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# NMR Method for Measuring Carbon-13 Isotopic Enrichment of Metabolites in Complex Solutions

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**Isotope-based methods are commonly used for metabolic flux analysis and metabolite quantification in biological extracts. Nuclear magnetic resonance (NMR) spectroscopy is a powerful analytical tool for these studies because NMR can unambiguously identify compounds and accurately measure $^{13}C$ enrichment. We have developed a new pulse sequence, isotope-edited total correlation spectroscopy (ITOCSY), that filters two-dimensional $^{1}H-^{1}H$ NMR spectra from $^{12}C$- and $^{13}C$-containing molecules into separate, quantitatively equivalent spectra. The ITOCSY spectra of labeled and unlabeled molecules are directly comparable and can be assigned using existing bioinformatics tools. In this study, we evaluate ITOCSY using synthetic mixtures of standards and extracts from *Escherichia coli*. We show that ITOCSY has low technical error (6.6% for metabolites ranging from 0.34 to 6.2 mM) and can detect molecules at concentrations less than 10 $\mu$M. We propose ITOCSY as a practical NMR strategy for metabolic flux analysis, isotope dilution experiments, and other methods that rely on carbon-13 labeling.**

Nuclear magnetic resonance (NMR) spectroscopy is one of the primary analytical tools used for investigating metabolites in complex biological extracts. Recent advances in NMR techniques,[1] data analysis software,[2,3] and spectroscopic libraries of metabolite standards[4,5] have considerably increased the number of metabolites that can be identified and quantified in routine studies. Currently, more than 80% of the signals observed in 2D $^{1}H-^{13}C$ spectra of biological extracts can be assigned,[1] and hundreds of spectra can be analyzed in a few hours.[2] However, these tools are primarily constrained to steady-state analyses of metabolites.

A wide variety of isotope-based methods have been developed for tracing metabolic pathways,[6] measuring reaction kinetics,[7] and quantifying molecules after extensive sample handling.[8] These techniques are of obvious utility to metabolomics but have not been widely applied to NMR-based studies because of complications related to signal overlap. NMR spectra of unenriched biological extracts can contain thousands of $^{1}H$ resonances. Spectra of $^{13}C$-enriched extracts are further complicated by $^{1}H-^{13}C$ *J*-couplings. In isotope-based metabolomics studies, which involve complex mixtures of both $^{13}C$-labeled and unlabeled metabolites, NMR spectra are too heavily overlapped to support comprehensive quantitative analyses. As a result, studies that have employed isotope-based methods have been restricted to the subset metabolites with well-isolated resonances.[9−11]

To make isotope-based techniques more accessible to comprehensive metabolic analyses, we have developed a homonuclear $^{1}H$ NMR experiment that separates signals from $^{12}C$- and $^{13}C$-containing molecules into distinct, quantitatively equivalent spectra. This pulse sequence, isotope-edited total correlation spectroscopy (ITOCSY), allows metabolomics studies to capitalize on isotope-based methods without increasing the complexity of spectra. ITOCSY is based on the established TOCSY pulse sequence,[12−14] which is desirable for metabolomics[11,15−19] because of its high sensitivity and compatibility with existing

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spectroscopic libraries and bioinformatics tools.[2−5,20] We introduce the ITOCSY pulse sequence and evaluate its performance under both controlled and biologically relevant conditions. We show that ITOCSY is a reliable quantitative tool, and we foresee its potential utility in metabolic flux analysis, isotope dilution experiments, and other methods that rely on isotopic labeling.

## EXPERIMENTAL SECTION

**Strategy for Isotope Ratio-Based Quantification.** This study uses an NMR adaptation of the traditional isotope dilution method for quantifying metabolites.[8] Isotope dilution studies derive concentration from the ratio of a metabolite's signal to the signal from an isotope-labeled standard. Multiplying the observed isotope ratio by the amount of standard added allows one to calculate the amount of metabolite present in the sample at the time the standard was added.[8] In metabolomics studies, each observable metabolite is standardized against an isotopically enriched version of the same molecule. Alternatively, isotopically enriched extracts can be standardized against unlabeled molecules.

We used both the $^{12}$C- and $^{13}$C-standardized isotope dilution approaches in this study. To evaluate the qualitative performance and quantitative accuracy of ITOCSY-derived isotope ratios, we used 24 complex synthetic mixtures of unlabeled metabolites containing varying amounts of $^{13}$C-labeled molecules (Table S-1 in Supporting Information). We calculated the concentrations of the $^{13}$C-labeled metabolites by isotope dilution and compared them to the known concentrations of each molecule. We then applied ITOCSY to a metabolic investigation of the osmotic stress response in *Escherichia coli*. We describe how a mixture of $^{13}$C-labeled metabolites isolated from *E. coli* cells grown on [U−$^{13}$C] glucose can be calibrated for use as a standard.

**ITOCSY Pulse Sequence.** ITOCSY data are collected as two $^1$H−$^1$H DIPSI-TOCSY spectra,[12−14] one specific to $^{12}$C and the other isotopically nonspecific ($^{12}$C + $^{13}$C). Signals from $^{13}$C-bound protons are removed from $^{12}$C-specific spectra by the use of 90°($^1$H)−1/4$^1J_{HC}$−180°($^1$H,$^{13}$C)−1/4$^1J_{HC}$−90°($^{13}$C)−PFG filter elements. These low-pass *J*-filters convert antiphase $^{13}$C-bound $^1$H magnetization to undetectable double- and zero-quantum coherence.[21] ITOCSY employs three consecutive filtering elements with 1/4$^1J_{HC}$ delays tuned to aromatic (170 Hz), aliphatic (140 Hz), and anomeric (125 Hz) coupling constants (Figure 1). Isotopically nonspecific spectra are collected using the same pulse sequence but with the carbon transmitters tuned 300 ppm off resonance to prevent the filtering elements from functioning. This approach ensures that the complementary spectra are collected under identical conditions. $^{12}$C + $^{13}$C and $^{12}$C data are collected in interleaved scans, and both utilize carbon decoupling to minimize spectral complexity. $^{13}$C-specific data are derived as a difference spectrum from the nonspecific ($^{12}$C + $^{13}$C) and $^{12}$C-specific spectra.

**Preparation of Synthetic Mixtures.** We evaluated the quantitative efficacy of ITOCSY by analyzing synthetic mixtures containing both $^{13}$C-labeled and unlabeled standards prepared

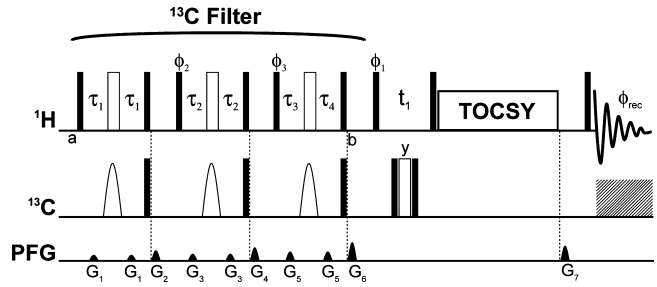


**Figure 1.** Pulse program for acquiring ITOCSY. Filled and open bars indicate nonselective 90 and 180° hard pulses, respectively. Shaped $^{13}$C pulses denote 1 ms (200 ppm) hyperbolic secant inversions.[28] Spectra are acquired with the $^1$H carrier set on resonance with the residual water signal (4.77 ppm). Isotope-specific and nonspecific data are collected on alternate scans; filtered data are collected with the $^{13}$C carrier set on resonance (70 ppm), and isotopically nonspecific data are collected with the $^{13}$C carrier set off resonance (300 ppm) for pulses between points a and b. The $\tau_1$, $\tau_2$, and $\tau_3$ delays are set to 1/4 $^1J_{HC}$ with the filtering elements optimized for 125, 140, and 170 Hz coupling constants. All $^{13}$C pulses are placed on resonance after point b for $^{13}$C decoupling; a composite pulse (90*x*−180*y*−90*x*) and a WURST CPD sequence[29] (140 ppm) are used for $^{13}$C decoupling during the $t_1$ and $t_2$ evolution periods, respectively. Isotopic mixing is achieved via the DIPSI-2 sequence[13] with a bandwidth of 4 kHz and a length of 80 ms. Pulses are applied along the *x*-axis unless otherwise indicated. Phase cycling for $\phi_1 = x,-x$; $\phi_2 = x,x,-x,-x$; $\phi_3 = x,x,x,x,-x,-x,-x,-x$; $\phi_{rec} = x,-x,-x,x,-x,x,x,-x$. To achieve quadrature detection for the $^1$H indirect dimension, a second FID is collected for each increment with the phase cycling for $\phi_1$ set to *y* and −*y*; states-TPPI requires the $\phi_1$ and $\phi_{rec}$ phases to alternate by 180° every other increment. Pulse field gradients (PFG) employ shaped *z*-axis WURST pulses applied for 1.0 ms. Gradients $G_1$, $G_3$, and $G_5$ select magnetization inverted by the 180° $^1$H pulses and are varied between 2 and 5 G/cm. Gradients $G_2$, $G_4$, $G_6$, and $G_7$ destroy *xy*-plane magnetization and are set to 7, 9, 11, and 5 G/cm, respectively. The Varian pulse program used in this study is available from www.nmrfam.wisc.edu/software.

at known concentrations. Synthetic mixtures ($N$ = 24) contained 30 biologically relevant compounds, six of which included both U−$^{13}$C-labeled and unlabeled species. These differentially labeled compounds were alanine, fructose, glucose, glutamate, glutamine, and lactate. All unlabeled molecules were prepared at 2 mM, and the concentrations of the U−$^{13}$C compounds ranged from 130 $\mu$M to 6.5 mM (Table S-1 in Supporting Information). All synthetic samples were prepared in $D_2O$ containing 300 $\mu$M $NaN_3$ (to inhibit microbial growth) and 300 $\mu$M DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid, the NMR chemical shift standard). Samples were titrated with DCl/NaOD as needed to achieve an observed (glass electrode) pH of 7.400 ± 0.004.

**In Vivo Isotopic Labeling Strategy.** ITOCSY-based quantification requires isotopically labeled standards, but it is impractical to purchase the number of enriched compounds required for comprehensive metabolic analyses. For the osmotic stress study (see below), [U−$^{13}$C] standards were produced in vivo from *E. coli* incubated in [U−$^{13}$C]-glucose under the control condition. To quantify metabolites in the labeled extracts, a second set of unlabeled cultures was grown from the same starter in a medium containing unlabeled glucose. Metabolites in the unlabeled cultures were quantified using established methods,[1] and these levels were used as a benchmark for the labeled extracts. Regression coefficients used for quantifying enriched

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metabolites (see below) were derived from solutions of enriched extracts prepared at three dilutions (1:0, 1:1, 1:3) in $D_2O$.

**Osmotic Stress Study.** As a biological application of ITOCSY, we investigated the metabolic response of *E. coli* incubated under varying levels of osmotic stress. Cultures (500 mL each; $N = 4$ per condition) of *E. coli* (MG1655) were grown in M9 media containing 22.8 mM glucose and variable concentration of salt: 0 (control), 150, 300, or 500 mM NaCl. To probe the dependence of the observed metabolic responses on osmolarity, we prepared additional cultures ($N = 4$) in M9 medium containing 500 mM NaCl and 10 mM glycine betaine, an established osmoprotectant.[22] Isotopically enriched cultures used for metabolite standards (250 mL each; $N = 20$) were grown in M9 medium containing 22.1 mM [U−$^{13}$C]-glucose (Cambridge Isotope Laboratories). All cultures were incubated on a shaking platform at 37 °C. When cultures reached an optical density of 0.80 (660 nm), they were transferred to an ice−water bath to minimize metabolic activity. All subsequent sample preparation was conducted in a 4 °C cold room. Chilled samples (500 mL for natural abundance, 250 mL for $^{13}$C cultures) were centrifuged (18 500*g*), and the supernatant was discarded. Pellets were washed with 10 mL of glucose-free M9 media osmotically adjusted with NaCl to match each of the culture conditions. Washed pellets were recentrifuged, and the resulting pellets were flash frozen in liquid $N_2$.

**Preparation of Biological Extracts.** Frozen *E. coli* pellets were resuspended in 16 mL of boiling water containing 250 $\mu$M MES. Samples were incubated in sealed reaction vials in a boiling water bath for 7.5 min, then centrifuged to remove cellular debris (8000*g*). The pellet was discarded, and extracts were microfiltered (Vivaspin 20; 3000 MWCO) to remove high molecular weight components. Filtrates were lyophilized and redissolved in 800 $\mu$L of $D_2O$ with 300 $\mu$M $NaN_3$ and 300 $\mu$M DSS. Samples were titrated with DCl/NaOD as needed to an observed pH of 7.400 (±0.004). All $^{13}$C-labeled samples were pooled to create a single $^{13}$C-enriched library. Labeled standards were mixed with unlabeled samples (300 $\mu$L each) and transferred to 5 mm NMR tubes (Wilmad) for spectroscopic analysis.

**NMR Spectroscopy.** All NMR spectroscopy was conducted at the National Magnetic Resonance Facility at Madison. Two-dimensional $^1$H−$^1$H ITOCSY spectra were collected on a 600 MHz Varian spectrometer equipped with a cryogenic probe. Spectra were collected with a 1.5 s repetition delay, 64 steady-state transits, 2 acquisition transits, 128 increments, and an acquisition time of 0.5 s (7530 points). Sweep widths for the direct and indirect dimensions were 7500 and 5400 Hz, respectively. $^{12}$C + $^{13}$C and $^{12}$C-specific spectra were collected concurrently using interleaved scans. Time-domain data were Fourier transformed with a shifted sine bell window function, zero-filled, phased, and referenced to DSS using automated NMRPipe[23] macros written in-house. $^{13}$C-specific difference spectra were derived from the $^{12}$C + $^{13}$C and $^{12}$C-specific data using custom NMRPipe macros.

**Metabolite Quantification.** All NMR data analyses were performed using the rNMR software package.[2] Metabolite signals in ITOCSY spectra were assigned using previously established methods.[1] Briefly, metabolites were identified by submitting peak lists to the Madison Metabolomics Consortium Database;[20] possible metabolite matches were verified by visually inspecting overlaid spectral standards from the BioMagResBank.[4] Dispersed resonances were selected for each of the assigned metabolites, and the peak intensities of these signals were measured.

Deriving accurate metabolite concentrations from multidimensional NMR signal intensities requires the use of calibration coefficients.[1] These isotope- and resonance-specific coefficients ($m_{12}$, $m_{13}$) are the linear regression slopes describing concentration as a function of intensity (Δconcentration/Δintensity) for metabolite signals in standards prepared at three concentrations. In the *E. coli* study, $m_{12}$ was calculated from the signals of unlabeled standards prepared at 2, 5, and 10 mM; $m_{13}$ was calculated from signals of $^{13}$C-enriched metabolite extracts prepared at 1:0, 1:1, and 1:3 dilutions. Calibration coefficients in the synthetic samples were calibrated independently using standards prepared at the minimum, median, and maximum concentration of each metabolite in the synthetic mixtures (Table S-1 in Supporting Information).

Metabolite concentrations were calculated from ITOCSY signals via three methods (eqs 1−3). The established fast metabolite quantification (FMQ) method,[1] which was used as a benchmark for *E. coli* study, determines the total concentration ($U_{tot}$) of unlabeled ($U_{12}$) and natural abundance $^{13}$C ($U_{na}$) resonances from signal intensities ($I_{12}$) observed in $^{12}$C-specific ITOCSY spectra (eq 1).

$$U_{tot} = U_{12} + U_{na} = I_{12} \times m_{12} \tag{1}$$

Metabolites were also quantified using the calibrated ITOCSY method (eqs 2 and 3), which relates unenriched signals ($I_{12}$) to the corresponding resonances from [U−$^{13}$C]-labeled molecules ($I_{13}$). The calibrated ITOCSY approach can be used to either (eq 2) calculate concentrations of enriched molecules ($U_{13}$) relative to known concentrations of unenriched standards ($S_{12}$) or (eq 3) calculate concentrations of unenriched molecules ($U_{tot}$) relative to concentrations of [U−$^{13}$C] standards ($S_{13}$). The equations used for calibrated ITOCSY calculations differ between $S_{12}$- and $S_{13}$-standarized experiments to account for the natural abundance levels of $^{13}$C (1.1%) in unenriched molecules. In this study, the $S_{12}$ protocol (eq 2) was used for calculating metabolite concentrations in synthetic mixtures whereas the $S_{13}$ protocol (eq 3) was used in the *E. coli* study.

$$U_{13} = S_{12} \times \left\{ \frac{I_{13} \times m_{13}}{I_{12} \times m_{12}} - 0.011 \right\} \tag{2}$$

$$U_{tot} = S_{13} \times \left\{ \frac{I_{12} \times m_{12}}{I_{13} \times m_{13} - (I_{12} \times m_{12} \times 0.011)} \right\} \tag{3}$$

## RESULTS AND DISCUSSION

We used complex mixtures of standards prepared with [U−$^{13}$C]-labeled and unlabeled compounds (Table S-1 in Supporting Information) to investigate the efficacy of ITOCSY-based analyses. As expected, ITOCSY was effective for separating resonances on

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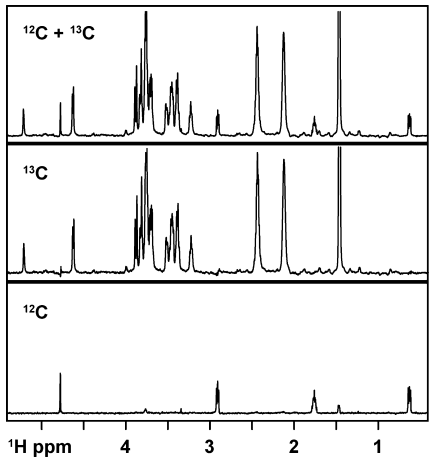


**Figure 2.** Isotopically nonspecific ($^{12}C + {}^{13}C$), difference edited ($^{13}C$), and isotope-filtered ($^{12}C$) 1D $^{1}H-{}^{1}H$ ITOCSY spectra of a mixture of unlabeled and uniformly $^{13}C$-labeled molecules. Signals in the $^{13}C$ spectrum correspond to [U−$^{13}C$]-glucose, [U−$^{13}C$]-glutamine, and [U−$^{13}C$]-alanine, whereas signals in the $^{12}C$ spectrum correspond to unenriched DSS.

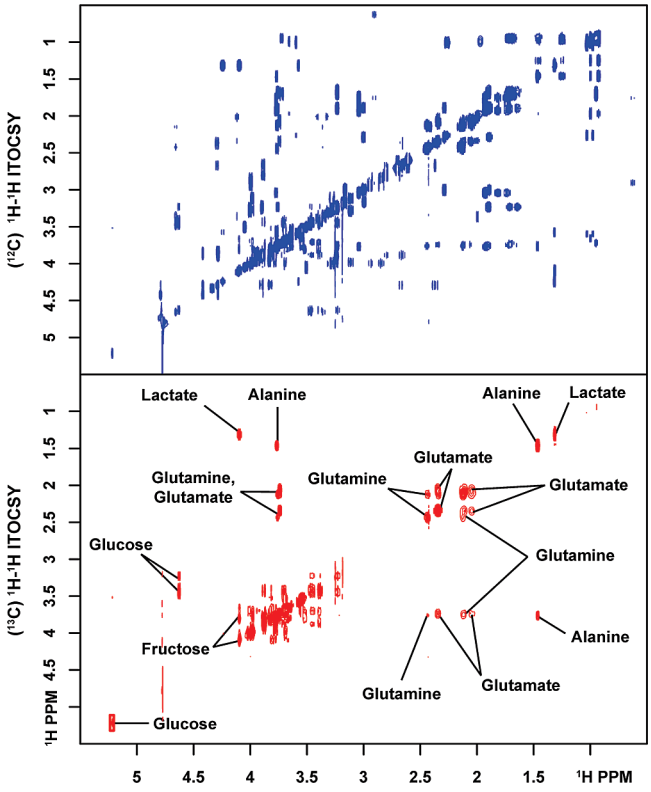


**Figure 3.** Isotope-filtered ($^{12}C$) and difference edited ($^{13}C$) 2D $^{1}H-{}^{1}H$ ITOCSY spectra of a synthetic mixture containing 30 unlabeled and six $^{13}C$-labeled metabolites. Resonance assignments for $^{13}C$-enriched compounds are shown. A complete listing of the unlabeled metabolites and the concentrations of the $^{13}C$-enriched compounds are provided in the Supporting Information (Table S-1, synthetic mixture 1).

the basis of isotopic composition (Figure 2 and Figure 3). We had hoped that quantification based on $^{13}C/^{12}C$ ratios for individual resonances would normalize the systematic quantitative defects inherent to multidimensional NMR[1] and eliminate the need for external signal calibration. Although enrichment-based quantification eliminated several major sources of

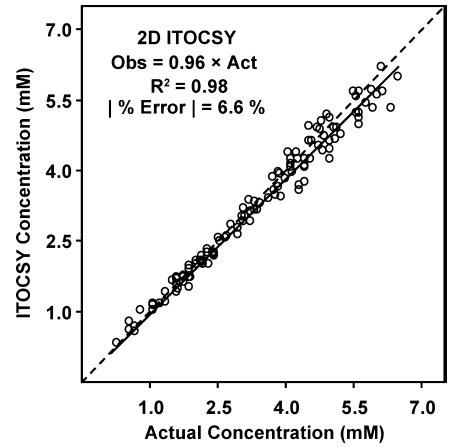


**Figure 4.** Metabolite concentrations ($N$ = 121) measured by calibrated 2D $^{1}H-{}^{1}H$ ITOCSY vs known values. The dotted line indicates the ideal regression line (slope = 1). The data represent 24 synthetic mixtures; each mixture contained 30 metabolite standards, 6 of which were supplemented with a [U−$^{13}C$]-labeled version. ITOCSY values were calculated from the observed $^{13}C/^{12}C$ ratio and were corrected for systematic quantitative errors using empirically derived coefficients. The error estimate reflects the average absolute error for all ITOCSY-derived metabolite concentrations. The compositions of the synthetic mixtures are listed in Supporting Information Table S-1.

systematic error, differential $T_1$ relaxation and other isotope-related phenomena prevented accurate quantification from the uncalibrated ITOCSY signals.

We previously reported a general method for correcting systematic quantitative defects in NMR pulse sequences using empirically determined calibration coefficients.[1] ITOCSY-derived isotopic ratios were calibrated in this manner using standards prepared at multiple dilutions. As expected, the corrected $^{13}C/^{12}C$ values were quantitatively reliable; regression of 121 ITOCSY-derived concentrations versus known levels yielded a slope of 0.96 (ideal slope of 1) with $R^2 = 0.98$. This translates to 6.6% average technical error for concentrations ranging from 0.34 to 6.2 mM (Figure 4).

To evaluate ITOCSY in the context of a biological study, *E. coli* cultures were prepared under varying levels of osmotic stress. Unlabeled extracts were mixed with a $^{13}C$ metabolite library, and compounds were quantified using the FMQ[1] and corrected ITOCSY protocols. FMQ-based values were calculated from signals observed in $^{12}C$-specific ITOCSY spectra, whereas ITOCSY-based values were calculated from $^{12}C/^{13}C$ ratios. Regression of concentrations ($N$ = 339; 0.009 to 11 mM) determined by ITOCSY versus FMQ showed a slope of 1.02 and an $R^2$ of 0.99 (Figure 5). Concentration-dependent error was observed, with values under 100 $\mu$M showing a substantial increase in error. These data indicate that the sensitivity limit of ITOCSY is less than 10 $\mu$M, but the limit for reliable quantification is 100 $\mu$M.

ITOCSY-based analyses of the *E. coli* extracts relied on isotopically enriched standards produced in vivo. These standards were quantified under the assumption that steady-state metabolite levels observed in labeled and unlabeled extracts are equivalent. The strong correlation observed between FMQ and ITOCSY values verifies this assumption. If steady-state levels differed

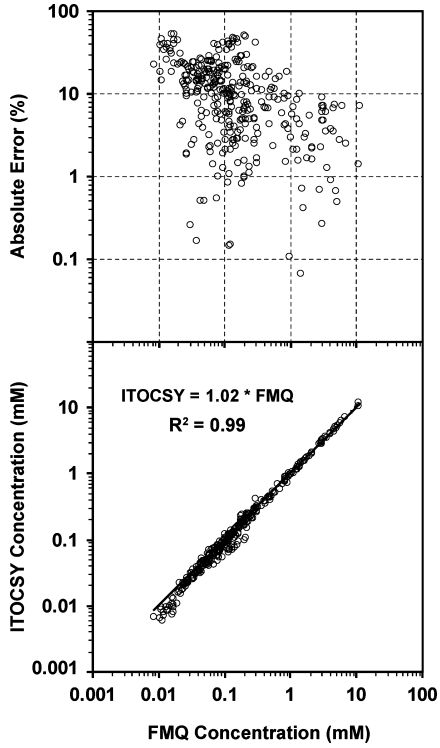


**Figure 5.** Metabolite concentrations ($N$ = 339) and quantitative errors observed in 20 *E. coli* extracts as measured by calibrated ITOCSY vs the established FMQ protocol.[1] Each extract was mixed 1-to-1 with a $^{13}$C-enriched *E. coli* extract. FMQ values were based on the intensities of $^{12}$C-specific spectra, whereas ITOCSY values were derived from $^{12}$C/$^{13}$C ratios observed for metabolites in each mixture. Quantitative errors observed in these data were concentration-dependent; metabolites with concentrations ranging from 0.010 to 0.1, 0.1 to 0.5, 0.5 to 1, and 1 to 11 mM showed average absolute errors of 17, 13, 8, and 4%, respectively. A total of 24 compounds were quantified in this study; glucose, glutamine, isoleucine, MES, phenylalanine, and trehalose were excluded from the figure because these compounds were not observable in ITOCSY spectra of the $^{13}$C-enriched library. A complete list of metabolite concentrations observed in the *E. coli* extracts can be found in Supporting Information Table S-2.

between labeled and unlabeled samples, then compound-specific systematic error would have been observed.

One limitation of ITOCSY evident from the *E. coli* study is that reliable quantification requires both the labeled and unlabeled molecules to be present at concentrations above the detection limit. Six of the 24 metabolites quantified by FMQ were not observable in ITOCSY spectra of the enriched extracts. Four of these compounds were present at concentrations near the limit of detection for FMQ and were unobservable in enriched extracts because of the faster $R_1$ relaxation of $^{13}$C-labeled molecules. The other two compounds, glucose and trehalose, were absent from the enriched library because these metabolites are only accumulated under high-salt conditions. The main biological findings of this study were salt-dependent increases in trehalose and glucose, diminished putrescine levels at high salt, and the reversal of these phenotypes by glycine betaine (Figure 6). These findings, which had been previously reported,[24−27] were

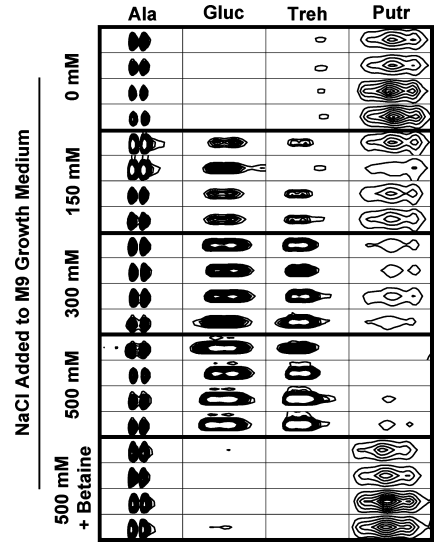


**Figure 6.** Osmolarity-dependent alterations in metabolites observed in 2D $^1$H−$^1$H ITOCSY spectra ($^{12}$C-specific) of *E. coli* extracts. Each box shows a metabolite-specific region of an NMR spectrum; columns denote the four selected compounds; rows denote the five conditions ($N$ = 4 each) used for *E. coli* cultures. All spectra are shown with the minimum and maximum contour thresholds set to 25 and 55 standard deviations above the thermal noise, respectively. Steady-state concentrations of glucose (Gluc) and trehalose (Treh) are elevated under osmotic stress, whereas putrescine (Putr) levels are diminished. Samples incubated in 500 mM NaCl with 10 mM glycine betaine (last four rows) show a metabolic phenotype similar to cultures grown in standard M9 medium (first four rows). Alanine (Ala) signals are shown as a reference. A complete listing of all of the metabolites quantified in this study can be found in Supporting Information Table S-2.

not quantifiable by isotope dilution because of our design of the in vivo labeling conditions. This limitation could be mitigated in future studies by including samples from each condition as standards.

## CONCLUSIONS

ITOCSY was effective in separating signals from labeled and unlabeled molecules. Although accurate quantification required calibration, the corrected signals had sufficiently low error to be useful for metabolomics studies. We foresee the primary applications of ITOCSY to be metabolic flux analysis, isotope dilution, and metabolite quantification using standards produced in vivo.

The in vivo labeling strategy used in this study has obvious applications beyond the limited case presented here. Enriched metabolites produced in cost-effective systems could be used to quantify extracts from unrelated species. The $^{13}$C-labeled metabolite library generated for this study, for example, could be used to quantify metabolites in human serum. The main limitation to this approach is that quantification requires metabolites to be present in both library and test samples. However, a more comprehensive library could be constructed using a variety of free-living organisms or under a range of conditions appropriate to a particular study.

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The ITOCSY quantification protocol is an NMR adaptation of the traditional radioisotope dilution method for measuring metabolite concentrations.[8] Isotope dilution methods are advantageous because they can correct sample-to-sample variations in extraction efficiency, chromatography, and other sample handling errors if standards are added prior to any manipulation of the samples. Although extracts were analyzed as a complex solution in this study, the ITOCSY quantification protocol presented here allows samples to be fractionated and concentrated without affecting quantification.

Capitalizing on enriched libraries depends on our ability to quantify the labeled metabolites. ITOCSY provides a simple mechanism for achieving this; labeled compounds can be identified by using existing bioinformatics tools and quantified by using unlabeled standards. In summary, ITOCSY is a convenient tool for differentiating between molecules on the basis of enrichment and provides the framework for extending metabolomics into comprehensive isotope-based studies.

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## SUPPORTING INFORMATION AVAILABLE

ITOCSY pulse sequence for Varian, along with related macros for using the pulse sequence, is available from www.nmrfam.wisc.edu/software. The rNMR analysis software and NMR data used in this study are available at http://rnmr.nmrfam.wisc.edu. A complete listing of metabolites and their concentrations in the synthetic mixtures and *E. coli* extracts can be found in the online Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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