

# Applying Low Field $^{13}\text{C}$ NMR Spectroscopy to Find the Isoelectric Points of Amino Acids

Jun H. Shin<sup>†</sup>, Sabrina Song<sup>†</sup>, Yoomi Kim<sup>†</sup>, and Gopal Subramaniam<sup>‡\*</sup>

<sup>†</sup>Department of Chemistry, Queensborough Community College -CUNY, Bayside, NY 11364; <sup>‡</sup>Department of Chemistry and Biochemistry, Queens College -CUNY, Flushing NY 11367, gopal.subramaniam@qc.cuny.edu

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**Abstract:** We describe a cost efficient  $^{13}\text{C}$  NMR spectroscopy experiment using a low-field NMR instrument and non-deuterated aqueous solutions that can be easily performed at teaching schools where high field instruments are not affordable. The experiment uses the effect of pH on the structure of amino acids, which affects the chemical shifts. By monitoring the changes in the  $^{13}\text{C}$  chemical shifts of the amino acid as a function of pH, we can find the isoelectric points (pI) of alanine, cysteine, glycine, proline, serine, threonine, valine and methionine. Other than the one-time cost of purchasing the instrument, ongoing reagent costs are minimal and comparable to other laboratory experiments. The method complements other biophysical methods to find the pI of amino acids and serves as an introduction to the application of the NMR spectroscopy technique to biological compounds with concepts learned in introductory chemistry courses. This experiment is designed for students who have finished the equivalent of the first two semesters of basic chemistry and it provides hands-on learning to a very fundamental structure determination technique.

## Introduction

Over the last three decades advances in high-field instruments has made NMR spectroscopy a very powerful structure determination technique for organic and biological molecules [1, 2]. In most research universities where high-field NMR instruments are common, NMR spectroscopy laboratory is introduced at the junior or senior year in conjunction with some advanced courses or undergraduate research. However, at community colleges and high schools, which are mainly teaching institutions, it is not cost-efficient to maintain a high-field NMR system. Fortunately, cheap low-field NMR instruments are still commercially available that can serve as an educational tool at such teaching institutions, but, there are not many experiments that can be adopted at a 2-year institution. A simple experiment to measure the isotope ratio of boron with a low-field instrument was described recently [3]. Another way to introduce NMR spectroscopy without the high cost of instrumentation is to use software programs that can generate NMR spectra from structures [4, 5]. While it is a good NMR interpretation exercise, it is not the same as getting a hands-on experience. Herein we use small biological molecules to introduce pulse FT-NMR concepts and NMR instrumentation. Students who pursue health-related professions after their 2-year degree will greatly benefit from this laboratory exercise.

The experiment described here uses a 60-MHz EFT-NMR instrument and solutions of amino acids in  $\text{H}_2\text{O}$  to find their isoelectric points. Because we use nondeuterated solvents, continuing experimental costs are comparable to other chemistry laboratory experiments. Even though  $^1\text{H}$  NMR is more sensitive, in aqueous solutions, the proton signal from the  $\text{H}_2\text{O}$  solvent is very large, making it difficult to monitor the proton signals from the solute. This is not the problem with the  $^{13}\text{C}$  nuclei because it is present only in the solute. The chemical shifts of  $^{13}\text{C}$  are sensitive to small structural changes, but the

low natural abundance of  $^{13}\text{C}$  requires a high concentration of solute to get a good signal in a short time.

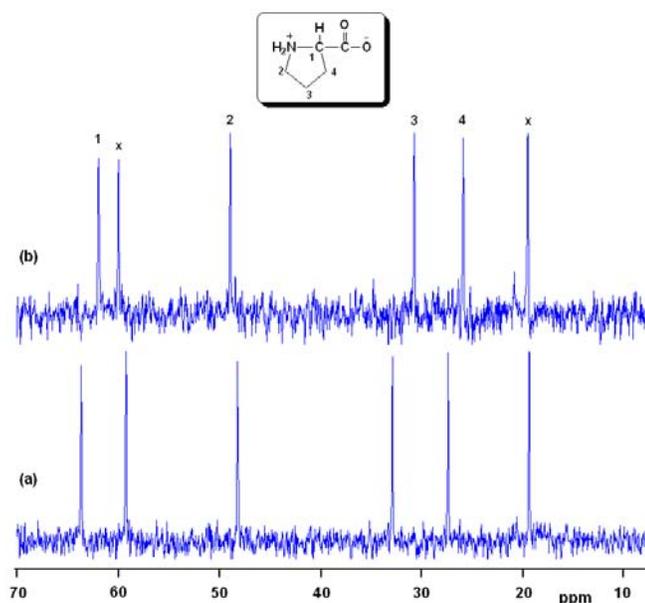
In this experiment, students learn about the basics of the NMR instrumentation, obtaining experimental NMR data, characterizing a chemical structure using  $^{13}\text{C}$  NMR chemical shifts, effect of chemical equilibrium on the observed chemical shifts, and the importance of pH in the structure of biological molecules. This experiment can be introduced as an organic laboratory experiment with an optional instrumentation laboratory with other instruments like GC-MS, HPLC, IR, etc.

## Experimental

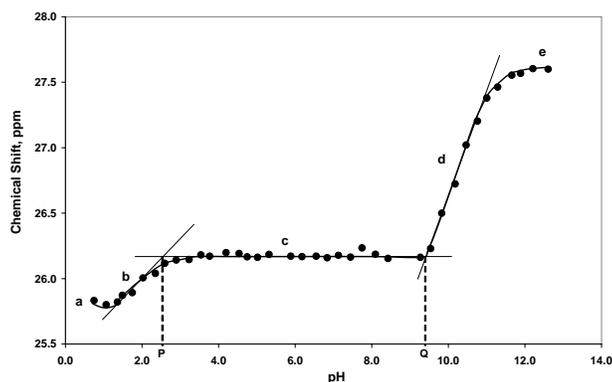
**Sample Preparation.** 1.00 g of an amino acid was added to a 50-mL beaker. Using a 10 mL graduated pipette, 10.0 mL of 3 M HCl solution was added to dissolve the amino acid. To the solution of the amino acid, 1.00 mL of ethanol was added to serve as an internal reference for the  $^{13}\text{C}$  NMR spectra. The procedure was repeated with 3 M NaOH solution to prepare a basic solution of the amino acid. The pH values of both acidic and basic amino acid solutions were measured using a pH meter (Model #PH77-SS, IQ Scientific Instrumentations), which was calibrated using pH 4.0 and 7.0 buffer standards.

**NMR Parameters.** The  $^{13}\text{C}$  NMR of the sample was recorded on an Anasazi EFT-60 60-MHz NMR instrument with the  $^1\text{H}$  frequency at 60.01 MHz and  $^{13}\text{C}$  frequency at 15.089 MHz at room temperature with the sample spinning at 20 Hz. The  $^{13}\text{C}$  NMR data was recorded with a 14.4 microsecond pulse, 8192 data points, 2.0 second relaxation delay, and 64 scans. The data was processed with a 2-Hz line broadening and Fourier-transformed using the Nuts-Pro utility software. Chemical shifts of the sample are internally calibrated to the methyl group of ethanol at 19.459 ppm. The total time for recording one  $^{13}\text{C}$  NMR spectrum was about 5 minutes.

**Titration.** About 2 mL of the acidic amino acid solution was placed in a 5-mL glass vial and the basic amino acid solution was added while stirring until the pH of the solution reached around 0. About 0.7 mL of the solution was transferred to an NMR tube and its  $^{13}\text{C}$  NMR spectrum was recorded. The solution was poured back into



**Figure 1.**  $^{13}\text{C}$  NMR spectra of proline at pH = 12.97 (a) and pH = 0.75 (b). Reference peaks from ethanol are marked with an x and the assignment of proline carbon signals 1, 2, 3, and 4 are indicated on top of the peaks.



**Figure 2.** Titration graph of the  $^{13}\text{C}$  chemical shift of C4 carbon of proline plotted against pH of the solution. There are five regions in the titration curve marked a through e, which are described in the text. The midpoint of the regions b, c, and d corresponds to  $\text{p}K_{a1}$ ,  $\text{pI}$  and  $\text{p}K_{a2}$  of proline. The midpoint of region c is measured using the two intersection points, P and Q, from the visually best-fit straight lines as shown in the figure.

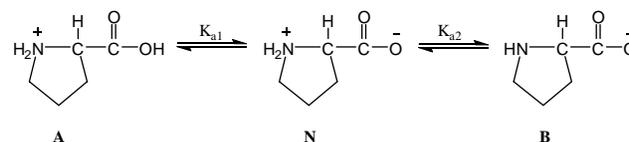
the same vial, and about 0.10 mL of the basic amino acid solution was added using a microsyringe, stirred, and the pH of the solution was recorded. About 0.7 mL of the solution was transferred to a clean NMR tube and its  $^{13}\text{C}$  NMR spectrum was recorded. The process was repeated with 0.10 mL additions of the basic amino acid solutions until the pH changes were small. When the pH changes increased, the amount of added solution was reduced to 0.050 mL. Between 20 and 30 titrations covering the entire pH range were required to calculate  $\text{pI}$ . Because the acidic and basic amino acid solutions were of the same concentration, there is no change in the total concentration of the amino acid during the titration.

**Hazards.** Concentrated acids and bases are used in this experiment. Lab coat, safety goggles, and rubber gloves are required. Used NMR solutions of amino acids are acidic or basic and considered corrosive. Hence, they should be properly labeled and disposed of as a hazardous waste.

## Results and Discussion

**$^{13}\text{C}$  NMR Spectrum of Proline.** The proton-decoupled  $^{13}\text{C}$  NMR spectra of proline in basic and acidic solutions are illustrated in Figure 1a and 1b. The assignment of the  $^{13}\text{C}$  signals can be easily determined based on their environment and from commercial programs or spectral databases [4, 6]. Comparing Figures 1a and 1b, we observe that the chemical shift of the C4 carbon changed from 25.83 ppm ( $\delta_a$ ) at low pH to 27.51 ppm ( $\delta_b$ ) at high pH. Scheme 1 shows the chemical structures of proline that can exist in an aqueous solution. The chemical shift observed in solution is the weighted average chemical shift of the equilibrating species **A**, **N**, and **B** whose concentrations vary with pH of the solution.

**$^{13}\text{C}$  NMR Titration Curves.** A plot of  $\delta_{\text{obs}}$  as a function of pH is shown in Figure 2 using the  $^{13}\text{C}$  chemical shift of C4 of proline. The titration curve shows a double sigmoidal feature corresponding to the neutralization of the two different functional groups. There are five regions marked a to e for identification (Figure 2). At extremely acidic pH, only structure **A** is expected to be present because the equilibrium is pushed to the far left (Scheme 1). This corresponds to region a in Figure 2.



**Scheme 1.** Structure of proline at various pH conditions. The neutral species, **N**, is present when  $\text{pH}=\text{pI}$ . **A** is present at pH below  $\text{pI}$  and **B** is present at pH above  $\text{pI}$ .

Addition of base disturbs the equilibrium by lowering the amount of **A** and increasing the amount of **N**. Since **A** and **N** exchange with each other faster than the NMR timescale, only one signal is obtained based on the weighted average of the concentrations of **A** and **N** (Figure 2, region b). The pH changes are minimal in this region because of the buffering action and governed by the Henderson-Hasselbach equation,

$$\text{pH} = \text{p}K_{a1} + \log \frac{[\text{N}]}{[\text{A}]}$$

Region c depicts a large pH change with little chemical shift change. In this region, the solution contains predominantly **N**, representing the complete neutralization of the carboxylic acid group. Region d corresponds to the neutralization of the protonated amino group which starts the formation of **B**. The buffering action minimizes changes to the pH while the chemical shift of C4 increases to its maximum because more **B** is formed with the addition of base. Region e of the titration curve corresponds to the formation of 100% **B** where there is pH change without chemical shift change when base is added.

**Table 1.** Observed  $^{13}\text{C}$  Chemical Shifts at Low pH and High pH of the Various Amino Acids Studied

Amino Acids	$\alpha$	$\beta$	$\gamma$	$\delta$
Proline	62.0–63.8	25.8–27.6 <sup>a</sup>	30.7–33.0	49.0–48.3
Glycine	42.8–47.3 <sup>a</sup>			
Cysteine	56.9–59.1 <sup>a</sup>	26.6–28.6		
Alanine	51.4–53.7 <sup>a</sup>	17.9–22.3		
Threonine	67.7–72.0	60.9–64.3 <sup>a</sup>	21.6–22.0 <sup>b</sup>	
Serine	62.0–67.3	57.3–60.0 <sup>a</sup>		
Valine	60.9–65.5	31.5–34.6	19.9–21.8 <sup>a</sup>	
Methionine	54.5–57.7	31.4–36.6 <sup>a</sup>	31.2–32.1	16.6–16.6

<sup>a</sup>These chemical shift changes are plotted in Figure 3 as a titration curve. <sup>b</sup>The titration curve for this carbon showed a bell shape with the chemical shift maximum at pH = 5.0.

**Table 2.** pI Values Obtained by the  $^{13}\text{C}$  NMR Titration Method

Amino Acid	Range of Measured pI Values	pI Values from Reference [10]
Threonine	5.4–5.7	5.6
Serine	5.4–5.6	5.7
Alanine	5.9–6.1	6.0
Proline	6.1–6.4	6.4
Cysteine	4.9–5.2	5.1
Glycine	5.8–6.0	6.1
Valine	6.0–6.2	6.0
Methionine	5.5–5.8	5.7

The midpoints of the regions b and d corresponds to  $\text{p}K_{\text{a}1}$  and  $\text{p}K_{\text{a}2}$ , respectively. The midpoint of the nearly flat region of c corresponds to pI. pI can also be calculated as the average of  $\text{p}K_{\text{a}1}$  and  $\text{p}K_{\text{a}2}$ . The chemical shift change in the region d is larger than b implying that C4 is affected more by the ionization of the  $\text{NH}_2$  group than the  $\text{COOH}$  group. This can be explained by the increase in the number of bonds that separates C4 and O atoms compared to C4 and N atoms.

#### Chemical Shift Changes for the Amino Acids Studied.

The chemical shift ranges for each of the carbons in the amino acids used in this study are shown in Table 1. As expected, the chemical shift changes are lower for  $^{13}\text{C}$ s that are farther away from the amino group. It is interesting to note that the  $^{13}\text{C}$  signals shift downfield at higher pH in spite of the increase in electron density. This can be explained by the change in hybridization of the nitrogen atom of the amino group as it ionizes affecting its inductive and spatial effects [7]. Using molecular orbital calculations, Quirt et. al. [8] reasoned that there is relatively a higher paramagnetic contribution to chemical shift causing deshielding at higher pHs. Threonine's  $\gamma$ -carbon exhibited a bell-shaped titration graph where the chemical shift went up and then down which can be explained by the changes in intramolecular H-bonding as the carboxylic acid ionizes. Measuring the changes in  $^{17}\text{O}$  chemical shifts and linewidth as a function of pH in glycine and alanine, Valentine et. al. [9] observed the dependence of  $^{17}\text{O}$  chemical shift on the intramolecular association at zwitterionic form and solute-solvent association at higher pHs.

The unusual nature of the chemical shift change for threonine  $\gamma$ -carbon precluded its use for calculating  $\text{p}K_{\text{a}}$ . Similarly  $^{13}\text{C}$ s that are less sensitive to pH changes were also excluded for  $\text{p}K_{\text{a}}$  calculations. Figure 3 shows the titration plots for glycine, cysteine, alanine, threonine, serine, valine, and methionine using their  $\alpha$ ,  $\beta$  or  $\gamma$   $^{13}\text{C}$  chemical shifts. The isoelectric points obtained by various student groups are

summarized in Table 2. The measured pI values are within  $\pm 0.3$  pH units of literature values [10].

#### Conclusions

We have demonstrated a NMR spectroscopy experiment that can be introduced using a low-field NMR instrument and non-deuterated aqueous solutions adoptable at a teaching institution where the cost of maintaining a high-field instrument is not feasible. Students get hands-on experience with the NMR instrument and use NMR to characterize the structure of molecules that are in equilibrium.

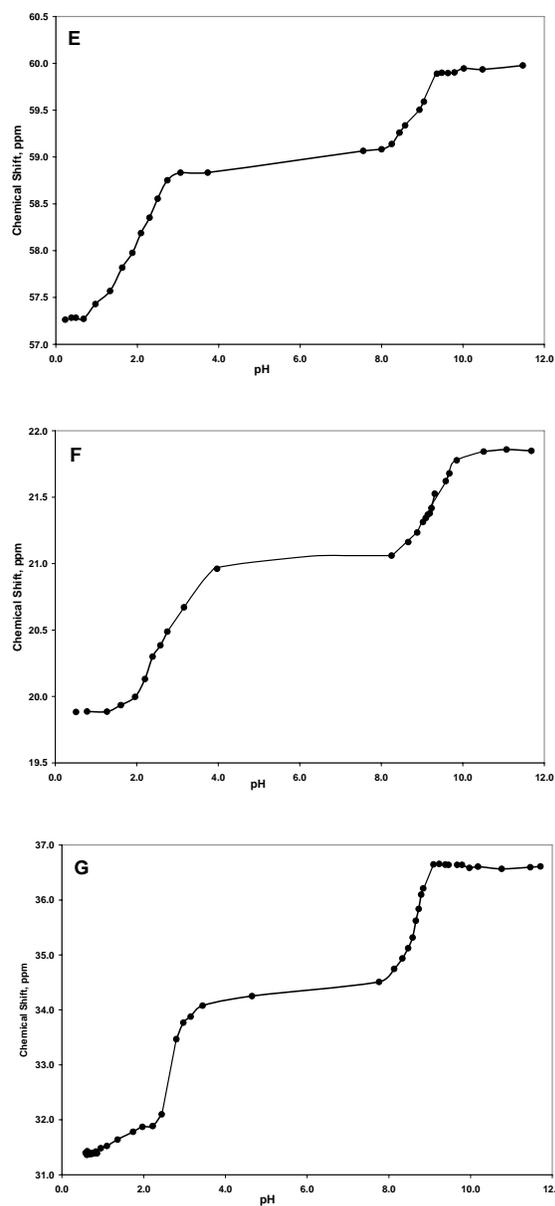
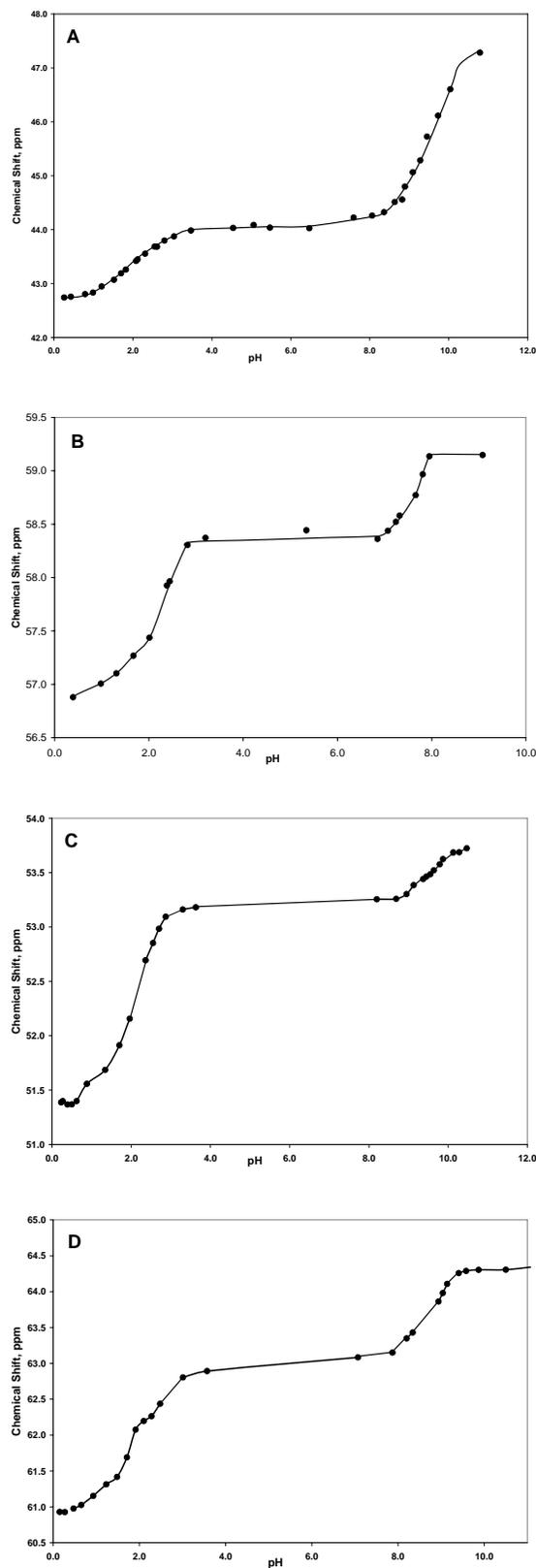
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**Supporting Materials.** An instructor note section detailing the chemicals and equipments, procedure, implementation, pre-lab, and post-lab exercises is available. A simple step-by-step operating procedure for acquiring NMR spectrum adopted for our organic chemistry laboratory is also available (<http://dxdoi.org/10.1333/s00897072059a>).

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**Figure 3.**  $^{13}\text{C}$  chemical shifts of various amino acids plotted as a function of pH: (A) glycine  $\alpha$ - $^{13}\text{C}$ , (B) cysteine  $\alpha$ - $^{13}\text{C}$ , (C) alanine  $\alpha$ - $^{13}\text{C}$ , (D) threonine  $\beta$ - $^{13}\text{C}$ , (E) serine  $\beta$ - $^{13}\text{C}$ , (F) valine  $\gamma$ - $^{13}\text{C}$ , (G) methionine  $\beta$ - $^{13}\text{C}$ .