Journal of Magnetic Resonance **137,** 448–450 (1999)
Article ID jmre.1998.1698, available online at http://www.idealibrary.com on IDEAL®

Separate Quantification of Doubly and Singly ^{13}C-Labeled Metabolites by HSQC-Filtered *J* Spectroscopy

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Received September 1, 1998; revised December 22, 1998

NMR detection of multiply labeled compounds in biological samples is often used to follow metabolic pathways. Detection of protons bound to ^{13}C atoms offers a more sensitive approach than direct ^{13}C detection, but generally results in the loss of carbon–carbon coupling information. We have modified an HSQC sequence to refocus the carbon chemical shifts in order to obtain a proton-correlated ^{13}C homonuclear *J* spectrum, which allows us to measure singly and doubly labeled compounds in the same spectrum.

Key Words: **HSQC; *J* spectroscopy; doubly ^{13}C-labeled metabolites; biofluids; ^{13}C NMR.**

INTRODUCTION

Carbon-13-labeled metabolites have been used extensively for following metabolic pathways (*1–5*). A great advantage that detection of the label by NMR has over detection of radioisotopes is that the position of the label within the molecule is immediately apparent. A further advantage is that when two adjacent carbons in the same molecule are labeled, both signals are split by *J* coupling. This allows the fate of labeled carbons to be followed in great detail, and allows us to determine whether particular bonds are formed or broken during a sequence of reactions.

While direct detection of the ^{13}C NMR spectrum is convenient and provides excellent chemical shift resolution, this approach is fairly insensitive. A more sensitive approach is to detect the presence of ^{13}C labels in proton spectra, for example, by using a HSQC filter (*6–8*) that selects only protons attached to ^{13}C. The disadvantage of this approach is that the distinction between singly labeled and doubly labeled compounds is lost, as the signal arising from ^{1}H–^{13}C–^{13}C is the same as that from ^{1}H–^{13}C–^{12}C. The distinction can be restored if ^{13}C decoupling is not applied during detection, as this allows the long-range coupling $^{2}J_{CH}$ to be detected, but this results in a significant loss of sensitivity. It would in principle be possible to detect the direct $^{1}J_{CC}$ coupling in a two-dimensional HSQC spectrum, but in practice the required digital resolution could not be achieved in any reasonable time. This is not an insuperable problem, however, as it is not usually necessary to determine the ^{13}C chemical shifts, and so it is possible to detect $^{1}J_{CC}$ couplings in a small spectral width, by using extensive folding, by selecting a narrow ^{13}C bandwidth (*9*), or by refocusing the ^{13}C chemical shift. This last approach does not require technically demanding selective ^{13}C pulses, and permits the unambiguous assignment of signals from singly and doubly labeled species with a wide range of ^{13}C chemical shifts, and is thus both simpler and more general than the previous methods.

We therefore modified a two-dimensional HSQC sequence, in which the ^{1}H 180° pulse in the center of the t_1 period, which acts to refocus proton–carbon couplings, is replaced by a ^{13}C 180° pulse. This will refocus both heteronuclear proton–carbon couplings and ^{13}C chemical shifts, but will retain homonuclear carbon–carbon couplings. The resulting spectrum will show ^{1}H chemical shifts in the direct dimension, and the carbon–carbon coupling pattern of the directly attached ^{13}C in the indirect dimension. Thus signals from singly labeled compounds will occur along the center of the spectrum, while signals from compounds with two adjacent ^{13}C labels will appear as a pair of peaks symmetrically placed around the center. This modification can be incorporated into any HSQC sequence; for simplicity we modified a standard gradient-selected HSQC experiment (Fig. 1).

RESULTS AND DISCUSSION

Figure 2 shows spectra obtained from a liver extract containing (among other compounds) a mixture of $^{13}C_2$-phosphoethanolamine (H_2PO_4–$^{13}CH_2$–$^{13}CH_2$–NH_2) and natural-abundance phosphoethanolamine. Figure 2a shows a portion of the direct carbon-detected spectrum, showing one of the labeled carbon atoms, in which the doubly labeled and the singly labeled (natural abundance) species can be clearly distinguished. The small coupling is between ^{31}P and ^{13}C. Figure 2b shows a row from a 2D HSQC *J* spectrum containing the corresponding signals, in which it can be seen that the doubly labeled (outer lines) and singly labeled (inner line) compounds can be readily distinguished and quantified. Figure 2c contains

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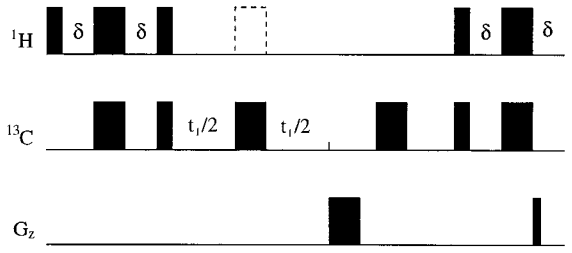

FIG. 1. Pulse sequences for conventional and modified gradient-selected HSQC experiments. Narrow boxes correspond to 90° pulses, while broad boxes correspond to 180° pulses. $\delta = \frac{1}{4}J_{CH}$, and the two gradient pulses have integrated intensities in the ratio 4:1. The conventional and modified sequences differ only in the 180° pulse in the center of the t_1 period. In a conventional HSQC experiment this pulse (shown with dashed lines) is applied to ^{1}H, to refocus proton–carbon couplings, while in our modified HSQC experiments it is instead applied to ^{13}C, thus acting to refocus both proton–carbon couplings and carbon chemical shifts, retaining only homonuclear carbon–carbon couplings.

the 2D HSQC *J* spectrum, showing the proton chemical shift on the vertical axis and the carbon *J* coupling on the horizontal axis. The natural-abundance compounds appear along the central column of this 2D spectrum, with any doubly labeled compounds appearing as pairs of peaks equidistant from the central column. Molecules with more than two adjacent ^{13}C would appear in the 2D spectrum with their C–C couplings intact. For example, the signal from H–^{13}C(–^{13}C)$_2$ would appear as a 1:2:1 triplet in the ^{13}C dimension (provided that the $^1J_{CC}$ values were equal), while H–^{13}C–^{13}C–^{13}C would appear as a doublet of doublets. The ^{31}P–^{13}C coupling is refocused like any other heteronuclear coupling, and so is not seen in the carbon dimension; this simplifies the spectrum further and improves the signal-to-noise ratio. Overlap of signals in the proton dimension may make quantification of the singly labeled signals difficult for some species. To alleviate this problem, the ^{13}C chemical shift could be partially reintroduced by

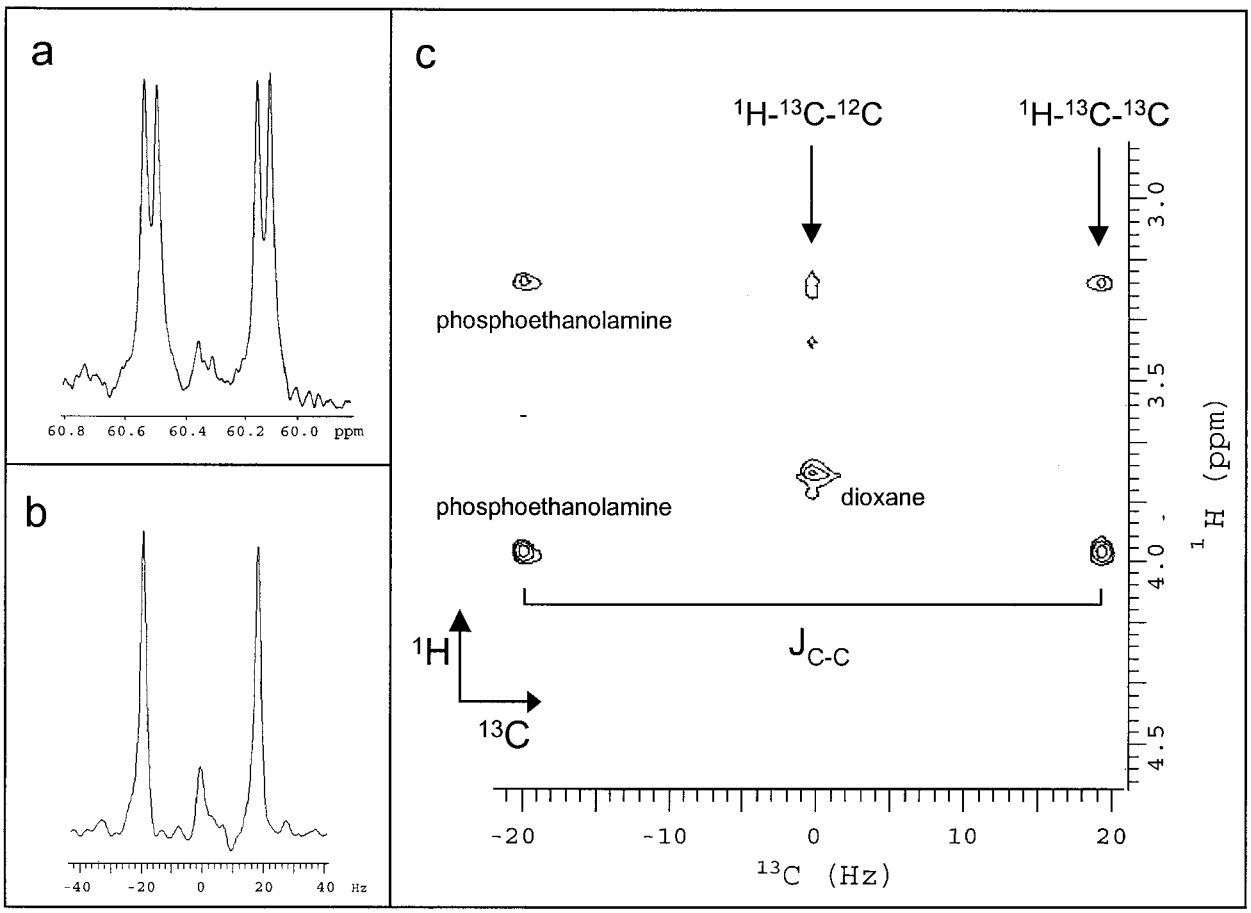

FIG. 2. Spectra of liver extract containing $^{13}C_2$-phosphoethanolamine. Spectra were obtained on a 7.35-T magnet interfaced to a Varian Inova spectrometer, operating at 400.15 MHz for proton and 100.62 MHz for carbon. (a) Directly detected ^{13}C signal from C1 of $^{13}C_2$-phosphoethanolamine. Proton irradiation was applied throughout the sequence, to give decoupling and the NOE. Parameters: number of transients, 1088; interpulse delay, 6 s; total time required, 110 min; signal-to-noise ratio, 24. (b) One row from the HSQC-filtered *J* spectrum, showing the same carbon atom as in (a). (c) 2D plot of the HSQC-filtered *J* spectrum. Parameters: interpulse delay, 1.6 s; number of t_1 increments, 64; zero-filled to 512 in t_1; spectral width in $f_1(^{13}C)$ dimension, 200 Hz; number of transients per increment, 64; total time required, 110 min; signal-to-noise ratio, 64.

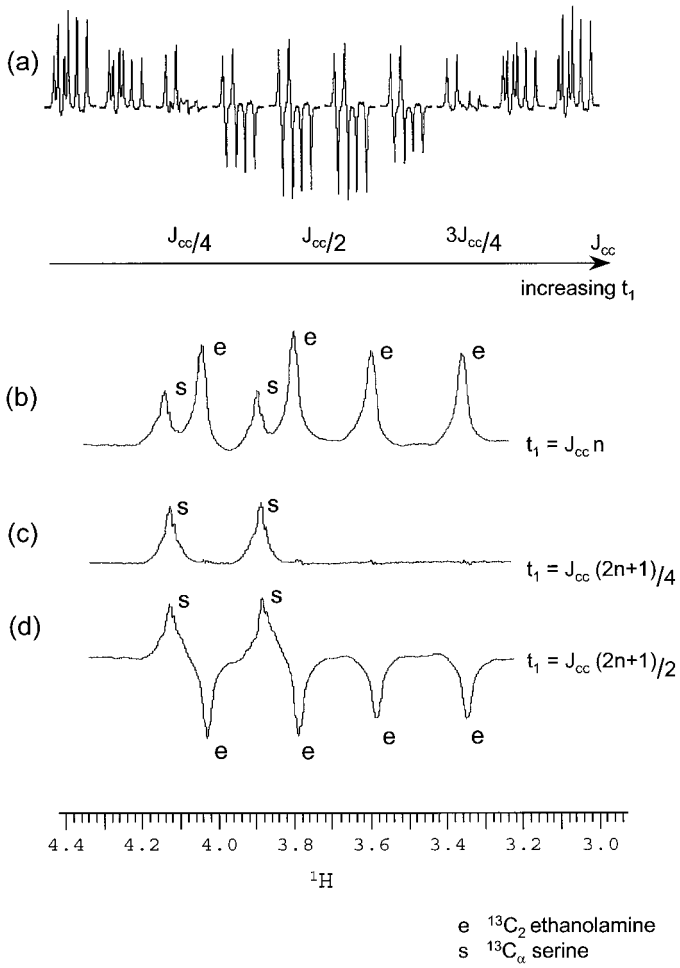

FIG. 3. 1D spectra from a sample containing a mixture of $^{13}C_\alpha$-serine and $^{13}C_2$-ethanolamine, obtained using the modified HSQC sequence at a series of t_1 times. Signals from the singly labeled serine are largely independent of t_1, while signals from the doubly labeled ethanolamine are modulated at a rate J_{CC}.

placing the carbon refocusing pulse asymmetrically in the t_1 period, but we have not evaluated the usefulness of this modification.

The signal-to-noise ratio of the HSQC-filtered J spectrum (as implemented here) is about three times that of direct detection of ^{13}C with proton decoupling and NOE enhancement (collected for an identical time), which while not spectacular, leads to a significant reduction in experiment time. A number of factors which reduce the theoretical sensitivity increase must be taken into account. Proton–proton coupling of the detected signals leads to losses due to increased effective linewidth and modulation during the pulse sequence, and gradient selection results in a decrease of the signal by a factor of 2. A gradient sensitivity-enhanced sequence would regain much of this loss.

A simpler, shorter version of this experiment involving only the collection of two 1D spectra is suggested by Fig. 3, which shows a series of spectra acquired at increasing values of t_1. The signals from singly labeled compounds remain almost constant, exhibiting only signal losses due to ^{13}C relaxation. By contrast signals from doubly labeled compounds are modulated at a rate J_{CC}. If only one doubly labeled compound is present with a known value of J_{CC}, it is possible to obtain spectra from singly and doubly labeled compounds directly. For example, choosing $t_1 = J_{CC}/4$ (when signals from doubly labeled compounds are passing through a null) gives a spectrum containing only signals from singly labeled compounds. Similarly, choosing $t_1 = J_{CC}/2$ gives a spectrum where signals from doubly labeled compounds are inverted; taking sums and differences with a spectrum at $t_1 = 0$ allows separate spectra corresponding to singly and doubly labeled compounds to be generated.

In conclusion, we have shown that a simple modification of the well-known HSQC experiment can be used to distinguish singly from multiply labeled compounds, with proton sensitivity. The technique is particularly relevant to metabolic studies of ^{13}C-labeled compounds, where detection of low levels of various isotopomers is often limited by the low sensitivity of direct ^{13}C detection.

ACKNOWLEDGMENT

J.A.J. is a Royal Society University Research Fellow.

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