

EDULAB FOR STUDENTS The Cola Chemistry

NMR of Cola Drink

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Keywords:

Food analysis, analytical chemistry, impurity analysis, benchtop NMR, ³¹P NMR, quantitative NMR, ERETIC, cola drinks.

Target group:

Advanced Undergraduate or Graduate, General Chemistry, Analytical Chemistry, Food Chemistry

Objectives:

NMR is typically introduced to students as a tool for structure determination, despite its routine use in other applications such as food analysis and quantitative impurity determination. In this EduLab, students will learn how to use NMR to analyze cola drinks, familiarizing with less-often taught concepts such as quantitative NMR and the analysis of nuclei other than ¹H and ¹³C. They will run NMR experiments, analyze data using two different calibration approaches - external calibration based on dilution series and sequential standard addition and learn how to use the ERETIC module available in TopSpin for quantification.

Background of the Experiment:

Cola drink is one of the most iconic beverages globally, known for its unique, refreshing taste. One of the key ingredients that gives cola its distinctive taste is phosphoric acid, which not only contributes to the tangy flavor but also plays a role in the beverage's preservation. This EduLab dives into the secrets of cola drinks by determining phosphorus content using NMR spectroscopy.

In this exercise, we will analyze cola drinks using ³¹P NMR spectroscopy, providing hands-on experience in applying NMR spectroscopy in the realm of food chemistry. It's important to note that the same approach can be employed in other fields, given the significance of ³¹P in other fields such as organic catalysis or biochemistry.¹⁻⁴ Using cola drinks as a case study, we will learn about quantitative NMR and compare the results obtained through different quantification approaches: external calibration, standard addition and ERETIC. This lab is based on a recent publication.⁵

The exercise can be easily tailored to distance education, as data analysis conveys the most important ideas even without observing measurements. The material can be further developed in various directions, such as qNMR, general spectroscopy, instrumental analysis, basic data analysis, or food chemistry, according to teaching staff or local curriculum needs. Extension to samples other than fizzy drinks is relatively straightforward.

To facilitate introducing the laboratory, it is possible to find additional information about phosphorus content determination, ³¹P NMR spectroscopy, and calibration approaches in the original paper.⁵

Preparation and Prerequisite:

During the laboratory practical, the students determine the phosphate content of cola drinks by both external calibration and standard addition. Figure 1 provides a general overview of the main steps.



Figure 1: Overview of the experimental procedure. Reproduced from Reference 5.

To ensure efficient completion of the experiments, it is recommended to form groups with a maximum of 4-6 students. The estimated time for the laboratory session is approximately 3 hours, including 1 hour of sample preparation and preliminary discussion and 2 hours for measurements, spectra acquisition and processing of the data.

To perform these experiments, it is required to have access to a Fourier 80 system that can measure ³¹P, and a PC that is connected to the Fourier 80 to acquire the data in TopSpin software. In addition, micropipettes, a sonicator, and prepared solutions of 80 mM NaH_2PO_4 and 1 M KH_2PO_4 are also required for sample preparation and standard addition.

Note that the same exercise can also be carried out with higher field instruments, with or without NMR automation. In the original paper the data was acquired on a Bruker Avance 250 MHz and a Bruker Avance Neo 400 MHz. If the exercise is adapted for a spectrometer not in automation mode, then the students might need to perform tuning, matching, locking, shimming and processing of the FID manually. This is very educational from a spectroscopic and technical point of view; however, carrying out all these steps and explaining the underlying concepts sufficiently takes time, therefore a 4-hour-long laboratory practical is preferred.

Glossary

NMR: Spectroscopic analytical technique based on radio frequency-induced transitions between energy levels that atomic nuclei adopt in an external magnetic field as a result of their own magnetic moment

T1: After excitation, the nuclear spins realign themselves along the external magnetic field. This process of realignment is referred to as longitudinal relaxation and characterized by the longitudinal relaxation time, T1.

D1: The amount of time that elapses after the signal is acquired, typically intended to allow the spins to return to equilibrium. To achieve this goal, it is recommended to set D1 to 5-7 times the longest T1.

P1: The length of a 90-degree pulse for your sample in the spectrometer

Experimental Setup:

Materials:

- NaH₂PO₄ and KH₂PO₄ samples
- HCl solution
- Phosphoric acid (85%, ³¹P NMR chemical shift reference)
- A cola drink sample/s
- 5 mm NMR tubes and caps
- A micropipette to perform standard additions
- A small beaker for pipetting the KH₂PO₄ solution
- pH meter suitable for small sample volumes (not compulsory)

Experimental set-up:

Set up in TopSpin the NMR experiments to acquire ³¹P, ¹H and ¹H with water suppression spectra. Use the suggested sequences and parameters given in the table below.

		Spectrum		
Parameter	Abbreviation/ unit	³¹ P{ ¹ H}, zgpg30	¹ H, zg30	¹ H wsupp, noesygppr1d
Spectral width	SW/ ppm	405	20	20
Transmitter offset	O1P/ ppm	-50	6	4.7
Number of transients	NS/ 1	256	64	4
Number of dummy scans	DS/ 1	4	2	2
Number of points in the time domain	TD/ 1	32k	8k	8k
Inter-scan delay	D1/s	2	3	3
Exponential line broadening	LB/ Hz	1	0.3	0.3
Size of real spectrum	SI/ 1	64k	16k	32k

Curly brackets around a nucleus indicate decoupling of the given nucleus during the measurement.

Abbreviations

NMR: Nuclear Magnetic Resonance

ERETIC: Electronic REference To access In vivo Concentrations

Sample Preparation:

The cola samples used in the experiment should be degassed. This should be done prior to the laboratory session by either boiling or sonication.

- 1. Prepare a 80 mM NaH_2PO_4 solution and a 1M KH_2PO_4 solution.
- 2. Prepare a 0.06 M HCl solution in water.
- 3. Prepare the calibration series of phosphate solutions directly in 5 mm NMR tubes using micropipettes according to the table below.

Preparation of calibration solutions and the cola drink sample

ID	c / mM	V(80 mMNaH ₂ PO ₄)/ µI	V(0.06 M HCI)/ µI	V(cola drink)/ µl
Cal 1	2.0	15	585	0
Cal 2	4.0	30	570	0
Cal 3	6.0	45	555	0
Cal 1	8.0	60	540	0
Cal 5	10.0	75	525	0
Sample	?	0	0	600

4. Each group prepares a single sample solution from a degassed cola sample.

Experimental Procedure:

- 1. Record 1D ¹H spectra of 2mM calibrant and cola sample with and without water suppression.
- 2. Record 1D ³¹P spectra of the calibrant series.
- 3. Record 1D ¹H spectra with water suppression and 1D ³¹P spectra of the cola sample
- 4. Perform 3 standard additions of 3 μ l of the 1M KH₂PO₄ solution to the cola sample. After each addition, acquire the 1D ³¹P spectra of the sample.
- 5. Process and analyze the data using the procedure described in the paragraph below.

Data Processing:

- 1. Process all the spectra using standard protocols, including phase and baseline correction.
- Reference the chemical shift to the ³¹P chemical shift of external 85% phosphoric acid (at 0 ppm).

- 3. For each ³¹P spectrum, extract the following parameters: chemical shift, peak height, peak integral and peak width at half height ('peak width' from here onward). These parameters can be extracted in TopSpin by peak integration and deconvolution. Accurately performing this step is critical for the success of the experiment. For the integration, use a 1 ppm wide window around the chemical shift of the single peaks in each spectrum to ensure reproducibility of integration. For the peak width, high and half heigh, use Lorentzian deconvolution.
- 4. Following peak parameter extraction, proceed with quantitative evaluation by extracting and analysing the data in an appropriate software of your choice (e.g. spreadsheet editor). Perform a total of 2 quantifications using the two calibration methods.
 - a. For external calibration, signal integrals are plotted against calibrant concentration and a linear fit is performed as shown in Figure 2. The concentration of the unknown sample is determined by interpolation according to

$$c_{\text{sample}} = \frac{I_{\text{sample}} - A}{B}$$

where c_{sample} is the total phosphorus concentration of the sample solution, I_{sample} is the intensity (integral) for the sample solution, while A and B are the y-intercept and the slope of the corresponding fitted line, respectively.

b. Quantitation via the sequential standard addition method is evaluated by plotting the measured intensities or areas against the increase of phosphorus concentration in the sample solution upon consecutive additions (Figure 2). The measured integral of the original sample solution belongs to the value zero on the x-axis. The 3 standard additions yield 4 points for linear fitting with y-intercept A' and slope B'. The concentration of the sample solution in sequential standard addition is calculated using

$$c_{\text{sample}} = \left| \Delta c_{\text{sample}} \right| = \left| -A'/B' \right|$$

5. ERETIC2 is a tool based on PULCON,⁶⁻⁸ a method that correlates the absolute intensities of two different spectra. Providing that the concentration of one of the samples is known (in this case, the concentration of the calibrant or standard addition), it is possible to quantify the concentration in the unknown sample using

$$C_{unk} = kC_{ref} \frac{A_{unk}T_{unk}\theta_{unk}NS_{ref}}{A_{ref}T_{ref}\theta_{ref}NS_{unk}}$$

Where A is the integral value, C is the concentration, T is the temperature, θ is the pulse length, NS is the number of scans used for the experiment and k is a correction factor which considers incomplete relaxation. This can be easily performed using the ERETIC2 module in TopSpin. The ERETIC2 manual included with TopSpin provides detailed explanation of how to use it for quantification.

Results & Discussion:

1. Analyze the ¹H spectra of the 2 mM calibrant and of the cola sample. What is the dominant specie in the samples? What differences do you observe in these spectra?

2. Compare the ¹H and ³¹P cola spectra acquired, what differences do you observe? Describe them and explain why you observe these differences.

3. Overlay the ³¹P spectra of the cola and of the different calibration solutions. What differences do you observe in the spectra? Describe them and explain why you observe these differences. Why do we detect only a single peak in each ³¹P spectrum? Use this data to determine the phosphate content in cola.

4. Overlay the ³¹P spectra of the cola and of the different standard addition's samples. What differences do you observe in the spectra? Describe them and explain why you observe these differences.

5. Compare the quantification results that you have obtained with the two methods and calculate the accuracy of the fit (R² score). What differences do you observe between external calibration and standard addition?

Notes

6. Both methods can be used effectively: the standard addition approach is particularly useful for eliminating matrix effects, as the calibration is performed within the same sample matrix. In contrast, external calibration is advantageous when a larger number of unknown samples need to be measured consecutively, as it requires fewer additions and simplifies the workflow. Depending on the experimental goals and resources, the instructor can choose the appropriate method or use both to compare their results.

7. Now choose one sample from the calibration curve as your external standard and perform ERETIC quantification on the cola sample. Repeat this with a different sample from the calibration curve and compare the results. Do they also differ from the results obtained using the previous two methods? If so, discuss why, and comment on the accuracy of ERETIC and how it can be improved.

Optional Questions for Students:

Pre-Laboratory Questions:

1. Which region of the electromagnetic spectrum can be used to excite nuclear spins in NMR?

2. List 4 NMR active nuclei.

3. List at least 3 advantageous properties of ³¹P as an NMR active nucleus.

4. Why are the calibrant solution samples acidified?

5.	Give at least one example for a material that can be used for NMR chemical shift
	referencing.

0		
6.	List at least 3 analytical methods that could be considered for determining phosphoric acid	
	content in a cola drink.	
Po	st-Laboratory Questions	
1.	List the steps preceding an NMR measurement that prepare the spectrometer for	
	spectrum acquisition	
~	Next second s	
2.	Name one spectral quantity that provides qualitative and one that provides quantitative	
	information during the practical.	

3.	What does matrix effect mean? How does the standard addition approach compensate for it?	Notes
4.	Why is peak area (integral) usually preferred for quantification in spectroscopy over peak height?	

Key Take Home Messages:

- See an example of benchtop NMR applications in food chemistry
 Learn how to use ³¹P NMR to quantify phosphates in cola drinks
 Apply different calibration approaches for quantitative NMR

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