EDULAB FOR INSTRUCTOR

Brewing Alcohol 101

A Toast to Benchtop NMR

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Experiment Hashtag: #Fermentation, #Educate2Resonate

Keywords:

Benchtop NMR, ERETIC, Fermentation, Low-Field NMR, Process Monitoring, Quantification, Reaction Kinetics, Undergraduate Education, Solvent Suppression

Target group:

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

Objectives:

- Learn sample preparation and techniques for spectral assignments of compounds within mixtures.
- Identify the relationship between high-field and low-field NMR and understand field strength impacts on J-coupling.
- Learn how to collect NMR data in a quantitative manner.
- Learn how to operate and collect NMR data using a benchtop spectrometer.
- Learn how to set up a solvent suppression NMR experiment on a benchtop spectrometer.
- Demonstrate how to quantify specific compounds in a one-step process using ERETIC.
- Relate process monitoring to reaction kinetics and identify the reaction order.
- Understand the potential of low-field NMR as an analytical tool for real-time sample monitoring.

Background of the Experiment:

Nuclear Magnetic Resonance (NMR) Spectroscopy stands as a cornerstone in modern analytical instrumentation, offering insights into diverse realms such as synthetic chemical structures, biomolecular arrangements, metabolic profiling, and molecular interactions.[1–4] Benchtop NMR, operating on similar principles to its high-field counterpart, diverges notably in its use of permanent magnets, rendering it more cost-effective without the need for cryogens.[5,6] Operating at lower frequencies, typically below 100 MHz and without the need for a deuterium lock, benchtop NMR simplifies sample handling and signal acquisition.[5,6]

Despite its accessibility, benchtop NMR remains underutilized in undergraduate laboratories, often confined to routine structure elucidation exercises.[2,4,6,7] The introduction of this undergraduate chemistry laboratory experiment seeks to address this gap by focusing on quantitative data acquisition through Benchtop NMR, while also honing students' proficiency in utilizing NMR processing software to enhance their experimental workflows. This NMR experiment introduces students to the process of simple fermentation in the production of alcohol and using quantitative NMR to measure the amount of alcohol produced.[8,9] This lab is based on a recent publication, which is referred below as "main paper".[10]

Experimental Setup:

- 200 mM D-Glucose in H_2O
- 5 mm NMR tube and cap
- Sparkling wine yeast (*Saccharomyces cerevisiae*)

In TopSpin, set up the following NMR experiments on the spectrometer (acquisition parameters are listed below): 1D PURGE[11] (i.e., 1D ^{1}H NMR with water suppression)

1D ^{1}H ZGPURGE

PULPROG	**zgpurge**
TD	4096
SW (ppm)	14.8562
AQ (sec)	1.72032
RG	1
D1 (sec)	10 s or 5*T1
DS	8
NS	360 (Based on D1 of 10 s -- if using a different D1, change NS accordingly such that it is divisible by 8)
O1 (ppm)	Determine from apex of water signal
D20, D21, D16	200 µs, 200 µs, 200 µs
PL9	60 dB
GPNAM1, GPNAM2, GPNAM3, GPNAM4	SINE.100
GPZ1 (%)	-13.17
GPZ2 (%)	52.68
GPZ3 (%)	-17.13
GPZ4 (%)	68.52
P16 (µsec)	1000

All NMR spectra are processed with a line broadening between 0.1 to 0.3 Hz.
(NOTE: due to the time required to determine T1 of your sample, it is necessary for the instructor to determine the T1 before the lab begins. Based on our experience, a D1 between 10 s to 20 s is enough to achieve the 5*T1 condition for quantification).

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

ERETIC: qNMR experimental technique to measure analytes based on the signal of the reference compound without additional hardware equipment

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

O1P: The transmitter frequency of the pulse, specified in ppm

Sample Preparation:

1. Create a 200 mM sample of D-glucose in H_2O. You will need at least enough sample to fill 4 NMR tubes (~600 µL each: one for your ERETIC standard, and three for the fermentation reaction, amounting to roughly 2.4 mL of total solution).

2. To 3 different Eppendorf tubes, place ~4 mg of yeast in each of them and be sure to note the weight.

3. To each Eppendorf tube, add 600 µL of 200 mM D-glucose solution and mix well.

4. Transfer the yeast/glucose solution to a 5 mm NMR tube and cap it.

5. To initiate the fermentation process, place the NMR tube in warm water at 30 °C for 5 min. Then, immediately transfer the NMR tube (wiped dry) to the spectrometer for acquisition.

6. The concentration for the ERETIC[12] standard used for this experiment can be found in Appendix I.

Experimental Procedure:

Insert sample in the spectrometer and determine the P1 and O1P, which in this case we want to match the chemical shift of the water signal. Setup a series of experiments, using these optimized parameters, that will take a total time of 24 hours. In our case, this was accomplished by determining the number of scans necessary for the total experiment time for one spectrum to equal roughly one hour and acquiring 24 spectra (at a rate of roughly one spectrum per hour) with such parameters. For instance, with a D1 of 10 seconds and acquisition time of 1.2 seconds, each scan takes 11.2 seconds. If NS is set to 360 with these parameters, we can expect a total experiment time of 67 minutes. With these parameters set in TopSpin, the experiment time can be checked easily by typing the command "expt" at the command line or by clicking on the stopwatch icon in the TopSpin flow bar.

Data Processing:

- All spectra were processed using standard protocols, including baseline correction and phasing, and were calibrated using the glucose doublet at 5.4181 ppm. More information can be found here: https://pubs.acs.org/doi/10.1021/ed086p360.
- After all the spectra are phased, baseline corrected and properly calibrated, integrate the regions corresponding to ethanol and α-glucose as shown in Figure 1.
- The students then use ERETIC to convert the integral value to concentrations.

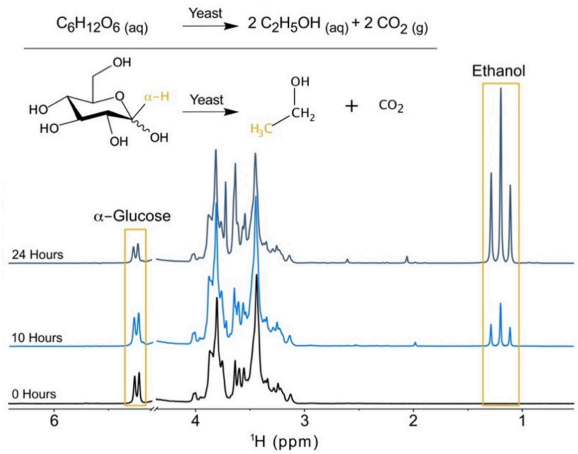

Figure 1 Examples of low-field NMR data during the fermentation process. The reaction equation and the structures involved are shown above the spectra.

Abbreviations

NMR: Nuclear Magnetic Resonance

PURGE: Presaturation Utilizing Relaxation Gradients and Echoes

ERETIC: Electronic REference To access In vivo Concentrations

Results & Discussion:

After processing their spectra, students utilized ERETIC[12] to derive values for each of the peaks over a 24-hour period. They were tasked with plotting these concentrations, as in the example shown in Figure 2. The experiment underwent three repetitions, enabling students to report errors and present associated error bars. A series of factors contributed to the noteworthy decrease in signal observed for D-glucose at point 2. During the initial warming of the yeast, the rate of D-glucose breakdown was accelerated and subsequently decelerated as the tube cooled to room temperature. Additionally, warming of the sample to activate the yeast caused both a temperature-dependent shift in the H_2O signal and the formation of bubbles due to the rapid production of CO_2. Cumulatively, these factors impacted the efficiency of water suppression, rendering the integration of glucose challenging at the second time point. This circumstance served as an educational opportunity to illustrate to students how chemical shifts may change with temperature, forming the basis for their response to question 4 in the assignment.

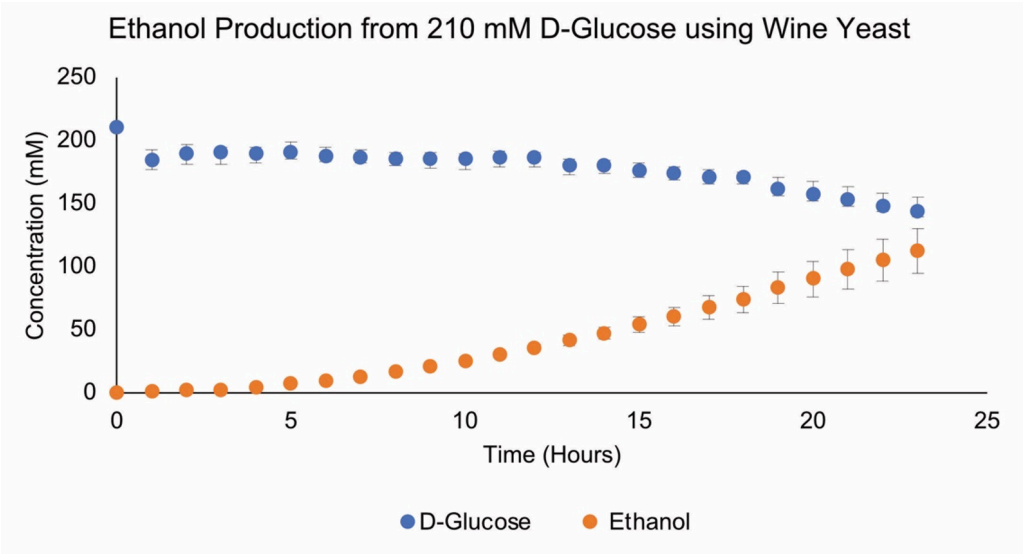

Figure 2 Example student quantification data. During the experiment, students used ERETIC[12] to quantify the α-glucose peak of D-glucose and the CH_3 of ethanol over 24 hours, which can then be used to determine the reaction kinetics. Note The graph has been corrected to reflect the concentration of both glucose anomers assuming an equilibrium ratio of 36:64 (alpha:beta). Experiments were conducted in triplicate and error bars represent standard error.

After using ERETIC to extract concentrations from the appropriate integrals, students graphed concentration over time for glucose and for ethanol, as depicted in Figure 2. They then extrapolated the concentration changes over time and related them to the overall reaction order. The reaction order may be observed in various ways to enhance student learning. Firstly, the overall mole ratio of 1:2 (as shown in Figure 2), where 1 mole of D-glucose yields 2 moles of ethanol, suggests fermentation operates as a first-order reaction with respect to ethanol. Students visually observed this trend over time, noting that the slope of the ethanol line is approximately twice that of glucose. Furthermore, this ratio was evident in concentration values, with students noting that after 24 hours, approximately 50 mM of D-glucose was consumed while approximately 100 mM of ethanol was produced, further supporting the 1:2 ratio observed in the overall reaction. By offering multiple avenues for engaging with the content, we aimed to demonstrate to students the connection between analytical measurements and fundamental physical chemical principles. Additionally, as these courses are analytical in nature, we emphasized the importance of obtaining quality analytical data through triplicate measurements and precise measurements with error bars. This approach enabled students to critically evaluate their work and verify the accuracy of their measurements.

ERETIC

Note that you are integrating the peak specific to α-glucose in class and this is only ~36 % of the total glucose. As α-glucose and β-glucose exist in a 36:64 ratio at equilibrium, if you plot the results directly from ERETIC[12] then the ethanol will increase at a ratio of 64/36 (anomer ratio) x 2 (mole ratio in equation) = 3.55 relative to α-glucose. Thus, to get the corrected graph shown in Figure 2, this adjustment must be conducted to the raw ERETIC data.

Upon the completion of the lab, the students will write a report outlining the experimental protocols and parameters (e.g., the T1 used to determine D1, the exact D-glucose concentration, NMR acquisition parameters, etc.) which they used for their experiment. The reports should be as detailed as possible, with the students writing in such a way that someone could read their report and replicate their experiments with no further information. The students will also be expected to answer the questions below with responses as detailed as possible.

Answer the following questions:

1. **Calculate the final ethanol concentration with respect to industry standards (% w/v). A store-bought beer has a measurement such as ~5 %, which is a % w/v measurement.**

   At 5 % alcohol content – for a 1L bottle of beer, assuming the density of water, we have 1 kg. Therefore, 5 % w/v, we have 0.05 kg or 50 g of ethanol.

2. **Provide the graphs of D-glucose and ethanol concentrations over time based on the ERETIC measurements. What can you determine from this graph with respect to the reaction kinetics? What reaction order is fermentation? Is the rate that glucose decreases, and ethanol increases balanced, or do you see differences in the slopes? Read about fermentation and if possible, write a reaction that explains the difference in moles "consumed" vs moles "produced."**

   The overall mole ratio of 1:2 (as shown in Figure 2), where 1 mole of D-glucose yields 2 moles of ethanol, suggests fermentation operates as a first-order reaction concerning ethanol. Secondly, students visually observed this trend over time, noting that the slope of the ethanol line is approximately twice that of glucose. Furthermore, this ratio was evident in concentration values, with students noting that after 24 hours, approximately 50 mM of D-glucose was consumed while approximately 100 mM of ethanol was produced, further supporting the 1:2 ratio observed in the overall reaction.

3. **Based on your starting D-glucose concentration, calculate the theoretical maximum yield of the ethanol. Were you able to achieve this? Why or why not?**

   For 100 mg of glucose, we have 0.55 mmols of glucose, and based on the reaction

   $C_6H_{12}O_6$ (aq) ————> $2C_2H_5OH$ (aq) + $2CO_2$(g) + 2ATP

   you should have 0.55*2 mmols of ethanol produced. However, it is likely you do not get full conversion due to temperatures, time, and toxicity of ethanol (can kill certain yeast strains) over the course of the reaction.

4. **Do you see any reproducibility issues with the graphed lines for glucose and/or ethanol? If so, can you look at the NMR data and try and explain why this may be so and what factor(s) could be the issue?**

   The student should discuss the temperature, mass of yeast used, and its activity (bubbles of CO_2 can form) which may deviate based on the exact experimental conditions.

5. **Provide at least 3 processed (start, middle, and end) labelled NMR spectra along with the rest of the data.**

   These spectra should be labelled at least with the alpha glucose and ethanol peaks.

6. **In what ways could the reaction time be decreased? Discuss a few practical methods for impacting the reaction time of ethanol fermentation.**

   Increase the amount of yeast, change strain of yeast used or increase the temperature to increase yeast activity.

Notes

Optional Questions for Students:

1. **What are we making in this reaction?**

   Ethanol from D-Glucose and sparkling wine yeast.

2. **What is the balanced chemical equation for this reaction?**

   $C_6H_{12}O_6\ (aq) \rightarrow 2CH_3CH_2OH\ (aq) + 2CO_2\ (g)$

3. **What is the expected reaction order?**

   1st order with respect to ethanol.

4. **How would we quantify these using internal standards?**

   Put in a known amount of a different compound which is present at a ppm shift different from the target compounds and integrate the area under the peak. This can then be compared to the area under the peaks of glucose and ethanol over time.

5. **How does the graph of the reaction relate to reaction kinetics?**

   The slope of the lines is related to the rate at which the glucose is being used and ethanol is being produced.

6. **What other processes could you monitor with NMR?**

   Open-ended for students.

Further Applications and Improvements:

For instructors who are interested in applying this lab to their courses, we present a range of potential enhancements and extensions. These suggestions are designed to not only enrich the current lab experience but also inspire the creation of future EduLabs:

1. One of the 'bottle necks' of this lab was the manual processing which has to occur in order to quantify data across a large array of spectra. Combining this experiment with additional learning tools such as automated integration using ERETIC across multiple spectra would be beneficial and educational. Such features are well developed in dedicated analysis packages such as MNova (Mestrelab Research).

2. Fermentation is only one example of a process which students can examine. Adaptations to this experiment could be made which investigate the formation of new products in an organic chemistry reaction, switching the system to a closer approximation to a natural system (e.g., using grape juice rather than pure glucose), or exploring a different process entirely. The principles applied here can be translated to any system so long as the ERETIC calibration spectra and the data are both collected under fully quantitative NMR conditions.

3. The approach also opens up future possibilities for flow-based NMR for real-time sampling, with the InsightMR flow unit for the Fourier RxnLab.

Notes

Tips/Tricks:

1. Experiment parameters: Experiments were collected with 8 dummy scans, 300 scans, and 4k time domain points. ERETIC parameters were set using a relaxation delay of 10 seconds (plus an additional 1.2 second AQ time) in order to achieve quantitative data. This can be adapted to fit your specific experiment, but these parameters worked in our experience.

2. Processing: it is important that the data is processed (Fourier transformed, phased, baseline corrected) and calibrated (this was done in the center of the α-glucose H1 at 5.2354 ppm in our experiment) prior to calculating the ERETIC concentrations. In our lab setting the instructor/TA shows the students this process as a refresher, but many students have processed data before.

3. Prior to starting the fermentation sample tube, the D-glucose solution should be warmed to just above room temperature (~30 °C) to help initiate the process. This can be done using a water bath (warm, not boiling) and the NMR tube should be placed inside and left for 2 minutes. Once warm, the yeast is added immediately and placed in the spectrometer for experimentation.

4. Students in our courses are in groups of 4-6, so this gives each of them 12 spectra to process and analyze themselves (4 per replicate) which we have noted is reasonable in terms of workload, but also enough to make them comfortable with processing and analyzing their data. If working with smaller groups, it may be useful to take spectra every 2 hours instead of every 1.

5. For assignment question 3, a suitable answer is the conversion is not complete and eventually the yeast may stop functioning when the alcohol gets too concentrated (depends on the strain used). However, students may also extrapolate from the equation of the lines on their graphs to determine the concentration when the slope of ethanol will reach steady state. For question 4, see figure caption of Figure 3 in main paper. For question 6, suitable answers are: increase concentration of yeast, increase temperature of reaction, change the strain of yeast.

Notes

Key Take Home Messages:

- Recent developments in NMR have expanded its applications beyond historical use as a tool for structural elucidation in organic chemistry.
- Benchtop NMR can be used to monitor the rate of a biochemical reaction.
- It is possible to calculate concentration in NMR experiments using ERETIC[12].
- Benchtop NMR can be used to quantify components under quantitative conditions.

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Appendix I – ERETIC Profile Setup:

An ERETIC[12] sample will need to be prepared that is reflective of the actual sample. In this case, 210 mM of D-glucose in H_2O is used for calibration. Once calibrated the approach is then tested on a mixture containing ~180 mM beta-glucose and ~240 mM ethanol. Once calibrated, ERETIC can be used to calculate concentrations in subsequent fermentation experiments. To assist in the assignment process, an example of D-Glucose collected at 500 MHz (top) and 80 MHz (bottom) are shown in Figure A1. The frequency range of both spectra were the same (1226 Hz) but due to the lower field, the 80 MHz spectra has lower dispersion leading to a smaller spectral window, and increased overlap, whereas the 500 MHz spectrum was fully assigned. Note, the artifact in the baseline in the 500 MHz spectrum at ~2420 Hz is the residual water remaining after water suppression. Additionally, the H1β is partially suppressed by the presaturation at 80 MHz, understandable given that the water is less than 15 Hz from the water resonance being suppressed.

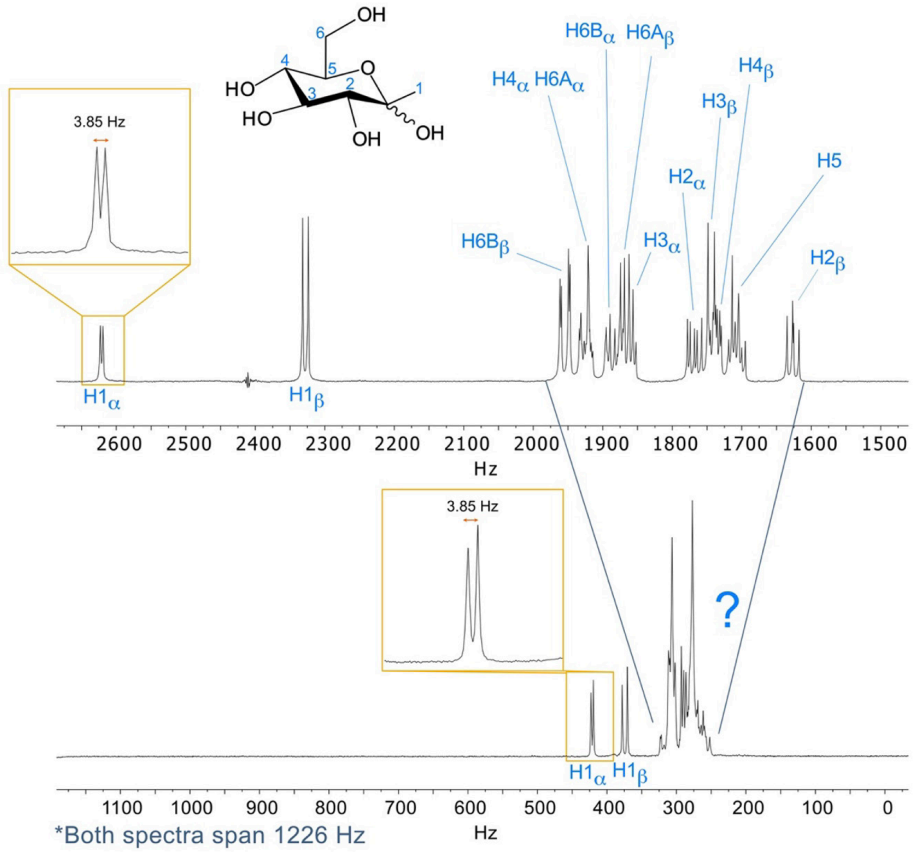

Figure A1 D-Glucose spectra collected at 500 MHz (top) and 80 MHz (bottom).

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