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# Real-Time Enzyme Kinetics by Quantitative NMR Spectroscopy and Determination of the Michaelis–Menten Constant Using the Lambert-W Function†

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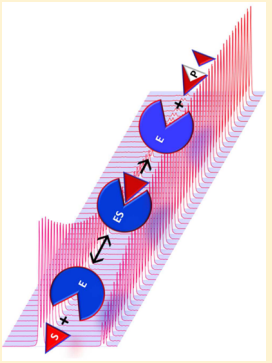
Supporting Information

**ABSTRACT:** Enzyme kinetics is an essential part of a chemistry curriculum, especially for students interested in biomedical research or in health care fields. Though the concept is routinely performed in undergraduate chemistry/biochemistry classrooms using other spectroscopic methods, we provide an optimized approach that uses a real-time monitoring of the kinetics by quantitative NMR (qNMR) spectroscopy and a direct analysis of the time course data using Lambert-W function. The century old Michaelis–Menten equation, one of the fundamental concepts in biochemistry, relates the time derivative of the substrate to two kinetic parameters (the Michaelis constant $K_M$ and the maximum rate $V_{max}$) and to the concentration of the substrate. The exact solution to the Michaelis–Menten equation, in terms of the Lambert-W function, is not available in standard curve-fitting tools. The high-quality of the real-time qNMR data on the enzyme kinetics enables a revisit of the concept of applying the progress curve analysis. This is particularly made feasible with the advent of analytical approximations of the Lambert-W function. Thus, the combination of NMR experimental time-course data with progress curve analysis is demonstrated in the case of enzyme (invertase) catalyzed hydrolysis reaction (conversion of sucrose to fructose and glucose) to provide students with direct and simple estimations of kinetic parameters of Michaelis–Menten. Complete details on how to implement the experiment and perform data analysis are provided in the Supporting Information.



**KEYWORDS:** *Upper-Division Undergraduate, Graduate Education/Research, Undergraduate Research, NMR Spectroscopy, Laboratory Instruction, Physical Chemistry, Biophysical Chemistry, Catalysis, Enzymes, Kinetics*

## INTRODUCTION

Enzyme kinetics is one of the essential topics to understand in physical chemistry and biochemistry curriculum. Enzymes catalyze biochemical reactions, speeding up the conversion from substrate to product molecules. More than 100 years ago, Leonor Michaelis and Maud Leonora Menten developed an approach to describe and characterize enzymatic rates;[1] the classic Michaelis–Menten equation remains the fundamental equation in enzyme kinetics.[2] Today, the quest for fundamental understanding of the working of enzymes continues with vigor.[3] Though spectroscopic based approaches are inherently used to study enzyme kinetics, quantitative NMR (qNMR) allows simultaneous quantification and detection of both substrate and product in the same experiment on a real time basis.

Michaelis and Menten's theory does not explain the catalytic function of the enzyme (how it accelerates a reaction), but it describes a kinetic scheme for the enzyme and its substrate molecule to form a complex before proceeding to the product:

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{E{\cdot}S} \xrightarrow{k_{cat}} \mathrm{E} + \mathrm{P} \qquad (1)$$

In this scheme (eq 1), the enzyme (E) and the substrate (S) react reversibly with a forward ($k_1$) and reverse ($k_{-1}$) rate constants forming an intermediate enzyme–substrate complex (E·S). The complex converts irreversibly to the enzyme and product (P) with a rate constant $k_{cat}$. Briggs and Haldane[4] introduced the steady-state approximation, $d[E{\cdot}S]/dt = 0$. In a conventional enzymatic assay, the steady-state condition for E·S is reached very rapidly because the concentration of S is much greater than that of E, after which the E·S concentration remains constant, while the substrate concentration decreases and product concentration increases. The steady-state approximation results in the same form of the Michaelis–Menten equation with $K_M = (k_{cat} + k_{-1})/k_1$. This derivation is presented in most physical or biochemistry textbooks. The Michaelis–Menten equation quantifies the kinetics of enzymatic reactions by providing practical means to characterize an enzyme in terms of $k_{cat}$ and $K_M$. A high $k_{cat}$ and a low $K_M$, or a high $k_{cat}/K_M$ ratio, are indicators for an enzyme's high effectiveness. Lineweaver and Burk[5] rearranged the Michaelis–Menten equation to facilitate the determination of $K_M$ and $k_{cat}$ using a double-reciprocal plot approach ($1/V$ vs $1/[S]$).

The purpose of the present note is to incorporate real-time NMR measurements of enzyme kinetics into the undergraduate





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physical chemistry laboratory curriculum to enable the students to learn the basics of the Michaelis–Menten equation upon analyzing the progress curve using Lambert-W function. High-resolution NMR spectroscopy is sensitive to measure the substrate and product concentration changes simultaneously in an enzymatic reaction as a function of time in a highly reproducible manner. Thus, eliminating the need for simplifying assumptions and afford qNMR analysis of the full progress curves.[6] In addition to the experience the student acquires on the use of NMR spectroscopy to collect real time data, this approach promotes the development of analytical reasoning skills to qNMR data to relevant kinetic parameters by nonlinear least-squares analyses.

This *Journal* has contributed significantly to the development of various aspects of enzyme kinetics experiments. Starting from the comprehensive introduction to the topic by Shaw in 1957,[7] there are several notable publications that address conceptual, theoretical and experimental aspects.[8] Over the past few years, NMR has been introduced to perform enzyme kinetics; Olson et al.[9] introduced an organic chemistry laboratory experiment on the kinetics of acylase 1-catalyzed hydrolysis of *N*-acetyl-DL-methionine using $^{1}$H NMR spectroscopy with a traditional approach to estimate the Michaelis constant using the Lineweaver–Burk plot. Eicher et al.[10] utilized a $^{31}$P based NMR time-course experiment to analyze the enzyme kinetics of phosphoglucose isomerase/phosphofructokinase in the production/consumption of NAD(P)H using a Hill equation approach. A real-time NMR experiment combined with linearization of Lambert-W function was introduced,[11] while more recently Kehlbeck and co-workers[12] studied the hydrolysis of sucrose under both pseudo-zero order reaction (excess substrate) and initial rate approximation. In this work, we demonstrate that an optimal combination of both real-time acquisition of NMR data on the enzyme kinetics and a *progress curve analysis* using the Lambert-W function allow the estimation of both $K_M$ and $V_{max}$ reliably from a single experiment.

Here, we provide a concise description of this approach that can be easily adopted in an undergraduate physical chemistry laboratory. The learning objectives of this experiment in an undergraduate physical or biophysical chemistry laboratory are to

- Understand the concept of Michaelis–Menten equation for enzyme kinetics by measuring the substrate concentration using NMR spectroscopy,
- Explore the concept of progress curve analysis using the Lambert-W function and how the fitted parameters relates to enzyme kinetics,
- Learn the basics of optimizing a NMR experiment, to quantify the peak intensities to molecular concentrations (qNMR) and
- Combine the above-mentioned key aspects to estimate the $K_M$ and $V_{max}$ of the Michaelis–Menten mechanism using nonlinear least-squares fitting procedures.

Further explorations may include an investigation of the difference between the traditional analyses (based on initial rates) vs progress curve analysis. The laboratory could be divided into multiple groups of students to perform the same experiment with varying substrate concentrations while each group will analyze all the data. The class could restrict the analysis to initial rates and perform the traditional Lineweaver and Burk[5] plot in comparison with results obtained using progress curve analysis. The kinetic parameters determined using this approach are comparable to both the traditional results obtained using polarimetry[13] as well as other NMR based experiments that use conventional analysis.[12] Students with focus on chemistry, biochemistry or biophysical chemistry will learn the process for obtaining kinetic data, become familiar with statistical analysis through the use of algorithms, and achieve a deeper understanding of enzyme kinetic concepts through graphical and mathematical data analyses. The experiment and data analysis can be completed typically in an upper-level physical or biochemistry laboratory course within a period of 3 h.

## DESCRIPTION OF THE EXPERIMENT

### Experimental Procedures

All experiments and analyses were performed by the students (first four authors) for their final project assignments in a Physical Chemistry Laboratory course (CHEM 111) at Fresno State Chemistry Department during the spring of 2013.

**Samples.** Invertase (EC 3.2.1.26, $\beta$-fructofuranosidase, *Saccharomyces cerevisiae*) was purchased from Sigma-Aldrich with a specific activity of 200–300 u/mg of enzyme (pH 4.6, 303 K). Sucrose, $D_2O$ (99.9 atom % D), and 3-(trimethylsilyl)-propionic-2,2,3,3-$d_4$ acid sodium salt (TSP) were purchased from Sigma-Aldrich.

A stock acetate buffer solution (24.0 mM acetate (not-deuterated), 20.3 mM acetic acid-$d_4$, 1.1 mM TSP, and pH 4.8) was made in $D_2O$. (All solutions and dilutions will use this stock acetate buffer in order to maintain the same pH 4.8 and the same concentration of TSP throughout.) With the acetate buffer, a 100.1 mM stock sucrose solution and a 34.3 $\mu$g/mL stock invertase solution were made. Two different samples in a standard 5 mm NMR tube were used. The first sample, containing only sucrose, was made in an Eppendorf tube using the 100.1 mM stock sucrose solution. The final concentration of the first sample was 40.0 mM at a final volume of 650 $\mu$L. A 600 $\mu$L portion of this was transferred into a NMR tube. This sample was initially used to set up the NMR parameters (see below). The second sample was the kinetics experiment sample containing 40.0 mM sucrose and 5.3 $\mu$g/mL invertase. The second sample was made by transferring 260 $\mu$L of the 100.1 mM stock sucrose solution along with 290 $\mu$L of stock acetate buffer solution into an Eppendorf tube. Then, 100 $\mu$L of the 34.3 $\mu$g/mL stock enzyme solution was added into the Eppendorf tube and 600 $\mu$L of the solution was transferred to an NMR tube for experimentation. A timer was started to keep track of the delay time before the start of the collection of the first NMR spectrum and the delay time (T0) was added into the later calculations (see Supporting Information).

**Real-Time NMR Measurements.** A one-dimensional NMR experiment was first performed on the first sample, containing only sucrose, at 30 °C to identify the distinct resonances of sucrose at ~5.41 ppm (doublet, $^{1}$H). NMR experiments were done by regulating the probe temperature at 30 °C and without spinning the samples. Standard pulse calibration was performed to determine the 90° pulse. All the 1D NMR experiments were performed at a pulse angle corresponds to Ernst angle (~70°).[14] All the chemical shifts are expressed in units of parts per million (ppm) with respect to TSP. Each one-dimensional NMR spectrum was collected in a Varian-Agilent 400 MHz NMR spectrometer, with a spectral width of 14.88 ppm (5952.4 Hz) over 32,768 points to provide


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an acquisition time of 2.75 s per experiment (see Supporting Information for additional details). A relaxation delay of 1 s was used between the scans and 24 scans were signal averaged and saved for further processing. This experiment was arrayed to collect FIDs continuously one after another and a total of 80 such experiments (80 × 1.5 min = 120 min) were performed. A relaxation delay of 1 s was chosen as it is necessary to sample as many intermediate spectra as possible before the complete enzymatic conversion of sucrose to the products. The NMR experiments are similar to the routine experiments performed in the laboratory including the earlier work presented by us in *Journal of Chemical Education*.[15]

**Data Analysis.** NMR data were processed using the vnmrJ (Operating and processing software for Varian NMR spectrometers). Areas under the curves from two distinct resonances, of sucrose (doublet at 5.41 ppm) and TSP (0.0 ppm), were calculated.

### Theoretical Basis for Progress Curve Analysis

Integrated form of the Michaelis–Menten equation using the Lambert-W function was presented by Schnell and Mendoza[16] with application developed by Goudar and co-workers.[17] As the description of this approach in textbooks is limited,[18] a brief description in relation to enzyme kinetics is given here: The Michaelis–Menten equation in the differential form can be used to describe the dynamics of substrate depletion as

$$\nu = \frac{\mathrm{d[P]}}{\mathrm{d}t} = -\frac{\mathrm{d[S]}}{\mathrm{d}t} = \frac{V_{\max}[\mathrm{S}]}{K_\mathrm{M} + [\mathrm{S}]} \tag{2}$$

As defined previously, [S] is the substrate concentration, and $V_{max}$ and $K_M$ are the maximal rate and Michaelis–Menten half-saturation constant, respectively. Equation 2 can be readily integrated to obtain the integral form of the Michaelis–Menten equation as[16,19]

$$K_\mathrm{M} \ln\left(\frac{[\mathrm{S}]_0}{[\mathrm{S}]}\right) + [\mathrm{S}]_0 - [\mathrm{S}] = V_{\max} t \tag{3}$$

The Lambert-W function is a mathematical function that has numerous well-documented applications in mathematics, physics, and computer science.[20] Its definition is probably most easily understood by analogy with the inverse relationship between the exponential function and the natural logarithmic function ln($x$). The Lambert-W function works similarly, with the difference being the initial expression that connects $x$ and $y$. $W(x)$ is defined as the inverse of the function satisfying $ye^y = x$ and its solution expressed by the Lambert-W(x) function as $y = W(x)$. By substituting $y = [\mathrm{S}]/K_\mathrm{M}$ in eq 3 and rearranging (detailed steps are provided in Supporting Information),

$$ye^y = x(t) = \exp\left(\frac{[\mathrm{S}]_0 - V_{\max} t}{K_\mathrm{M}} + \ln\left(\frac{[\mathrm{S}]_0}{K_\mathrm{M}}\right)\right)$$

$$= \frac{[\mathrm{S}]_0}{K_\mathrm{M}} \exp\left(\frac{[\mathrm{S}]_0 - V_{\max} t}{K_\mathrm{M}}\right) \tag{4}$$

The left-hand side of eq 4 is analogous to Lambert-W function.[20b] Thus, using the definition of Lambert-W function ($y = W(x)$), an expression for $y$ could be obtained as

$$y = W\left\{\frac{[\mathrm{S}]_0}{K_\mathrm{M}} \exp([\mathrm{S}]_0 - V_{\max} t/K_\mathrm{M})\right\} \tag{5}$$

Further substituting $y = [\mathrm{S}]/K_\mathrm{M}$ back in eq 5,

$$[\mathrm{S}] = K_\mathrm{M} W\left\{\frac{[\mathrm{S}]_0}{K_\mathrm{M}} \exp\left(\frac{[\mathrm{S}]_0 - V_{\max} t}{K_\mathrm{M}}\right)\right\} \tag{6}$$

Equation 6, derived from eq 3, relates the substrate concentration at any time ([S]) to its initial concentration ($[\mathrm{S}]_0$), the Michaelis–Menten kinetic parameters $V_{max}$ and $K_M$. As most of the available nonlinear regression curve-fitting packages in classrooms may not handle the full nonlinear fitting process of the Lambert-W function (eq 6), a simplified procedure developed by Goličnik[21] is used to the fitting process. These modifications can be incorporated in a straightforward manner by the students starting from the raw real time experimental data. Supporting Information provides a step-by-step demonstration of the data analysis using either Sigma Plot or R-Statistical environment.

## HAZARDS

All materials used in this experiment are level 1 health hazards except for acetic acid-$d_4$ which is a level 3 health hazard. Precautions to prevent skin contact, inhalation, and ingestion should be taken. Sodium acetate is an irritant and is slightly hazardous in case of skin or eye contact. Deuterium oxide is hazardous in case of ingestion. Acetic acid-$d_4$ is highly concentrated so direct skin contact should be avoided; also acetic acid-$d_4$ should be handled in the fume hood due to vapor. Students should wear protective eyewear and can use protective gloves to prepare solutions. Students are instructed to collect used solutions in properly labeled containers so they can be collected by environmental health and safety at your institution.

## RESULTS

The real-time enzyme kinetics of the substrate (S, sucrose) converted to the product ($P_\alpha$, glucose) by invertase via hydrolysis is shown in Figure 1. The sucrose resonance (5.41 ppm) completely converted into that of the glucose molecules by the end of the experiment (~120 min). As the product $\alpha$-D-glucose at 5.22 ppm forms, it immediately starts to convert into $\beta$-D-glucose at 4.64 ppm (Figure S1). At equilibrium, the ratio of $\alpha$:$\beta$ anomer of D-glucose is approximately 1:2.

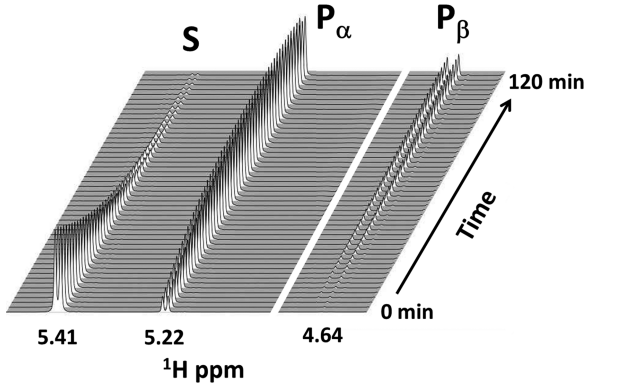


**Figure 1.** Real-time $^1$H quantitative NMR (qNMR) spectra of the enzyme kinetic at 400 MHz. Hydrolysis of sucrose (substrate, 5.41 ppm) as it is converted to glucose (product, $P_\alpha$, and 5.22 ppm) is shown as a function of increasing time. The delayed anomerization of $\alpha$-glucose form to $\beta$-glucose form is also shown ($P_\beta$, 4.64 ppm).

Figure 2 shows the progression of the kinetics for the substrate concentration as a function of time for three independent trials. The production of the glucose molecules


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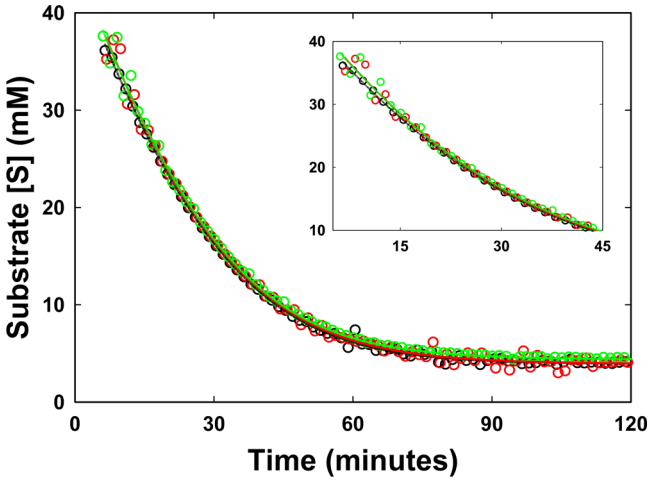


**Figure 2.** Real-time qNMR results of substrate depletion ([S]) obtained using independent trails. Three independent samples were prepared and NMR experiments were performed (black, red, and green symbols). The concentration of sucrose (substrate) is shown as a function of time due to hydrolysis process of the enzyme invertase. Time dependence of the substrate concentration, fitted to Lambert-W function (eq 6 and Supporting Information eq 9), is shown by the continuous curves. For the average initial sucrose concentration of 42.08 ± 0.67 mM ($[S]_0$), the nonlinear least-square fit yielded an average $K_M$ = 38.57 ± 3.72 mM and an average $V_{max}$ = 2.54 ± 0.118 $\mu$M/min. The inset shows the same data points during the initial time points.

in a similar experiment is given in the Supporting Information (Figure S2). The continuous curves in Figure 2 show the nonlinear least-square fit to the time dependent substrate concentration. Table 1 lists the estimated Michalis–Menten constants and the initial concentration according to the progress curve analysis. The average value (estimated from three independent trials) for the Michalis–Menten constant ($K_M$) and the maximal rate obtained ($V_{max}$) are 38.57 ± 3.72 mM and 2.54 ± 0.118 $\mu$M/min, respectively, using R. The results are similar (<0.01% error) when a similar routine using Sigma Plot is used (complete instructions including an R-code are given in the Supporting Information). In the traditional approach that measures the concentration of the product, it would be necessary to include the peak intensities at both the $\alpha$ and $\beta$ anomers particularly at longer experiments. We find that using the substrate concentration ([S]) to fit the Lambert-W function is preferred in this case as it does not include the factors due to anomerization of the glucose. Furthermore, the sucrose peak at 5.41 ppm from the $^1H\gamma$ proton is well resolved even at proton resonance frequencies as low as 60 MHz.[12]

The quality and reproducibility of the NMR based approach critically depends on how reliably the peak areas can be converted into concentrations. As NMR spectroscopic methods take a central role in many applications, such as metabolomics, quantitative NMR (qNMR) spectroscopy are becoming a routine.[22] Current protocols of qNMR rely on the quantitative feature of $^1H$ NMR resonance area corresponding to the number of protons of the same type in a molecule to an internal standard, such as TSP. Here the peak intensities were converted into concentrations using the known concentration of TSP added to the sample (Supporting Information). Determining the molecular concentration using this approach tends to reduce uncertainties in quantifying the peak areas as each sample has its own internal reference (known concentration of TSP spiked to the solvent $D_2O$). This approach further allows a comparison of the data collected between the various student groups.

The integration of NMR spectra can be carried out with high accuracy, but this is only possible if a number of sources of error are properly handled. On a modern spectrometer the accuracy of ±5% can be achieved easily if relaxation issues are handled properly. NMR spectroscopy has a feature unique among spectroscopic methods, that *relaxation* processes are relatively slow (on the order of seconds), compared to other spectroscopic techniques (UV or IR). If the pulse angle and repetition rates are too high, then the spectra can become partially *saturated*, causing the integrations to be less accurate, because the relaxation rates of various protons in the sample are different. Saturation effects are particularly severe for small molecules in mobile solvents because these typically have the longest $T_1$ relaxation times. In samples that do not change with time, typically one could use a relaxation delay (interpulse delay) of 5 times the $T_1$. However, in kinetics experiments such as the current experiments, we expect the relaxation effects on the measured Michalis–Menten constants would not be highly significant. One option to reduce the saturation effects due to the relaxation process is the use of Ernst angle (see Experimental Procedure).[14]

The quality of the NMR data obtained in each trial (Figure 2) is highly reproducible. The Michaelis–Menten enzyme kinetic parameters obtained are similar to the results obtained using other experimental approaches (enzyme activity >300 u/mg)[12] while reproducible with the enzyme activity of 200–300 u/mg.[11] To estimate the overall reproducibility of the measurement, we repeated the experiments three times independently and the coefficient of variation of the mean was determined (Table 1). The coefficient of variation of the $V_{max}$ was lower (8.5%), while that of the $K_M$ was found to be higher (11.8%). Careful preparation of the samples and consistent experimentation is expected to produce a coefficient of variation of the Michalis–Menten constants within ∼10%.

## CONCLUSIONS

We here present an alternate approach that is a relatively rapid method for obtaining enzyme-kinetic parameters from metabolite time-course data generated using NMR spectroscopy. The method requires fewer runs than traditional initial-rate studies and yields more information per experiment, as whole time-courses are analyzed and used for parameter fitting.

**Table 1. Estimated Michalis–Menten Parameters Using qNMR Data**[a]

| Parameters | Trial 1 | Trial 2 | Trial 3 | Average | Coefficient of Variation (%) |
|---|---|---|---|---|---|
| $K_M$ (mM) | 34.69 ± 3.96 | 43.59 ± 11.90 | 37.44 ± 7.79 | 38.57 ± 3.72 | 11.81 |
| $V_{max}$ ($\mu$M/min) | 2.34 ± 0.18 | 2.77 ± 0.54 | 2.51 ± 0.36 | 2.54 ± 0.18 | 8.52 |
| $[S]_o$ (mM) | 41.27 ± 0.39 | 42.91 ± 0.88 | 42.05 ± 0.69 | 42.08 ± 0.67 | 1.94 |

[a]Progress curve analysis using either Sigma Plot or R produced results with an error of <0.01%.



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Additionally, this approach allows real-time simultaneous quantification of both the substrate and product(s) present in the assay system, which demonstrates the superiority of qNMR over traditional spectrophotometric coupled enzyme assays. The methodology presented is applied to the elucidation of kinetic parameters for invertase catalyzed conversion of sucrose to glucose and fructose. $^1$H NMR time-course data were collected in real time by qNMR, and the kinetic data were subsequently processed using a nonlinear least-square fit procedure by using the Lambert-W function. Detailed information on the data analysis using Sigma Plot and R-statistical environment is provided in the Supporting Information.

The calculation of kinetic parameters of Michalis−Menten equation using qNMR is an excellent way to introduce students to a spectroscopic method that may be utilized in both their current and future education as well as their research efforts. The complete experiment does not require a substantial length of time (2 h), and some portions can be performed in an automated manner. Analysis of the resultant data is straightforward enough that no detailed knowledge of NMR spectroscopy is necessary.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.5b00136.

Instructions for the students and notes for the instructor (PDF, DOCX)

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### Notes

The authors declare no competing financial interest.

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## ■ DEDICATION

†This article is dedicated to Professor K. V. Ramanathan who is retiring in 2015 from the Indian Institute of Science, Bangalore, India, after an outstanding career in the field of NMR.

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