EDULAB FOR STUDENTS

Brewing Alcohol 101

A Toast to Benchtop NMR

Authors & Affiliation:

Amy Jenne, Ronald Soong, Katelyn Downey, Rajshree Ghosh Biswas, Myrna J. Simpson, and Andre J. Simpson
Environmental NMR Center, University of Toronto, 1265 Military Trail, Toronto, ON, Canada, M1C 1A4

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.[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 ^1H 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) of the sample. With the assistance of the instructor, setup a series of experiments using these optimized parameters that will take a total time of 24 hours. Then, start the acquisition once you are ready to do so.

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.
- Finally, use ERETIC to convert the integral value to concentrations.

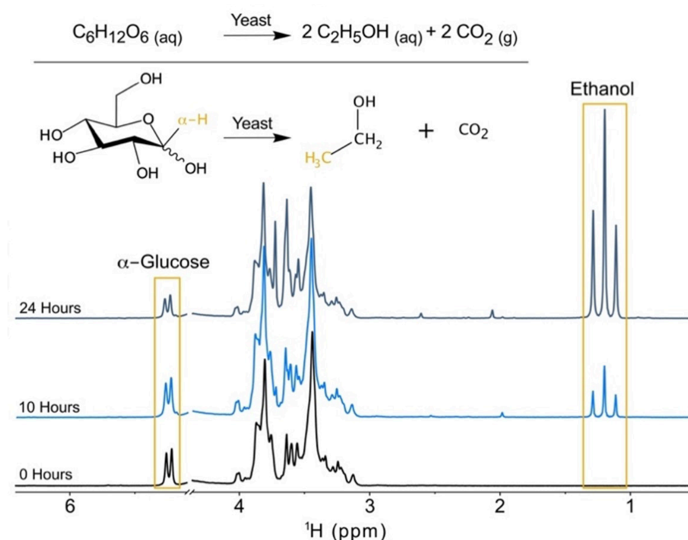

Figure 1 Examples of low-field NMR data during the fermentation process. The reaction equation and the structures involved are shown above the spectra.

Abbreviations

NMR: Nuclear Magnetic Resonance

PURGE: Presaturation Utilizing Relaxation Gradients and Echoes

ERETIC: Electronic REference To access In vivo Concentrations

Results & Discussion:

- Using ERETIC, obtain the concentration of ethanol and glucose for each time point for each trial
- Obtain the average concentration calculated using the three trails.
- Plot the average concentration of ethanol and glucose as a function of time, with error bars, as shown in Figure 2.

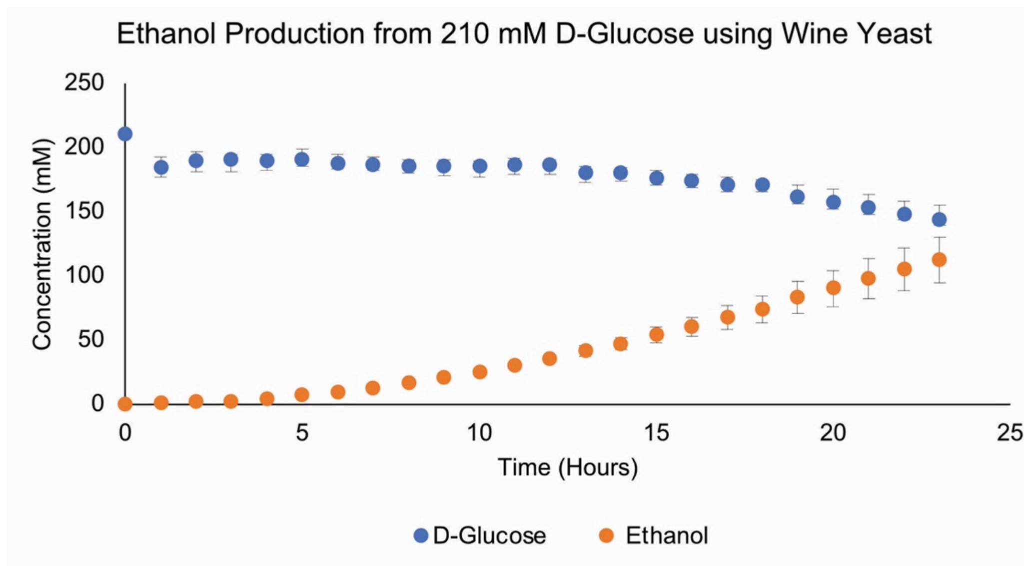

Figure 2 Example student quantification data. During the experiment, students used ERETIC[12] to quantify the α-glucose peak of D-glucose and the CH_3 of ethanol over 24 hours, which can then be used to determine the reaction kinetics. Note The graph has been corrected to reflect the concentration of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

- Using the kinetic plot you created, determine the rate of reaction for this fermentation reaction.

Upon the completion of the lab, 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.) Please be as detailed as possible; you should be writing it in such a way that someone could read your report and replicate your experiments with no further information. You are also expected to complete the questions below. These responses should be as detailed as possible.

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.

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.

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."

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?

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?

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

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

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.

References:

1. Beek, T. A. *Low-field Benchtop NMR Spectroscopy: Status and Prospects in Natural Product Analysis †*. Phytochem. Anal. (2021), 32 (1), 24–37. https://doi.org/10.1002/pca.2921.

2. Singh, K.; Blümich, B. *Desktop NMR for Structure Elucidation and Identification of Strychnine Adulteration.* The Analyst (2017), 142 (9), 1459–1470. https://doi.org/10.1039/C7AN00020K.

3. Shine, T. D.; Glagovich, N. M. *Organic Spectroscopy Laboratory: Utilizing IR and NMR in the Identification of an Unknown Substance.* J. Chem. Educ. (2005), 82 (9), 1382. https://doi.org/10.1021/ed082p1382.

4. Zivkovic, A.; Bandolik, J. J.; Skerhut, A. J.; Coesfeld, C.; Zivkovic, N.; Raos, M.; Stark, H. *Introducing Students to NMR Methods Using Low-Field 1 H NMR Spectroscopy to Determine the Structure and the Identity of Natural Amino Acids.* J. Chem. Educ. (2017), 94 (1), 115–120. https://doi.org/10.1021/acs.jchemed.6b00168.

5. Blümich, B. *Low-Field and Benchtop NMR.* J. Magn. Reson. (2019), 306, 27–35. https://doi.org/10.1016/j.jmr.2019.07.030.

6. Bonjour, J. L.; Hass, A. L.; Pollock, D. W.; Huebner, A.; Frost, J. A. *Bringing NMR and IR Spectroscopy to High Schools.* J. Chem. Educ. (2017), 94 (1), 38–43. https://doi.org/10.1021/acs.jchemed.6b00406.

7. Guthausen, G. *Analysis of Food and Emulsions*. TrAC Trends Anal. Chem. (2016), 83, 103–106. https://doi.org/10.1016/j.trac.2016.02.011.

8. Giles, B. J.; Matsche, Z.; Egeland, R. D.; Reed, R. A.; Morioka, S. S.; Taber, R. L. *An in Vivo 13C NMR Analysis of the Anaerobic Yeast Metabolism of 1-13C-Glucose*. J. Chem. Educ. (1999), 76 (11), 1564. https://doi.org/10.1021/ed076p1564.

9. Hamper, B. C.; Meisel, J. W. *Introducing Nonscience Majors to Science Literacy via a Laboratory and Lecture Beer Brewing Course.* J. Chem. Educ. (2020), 97 (5), 1289–1294. https://doi.org/10.1021/acs.jchemed.9b00870.

10. Jenne, A.; Soong, R.; Downey, K.; Biswas, R. G.; Decker, V.; Busse, F.; Goerling, B.; Haber, A.; Simpson, M. J.; Simpson, A. J. *Brewing Alcohol 101: An Undergraduate Experiment Utilizing Benchtop NMR for Quantification and Process Monitoring.* Magn. Reson. Chem. (2024), 1–10. https://doi.org/10.1002/mrc.5428.

11. Simpson, A. J.; Brown, S. A. *Purge NMR: Effective and Easy Solvent Suppression.* J. Magn. Reson. (2005), 175 (2), 340–346. https://doi.org/10.1016/j.jmr.2005.05.008.

12. Akoka, S.; Barantin, L.; Trierweiler, M. *Concentration Measurement by Proton NMR Using the ERETIC Method.* Anal. Chem. (1999), 71 (13), 2554–2557. https://doi.org/10.1021/ac981422i.

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.

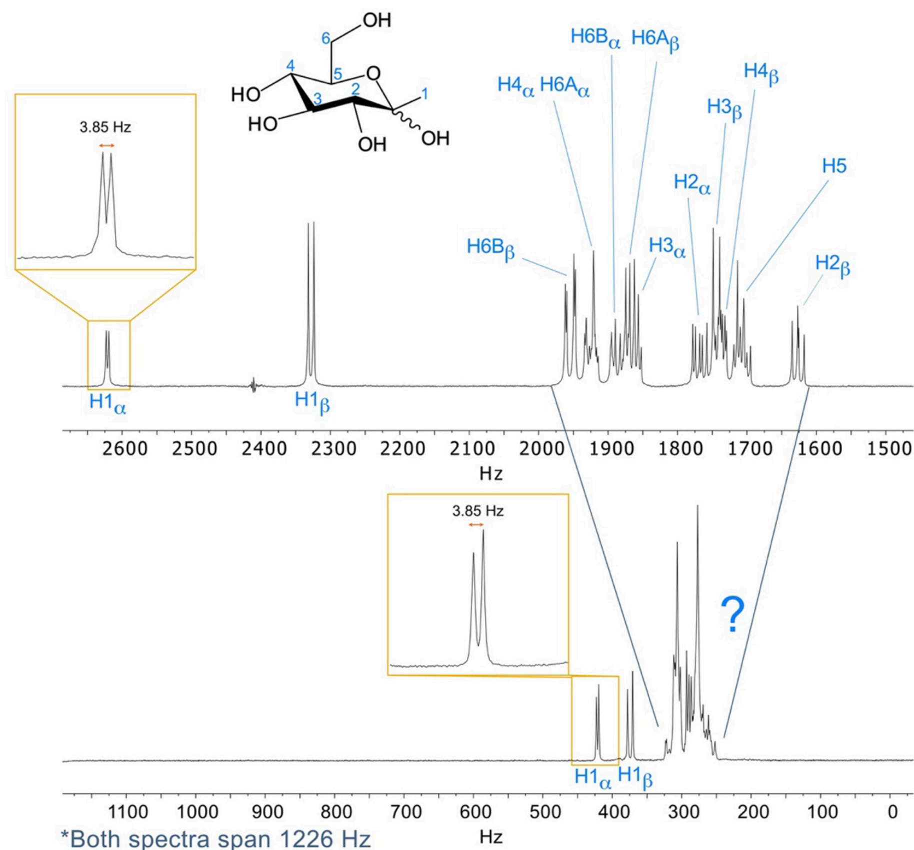

Figure A1 D-Glucose spectra collected at 500 MHz (top) and 80 MHz (bottom).

© 2024 Bruker BBIO

Bruker BioSpin
educate2resonate.bbio@bruker.com

Worldwide offices
bruker.com/

Educate2Resonate
Community Page