EDULAB FOR INSTRUCTORS

The Cola Chemistry

NMR of Cola Drink

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Keywords:

Food analysis, analytical chemistry, impurity analysis, benchtop NMR, ^{31}P 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 ^{1}H and ^{13}C. 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 ^{31}P 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 ^{31}P 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, ^{31}P 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.

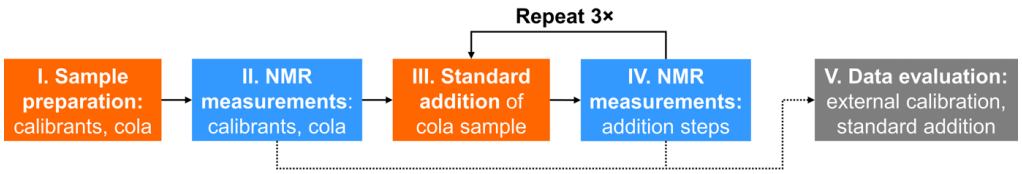


Figure 1: Overview of the experimental procedure. Reproduced from Reference 5.

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 ^{31}P, 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:

- NaH_2PO_4 and KH_2PO_4 samples
- HCl solution
- Phosphoric acid (85%, ^{31}P NMR chemical shift reference)
- A cola drink sample/s (multiple drinks could be analyzed if time allows)
- 5 mm NMR tubes and caps
- A micropipette to perform standard additions
- A small beaker for pipetting the KH_2PO_4 solution
- pH meter suitable for small sample volumes (not compulsory)

Note that it is not necessary to include both NaH_2PO_4 and KH_2PO_4. Either one of these can be used for both external calibration and standard additions as the cation complementing the H_2PO_4- plays no role in the practical.

Experimental set-up:

Set up in TopSpin the NMR experiments to acquire ^{31}P, ^{1}H and ^{1}H 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.

For ^{31}P acquisition we suggest the zgpg30, power gated decoupling pulse sequence ($^{31}P\{^{1}H\}$), which makes use of the nuclear Overhauser effect (NOE) to increase sensitivity. This can be set-up with a 30- or 45-degree hard pulse. It is important to highlight the role of the relaxation delay for quantification under different conditions. Theoretically, for a 90 degree pulse the relaxation delay should be $5*T_1$ in order to recover 99% of the equilibrium magnetization, and even longer when there is NOE buildup. Further on, T_1 values depend on the B_0 field and the temperature, so that proper T_1 measurements may be necessary under the specific conditions applied. With a T_1 time of 8 s (the value measured for phosphate in cola drink at 400 MHz and 298 K, likely longer at 80 MHz) this would lead to long experiment times with at least 40 s relaxation delay between scans. However, this sensitivity to T_1 times only applies in cases, where signals with

Abbreviations

NMR: Nuclear Magnetic Resonance

ERETIC: Electronic REference To access In vivo Concentrations

potentially differing T_1 times are integrated and quantified. In our case, the same phosphorous signal of phosphate is the only signal we measure both using the dilution series and the standard addition approach. As T_1 times are identical in all measurements, if there is incomplete recovery, this will only result in a single scaling factor applicable to all spectra and corresponding integrals. As such, much shorter recovery delays and the Ernst-angle approach can be applied to reliably obtain the desired measurement series.

The ^{31}P spectra were acquired with 256 scans (15 min experiment time) to allow all spectra to be recorded in one laboratory session, however if an autosampler is available, longer experiments (e.g. with 2048 scans) can be set up to increase the signal-to-noise ratio and hence the accuracy of the quantification.

The ^{1}H with water suppression experiments can easily be set up using the *MULTISUPP_f* parameter set available in TopSpin 4.4.1 and higher. The experimental parameters will be set up automatically when executed with the acquisition AU program (command xaua).

Note that if a sugar-free cola drink is analysed, the water-supressed ^{1}H spectrum will contain little to no signal, due to the significantly lower concentration of sweeteners compared to sugar in regular cola.

Notes

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 mM NaH_2PO_4)/ µl	V(0.06 M HCl)/ µl	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.

The use of dilute HCl is necessary for the calibration samples due to signal broadening caused by slow chemical exchange between the different phosphate species at neutral pH. At acidic pH, such as in Coke (pH approx. 2.7) the signals are narrower, resulting in a better signal-to-noise ratio. Due to the buffer capacity of phosphate, different amounts of acid would need to be added to the calibration samples to achieve the same pH. To make the sample preparation easier, a high enough concentration of acid (0.06 M) to achieve an acidic pH – and hence a narrow signal – in the most concentrated sample is used. The amount of acid needed might need to be adjusted; the ^{31}P signal in the cola sample serves as a good guide for the desired line shape. If the calibration sample signals are still broad, a small addition of concentrated acid could be beneficial.

Experimental Procedure:

1. Record 1D ^{1}H spectra of 2mM calibrant and cola sample with and without water suppression.
2. Record 1D ^{31}P spectra of the calibrant series.
3. Record 1D ^{1}H spectra with water suppression and 1D ^{31}P spectra of the cola sample
4. Perform 3 standard additions of 3 µl of the 1M KH_2PO_4 solution to the cola sample. After each addition, acquire the 1D ^{31}P spectra of the sample.
5. Process and analyze the data using the procedure described in the paragraph below.

Notes

Data Processing:

1. Process all the spectra using standard protocols, including phase and baseline correction.

2. Reference the chemical shift to the ^{31}P chemical shift of external 85% phosphoric acid (at 0 ppm).

3. For each ^{31}P 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.

Notes

Results & Discussion:

After processing the data, answer the following questions:

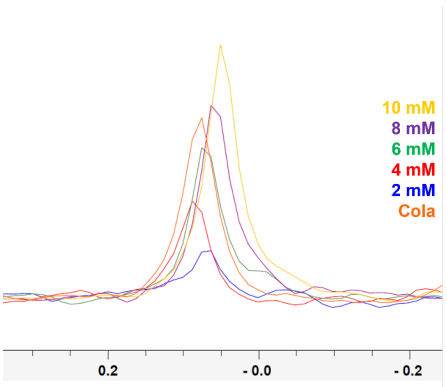
1. **Analyze the ^{1}H spectra of the 2 mM calibrant and of the cola sample. What is the dominant specie in the samples? What differences do you observe in these spectra?**
   In these spectra students should notice that (i) the dominant species in a sample is usually not the analyte but the solvent (water) and (ii) how dramatically the spectrum of the cola drink sample differs from that of the most dilute calibrant because of other compounds being present in the matrix.

2. **Compare the ^{1}H and ^{31}P cola spectra acquired, what differences do you observe? Describe them and explain why you observe these differences.**
   In these spectra students should notice that the ^{31}P spectra are much simpler than the ^{1}H spectra. The cola sample ^{1}H spectrum is crowded because of the presence of various organic substances that contribute to matrix effect. Sugars can be identified by the ^{1}H signals of the hydrogen at the anomeric position (e.g., sucrose at 5.4 ppm, glucose at 5.2 ppm). In contrast, the ^{31}P spectrum is simple, consisting of a single resonance peak - which is analytically advantageous. Students should also observe that the signal-to-noise ratio of the $^{31}P\{^{1}H\}$ spectra is much worse than that of the corresponding ^{1}H spectra, due to lower ^{31}P concentrations and lower gyromagnetic ratio of ^{31}P compared to ^{1}H

3. **Overlay the ^{31}P 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 ^{31}P spectrum? Use this data to determine the phosphate content in cola.**
   Students should notice a different ^{31}P chemical shift in the various samples. This single ^{31}P resonance – referred to as 'phosphate peak' - is the concentration-weighted average signal of the phosphate species (PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^{-}$, H_3PO_4). As the 0 ppm reference is the 85% H_3PO_4 solution, the smaller the pH, the smaller the chemical shift of the phosphate peak becomes.

   These spectra are used to explain the effects of signal width on peak height and peak integral. A suboptimal shim, and chemical exchange contribute to line broadening, therefore biasing quantitation based on peak height. On the other hand, integrals are insensitive to this effect as long as the integration window is wide enough.

   Example data: Upon concentration increase the chemical shift value of the peak decreases. This is expected, as the pH of the solutions is slightly decreasing. The line-width values slightly vary; therefore, not the peak intensities but the integral values were used for the quantitative evaluation. Integration was performed using a 1 ppm-wide window.



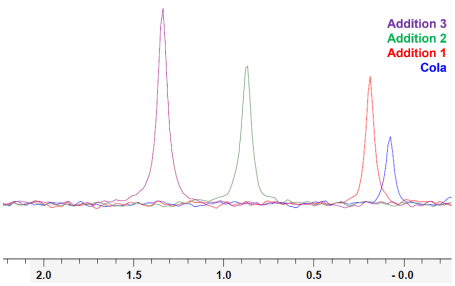
Overlaid $^{31}P\{^{1}H\}$ spectra of the calibrant solutions and the cola drink sample.

Sample/ mM	δ/ppm	LW/Hz	Integral/ AU	Conc/mM
2	0.07	1.8	3.64E+07	
4	0.08	1.5	5.37E+07	
6	0.07	1.6	9.69E+07	
8	0.05	1.6	1.33E+08	
10	0.04	1.6	1.59E+08	
Coke	0.07	1.6	1.13E+08	6.98

The concentration of the cola drinks was determined from the obtained calibration curves

Notes

4. **Overlay the ^{31}P 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.**
In this case small amounts of stock solutions were added to the soft drink samples. Students should observe different in peak intensity/area due to different concentration, as well as differences in chemical shifts. During standard additions, the phosphate protonation equilibrium is shifted because of changes in the concentration ratio of $H_2PO_4^-$ and H_3PO_4. Dilution caused by the additions also increases the pH but to a much smaller extent.



Overlaid $^{31}P\{^1H\}$ spectra of the cola drink sample and the three standard additions.

Sample/mM	δ/ppm	LW/ Hz	Integral/ AU	Conc/mM
Coke	0.07	1.6	1.13E+08	6.04
Addition 1	0.18	1.7	2.14E+08	
Addition 2	0.87	2.0	2.86E+08	
Addition 3	1.33	2.0	4.01E+08	

5. **Compare the quantification results that you have obtained with the two methods and calculate the accuracy of the fit (R^2 score). What differences do you observe between external calibration and standard addition?**

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.**
In the comparison of the external calibration and standard addition, students should notice that:

   - Spectra evaluation show that the standard addition method yields better results.
   - Phosphorus content determination from spectra recorded with NS = 2048 is more accurate, but for practical purposes also the NS =256 parameter can be used.
   - the obtained values are in accordance with values obtained at higher field instruments (see Kovács et al., 2024, JCE)

8. **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.**
In case of ERETIC2 module, two spectra are chosen: one from the calibration series and the other containing the unknown concentration. Calculation is done based on the above presented equation. Note, the sample volume (600 µL) and the molecular weight (91.994 g/mol) also need to be provided.

Notes

The concentration will be obtained by initializing the ERETIC2 module in TopSpin. This is done by right-clicking on the previously defined integration region. The same input parameters (number of atoms, molecular weight, sample volume) must be re-entered at this stage.

Example data: results of phosphate content determination on regular Coke with ERETIC2 module using different concentrations samples as the ERETIC reference.

Standard concentration/ mM	2.0	4.0	6.0	8.0	10.0
Cola phosphate concentration / mM	6.0	8.1	7.0	6.8	7.1

In this case, the results obtained with ERETIC are close to but not identical to the previous methods. Additionally, there is significant variation depending on which sample is chosen as an ERETIC reference. This is expected, as a single point calibration will always be less accurate than a calibration using multiple points. The effect of random errors such as a dilution error of a single sample (incorrect dilution of all samples would cause a systematic error) or instrumental fluctuations will be diminished in calibration with multiple data points. By using the same logic, acquiring multiple replicates of the quantification standard and cola sample would increase the reliability of the quantification results. This would also allow the precision of the method to be determined. Finally, NMR quantification is always more accurate when there is a higher signal-to-noise ratio, so acquiring more scans would be beneficial.

Optional Questions for Students:

Pre-Laboratory Questions:

1. **Which region of the electromagnetic spectrum can be used to excite nuclear spins in NMR?**
   Radiofrequency waves with the MHz domain (wavelengths in the cm to m range) are used.

2. **List 4 NMR active nuclei.**
   NMR active nuclei utilized during the practical are ^{1}H, ^{2}H, and ^{31}P. Furthermore, ^{13}C is abundantly studied in organic chemistry. Any other, NMR active nucleus is acceptable. More common examples are ^{19}F, ^{14}N, ^{15}N, ^{3}H and ^{17}O

3. **List at least 3 advantageous properties of ^{31}P as an NMR active nucleus.**
   As an NMR active nucleus, ^{31}P
   - has a large gyromagnetic ratio (17.235 MHz/T compared to 42.577 MHz/T of ^{1}H),
   - its natural abundance is 100% and
   - is only present a limited number of chemical forms in most samples.
   - The large spread (dispersion) of ^{31}P chemical shifts could also be mentioned.

4. **Why are the calibrant solution samples acidified?**
   To reduce the signal broadening caused by slow chemical exchange between the different phosphate species at neutral pH. At acidic pH, such as in Coke (pH approx. 2.7) the signals are narrower, resulting in a better signal-to-noise ratio.

5. **Give at least one example for a material that can be used for NMR chemical shift referencing.**
   During the laboratory practical, external 85% phosphoric acid is used for chemical shift referencing for ^{31}P. In organic applications (^{1}H and ^{13}C NMR), tetramethylsilane (TMS) and its variants are popular for ^{1}H and ^{13}C referencing.

Notes

6. **List at least 3 analytical methods that could be considered for determining phosphoric acid content in a cola drink.**
   Methods expected for listing are alkalimetric and gravimetric titration, visible spectrophotometry and electrochemical methods (amperometric and potentiometric titration).

   More advanced instrumental methods like ion chromatography, atomic absorption spectroscopy or inductively coupled plasma (ICP) techniques are obviously also acceptable.

Post-Laboratory Questions:

1. **List the steps preceding an NMR measurement that prepare the spectrometer for spectrum acquisition**
   Preparation steps of an NMR measurement are tuning the probe-head, locking to the appropriate signal and shimming. It is also recommended to wait until thermal equilibrium is established

2. **Name one spectral quantity that provides qualitative and one that provides quantitative information during the practical.**
   Chemical shifts and peak widths carry qualitative information about the sample. On the other hand, peak height and peak integral are used for quantitative purposes.

3. **What does matrix effect mean? How does the standard addition approach compensate for it?**
   Matrix effect is any increase or decrease in the analytical signal that can be attributed to materials in the sample other than the analyte. Matrix effect emerges if the medium in calibrant solutions is very different from the medium of the sample. In the case of the standard addition approach, the matrix remains almost unchanged, however, the concentration of the analyte is increased significantly. This ensures that a calibration valid in the given medium is obtained.

4. **Why is peak area (integral) usually preferred for quantification in spectroscopy over peak height?**
   The integral is more robust to matrix-related changes in peak width than peak height. This is because even if line broadening occurs, the area under the peak remains the same. Also, the numerical uncertainty of an integral is generally smaller than that of the individual values of the peak height. Theoretically, the peak integral is the quantity that should be linearly related to the concentration and is therefore the intensity-type quantity to be trusted more. Also, in any spectroscopy, the relative random error associated with a peak integral is always smaller than that of the corresponding peak height. Students can try to perform quantification using a set of peak height data for comparison. Usually, R2-values describing the goodness-of-fit of the linear models are also higher for the integral than for peak height. Results based on peak height are expected to overestimate the phosphate content. They would match those based on integral if the variation of peak width is relatively small, which is not the case in this experiment.

Notes

Key Take Home Messages:

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

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