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Optimised NMR detection of $^{13}C–^{2}H$ double labelling in small molecules

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Abstract

[$^{13}C^{2}H_3$-*methyl*]-nicotine was used to optimise the selective NMR detection of the double $^{13}C–^{2}H$ label with a view towards subsequent metabolism studies. It is concluded that the INEPT transfer from ^{2}H to ^{13}C offers the best choice with regards to sensitivity, resolution and generality for different types of doubly labelled molecules. The necessary experimental arrangements are described. ***To cite this article: T. A. Bartholomeusz et al., C. R. Chimie 9 (2006)***.
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Résumé

De la [$^{13}C^{2}H_3$-*méthyl*]-nicotine a été utilisée pour optimiser la détection sélective du double marquage $^{13}C–^{2}H$ par RMN dans la perspective d'études du métabolisme. Il est conclu que le transfert INEPT du ^{2}H au ^{13}C est le meilleur choix en ce qui concerne la sensibilité, la résolution et la validité générale pour différents types de molécules doublement marquées. Les arrangements expérimentaux nécessaires sont décrits. ***Pour citer cet article : T. A. Bartholomeusz et al., C. R. Chimie 9 (2006)***.
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Mots clés : RMN ; Carbone-13 ; Deutérium ; INEPT ; Marquage isotopique stable ; Nicotine

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1. Introduction

Isotope labelling is a powerful tool to elucidate metabolic pathways [1] and to determine metabolic fluxes [2]. Nuclear magnetic resonance (NMR) is widely employed to detect and quantify labelled compounds resulting from the metabolisation of a fed stable isotope labelled substrate. A major advantage of NMR compared to mass spectroscopy or radioactivity measurements is that crude cell extracts can be analysed without further sample purification or derivatisation, and that the detection is essentially indiscriminate with respect to chemical properties of the metabolites. Thus it is possible to detect all sufficiently abundant small soluble metabolites in their chemical diversity. The main drawback of NMR, however, is its rather low sensitivity restricting analysis to the more abundant metabolites, though this is sufficient for many purposes.

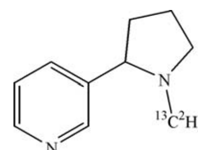
The most useful stable isotopes for NMR detected metabolic labelling experiments are ^{13}C and ^{2}H, elements which occur in nearly all metabolites, and ^{15}N which is profitable for specifically following the metabolism of nitrogen containing compounds. Between ^{13}C and ^{2}H, the former is much more commonly employed. It has a wide chemical shift range spreading the signals out and reducing overlap between different peaks. Its sensitivity can often be improved by employing indirect detection schemes via attached protons. However, ^{13}C has a natural abundance of 1.1%, which means that it can be difficult to distinguish small labelled metabolite pools from the natural abundance signals of major metabolites. On the contrary, ^{2}H has a very low natural abundance, only 0.02%, so that even small signals from labelled compounds are easily picked out. However, when there are several labelled compounds present, peak overlap rapidly becomes a problem since the chemical shift range is narrow, and moreover, peaks are relatively large owing to the fact that ^{2}H is a spin 1 nucleus with an albeit small quadrupole moment. Thus the strengths and weaknesses of ^{13}C and ^{2}H are quite complementary, and in certain cases it might be a good strategy to combine both labels.

In recent work [3] investigating the *d*emethylation of nicotine in plants, we fed (*R,S*)-[^{13}C-*methyl*]-nicotine to *Nicotiana plumbaginifolia* cells, and analysed extracts taken from cells after several days of culture with the labelled substrate. Several small peaks resulting from the metabolisation of the fed labelled nicotine could indeed be identified in 1D ^{13}C and 2D ^{1}H–^{13}C HMQC spectra. Nevertheless this result was obtained only after careful comparison with spectra obtained from cell extracts after feeding of unlabelled nicotine, and by showing that the peaks of interest increased with the duration of the feeding experiment. Parallel feeding experiments with (*R,S*)-[$^{2}H_3$-*methyl*]-nicotine showed the unequivocal appearance of ^{2}H-labelled metabolisation products but these could not be identified from the ^{2}H NMR spectra. In order to push the investigation further we are now conducting feeding experiments with doubly labelled (*R,S*)-[$^{13}C^{2}H_3$-*methyl*]-nicotine instead of using the isotopes separately. Of particular interest will be the question of whether the methyl-group is transferred as a whole, leading to $^{13}C^{2}H_3$-containing compounds, or only after oxidation, leading to $^{13}C^{2}H_2{}^{1}H$- or $^{13}C^{2}H^{1}H_2$-containing compounds.

The feasibility of a NMR detected $^{13}C^{2}H_3$ double labelling strategy for plant metabolic studies has recently been shown in the clarification of the biosynthesis pathway for artemisinin in *Artemisia annua* [4]. The NMR method chosen for this work [5] was ^{13}C-detected heteronuclear ^{13}C–^{2}H 2D correlation spectroscopy (COSY, sometimes also termed HETCORR). However, this method does not produce pure absorption peaks and has to be treated in absolute mode. A gain in sensitivity might therefore be expected from other methods, and the aim of the present paper is to explore different possible pulse sequences in order to choose the most sensitive for the planned metabolic study.

2. Material and hardware considerations

(*R,S*)-[$^{13}C^{2}H_3$-*methyl*]-nicotine (see below) was synthesised from nornicotine and [$^{13}C^{2}H_3$]-methyliodide as described by Mesnard et al. [3]. 6 µl of labelled nicotine were dissolved in 500 µl of H_2O and used directly for the NMR measurements.



NMR analysis was carried out on a Bruker Avance 500 spectrometer operating at 500.13 MHz,

125.69 MHz and 76.77 MHz for ^1H, ^{13}C and ^2H respectively. Two probeheads were used with similar results, a 5-mm broadband inverse detection (BBI) probehead and a 5-mm triple-resonance ^{13}C–^{15}N (TXI) probehead. In both cases, the lock circuit was used for ^2H and the broadband or ^{13}C circuit for ^{13}C. Spectra were run in unlocked mode, since the spectrometer was not equipped for using another lock nucleus than ^2H. 90° pulse lengths for ^{13}C were 14.6 µs (powerlevel –3 dB) with the BBI probehead and 10.8 µs (powerlevel –5 dB) with the TXI probehead. The ^2H circuit was quite prone to arcing on both probeheads and powerlevels of +3 dB had to be used leading to 90° pulse lengths of 25.1 and 23.0 µs on the BBI and TXI probehead, respectively. Trials to tune and match the ^2H circuit, which can be done only when the probehead is taken out of the magnet did not lead to any great improvements.

The broad-band preamplifier was used for both ^{13}C and ^2H detection. The ^2H preamplifier had to be physically disconnected to avoid a leakage lock signal in the ^2H spectrum (even when the lock mechanism was switched off by software control).

For experiments using both nuclei ^{13}C and ^2H, the spectrometer had to be equipped with a third radiofrequency channel, since on this Bruker spectrometer series, either channel 1 or channel 2 has to be on ^1H. Both ^{13}C and ^2H channels also had to be equipped with filters to avoid spectral noise at the detection frequency coming from the decoupling channel. The ^{13}C line was equipped with a standard ^2H-stop ^{13}C-pass filter and also a standard ^1H-stop. Not disposing of a standard filter for the ^2H line, we used instead a ^{13}C-pass ^{31}P-stop filter from a 300 MHz spectrometer. The frequencies were sufficiently close (75.47 vs. 76.77 MHz and 121.49 vs. 125.69 MHz, respectively) to those required that the ^2H pulse length was not affected by more than 0.4 µs and that the noise introduced at the ^{13}C frequency from ^2H-decoupling completely vanished. Without the filter, the decoupling noise was about 150 times stronger than the normal noise level. The ^1H line contained the usual ^1H high pass but this was not strictly necessary.

3. Spectroscopy results and discussion

The one-pulse undecoupled ^{13}C spectrum (Fig. 1) of the doubly labelled nicotine showed the expected

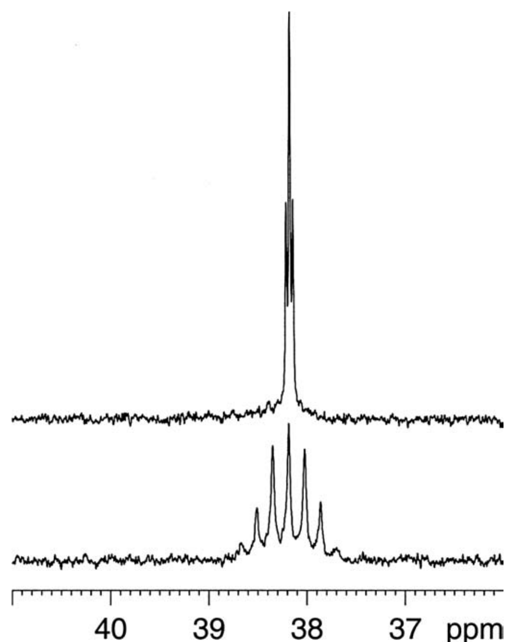


Fig. 1. Undecoupled (bottom) and ^2H-decoupled (top) ^{13}C-spectra of 0.6% v/v (*R,S*)-[$^{13}C^2H_3$-*methyl*]-nicotine in H_2O. NMR parameters: 90° pulse, 2.7 s interpulse delay, 64 scans, no ^1H-decoupling, WALTZ-16 ^2H-decoupling where appropriate.

1-3-6-7-6-3-1 septuplet due to scalar coupling with $^1J_{C-^2H}$ = 20.3 Hz. With ^2H-decoupling and the proper frequency filters in the rf lines the septuplet collapsed and the ^{13}C peak narrowed showing a pseudo-triplet of 4.3 Hz due to two distinct $^3J_{C-H}$ couplings of respectively 4.2 and 4.4 Hz (as taken from ^1H spectra (data not shown)). When ^1H-decoupling was applied simultaneously, the pseudo-triplet collapsed into a narrow singlet line.

Similarly, the ^2H spectrum in Fig. 2 shows the expected doublet separated by $^1J_{C-2H}$ and decoupling collapsed it as well. Compared to the ^{13}C spectrum, the ^2H spectrum had a roughly 10% better signal-to-noise ratio when taking into account the different number of scans. Indeed, the ^2H equilibrium magnetisation M_0 is expected to be higher than the ^{13}C one due to the fact that ^2H is a spin $I = 1$ nucleus which more than compensates for the lower gyromagnetic ratio γ. In fact, M_0 is proportional to γ I (I + 1), giving a ratio $M_0(^2H)/M_0(^{13}C)$ = 1.63, and furthermore the methyl group contains one ^{13}C but three ^2H. However, detection is for a given quality factor 2.37 times more sensitive for ^{13}C than for ^2H as it is proportional to $\gamma^{7/4}$. Finally the quality factor of the resonance circuits

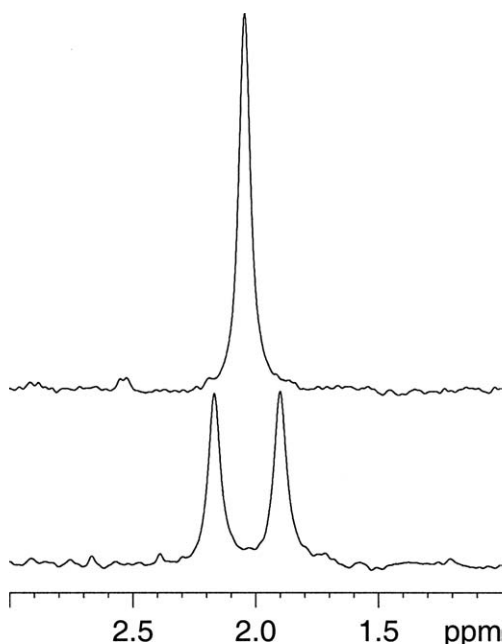


Fig. 2. Undecoupled (bottom) and ^{13}C-decoupled (top) ^{2}H-spectra of 0.6% v/v (*R,S*)-[$^{13}C^{2}H_3$-*methyl*]-nicotine in H_2O. NMR parameters: 90° pulse, one scan, ^{13}C decoupling with the GARP sequence.

depends on the fill factor, which is better for the inner coil (in favour of ^{2}H in our case as the probeheads are for inverse detection with ^{13}C on the outer coil and ^{1}H and ^{2}H on the double tuned inner coil) and on the quality of the resonance circuit [6]. The latter is usually good for the ^{13}C circuit but not necessarily for the ^{2}H circuit since mostly the quality of the ^{1}H circuit is optimised at the expense of the ^{2}H lock circuit.

The relaxation times for both nuclei were roughly evaluated. For ^{2}H, a T_1 of less than 0.2 s was found, whereas for ^{13}C, T_1 was around 3 s. This fast relaxation adds to the higher equilibrium magnetisation for ^{2}H, and it can be argued that an optimal pulse sequence should start with the higher ^{2}H magnetisation after only a short relaxation delay, and then transfer the polarisation to ^{13}C for more sensitive detection, preferably with a direct detection probehead which however we do not have. It might seem tempting to also make use of the $^{3}J_{C-H}$ couplings but besides their relatively small value not allowing for an efficient transfer, this coupling is specific for nicotine, and is not general for other molecules that take over the methyl group : for example, methionine, which was shown to incorporate the methyl group from nicotine [3], does not have such a coupling.

Given the previous arguments for a transfer from ^{2}H to ^{13}C which was already chosen for the artemisin in work [5] and the advantage of having pure absorption peaks, the nearest possibility was to expand the heteronuclear COSY sequence by adding 180° refocusing pulses in the middle of the delays thus getting the INEPT sequence [7] (Fig. 3). This sequence has indeed already been used earlier in its 1D version for investigating different aspects of polymerisation in plastics after ^{2}H labelling and polarisation transfer onto natural abundance ^{13}C [8] and then in its 2D version for assigning ^{2}H resonances of lowly ^{2}H-enriched molecules in solution [9]. More recently, the technique has found quite widespread use for assigning and measuring quadrupolar couplings for ^{2}H in oriented media using either cross polarisation [10], HMQC-type experiments [11] or INEPT and DEPT transfers [12]. Also preliminary work on the metabolisation of the herbicide glyphosate by soil micro-organisms has been presented [13] but this does not seem to have been followed up.

We first optimised the transfer τ and refocusing τ' delays used in the INEPT sequence. For the transfer delay τ during which $2^{13}C_z{}^{2}H_x$ evolves, the theoretical value of $1/(2\ J) = 24.6$ ms for the whole delay (i.e. before and after the 180° pulse) worked best which means that relaxation losses were negligible. According to Brown [5] the optimal refocusing delay τ' for the COSY sequence was close to $1/(7\ J)$, and we found indeed the optimum between 5.5 and 7 ms, i.e. between $1/(9\ J)$ and $1/(7\ J)$. The optimised 1D INEPT sequence was then 50% more sensitive than the one-pulse ^{13}C spectroscopy as can be seen in Fig. 4. The small natural abundance peaks from the pyrrolidine ring that are visible in the one-pulse spectrum are effectively suppressed as are those from the aromatic pyridine ring.

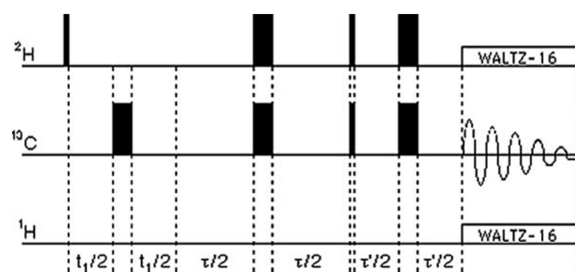


Fig. 3. The ^{1}H-decoupled ^{2}H–^{13}C 2D INEPT pulse sequence. Quadrature detection in the f_1 dimension uses the TPPI scheme ($\pi/2$ increment of the phase of the second ^{2}H 90° pulse). The 1D version is obtained by omitting the t_1 block.

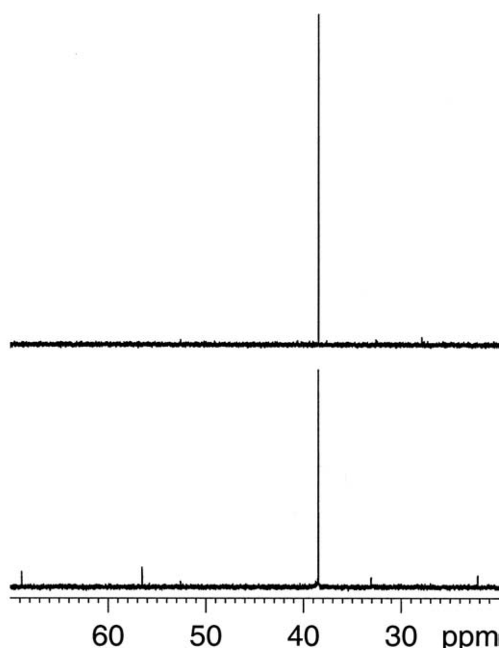


Fig. 4. Single-pulse ^{13}C (bottom) and 1D ^{2}H–^{13}C INEPT (top) spectra of 0.6% v/v (*R,S*)-[$^{13}C^{2}H_3$-*methyl*]-nicotine in H_2O. NMR parameters: 90° pulse, 1.7 s interpulse delay, 16 scans, WALTZ-16 ^{2}H- and ^{1}H-decoupling; specifically for the INEPT: 24.6 ms transfer delay τ, 7.0 ms refocusing delay τ'.

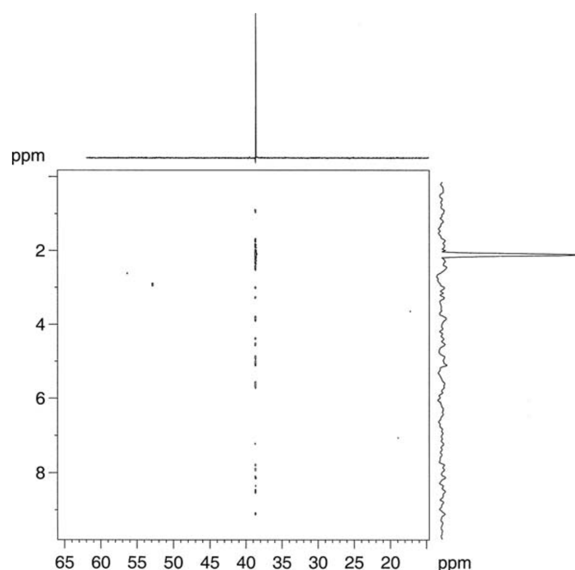


Fig. 5. 2D ^{2}H–^{13}C INEPT spectrum of 0.6% v/v (*R,S*)-[$^{13}C^{2}H_3$-*methyl*]-nicotine in H_2O. The full spectrum is shown, and the projections correspond to the respective 1D spectra at the frequency of the nicotine methyl cross peak. NMR parameters: 0.1 s relaxation delay, 24.6 ms transfer delay τ, 7.0 ms refocusing delay τ', four scans per increment, 256 increments for 10 ppm spectral width giving an FID resolution of 3.0 Hz, TPPI quadrature, WALTZ-16 ^{1}H- and ^{2}H-decoupling during acquisition, 8 min total duration.

The spectrum resulting from the corresponding 2D INEPT sequence can be seen in Fig. 5. Some t_1-noise is apparent, probably due to the fact that the spectrometer was run in unlocked mode. Its magnitude can be appreciated from the projections, which show the 1D spectra corresponding to the frequency of the peak maximum. It should be noticed that sufficient resolution was achieved with relatively few increments since the ^{2}H chemical shift range is small. When ^{13}C is the indirect dimension, good resolution takes many more increments thus increasing problems with t_1-noise. We also tried HSQC spectra in both directions ($^{13}C \rightarrow {}^{2}H \rightarrow {}^{13}C$ or $^{2}H \rightarrow {}^{13}C \rightarrow {}^{2}H$) and heteronuclear COSY sequences but never got as good results as with the INEPT sequence (data not shown). This is not surprising as shown by the sensitivity considerations above and by previous workers with other systems [8,9,12]. $^{13}C \rightarrow {}^{2}H \rightarrow {}^{13}C$ HSQC spectra can't be more sensitive than one-pulse ^{13}C spectra as the excited and detected nucleus are the same, and there will rather be transfer losses so the demonstration of Fig. 4 is also valid for this case. The opposite transfer $^{2}H \rightarrow {}^{13}C \rightarrow {}^{2}H$ suffers from the lower sensitivity for detection compared to ^{13}C but depending on the probehead this effect is not very strong. The advantage of HSQC-type transfers is that their efficiency does not depend on the number of ^{2}H attached to the ^{13}C so that quantification of relative populations of $^{13}C^{2}H_3$, $^{13}C^{2}H_2{}^{1}H$ and $^{13}C^{2}H^{1}H_2$ isotopomers is more reliable. Identification of these groups should be possible just from the ^{2}H induced isotope shift of the ^{13}C resonance but if this is not sufficient then DEPT transfers might also be of interest.

The 2D ^{2}H–^{13}C INEPT sequence, if necessary completed by other transfer sequences, thus seems a promising tool to analyse the metabolic fate of the doubly labelled methyl group from nicotine in plant cell suspensions and hopefully to learn more about the involved reaction mechanisms.

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