}C NMR experiment RESTORE, detailed below, strongly suggest that non-uniformity in $T_{1\rho}$H relaxation rates does not contribute to the low N_{obs}(uncorrected) values for the soils.

We have shown previously for our ^{13}C CP NMR spin counting method that the effect of differences in $T_{1\rho}$H relaxation rates between the reference and the soil sample on signal observability can be corrected for. Equation (3) incorporates a correction factor for ^{15}N CP NMR spectra acquired with a contact time of 1 ms, analogous to the one used in Smernik and Oades (2000a).

$$N_{obs}(corrected) = N_{obs}(uncorrected) \div \frac{\exp\left[-1/T_{1\rho}H\right]_{sample}}{\exp\left[-1/T_{1\rho}H\right]_{reference}} \qquad (3)$$

Values of N_{obs}(corrected), calculated using Equation (3), ranged from 44–74% (Table 3), and were 1–3% higher than the corresponding uncorrected values for each of the four soils. The small magnitude of the difference between uncorrected and corrected N_{obs} values is a consequence of the similarity of $T_{1\rho}$H values (Table 3) between gliadin (3.45 ms) and each of the four soils (2.97–3.25 ms). This contrasts with the much larger differences between uncorrected and corrected C_{obs} values we reported for ^{13}C spin counting experiments which used glycine ($T_{1\rho}$H = 26 ms) as a reference (Smernik and Oades 2000a).

Cross polarization dynamics in ^{15}N CP NMR spectra of glycine and caffeine

The previous section showed that the cross polarization dynamics of gliadin and soil organic matter amide ^{15}N CP NMR resonances were very similar. This

suggests that amide N produces essentially an equivalent amount of ^{15}N CP NMR signal whether it is in organic matter or in the reference (gliadin). In this section we show that other N types, in particular the amine N of glycine and the non-protonated heterocyclic N of caffeine, produce significantly less ^{15}N CP NMR signal at a contact time of 1 ms.

The ^{15}N CP NMR spectra of glycine and caffeine are shown in Figure 4. The ^{15}N CP NMR spectrum of glycine contains a single, sharp resonance at −347 ppm (Figure 4a). The ^{15}N CP NMR spectrum of caffeine contains four sharp resonances, at −152 ppm (partially split), −223, −230 and −265 ppm, one for each of the four N nuclei found in different chemical environments.

Figure 5 shows the results of VCT experiments on glycine and caffeine. Both signal build-up and signal decay are much slower for these two compounds than they are for gliadin and the Mt Schank soil (Figure 3). The glycine signal reaches maximum intensity at a contact time of 3 ms, whilst the caffeine signal reaches maximum intensity at a contact time of 15 ms. Signal build-up is slow for caffeine because its four nitrogens have no directly attached hydrogens. Although glycine has directly attached hydrogens, the molecular motion of the amine group reduces the strength of the N–H dipolar coupling that enables cross-polarization. A similar, though less severe, effect is observed for methyl

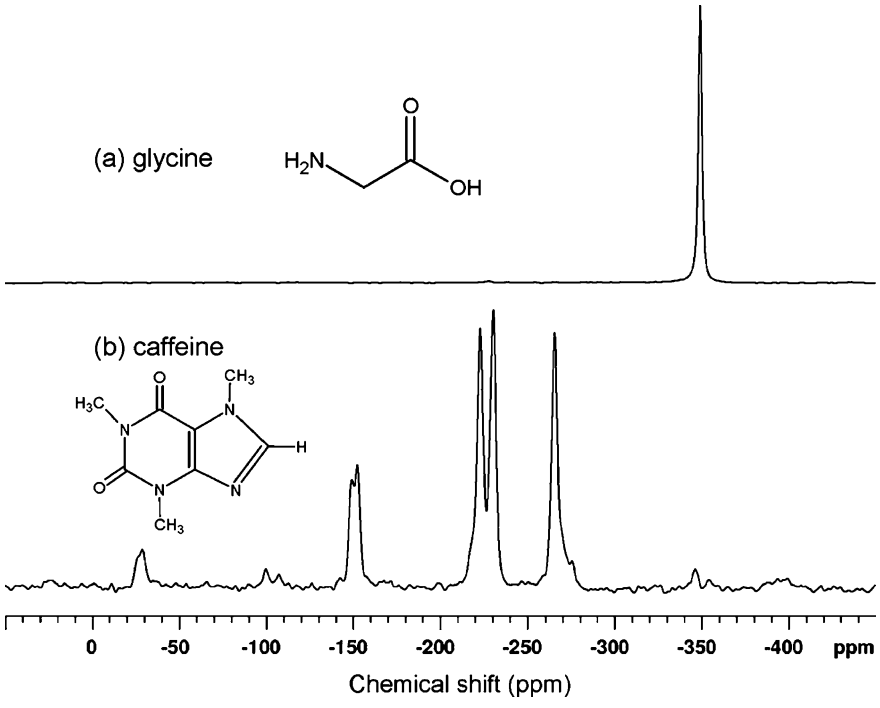

Figure 4. Solid-state ^{15}N CP NMR spectra of glycine (26368 scans, 1 ms contact time, 2 s recycle delay) and caffeine (18912 scans, 8 ms contact time, 3 s recycle delay).

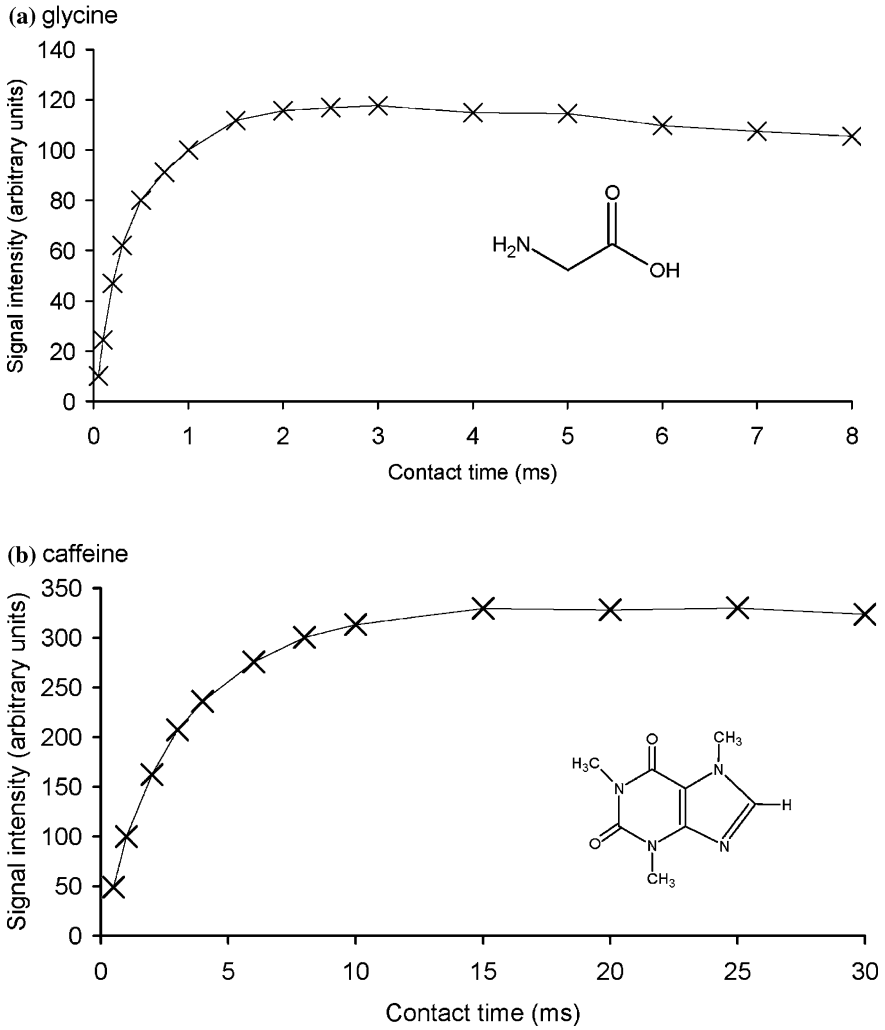

Figure 5. Plots of signal intensity versus contact time for ^{15}N variable contact time (VCT) NMR experiments on (a) glycine and (b) caffeine.

carbons in ^{13}C CP NMR spectra. The reason that high signal intensities are achieved at long contact times is that both of these compounds have long $T_{1\rho}H$ values (Table 3). If slowly cross-polarizing ^{15}N nuclei such as these exist in soil organic matter, they would not give strong signals at long contact times because $T_{1\rho}H$ relaxation in soils is much faster.

The results of ^{15}N CP NMR spin counting for caffeine and glycine are shown in Table 3. Values of N_{obs}(uncorrected) for glycine and caffeine were 101% and 22%, respectively, indicating that glycine N produces as much signal at a 1 ms contact time as does gliadin, whilst caffeine N produces less than one-quarter

as much signal. The $T_{1\rho}$H correction made a considerable difference for glycine and caffeine; N_{obs}(corrected) values were 78% and 16%, respectively. The N_{obs}(corrected) values are the relevant values when considering the biases of ^{15}N CP NMR spectra of the soil clay fractions.

It is evident from the VCT results for glycine that any amine N present in soil organic matter would be moderately under-represented (relative to amide N) under the conditions employed to obtain the ^{15}N CP NMR spectra of the soils. However, given that amine N resonances cover a relatively narrow range of chemical shifts around −350 ppm, and that the bias is only moderate, it is unlikely that amine N accounts for anything but a small proportion of the N that is not detected in the ^{15}N CP NMR spectra of the soils. It is further evident from the VCT results for caffeine that any non-protonated N present in soil organic matter would be severely under-represented under these acquisition conditions. Given that heterocyclic N resonances cover a broad range of chemical shifts (Schulten and Schnitzer 1998), it is not implausible that 25–50% of soil organic N could go undetected by ^{15}N CP NMR spectroscopy if it existed in heterocyclic structures.

^{13}C NMR spectra of the soil clay fractions

In this section we compare results from ^{13}C CP NMR spin counting to the ^{15}N CP NMR spin counting results, and employ ^{13}C direct polarization (DP) and RESTORE techniques to determine whether or not the N 'missing' from the ^{15}N CP NMR spectra of the soil clay fractions can be attributed to protein N that is under-represented by the CP technique.

The ^{13}C CP NMR spectra of the four soil clay fractions are shown in Figure 6. Of particular interest are resonances that can be attributed to protein structures. Each amino acid residue contains a carbon adjacent to N (α-amino C) that resonates at around 55 ppm and an amide carbon that resonates at around 173 ppm. Amino acid side-chains, which contain carbons in a wide variety of chemical environments, produce further resonances across the ^{13}C NMR spectrum. The amide carbon resonance coincides with resonances for other carbonyl carbon types such as carboxylic acids and esters, and it is not possible to determine how much of the carbonyl resonance in the ^{13}C CP spectra of the soil clay fractions (Figure 6) can be attributed to amide structures. The α-amino C resonance coincides with the resonance for methoxyl C. Methoxyl C in organic matter is mostly due to the presence of lignin residues. These soil clay fractions appear to contain very little in the way of lignin residues, as evidenced by the lack of a distinguishable O-aryl resonance at around 150 ppm in the ^{13}C CP NMR spectra (Figure 6). Therefore, it can be assumed that most of the signal in the 50–60 ppm region of the ^{13}C spectra is due to α-amino C in protein, and that the size of this resonance should give at least a qualitative gauge of the protein content of the soil clay fractions. The α-amino C (55 ppm) resonance is clearly strongest for the Millicent soil clay

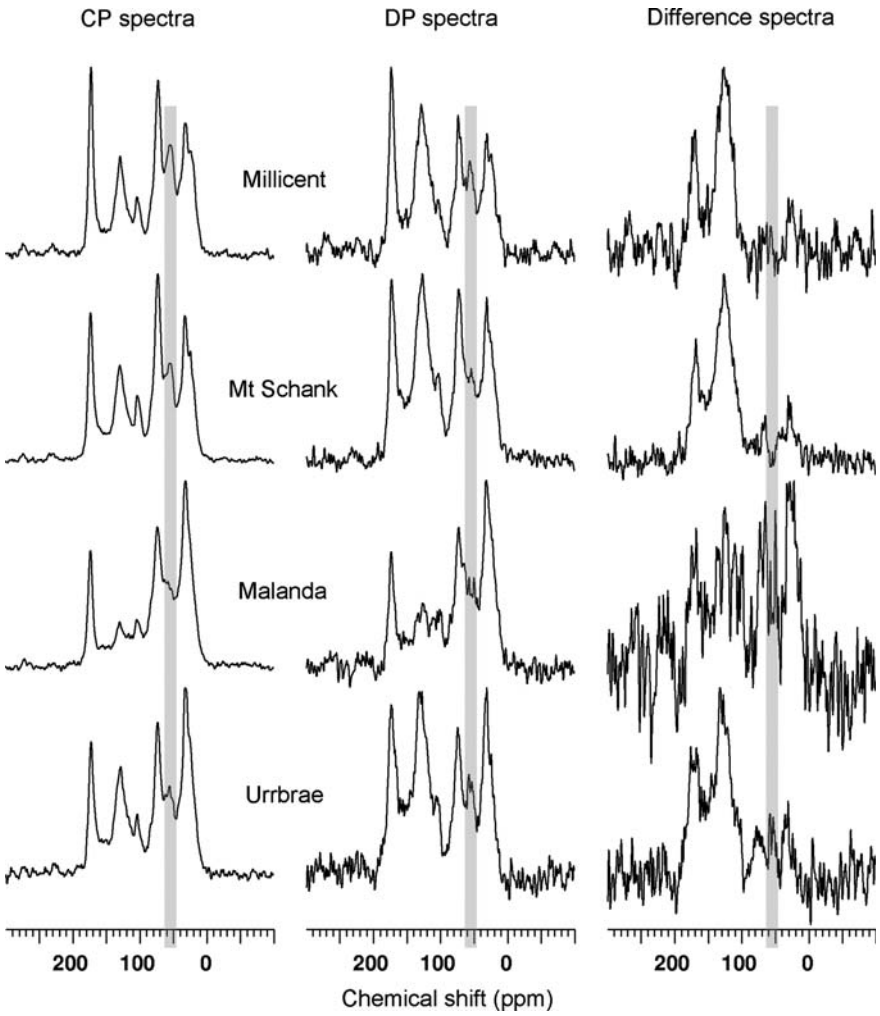

Figure 6. Solid-state ^{13}C CP and DP NMR spectra of the HF-treated soil clay fractions. The difference spectra were generated by subtracting corresponding CP from DP ^{13}C NMR spectra, after scaling for differences in the sensitivity of the two techniques. The difference spectra represent the signal detected by DP but not by CP. The shaded region around 55 ppm is where the resonances of the α-amino C of amino acid residues are expected.

fraction, which is consistent with the higher ^{15}N NMR observability for this soil. Note that the C:N ratios of the organic matter contained in the four soil clay fractions are all similar, in the range 9.3–10.9 (see Table 2, Materials and Methods section).

The ^{13}C CP NMR observabilities of the four soil clay fractions are presented in Table 4. Except for the Millicent soil clay fraction, the ^{13}C CP NMR observabilities are higher than the corresponding ^{15}N CP NMR observabilities

Table 4. Results of ^{13}C spin counting for the HF-treated 0.2–2 μm soil fractions and gliadin.

Soil	C_{obs}(CP) %	C_{obs}(DP)%
Millicent	70	104
Mt Schank	63	94
Malanda	61	86
Urrbrae	61	99
Gliadin	95	nd

(Table 3). The higher NMR sensitivity of the ^{13}C nucleus makes feasible the use of the direct polarization (DP), or Bloch decay (BD), technique. The DP technique is less sensitive than CP because DP produces less signal per scan and requires a longer delay between scans. The ^{13}C DP NMR spectra are shown in Figure 6 and ^{13}C DP NMR observabilities of the four soil clay fractions are presented in Table 4. For all four soil clay fractions, the DP observabilities were higher than the corresponding CP observabilities. In fact, the DP observabilities of 86–104% indicated that the DP spectra were close to quantitative for all four soil clay fractions.

Since the ^{13}C DP spectra are practically quantitative, the nature of the organic carbon 'missing' from the ^{13}C CP spectra can be determined by subtracting the CP spectra from the corresponding DP spectra, after accounting for the enhancement of signal intensity inherent with the CP technique. The 'difference spectra' so produced are presented in Figure 6. For three of the soil clay fractions (Millicent, Mt Schank and Urrbrae), the difference spectra were very similar in appearance, and closely resemble charcoal fractions isolated from soils (Skjemstad et al. 1996; Smernik et al. 2000). The strongest resonance in the difference spectra of these three soils was in the aromatic region, and there was also a substantial carbonyl resonance (Figure 6). This finding is consistent with the known low CP observability of charcoal C. The difference spectrum of the Malanda soil (Figure 6) was quite different to those of the other three soil clay fractions. The distribution of signal intensity in the difference spectrum of the Malanda soil clay fraction appeared similar to that of the Malanda CP and DP ^{13}C NMR spectra themselves. In other words, the distribution of carbon types 'missing' from the CP spectrum was similar to the distribution of carbon types detected by CP, i.e. CP signal loss was non-selective. The Malanda soil contains very little char, as evidenced by the low intensity of aromatic signal in its DP spectrum (Figure 6). The Malanda soil is an oxisol and contains large quantities of iron oxide. It may be that some of the iron survived the HF-treatment and was responsible for signal loss in the CP spectrum. Note that the DP observability for Malanda is the lowest of the four soils. It has been found that paramagnetic minerals can cause non-selective signal loss in both CP and DP spectra, with the CP spectra being considerably more sensitive to the presence of paramagnetic impurities (Smernik and Oades 2000a).

Importantly, the comparison of CP and DP ^{13}C NMR spectra provides no evidence that the resonance for α-amino C in protein at 55 ppm is underrepresented in the ^{13}C CP NMR spectra of the soils. In fact, for three of the four soil clay fractions, this resonance is considerably stronger in the CP spectrum than in the difference spectrum (Figure 6). If the vast majority of organic N were protein, then the expectation based on this result would be that ^{15}N CP observabilities should be higher than corresponding ^{13}C CP observabilities. This was only true for the Millicent soil clay fraction. That this was not the case for the other three soil clay fractions suggests that a substantial proportion of organic N in these soils is not in protein structures and is in a

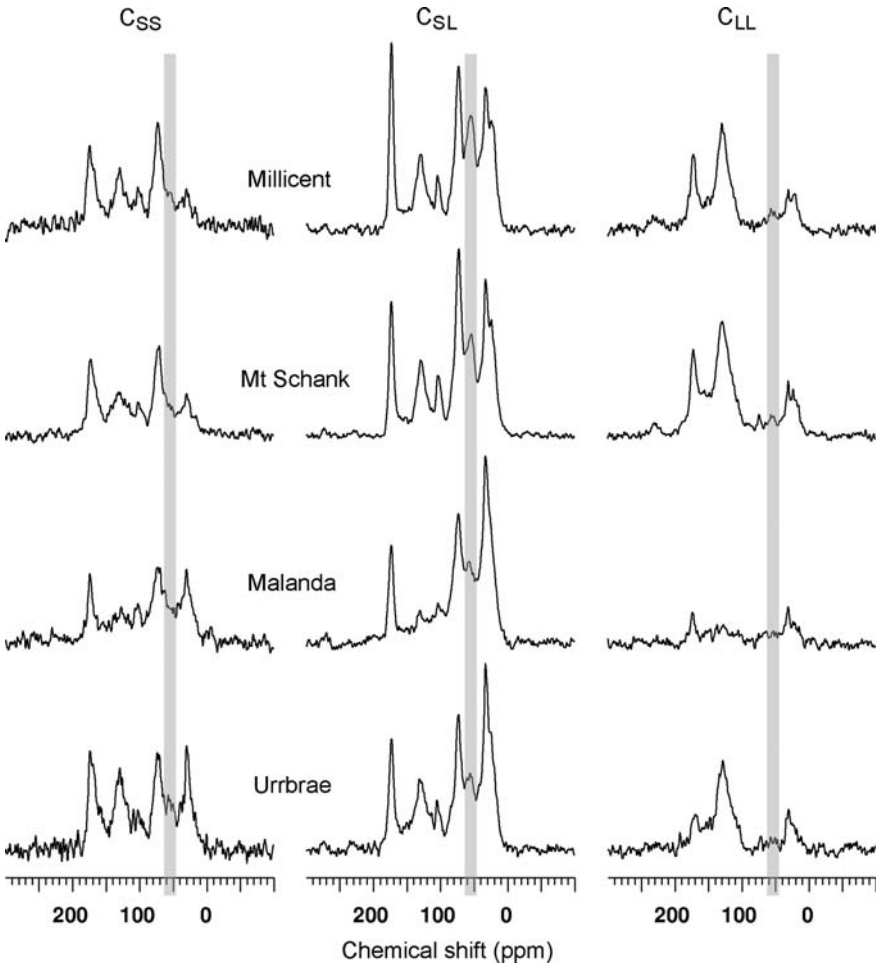

Figure 7. Solid-state ^{13}C RESTORE NMR subspectra of the HF-treated soil clay fractions. The shaded region around 55 ppm is where the resonances of the α-amino C of amino acid residues are expected.

Table 5. Results of RESTORE fit for HF-treated soil clay fractions.

	T_{CH}(short) (ms)	T_{CH}(long) (ms)	$T_{1\rho}$H(short) (ms)	$T_{1\rho}$H(long) (ms)	C_{SS} (%)[b]	C_{SL} (%)[b]	C_{LL} (%)[b]
Millicent	0.2[a]	3.61	0.79	3.30	27	48	25
Mt Schank	0.2[a]	4.27	0.80	2.97	20	56	24
Malanda	0.2[a]	4.09	0.47	3.36	36	52	11
Urrbrae	0.2[a]	3.23	0.80	3.49	40	46	14

[a]The value of T_{CH}(short) is fixed at 0.2 ms in the RESTORE fit (Smernik and Oades 2003).
[b]Percentage of total carbon detected by RESTORE in the RESTORE component.

form that is severely under-estimated by, or invisible to, ^{15}N CP NMR spectroscopy.

The RESTORE technique is another method that allows the biases of the CP technique to be gauged (Smernik and Oades 2003; Smernik et al. 2003b). The ^{13}C nuclei in organic matter exist in a wide range of chemical environments, which usually results in a range of different T_{CH} and $T_{1\rho}$H values. The RESTORE technique identifies and quantifies ^{13} C nuclei characterized by long T_{CH} values and short $T_{1\rho}$H values, which are under-represented in normal ^{13}C CP spectra. RESTORE generates three subspectra characterized by different combinations of T_{CH} and $T_{1\rho}$H. RESTORE subspectra of the four soil clay fractions are presented in Figure 7, and results of the RESTORE analysis are presented in Table 5. Component SS (C_{SS}) is characterized by short T_{CH} and $T_{1\rho}$H values and represents ^{13}C nuclei that are under-estimated in CP spectra due to rapid $T_{1\rho}$H relaxation. Component SL (C_{SL}) is characterized by a short T_{CH} value and a long $T_{1\rho}$H value and represents ^{13}C nuclei that are most easily observed in CP spectra. Component LL (C_{LL}) is characterized by long T_{CH} and $T_{1\rho}$H values and represents ^{13}C nuclei that are under-estimated in CP spectra due to slow cross polarization. Therefore, C_{SL} represents ^{13}C nuclei easily seen by CP, and C_{SS} and C_{LL} represent ^{13}C nuclei under-represented by CP.

For the Millicent and Mt Schank soil clay fractions, it is clear that the resonance at 55 ppm, which is due to α-amino C in protein, is strongest in the C_{SL} subspectra. This confirms the result from the comparison of ^{13}C CP and DP spectra above that protein structures are not under-represented in ^{13}C CP NMR spectra of the soil clay fractions and suggests that protein structures should not be under-represented in ^{15}N CP NMR spectra either. For the Malanda and Urrbrae soils, the situation is less clear, but there is certainly no suggestion that the resonance at 55 ppm is stronger in the C_{SS} and C_{LL} subspectra.

Conclusions

The vast majority of nitrogen detected by ^{15}N CP NMR analysis of four HF-treated soil clay fractions was amide N. This is consistent with many previous

studies. However, the soil clay fractions produced substantially less signal per unit of N than did a wheat protein, gliadin. Cross polarization dynamics were very similar for the amide signal detected for gliadin and the amide signal detected for the soil clay fractions, suggesting that undetected soil N is not in amide structures. Quantitative evaluation of ^{13}C NMR spectra provided further evidence against soil amide N being under-represented in ^{15}N CP NMR spectra of the soil clay fractions. On the other hand, the very slow rates of cross polarization observed for caffeine N showed that if non-protonated heterocyclic N was present in the soil clay fractions, it may have gone undetected, even if it represented a substantial proportion of total N. Furthermore, it was conclusively shown that for three of the four soil clay fractions, most of the ^{13}C under-represented by the CP technique was aromatic C. Whilst this does not prove that the 26–56% of N 'missing' from ^{15}N CP NMR analyses is heterocyclic, it certainly confirms this as a plausible explanation.

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