QUANTITATIVE NMR

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About this Book

Goal

To provide instruction in the use of NMR as a tool for quantitative analysis

Learning Objectives

- Develop sufficient background in basic NMR theory to understand how it is used for quantitative measurements
- Understand the role of key acquisition parameters and how they affect quantitative NMR measurements
- Apply NMR to real problems requiring quantitative analysis

Description

To make correct use of NMR for quantitative analysis, it is necessary to begin with the Basic Theory that underlies the NMR experiment. The Practical Aspects section discusses the experimental requirements for performing quantitative measurements. These concepts are reinforced in a Virtual Experiment that uses an NMR simulation tool developed by Dr. Harold Bell. A Q-NMR Laboratory experiment is provided for those who have access to an NMR spectrometer. Alternatively, students can download and analyze FIDs acquired for the quantitative analysis of malic acid in apple juice. The Q-NMR Applications section presents a summary of the types of applications where NMR works well for quantitative measurements.

TABLE OF CONTENTS

6: Instructor's Guide

Licensing

*A detailed breakdown of this resource's licensing can be found in **Back Matter/Detailed Licensing**.*

CHAPTER OVERVIEW

1: Basic NMR Theory

This section presents a basic overview of the theory of modern NMR. Readers interested in more in-depth treatments of this subject are encouraged to utilize the resources listed in the reference page at the end of this section. The embedded animations in the web book http://www.cis.rit.edu/htbooks/nmr/ authored by Professor Hornak makes this site especially useful for students learning about NMR.

This section will help you answer the following questions:

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1.1: What is spin?

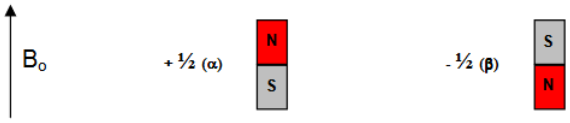
The fundamentals of NMR begin with the understanding that a nucleus belonging to an element with an odd atomic or mass number has a nuclear spin that can be observed. Examples of nuclei with spin include ^{1}H, ^{3}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P and ^{29}Si. All of these nuclei have a spin of ½. Other nuclei like ^{2}H or ^{14}N have a spin of 1. Nuclei with even atomic and mass numbers like ^{12}C and ^{16}O have spin of 0 and cannot be studied by NMR. The following introductory discussion of NMR is limited to spin ½ nuclei.

Nuclei that possess spin have angular momentum, ρ. The maximum number of values of angular momentum a nucleus can have is described by the magnetic quantum number, I. The possible spin states can vary from +I to –I in integer values. Therefore, there are 2I +1 possible values of ρ.

? Exercise 1.1.1

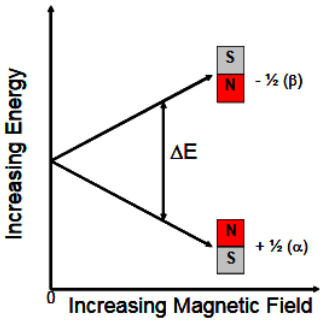
How many spin states would you predict for ^{2}H?

For spin ½ nuclei, the angular momentum can have two possible values: +½ or –½. Since spin is a quantum mechanical property, it can be difficult to visualize. One way to imagine spin is by thinking of spin ½ nuclei as tiny bar magnets that can have two possible orientations with respect to a larger external magnetic field. It is important to note that in the absence of an external magnetic field, these discrete spin states have random orientations and identical energies.



1.2: How does absorption of energy generate an NMR spectrum?

In the absence of an external magnetic field the two spins in the previous figure would be randomly oriented and their energies degenerate, in other words they would have identical energies. However in the presence of an applied magnetic field, the energies of the two spin states diverge and the spins orient themselves with respect to the applied field. The larger the magnetic field, the greater the difference in energy between the spin states. For most spin ½ nuclei, the + ½ (α) spin state is of lower energy and corresponds to having the spin aligned with the applied field while the - ½ (β) spin state can be thought of as having the spin opposed to the applied field.



The difference in energy between the states, ΔE, depends on the strength of the applied magnetic field, B_o, according to Eq. 1.2.1. In this equation γ is the gyromagnetic ratio, a fundamental property of each type of nucleus and h is Plank's constant. Table 1 shows values of the gyromagnetic ratio for several common NMR nuclei.

$$\Delta E = \frac{\gamma h B_o}{2\pi} \tag{1.2.1}$$

Table 1. Properties of Nuclei Commonly Studied by NMR[1]

Element	Atomic Number	Mass Number	Spin	Natural Abundance	Gyromagnetic Ratio γ (10^7 rad·s^{-1}·T^{-1})	Reference Compound
Hydrogen	1	1	½	99.985%	26.7522128	Me_4Si
Deuterium	1	2	1	0.0115	4.10662791	$(CD_3)_4Si$
Carbon	6	13	½	1.07	6.728284	Me_4Si
Nitrogen	7	15	½	0.368	-2.71261804	$MeNO_2$
Fluorine	9	19	½	100	25.18148	CCl_3F
Silicon	14	29	½	4.6832	-5.3190	Me_4Si
Phosphorus	15	31	½	100	10.8394	H_3PO_4
Selenium	34	77	½	7.63	5.1253857	Me_2Se
Cadmium	48	113	½	12.22	-5.9609155	Me_2Cd

1. R.K. Harris, E. D. Becker, S. M. C. De Menezes, R. Goodfellow, P. Granger, Pure. Appl. Chem. 73:1795-1818 (2001). http://www.iupac.org/publications/pa.../7311x1795.pdf

The signal in NMR is produced by absorption of electromagnetic radiation of the appropriate frequency. Energy absorption causes the nuclei to undergo transitions from the lower energy (α) to the higher energy (β) spin states. If we think about the spins as bar magnets, absorption of energy at the right frequency causes the spins to flip with respect to the applied field. As is the case with other spectroscopic methods, the difference in population of these two quantized states can be expressed by the Boltzmann equation, Eq. 1.2.2 where k is Boltzmann's constant, 1.38066 x 10^{-23} J·K^{-1}, and T is the temperature in degrees Kelvin.

$$\frac{N_{upper}}{N_{lower}} = e^{-\frac{\Delta E}{kT}} \tag{1.2.2}$$

Equation 1.2.2 relates the ratio of the number of nuclei in the upper (higher energy) spin state and the lower energy spin state to the energy difference between the spin states, ΔE, and therefore, the magnitude of the applied magnetic field, B_o (Eq. 1.2.1). In NMR the difference in energy in the two spin states is very small therefore the population difference is also small (about 1 in 10,000 for ^{1}H in an 11.74 T magnetic field). Because this population difference is the source of our signal, NMR is inherently a less sensitive technique than many other spectroscopic methods.

? Exercise 1.2.2

Given the same magnetic field and temperature, how would the difference in population for ^{1}H and ^{31}P compare?

1.3: How does the population difference in NMR compare to the difference between electronic ground and excited states?

Up to this point in our discussion, the theory of NMR seems similar to that for other common spectroscopic methods. However there are some differences that should be considered. For example in UV-visible absorption spectroscopy, which occurs as a result of electronic transitions, at room temperature essentially all of the molecules will be in the ground electronic state because the energy difference between the ground and excited states is large. However, in NMR the difference in energy in the two spin states is very small, therefore the population difference is also small (about 1 in 10,000 for ^{1}H in an 11.74 T magnetic field). Because this population difference is the source of our signal, NMR is inherently a less sensitive technique than many other spectroscopic methods.

Let's think now about the energy difference between the nuclear spin states in NMR. Do you recall the relationship between energy and frequency? Say we are interested in a compound with an absorption maximum at a wavelength, λ, of 600 nm. What would be the frequency, ν, of the light absorbed?

The frequency of light absorbed is inversely proportional to the wavelength as shown in the equation below, where c is the speed of light, 3.0 x 10^{8} m/s.

$$\nu = \frac{c}{\lambda} \tag{1.3.1}$$

Therefore, light with a wavelength of 600 nm has a frequency of 5 x 10^{14} Hz (cycles per second). The energy, E, of this light is directly proportional to the product of its frequency and Planck's constant (h), 6.626 x 10^{-34} J·s.

$$E = h\nu \tag{1.3.2}$$

Our 600 nm light has an energy of 3.31 x 10^{-19} J. The energy of the light absorbed by our molecule roughly corresponds to the energy difference between the ground and excited electronic states of our molecule. How does the energy absorbed in NMR compare with this value? We already indicated that we expect the energy difference between the ground and excited spin states in NMR to be much less than for absorption of visible light. We can calculate the energy of the NMR transition using Equation 2.1 for a particular nucleus in a given magnetic field strength. Let's do this calculation for the protons (hydrogen nuclei) in a sample placed in an 11.74 T magnet, using the value of γ for hydrogen (normally referred to as proton) in Table 1. We can now calculate the energy difference of the spin states, as in Equation 1.3.3.

$$\Delta E = \frac{26.75222127 \times 10^{7}\ rad \cdot s^{-1}T^{-1} \times 6.626 \times 10^{-34}\ J-s \times 11.74T}{2\pi} = 1.054 \times 10^{-25}\ J \tag{1.3.3}$$

This energy may not seem like it is that much less than the energy of our visible absorption transition at 600 nm, after all the numbers only differ by a factor of 10^{6}. However, if we think about the thermal energy of our sample in terms of kT (1.38066 x 10^{-23} J·K^{-1} x 298 K = 4.11 x 10^{-21} J) we can see that the thermal energy of our sample is about 100 fold less than the energy of the visible absorption of 600 nm light but is about 10,000 times greater than the energy of our proton NMR transition. This is why there is only a very small difference in population between the ground and excited states in NMR.

Having compared the energies of these two spectroscopic methods we might now ask how do the frequency and wavelength in NMR compare with our 600 nm light? We can calculate the NMR frequency, known as the Larmor frequency, using Equation 1.3.4

$$\upsilon = \frac{\Delta E}{h} = \frac{\gamma B_o}{2\pi} \tag{1.3.4}$$

For our example of protons in an 11.74 T magnetic field, ν is 500 x 10^{6} Hz or 500 MHz. This is in the radio frequency range of the electromagnetic spectrum. It is common to refer to NMR instruments by the frequency of protons in the magnetic field associated with a given spectrometer, therefore a spectrometer with an 11.74 T magnet is referred to as a 500 MHz instrument.

? Exercise 1.3.3

Calculate the wavelength of electromagnetic radiation corresponding to a frequency of 500 MHz.

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1.4: What is chemical shift and how does it relate to resonance frequency?

If each type of nucleus (e.g. all protons) gave a single resonance frequency, as implied by Equation 3.4, NMR would not be of much use to chemists. The actual nuclear resonance frequency is highly dependent on the local chemical environment. The effective magnetic field, B_{eff}, felt by a nucleus differs from that of the applied magnetic field, B_o, due to shielding by the motion of the electron clouds surrounding the nucleus. The greater the electron density around the nucleus, the larger is this shielding effect. The amount of shielding is expressed as the magnetic shielding constant σ, where $B_{eff} = (1-\sigma)B_o$. Therefore, the resonance frequency of each nucleus differs depending on the value of B_{eff}.

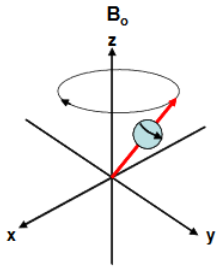
$$\nu = \frac{\gamma(1-\sigma)B_o}{2\pi} = \frac{\gamma B_{eff}}{2\pi} \tag{1.4.1}$$

The chemical shift of a nucleus reveals much about the structure of a molecule as shielding constants are well correlated with local chemical environment. For example I can know whether a molecule contains a methyl group or an aromatic ring depending on the chemical shifts of the protons in my NMR spectrum.

Early NMR spectrometers were scanning instruments in which the radio frequency was scanned through the proton chemical shift range until a frequency was reached at which energy was absorbed by the sample; this is the resonance condition. Modern instruments irradiate the sample with a broad band, or range, of frequencies and excite all of the protons at the same time.

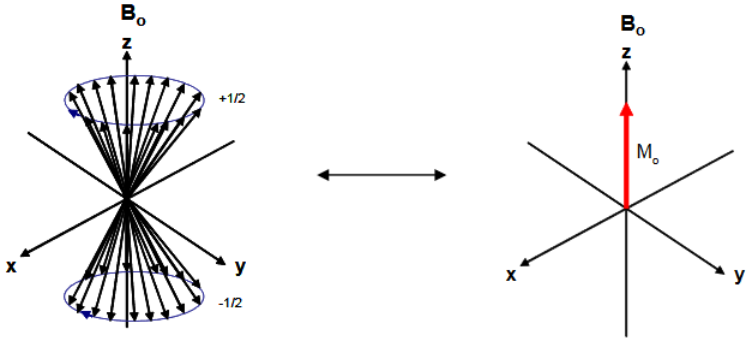
1.5: What is Precession?

A spinning charged particle creates a magnetic field, called the magnetic moment, μ. This magnetic moment is a vector quantity that is proportional to the angular momentum: $\mu = \gamma p$. Because our nucleus has angular momentum, the magnetic moment, depicted as the red vector in the figure below, will appear to precess (or rotate) about the applied magnetic field B_o. This precession is analogous to the motion of a spinning top. The frequency of precession is dependent only on the type of nucleus (defined by the gyromagnetic ratio, γ) and the value of B_{eff}, as defined in Equation 4.1. The precession of a single nucleus, depicted as a blue sphere spinning about its axis, is shown here.



1.6: How does precession generate the macroscopic magnetization (Mo)?

If we now examine what we would expect for an ensemble of nuclei, the magnetic moments of the +½ spins will be aligned with the applied magnetic field, while the moments of the higher energy –½ spin state will be opposed to B_o. However, all the spins in our sample will be precessing randomly about B_o at their Larmor frequency as illustrated in the figure below. Because slightly more of our nuclei are in the lower energy +½ spin state, if we take the vector sum of all the magnetic moments we will realize a single vector pointing in the direction of the applied magnetic field called the macroscopic magnetization, M_o. The macroscopic magnetization then provides a way to visualize the population difference of our spins. It is this macroscopic magnetization vector that is manipulated in the NMR experiment.



1.7: How can the nuclear spins be manipulated to generate the NMR spectrum?

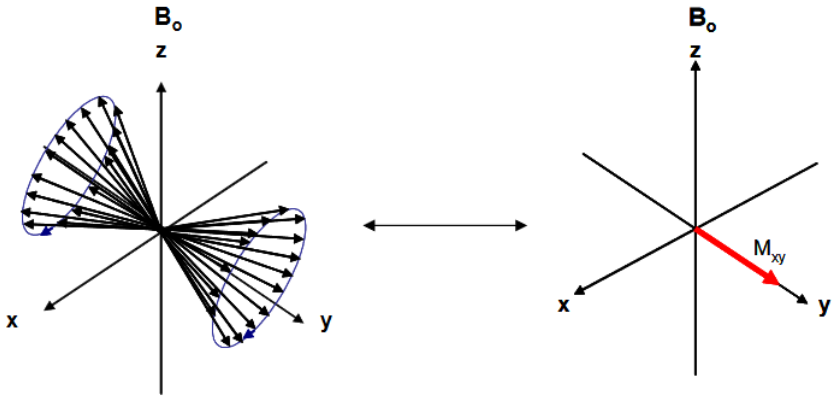
The previous figure shows the system at equilibrium. In order to generate a NMR signal, we must do something to perturb the populations of our spin states. As in other spectroscopic measurements, this is done through the absorption of radiant energy (light) of the appropriate frequency. In NMR, this transition is in the radio frequency (rf) range, corresponding to the Larmor frequency of the nucleus we are interested in. We cause this transition by irradiating our sample at a single radio frequency. An AC current oscillating at the desired rf frequency is applied to a coil wound around our sample. This oscillating current creates an additional magnetic field (called the B_1 field) that acts upon our macroscopic magnetization vector and tips it away from its equilibrium position aligned with B_o. This B_1 pulse creates the signal that we detect in NMR. In order to excite all of the different types of a single nucleus in our sample (e.g. all of the different types of protons or carbons), this pulse of rf radiation is kept short (typically ~10 μs). By the Heisenberg uncertainty principle, a short pulse will excite a broad range of frequencies; $\Delta f = 1/\Delta t$.

? Exercise 1.7.4

What range of frequencies would be excited by a 10 μs rf pulse?

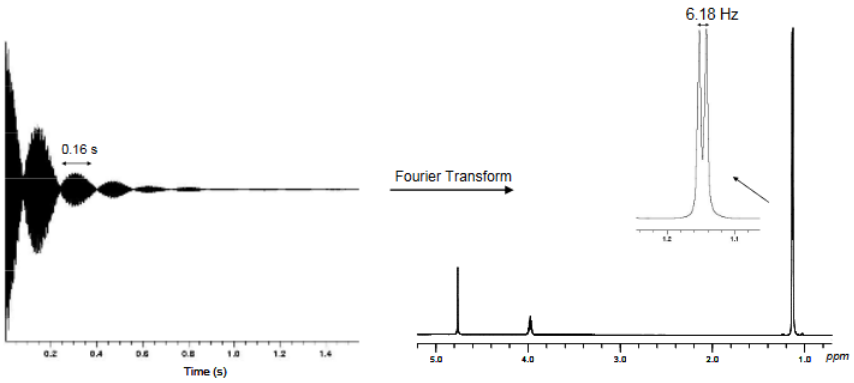
1.8: What is the tip angle?

The angle that the B_1 pulse tips the magnetization through depends on the power of the pulse and its length. For a given power setting, a tip angle, θ, (in radians) can be defined as $\theta = \gamma B_1 \tau$, where γ is the magnetogyric ratio and τ is the length of time the pulse is on. What we detect in the NMR experiment is the projection of the macroscopic magnetization vector, M_{xy}, into the xy plane of the NMR coordinate system. A 90^o pulse will produce the greatest signal in the xy plane. The Figure below shows the effect of a 90^o pulse on the spins.



1.9: What is the Free Induction Decay?

The signal we detect is called a **Free Induction Decay** (FID). The FID is produced by the macroscopic magnetization after the pulse. The magnetization will undergo several processes as it returns to equilibrium. First immediately after the pulse, the transverse component of the macroscopic magnetization, M_{xy}, will begin to precess at its Larmor frequency. This precessing magnetization will induce an alternating current in a coil (the same one used to generate the rf pulse) wound round the sample. This induced AC current is our FID, such as the one shown below.



The FID contains all of the information in the NMR spectrum, but it is difficult for us to discern the information in this format. Fourier transformation of the FID, a time domain signal, produces the frequency domain NMR spectrum. The resonance frequencies of the signals in the transformed spectrum correspond to the frequency of oscillations in the FID. In this FID measured for isopropanol, the 0.16 modulation of the FID is due to the 6.18 Hz difference in frequency of the resonances of the intense methyl doublet. The intensity information of each component is contained in the intensity of the first point of the FID. The signals that comprise the FID decay exponentially with time due to relaxation processes discussed in the next section. The rate of decay for each component of the FID is inversely proportional to the width of each NMR resonance.

1.10: How do T_1 and T_2 relaxation affect NMR spectra?

The decay of the FID corresponds to the loss of intensity of the macroscopic magnetization vector in the xy plane (called the transverse plane) by a process called spin-spin relaxation (or transverse or T_2) relaxation. T_2 relaxation occurs when a nucleus in a –½ spin state transfers its spin to a nearby nucleus in a + ½ spin state, and vice versa. Since T_2 relaxation occurs through mutual spin flips, the energy of the system is unaffected, it is an entropic process. In terms of our vector model, T_2 relaxation corresponds to a loss of coherence or dephasing of the magnetization vector. The recovery of magnetization along the z (longitudinal) axis (aligned with B_o) to its equilibrium position occurs by a process called spin lattice (or longitudinal or T_1 relaxation). T_1 relaxation occurs through interactions of the nuclei with the lattice (or the nuclei that surround our sample). Lattice motions at the same frequency as the Larmor frequency stimulate the magnetization in the higher energy – ½ spin states to lose this excess energy by transferring it to the lattice via a process called radiationless decay. Since T_1 relaxation involves a loss of energy by the system as the spins return to their equilibrium populations, it is an enthalpic process. These relaxation processes are first order processes characterized by the relaxation time constants T_1 and T_2. The width at half-height of a resonance is inversely related to the T_2 relaxation time of the nucleus, $w_{1/2} = (\pi T_2)^{-1}$. Because the magnets we use are not perfectly homogeneous, there is a secondary contribution to the line width that comes from magnetic field inhomogeneity. Therefore, the apparent spin-spin relaxation time constant or T_2* observed in the FID includes both the natural T_2 relaxation time of the nucleus as well as the effect of magnetic field inhomogeneity, $w_{1/2} = (\pi T_2^*)^{-1}$. If you want to know the real T_2 value for a nucleus, a special experiment, called the spin echo can be used.

? Exercise 1.10.5

What are the resonance line widths of nuclei that have apparent T_2 relaxation times (i.e.T_2* values) of 1 and 2 sec.

The effects of T_1 relaxation are more difficult to observe directly, because it corresponds to the return to equilibrium populations following the pulse. However, if several FIDs are coadded, as is usually the case in NMR, and if the time between successive pulse and acquire steps is insufficient for complete T_1 relaxation, the resonances in the resulting NMR spectrum will be less intense than they would otherwise appear. Because quantitative NMR measurements rely on resonance intensity, understanding the effects of T_1 relaxation is very important for obtaining accurate qNMR results. Therefore this subject is treated in greater depth in the Practical Aspects section of this module.

1.11: Where should I look to learn more about NMR?

It is hoped that this brief tutorial has provided sufficient background for you to understand the next section, focusing on practical aspects of quantitative NMR measurements. For further insights into NMR, the following websites and books are recommended. Many students find the e-book written by Professor Joseph Hornak at RIT to be especially useful since it contains embedded animations that illustrate many of the concepts introduced here.

“The Basics of NMR” by Joseph P. Hornak, http://www.cis.rit.edu/htbooks/nmr/

“Georgetown Graduate Course on NMR Spectroscopy” by Angel de Dios, http://bouman.chem.georgetown.edu/nmr/syllabus.htm

"2D NMR Spectroscopy” by Marc Bria, Pierre Watkin and Yves Plancke, http://rmn2d.univ-lille1.fr/rmn2d_en..._RMN2D_en.html

“Understanding NMR Spectroscopy” by James Keeler, John Wiley & Sons, 2005

“High-Resolution NMR Techniques in Organic Chemistry” by Timothy D. W. Claridge, Pergamon, Oxford, 1999.

“Spin Choreography: Basic Steps in High Resolution NMR” by Ray Freeman, Oxford University Press (1999).

“Modern NMR Spectroscopy: A Guide for Chemists”, 2nd Edition, by Jeremy K. M. Sanders and Brian K. Hunter, Oxford University Press, 1993.

"200 and More NMR Experiments: a Practical Course” by Stephan Berger and Siegmar Braun, Wiley-VCH, 2004.

“Basic One- and Two-Dimensional NMR Spectroscopy" by Horst Friebolin, Wiley-VCH, 2004.

"Experimental Pulse NMR: A Nuts and Bolts Approach" by Eiichi Fukushima and Stephen B. W. Roeder, Perseus Publishing, 1993.

CHAPTER OVERVIEW

2: Practical Aspects of Q-NMR

This discussion presumes that you already have an understanding of the basic theory of NMR. There are a number of issues that should be considered when measuring NMR spectra for quantitative analysis. Many of these issues pertain to the way that the NMR signal is acquired and processed. It is usually necessary to perform Q-NMR measurements with care to obtain accurate and precise quantitative results.

This section is designed to help you answer the following questions:

Topic hierarchy

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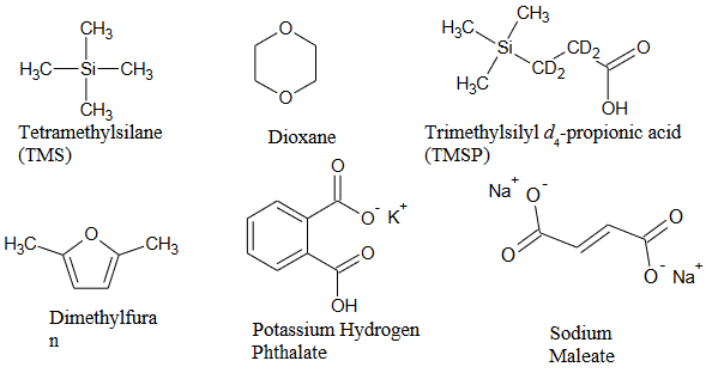
2.1: How do I choose a reference standard for my Q-NMR analysis?

With NMR, we need only to have available any pure standard compound (which can be structurally unrelated to our analyte) that contains the nucleus of interest and has a resonance that does not overlap those of our analyte. The analyte concentration can then be determined relative to this standard compound. The requirement for lack of overlap means that most standards have simple NMR spectra, often producing only singlet resonances. Additional requirements for standards to be used for quantitative analysis are that they:

- are chemically inert
- have low volatility
- have similar solubility characteristics as the analyte
- have reasonable T_1 relaxation times

The structures of several common NMR chemical shift and quantitation standards are shown in the figure below.



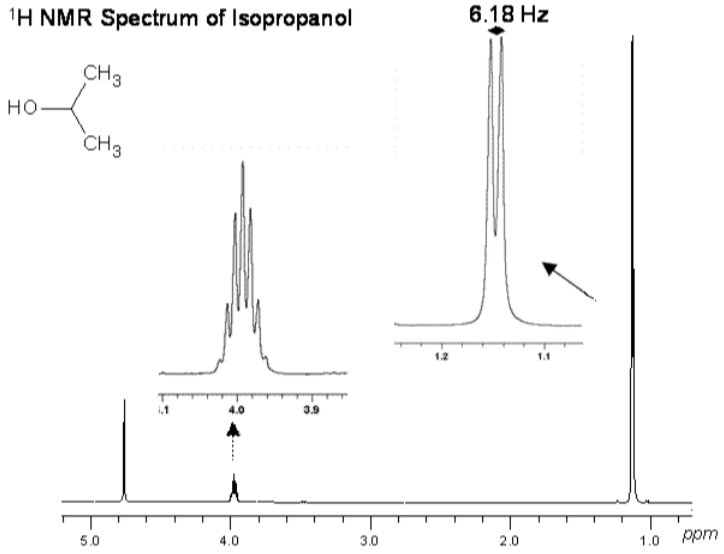
TMS and dioxane are chemical shift reference compounds commonly used in organic solvents. However they do not make good quantitation standards because they suffer from high volatility. Therefore it is difficult to prepare a standard solution for which the concentration is known with high accuracy. TMSP is a water soluble chemical shift reference. While it has improved performance as a quantitation standard compared with TMS or dioxane, it has been shown to absorb to glass so stock solutions may have stability problems.[1] In addition to the criteria listed above, it is helpful for quantitation purposes if the compound selected as the standard also has the properties of a primary analytical standard, for example potassium hydrogen phthalate (KHP), which is available in pure form, is a crystalline solid at room temperature and can be dried to remove waters of hydration.

2.2: How is the internal standard used to quantify the concentration of my analyte?

If an NMR spectrum is measured with care, the integrated intensity of a resonance due to the analyte nuclei is directly proportional to its molar concentration and to the number of nuclei that give rise to that resonance.

$$\frac{\text{Integral Area}}{\text{Number of Nuclei}} \propto \text{Concentration} \tag{2.2.1}$$

For example the ^{1}H NMR resonance of a methyl group would have 3 times the intensity of a peak resulting from a single proton. In the spectrum below for isopropanol, the 2 methyl groups give rise to a resonance at 1.45 ppm that is 6 times greater than the integrated intensity of the CH resonance at 3.99 ppm. Since this spectrum was measured in D_2O solution, only the resonances of the carbon-bound protons were detected. The OH proton of isopropanol is in fast exchange with the residual water (HOD) resonance at 4.78 ppm, therefore a separate resonance is not observed for this proton.



In this example we compared the relative integrals of the proton resonances of isopropanol. This information can be very useful for structure elucidation. If we instead compare the integral of an analyte resonance to that of a standard compound of known concentration, we can determine the analyte concentration.

$$\text{Analyte Concentration} = \frac{\text{Normalized Area Analyte} \times \text{Standard Concentration}}{\text{Normalized Area Standard}} \tag{2.2.2}$$

The direct proportionality of the analytical response and molar concentration is a major advantage of NMR over other spectroscopic measurements for quantitative analysis. For example with UV-visible spectroscopy measurements based the Beer-Lambert Law, absorbance can be related to concentration only if a response factor can be determined for the analyte. The response factor, called the molar absorptivity in UV-visible spectroscopy, is different for each molecule therefore, we must be able to look up the absorptivity or have access to a pure standard of each compound of interest so that a calibration curve can be prepared. With NMR we have a wide choice of standard compounds and a single standard can be used to quantify many components of the same solution.

? Exercise 2.2.1

Question 1. A quantitative NMR experiment is performed to quantify the amount of isopropyl alcohol in a D_2O solution. Sodium maleate (0.01021 M) is used as an internal standard. The integral obtained for the maleate resonance is 46.978. The isopropanol doublet at 1.45 ppm produces an integral of 104.43. What would you predict for the integral of the isopropanol CH resonance as 3.99 ppm. What is the concentration of isopropanol in this solution?

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2.3: What sample considerations are important?

What nucleus should I detect?

Just as you might make a choice between measuring a UV or an IR spectrum, in NMR we often have a choice in the nucleus we can use for the measurement. A wide range of nuclei can be measured, with the spin ½ nuclei ^{1}H, ^{31}P, ^{13}C, ^{15}N, ^{19}F, ^{29}Si, and ^{31}P among the most common. However, most quantitative NMR experiments make use of ^{1}H, because of the inherent sensitivity of this nucleus and its high relative abundance (nearly 100%). In addition, as we will see in the next section, the relaxation properties of nuclei are also important to consider in quantitative NMR experiments, and compared with many other nuclei like ^{13}C, ^{1}H nuclei have more favorable T_1 relaxation times. The choice of the observe nucleus can depend on whether one seeks universal detection (for organic compounds ^{1}H and ^{13}C fall into this category) or selective detection. For example fluoride ions can be easily detected in fluorinated water at the sub-ppm level, in large part because of the selectivity of the measurement – one expects to find very few other sources of fluorine in water. Similarly phosphorous containing compounds like ATP, ADP, and inorganic phosphate can be detected and even quantified in live cells, tissue or organisms.

How concentrated is my sample?

In the Beer-Lambert law you are probably familiar with from UV-visible spectroscopy, absorbance is directly related to the concentration of the analyte. Similarly, in NMR the signal we detect scales linearly with concentration. Since NMR is not a very sensitive method, you would ideally like to work with reasonably concentrated samples, for protons this means analyte concentrations typically in the millimolar to molar range, depending on the instrument you will be using. Other nuclei are less sensitive than protons. The sensitivity issue has two components, the inherent sensitivity, which depends on the magnetogyric ratio (γ), and the relative abundance of the nucleus (for example ^{19}F is 100% abundant, but ^{13}C is only 1.1% of all carbon atoms)

What other practical issues do I need to consider?

The sensitivity of an NMR experiment can also be affected by the homogeneity of the magnetic field that the sample feels. It is normal to adjust the field homogeneity through a process known as shimming. NMR samples should be free of particulate matter, because particles can make it difficult to achieve good line shape by shimming. You will also have better luck with shimming if you have a sample volume sufficient to meet or exceed the minimum volume recommended by your instrument manufacturer.

SECTION OVERVIEW

2.4: How do I choose the right acquisition parameters for a quantitative NMR measurement?

This may not be a big consideration in measuring a UV-visible or IR spectrum; you generally just walk up to the instrument, place your sample in a sample holder and make a measurement. However, with NMR there are several parameters, summarized below, that can have a huge impact on the quality of your results and whether or not your results can be interpreted quantitatively.

Number of Scans

An important consideration is the number of FIDs that are coadded. Especially for quantitative measurements it is important to generate spectra that have a high signal-to-noise ratio to improve the precision of the determination. Because the primary noise source in NMR is thermal noise in the detection circuits, the signal-to-noise ratio (S/N) scales as the square root of the number of scans coadded. To be 99% certain that the measured integral falls within + 1% of the true value, a signal-to-noise ratio of 250 is required. Acquisition of high quality spectra for dilute solutions can be very time consuming. However, even when solutions have a sufficiently high concentration that signal averaging is not necessary to improve the S/N, a minimum number of FIDs (typically 8) are coadded to reduce spectral artifacts arising from pulse imperfections or receiver mismatch.

? Exercise 2.4.1

A solution prepared for quantitative analysis using NMR was acquired by coaddition of 8 FIDs produces a spectrum with an S/N of 62.5 for the analyte signals. How many FIDs would have to be coadded to produce a spectrum with an S/N of 250?

Acquisition Time

The acquisition time (AT) is the time after the pulse for which the signal is detected. Because the FID is a decaying signal, there is not much point in acquiring the FID for longer than 3 x T_2 because at that point 95% of the signal will have decayed away into noise. Typical acquisition times in ^{1}H NMR experiments are 1 – 5 sec.

An interesting feature in choosing an acquisition time is the relationship between the number of data points collected and the spectral width, or the range of frequencies detected. Although the initial FID detected in the coil is an analog signal, it needs to be digitized for computer storage and Fourier transformation. According to Nyquist theory, the minimum sampling frequency is at least twice the highest frequency detected. The dwell time (DW) or time between data point sampling is a parameter that is not typically set by the user, but determined by the spectral width (SW) and the number of data points (NP).

$$DW = \frac{1}{2SW} \tag{2.4.1}$$

$$AT = DW \times NP \tag{2.4.2}$$

Another feature of the acquisition parameters that is important for quantitative measurements is the digital resolution (DR).

$$DR = \frac{SW}{NP(real)} \tag{2.4.3}$$

Almost all spectrometers are designed with quadrature phase detection, which in effect splits the data points into real and imaginary datasets that serve as inputs for a complex Fourier transform. It is important to have sufficient digital resolution to accurately define the peak. Since a typical ^{1}H NMR resonance has a width at half height ($w_{1/2}$) of 0.5 to 1.0 Hz, 8-10 data points are required to accurately define the peak. The total number of data points used in the Fourier transformation and contributing to the digital resolution can be increased by zero-filling, as described in the section on data processing.

? Exercise 2.4.2

A ^{1}H NMR spectrum was measured using a 400 MHz instrument by acquisition of 16,384 total data points (8192 real points) and a spectral width of 12 ppm. What was the acquisition time? Calculate the digital resolution of the resulting spectrum? Is this digital resolution sufficient to accurately define a peak with a width at half height of 0.5 Hz?

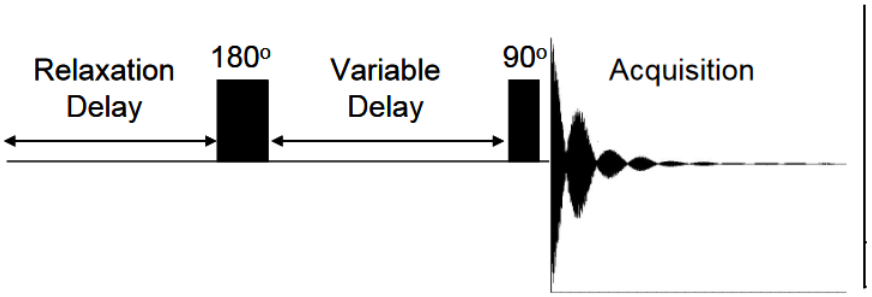
Receiver Gain

The receiver includes the coil and amplifier circuitry that detects and amplifies the signal prior to digitization by the analog-to-digital converter (ADC). It is important to set the receiver gain properly so that the ADC is mostly filled, without overflowing. ADC's used in NMR typically have limited dynamic range of 16 -18 bits. If the receiver gain is set too low, only a few bits of the ADC are filled and digitization error can contribute to poor S/N. If the receiver gain is set too high, (called clipping the FID) the initial portions of the FID will overflow the ADC and will not be properly digitized. In this case, resonance intensity can no longer be interpreted in a quantitative manner. In addition, a lot of spurious signals will appear in the spectrum. For most experiments the autogain routine supplied by the NMR manufacturer will work well for the initial setup of the experiment.

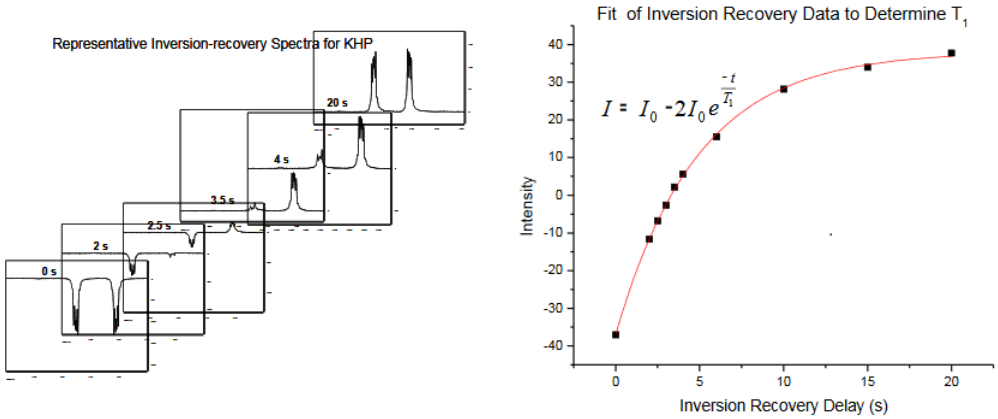
Repetition Time

The repetition time is the total time between the start of acquisition of the first FID and the start of acquisition of the second FID. The repetition time is the sum of the acquisition time and any additional relaxation delay inserted prior to the rf pulse. Recall that there are two relaxation times in NMR, T_1 and T_2 (with $T_1 \geq T_2$). If a pulse width of 90° is used to signal average multiple FIDs to improve S/N or reduce artifacts, we generally need to wait 5 x T_1 between each acquisition so that the magnetization can relax essentially completely (by at least 99%) to its equilibrium state. If the repetition time is less than $5T_1$, the resonances in the spectrum cannot be simply interpreted in a quantitative manner and resonance intensity is scaled according to T_1.

The inversion-recovery pulse sequence can be used to measure T_1 relaxation times. In this pulse sequence, diagrammed below, the magnetization is inverted by a 180° pulse. The relaxation delay at the start of the experiment is selected to assure complete relaxation between acquisitions.



During the variable delay, magnetization relaxes by spin-lattice (T_1) relaxation and is tipped into the transverse plane by the 90° read pulse. The intensity of the resonances is measured and then fit to an exponential function to determine the T_1 relaxation time. The figure below shows selected spectra measured for the KHP protons using the inversion-recovery experiment and the fit of the integral of one of the resonances to determine the T_1 relaxation time of the corresponding proton.



Pulse Width

As described in the Basic Theory section, the NMR signal is detected as a result of a radio frequency (rf) pulse that excites the nuclei in the sample. The pulse width is a calibrated parameter for each instrument and sample that is typically expressed in µs. The

pulse width can also be thought of in terms of the tip angle, θ, of the pulse

$$\theta = \gamma B_1 \tau \tag{2.4.4}$$

where γ is the gyromagnetic ratio, B_1 is the strength of the magnetic field produced by the pulse and τ is the length of the pulse. For quantitative NMR spectra, 90^o pulses with a repetition time ≥ $5T_1$ are typically used since this pulse produces the greatest S/N in a single scan, although other pulse widths can also be used. For spectra where qualitative, rather than quantitative analysis is desired, significant time savings can be obtained by using shorter pulses (i.e. 30^o) since the magnetization takes less time to recover to its equilibrium state after the pulse. For a more detailed analysis of the effects of tip angle in quantitative NMR experiments, visit the following page.

Topic hierarchy

Template:HideTOC

2.5: Effects of Tip Angle in Quantitative NMR Experiments

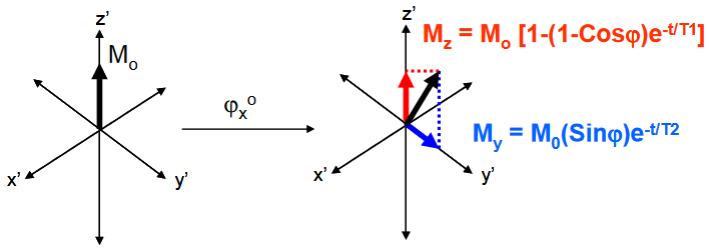
In experiments where the goal is qualitative analysis, it is not necessary to acquire the spectra in a manner that produces fully relaxed spectra. In this case, one can use the Ernst Angle relationship to calculate the tip angle that will maximize S/N for a given repetition time.[1] In such experiments, it is typically most efficient to eliminate the relaxation delay and use a repetition time equivalent to the acquisition period. Typically the acquisition time is set to allow the FID to decay to noise. This is often approximated by setting the acquisition time to about 3 x T_2*, which will allow the magnetization to decay by 95% of its initial value.

$$\text{Ernst Angle Relationship: } \cos\vartheta = e^{\frac{-T_r}{T_1}} \tag{2.5.1}$$

In the Ernst angle equation, ϕ is the tip angle, T_r is the repetition time and T_1 is the spin-lattice relaxation time of the resonance of interest. For example, with a repetition time of 3 sec and a T_1 relaxation time of 5 sec, we calculate an Ernst angle of 56.7°. While this repetition time and tip angle maximizes S/N, it will not give us integrals that can be interpreted quantitatively in a straight forward way. It is possible to correct for incomplete T_1 relaxation, however this introduces greater error in the result and is not always practical, since analyte solutions.

Where quantitative integrals are desired there is generally not that great of time savings by using a tip angle less than 90°. To see why, let's look at a concrete example. Assume that for an analyte resonance with a T_1 relaxation time of 5 sec, we have to coadd 100 FIDs to achieve a S/N of 250:1 using a 90° pulse. In this experiment, the shortest repetition time we should use is 5 x T_1, or 25 sec, which will allow the magnetization to relax to 99% of its initial value. This means that it would take 2500 sec (or 0.694 hr) to complete this experiment.

What would happen if we used a shorter tip angle? By using a smaller tip angle, the magnetization will take less time to recover following the pulse however we will also detect less intensity following the pulse.



The intensity of the magnetization following a pulse, M_y, can be described by the equation below, where Mo is the intensity of the fully relaxed magnetization, t is the time following the pulse, for a tip angle of ϕ, and T_2* is the apparent spin-spin relaxation time.

$$M_y = M_0 \sin\vartheta e^{\frac{-t}{T_2^*}} \tag{2.5.2}$$

Immediately following the pulse, t = 0. For a 56.7° tip angle, M_y would be 0.836 M_o immediately following the pulse. This means that we would have 83.6% of the signal that we would have achieved with a 90° pulse. However, unlike a 90° pulse, the value of M_z is not zero immediately following a 56.7° pulse. In general, the value of M_z following a pulse with a tip angle, ϕ, can be described by the equation below, where t is the time following the pulse, for a tip angle of ϕ, and T_1 is the apparent spin-lattice relaxation time.[2]

$$M_z = M_0[1-(1-\cos\vartheta)e^{\frac{-t}{T_1}}] \tag{2.5.3}$$

This means that immediately following a 56.7° pulse, t = 0 and the value of M_z, governed by $\cos\phi$ is already 0.549 x M_o. Since we want to acquire fully relaxed spectra, the question is how long will we have to wait until M_z = 0.99 x M_o if the T_1 relaxation time is 5 sec? Substitution into the equation above we calculate that t = 19.04 sec. This is a significant time savings over the 25 sec we would have to wait for T_1 relaxation if a 90° pulse were used. However, using a 56.7° pulse we have generated only 83.6% of the signal we had if a 90° pulse had been used. Since we gain S/N as the square root of the number of FID's coadded we would have to acquire 1.43 times as many FIDs as we did with a 90° pulse to obtain the desired S/N of 250:1. Therefore, the total experiment time using a 56.7° pulse would be 19.04 sec x 143 or 2723 sec or 0.756 hr, a slightly longer total acquisition time than was required

using a 90^o pulse. For this reason, most NMR spectroscopists simply use a 90^o pulse and with a repetition time of at least 5 x T_1 for quantitative NMR experiments.

References

1. R. R. Ernst and W. A. Anderson “Application of Fourier Transform Spectroscopy to Magnetic Resonance” *Rev. Sci. Inst.* (1966) **37**:93-102.
2. D. L. Rabenstein “Sensitivity Enhancement by Signal Averaging in Pulsed/Fourier Transform NMR Spectroscopy” *J. Chem. Ed.* (1984) **61**:909-913.

2.6: What data processing considerations are important for obtaining accurate and precise results?

Data processing describes operations that are performed to improve spectral quality after the data has been acquired and saved to disk.

Zero-Filling

Zero-filling is the addition of zeros to the end of the FID to increase the spectral resolution. Because zeros are added, not additional real data points carrying with them an overlay of noise, zero-filling can improve digital resolution without decreasing S/N. Another option is to use linear prediction to add data points calculated from the beginning of the FID where S/N is at its highest.

Apodization

Apodization is the multiplication of the FID by a mathematical function. Apodization can serve several purposes. Spectral resolution can be improved by emphasizing the data points at the end of the FID. S/N can be improved by multiplying the FID by a function that emphasizes the beginning of the FID relative to the later data points where S/N is poorer. For quantitative NMR experiments, the most common apodization function is an exponential decay that matches the decay of the FID (a matched filter) and forces the data to zero intensity at the end of the FID. This function is often referred to a line broadening, since it broadens the signals based on the time constant of the exponential decay. This trade-off between S/N and spectral resolution is not restricted to NMR and is common to many instrumental methods of analysis.

Integration Regions

Because NMR signals are Lorentzians, the resonances have long tails that can carry with them significant amounts of resonance intensity. This is especially problematic when the sample is complex containing many closely spaced or overlapped signals, or when the homogeneity of the magnetic field around the sample has not been properly corrected by shimming. For a Lorentzian peak with a width at half-height of 0.5 Hz, integration regions set at 3.2 Hz or 16 Hz on either side of the resonance would include approximately 95% or 99% of the peak area, respectively. Note that this analysis does not include the ^{13}C satellites which account for an additional 1.1% of the intensity of carbon-bound protons in samples containing ^{13}C at natural abundance. In cases where resonances are highly overlapped, more accurate quantitative analysis can often be achieved by peak fitting rather than by integration.

An alternative approach utilizes ^{13}C decoupling during the acquisition of the proton spectrum to collapse the ^{13}C satellites so that this signal is coincident with the primary ^{1}H-^{12}C resonance.[2, 3] This relatively simple approach requires only that the user has access to a probe (for example a broadband inverse or triple resonance probe) that permits ^{13}C decoupling.

Baseline Correction

NMR integrals are calculated by summation of the intensities of the data points within the defined integration region. Therefore, a flat spectral baseline with near zero intensity is required. This can be achieved in several ways; the most common is selecting regions across the spectrum where no signals appear, defining these as baseline and fitting them to a polynomial function that is then subtracted from the spectrum.

2.7: References

1. D.A. Jayawickrama, C.K. Larive, *Anal. Chem.* **71**:2117-2112 (1999).
2. The Quantitative NMR Portal, http://tigger.uic.edu/~gfp/qnmr/
3. G. F. Pauli, B. U. Jaki, D. C. Lankin "A Routine Experimental Protocol for qHNMR Illustrated with Taxol" *J. Nat. Prod.* **70**:589-595 (2007).

CHAPTER OVERVIEW

3: Virtual Experiment

This experiment, available in pdf format, uses FTNMR Simulator, a program written by Dr. Harold Bell, professor emeritus at Virginia Tech to simulate an NMR experiment. Instrument parameters such as spectral width, number of data points, pulse width, noise, etc., are selected by the user. Once the FID is displayed, it can be treated by exponential smoothing or resolution enhancement. After the Fourier transform, phase corrections and baseline flattening may be applied. Spectra may be printed, or saved as Windows metafiles.

To download the software, you can access the following website and download the FTNMR Simulator, newfid.zip(2.9 mBytes). http://www.asdlib.org/onlineArticles...retextpage.htm

The program is also available in Spanish, fidsp.zip, and French, fidwinfr.zip. A tutorial, wintutor.pdf (255 kBytes), to accompany this software is also available. It contains more than 20 exercises selected to help novices learn about FTNMR.

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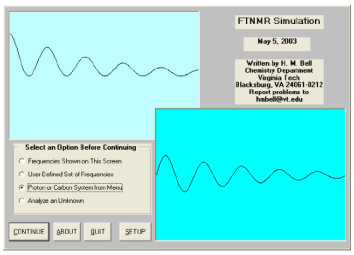
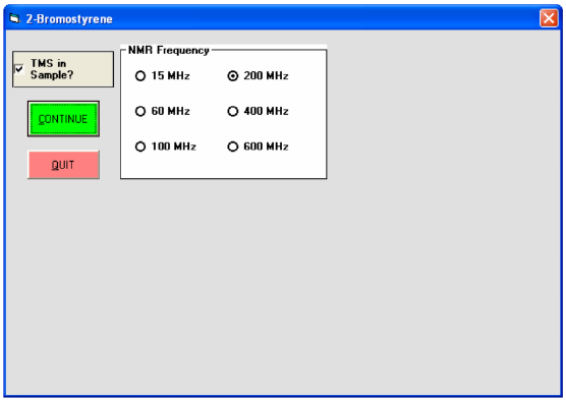
3.1: Virtual Laboratory

This virtual laboratory makes use of the FTNMR simulation program written by Harold Bell to explore the effects of the parameters discussed in the Practical Aspects section of this module on the results of simulated NMR data. Although you are encouraged to explore this program more fully, the focus here is on the effects of applied magnetic field strength, signal averaging, relaxation times, repetition times, the number of data points and the receiver gain.

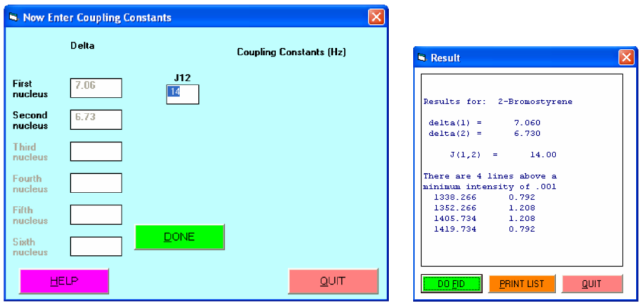
1. Run the simulation program and select “Proton or Carbon System from the Menu” and click “Continue”.



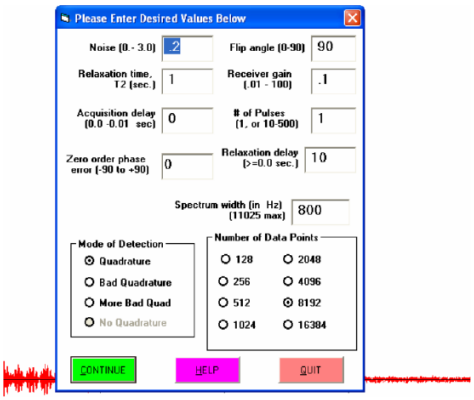
Choose one of the molecules from the proton menu. Compounds with a range of complexity are available and you may wish to investigate more than one molecule. In the new menu that appears, click the box that says “TMS in Sample?” Choose a NMR Frequency (magnetic field strength) from the list and click continue.



A new menu will appear (see figure below) that contains the resonance frequencies for the compound you selected. Click “Continue” and the coupling constants for these nuclei will appear. Clicking “Done” generates a list of the spectral frequencies. Click “DO FID” button to begin the process of simulating the FID.



2. A new menu should appear that contains many of the acquisition parameters that we would like to investigate.



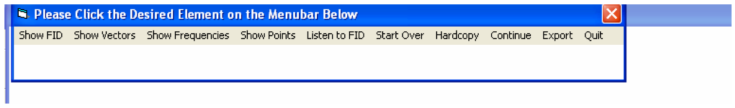
At this point there are some notable differences between the simulation and real data acquisition. This program allows you to add noise; this is not a feature of real spectrometers. Another difference here is that you can select the pulse width in degrees. If you were using a real spectrometer you would select the pulse power and calibrate the pulse width in μs that corresponded to the tip angle you desire. Start with these initial default parameters:

Default Parameters

- Noise 0.2
- T_2 Relaxation time 1.0 s
- Acquisition delay 0.0 s
- Zero order phase error 0.0
- Under “Mode of Detection”, Click the box that says “Quadrature”
- Flip angle 90º
- Receiver gain 0.1
- Number of pulses 1
- Relaxation delay 10 s
- Use the default spectral window selected for your compound
- Number of data points 8192

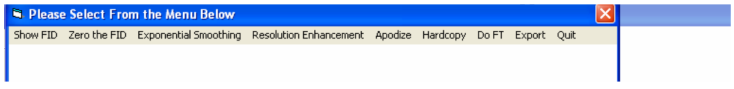
Click the box at the bottom of the page to “Continue” and simulate the FID

3. A new menu should appear along the top of the page.



Click “Show FID” to display the FID you simulated. You will see that there are choices to show the whole FID or expansions of the FID with real or imaginary data points. If you click on “Show Points” you will see the individual data points that would have been acquired. Although the pulses we apply in NMR are in the radio frequency range, the digitized signals are on the order of a few thousand Hz. This places them in the audio range and you can hear your FID by clicking on “Listen to FID” button.

4. Click Continue and select “zero fill” and a new menu will appear.



This menu accesses the data processing part of the simulation program. Choose “Exponential Smoothing” and use the value of 0.5 Hz line broadening and choose “Done”. Answer “YES” to accept the smoothing function and apodize the FID. Select “Do FT” to Fourier transform the FID and generate the spectrum.

5. Now let’s investigate the effect of the receiver gain. Choose “Start Over” and choose “Same Frequencies, Intensities and T_1’s”. Select gain values of 0.01, 0.1, 1, 10 and 100 and examine the effect on the FID and on the Fourier transformed spectrum. What is the optimum value of the gain? Can the gain be set too high? What is the effect of too high of gain on the FID? On the spectrum?

6. Using the gain you determined to be the optimum value, let's explore the effects of the number of pulses. Set the noise to 3.0 and repeat the simulation for 1 pulse. Now compare this result with 100 pulses. After selecting “100 pulses” and “Continue”, a dialog box called “Pause” will pop up showing the FID after 1 pulse, select continue. You will now get more dialog boxes showing you how the FID looks after 10, 50 and 100 pulses. Do you see how the signal-to-noise ratio (S/N) improves with the number of FIDs coadded? How does the Fourier transformed spectrum compare with the one you measured using a single pulse? In this simulation, it is nearly just as rapid to coadd 100 FIDs as to measure 1, but with a real measurement it would take 100 times as long. In this case do you think that the S/N improvement would be worth the extra time? How would the S/N improvement for quantitative data affect the quality of the results you would obtain?
7. Now go back to 1 pulse leaving all the other parameters the same including a noise level of 3.0 and let’s evaluate the effect of the exponential smoothing function. This time when you choose “Start Over” just select “Same FID” since this is a post-acquisition processing parameter and does not affect the saved data. Evaluate the transformed spectra using values of 0. 0.5, 1, 5 and 10 Hz line broadening. Which value do you think is the optimum? Why?
8. Click on "Start Over" and select "Complete Restart". This time choose “User-Defined Set of Frequencies” and press “Continue”. A new menu should appear. Select 1-5 Single Frequencies”. Enter a single frequency of 1.28 Hz. Choose a T_1 of 2 and intensity of 2, and press “Continue” and enter the default parameters from question #2 above. Click “Continue” to simulate the FID and then click "Show Vectors" and examine how the vectors relate to the FID. You may want to click “slow” on the vector speed bar to slow down the motion of the vector and the evolution of the FID. Fourier transform the FID and you should see a single resonance at 1.28 Hz.
9. Choose “Start Over” with the same frequencies, intensities and T_1’s. Change the T_2 to lower and higher values. Examine the effect of T_2 on both the FID and the Fourier transformed spectrum.
10. In this exercise, we will explore the effects of the flip angle. Use the Default FID Parameters from question #2 above, except select a T_2 of 2 sec. Now investigate the following flip angle values: 30, 45, 60 and 90^o. What is the effect of flip angle on the intensity of the FID and in the Fourier transformed spectrum? Can you use the vector model of NMR to explain the effect of flip angle on resonance intensity?
11. Now let’s explore the effect the number of data points (this is related to the acquisition time in a real spectrometer). Choose “Start Over” with the same frequencies, intensities and T_1’s. Use the Default FID Parameters except select a T_2 of 2 sec and a noise value of 0.1. Investigate the effect of the number of data points by choosing values less than and greater than 1024. What is the effect on the transformed spectrum? Why does the use of too few data points produce artifacts in the transformed spectrum?
12. With quadrature detection, the analog signal is split into two FIDs that differ by a 90^o phase shift. These FIDs form the sine and cosine inputs to the complex Fourier Transform. Choose 512 points and click on "Show FID". You should have two possibilities, real and imaginary. How does the real FID differ from the imaginary one?
13. Now let's see what happens if we have more than one resonance in our FID. Click on "Start Over" and select "Complete Restart". This time enter two frequencies 1.28 Hz, and 2.56 Hz choose an intensity of 1 and a T_1 of 2 sec for each resonance. Click “Continue”. Follow the instructions above for "Default FID Parameters" except choose 2048 data points and generate a new FID. Click on "Show Frequencies". You should see the individual components of the FID. Can you see how the two waves add to produce the FID? Now click on "show vectors" and examine how the vectors relate to the FID. Perform the Fourier transform and examine the spectrum. Does this spectrum make sense to you? Start over and choose some other frequencies. How does the choice of frequency affect the FID, vectors and the transformed spectrum?
14. Now let’s explore the effect of Spectral Width. Choose “Start Over” and “Complete Restart”. Choose a single frequency of 2.56 Hz, intensity of 2 and a T_1 of 2. Begin by using the "Default FID Parameters" listed above with a 100 Hz Spectrum Width to generate the FID. Now click on "Show Points". At this level of expansion, it is not possible to really see the individual data points. Select “Show FID” and choose "0.8 sec". Now you can better see that there are discrete data points that sample the FID. Now choose "Start Over" and choose "Same Frequencies, Intensities, T_1" and examine the effect the spectral width has on the FID. Reduce the value of the “Spectrum Width” to 20, 10, 5, 2 and 1 Hz expanding the FID (show the first 0.8 sec) to show the individual data points. What is the effect of changing the spectral width on the transformed spectrum? What happens if you choose a spectral width of 2 or 1 Hz? Is the frequency of the peak the expected value of 2.56 Hz?
15. Now let’s examine what happens if our acquisition parameters do not allow for complete relaxation of the magnetization. Start completely over and again let's use 3 resonances with frequencies of 0 Hz (20sec), 5 Hz (5sec), and 10 Hz (1sec) all with intensities of 0.5 and with the T_1 values given in parentheses after the frequency. Use the "Default FID Parameters" except choose a relaxation delay of 100 sec, 10 pulses, a T_2 of 0.3, a spectrum width of 100 Hz and 1024 points. Fourier transform the FID to examine the relative intensities of the resonances in the NMR spectrum. Choose “Start Over” selecting the “Same

Frequencies, Intensities and T1's" and examine the effect of signal averaging on the intensity of the resonances by reducing the relaxation delay to 20, 10, 5, 2, and 0 sec, and Fourier transforming each FID (remember that in each case, the magnetization does relax during the acquisition time). What is the effect of reducing the relaxation delay on the relative intensities and on the overall S/N ratio.

CHAPTER OVERVIEW

4: Q-NMR Experiment

Laboratory Experiment: Determination of Malic Acid Content in Apple Juice by NMR

This laboratory experiment contains 4 sections, a set of *prelab* exercises, introductory material to provide *background* in addition to the basic theory, practical aspects and virtual laboratory sections of this module, a *dry lab* that allows students to download and process Q-NMR data and a *wet lab* experiment in which students make their own measurements to quantify the malic acid content of apple juice. Additional background information about malic acid's role in fermentation and wine production, and its measurement by Q-NMR can be found in the Q-NMR Applications section of this module.

Topic hierarchy

Template:HideTOC

4.1: Prelab Exercises

1. Malic acid is a diprotic acid containing 2 carboxylate groups. Draw the structures of malic acid.
2. What other analytical methods might be used for quantitative analysis of malic acid in fruit juices?
3. What properties would you consider in choosing a reference standard for quantitative analysis by NMR?
4. How will changing pH affect the chemical shifts of malic acid? What potential problems might arise from these pH effects?

Follow the instructions provided in the Virtual Lab to help answer questions 5 and 6.

5. What acquisition parameters are important for a quantitative NMR measurement? How do you select the values of these parameters?
6. What data processing considerations are important for obtaining accurate and precise results?

4.2: Background

There are a number of issues that should be considered when measuring NMR spectra for purposes of quantitative analysis. Many of these issues pertain to the way that the NMR data is acquired and processed. It is usually necessary to perform quantitative NMR measurements with care to obtain accurate and precise quantitative results. The advantages of NMR over other spectroscopic methods are that no response factor is needed and all the resonances generated by a particular nucleus (for example ^{1}H, ^{31}P or ^{19}F) have an integrated intensity directly proportional to the molar concentration of the analyte and to the number of nuclei that give rise to that resonance.

$$\frac{\text{Integral Area}}{\text{Number of Nuclei}} \propto \text{Concentration} \tag{4.2.1}$$

The ^{1}H NMR resonance of a methyl group would have 3 times the intensity of a peak resulting from a single proton. For example, sodium 3-(trimethylsilyl)tetrapropionate TSP, has a methyl resonance equivalent to 9 protons (from the three methyl groups) and therefore would give rise to a resonance that is 9 times greater than the intensity resulting from a single proton.

For this experiment, KHP (potassium hydrogen phthalate) will be used as an internal quantitation standard. KHP has the advantage of being a primary standard, meaning that after drying, its solution concentration can be calculated directly from its mass. You may also wish you use an internal chemical shift reference, like TSP-d_4 in the preparation of your solutions. TSP is not a primary standard, and is known to adsorb to glass surfaces which can change its solution concentration over time, therefore it is not a useful quantitation standard. As a chemical shift reference, TSP-d_4 has the advantage of producing a single sharp resonance with a defined chemical shift of 0.00 ppm.

Malic acid and citric acids are the major organic acids in fruits. Q-NMR is a valuable technique for determining the quantities of major and minor compounds in fruit juices. By comparing the resonance integral of an analyte to that of a standard compound of known concentration, we can determine the analyte concentration according to the equation below:

$$C_{analyte} = \frac{I_{analyte} \times C_{std}}{I_{std}} \tag{4.2.2}$$

where $C_{analyte}$ is the analyte concentration, C_{std} is the quantitation standard concentration, and $I_{analyte}$ and I_{std} are the areas of the resonances of the analyte and the standard, respectively, normalized to the number of protons giving rise to each resonance.

The accuracy and precision of the integral measurements are affected by the following experimental factors.

- spectral S/N
- line shape
- quality of shimming
- baseline
- apodization window functions
- phasing, baseline-, and drift -corrections

Resonance overlap is a potential problem in accurate quantitation by NMR. This problem can sometimes be solved by careful selection of pH, using a different solvent, or adding a reagent to change the analyte chemical shift (i.e. lanthanide shift reagents). In some cases, the ^{1}H NMR spectrum may be too crowded for accurate quantitation, but another nucleus, for example ^{19}F or ^{13}C, that has a larger chemical shift window might produce well-separated resonances of the mixture components.

Field-frequency lock

The fields of superconducting magnets tend to drift over a period of minutes to hours causing loss of resolution. Most modern spectrometers are equipped with a lock channel that regulates the spectrometer field by monitoring the chemical shift of a deuterium resonance of the solvent. As the magnetic field drifts, the change in the deuterium resonance frequency generates an error signal that indicates both the magnitude and the direction of the field change, allowing compensation by a feedback circuit. Non-viscous deuterated solvents provide the best field-frequency lock because of their sharp and intense resonances. In ^{1}H NMR experiments an additional advantage of preparing samples in deuterated solvents is that the intensity of the solvent proton resonance is reduced. The resonance of protic solvents (e.g., H_2O, or CH_3CN) can obscure analyte ^{1}H NMR resonances and reduce the dynamic range of the measurement. While it is common to suppress the ^{1}H NMR resonances of protic solvents, analyte resonances with similar chemical shifts will also be suppressed. Sometimes it is not possible to make the sample solution in a

deuterated solvent, for example when the sample is already a liquid (i.e. blood plasma, urine or fruit juice). In such a case, a sufficient quantity of a deuterated solvent, like 10% D_2O, is added to the sample to provide the lock signal.

Solvent Suppression

Apart from accurate tuning of the probe and pulse width calibration, effective suppression of the solvent resonance is often crucial for the analyte resonance to be observed in aqueous samples. There are a number of solvent suppression methods available for use in NMR experiments, the simplest of which is presaturation. Presaturation uses a selective pulse to equalize the populations of the solvent spins. It is important to evaluate the effect of the solvent presaturation parameters on the resolution and sensitivity of the experiment to ensure good results. The presaturation power should be selected such that the solvent resonance is significantly attenuated without reducing the intensity of neighboring analyte resonances.

Repetition Time

The time between successive acquisitions is crucial in Q-NMR. To determine the longitudinal relaxation delay of a given analyte proton, the inversion recovery pulse sequence is used to measure T_1 relaxation times. The pulse sequence uses a calibration program which fits data to the exponential decay equation

$$I(\tau) = I_0 \left(1 - 2 \times e^{\frac{-\tau}{T_1}} \right) \tag{4.2.3}$$

where I(τ) is the intensity of the selected proton resonance for a given τ value, I_0 the intensity at equilibrium (infinite τ), τ is the value of the inversion delay, and T_1 is the first order time constant for longitudinal relaxation.

Malic and citric acid content of fruits

The table below summarizes the results obtained from quantitative NMR analysis of malic and citric acid content of various fruits.[1]

	Malic Acid	Citric Acid
Apple*	3.42-10.12 g/L	0.09-0.36 g/L
Apricot	4.59	4.13
Pear	2.55	1.05
Kiwi	2.66	11.00
Orange	2.13	11.71
Strawberry	1.74	7.13
Pineapple	1.33	5.99

*Data obtained from three apples ranged between the values given.

4.3: Dry Lab

Drylab Procedure:

This section contains FIDs measured using a 600 MHz Bruker Avance spectrometer. The FIDs can be downloaded and processed to quantify the concentration of malic acid in a D_2O solution and in apple juice. Inversion-recovery spectra measured for KHP are provided so that you can calculate the T_1 relaxation time using the resonance intensity. The FIDs are provided in JCAMP format which can be processed using most modern vendor software programs. If you do not have access to an NMR spectrometer, a free NMR processing software package wxNUTS can be downloaded to use with Mac OSX and Windows:

http://www.acornnmr.com/nuts.htm

A. Preparation of KHP solution and determination of the T_1 relaxation times of the KHP protons

A small amount of potassium hydrogen phthalate (KHP) was placed a beaker put into an oven at 110 ºC for 4 hrs. The beaker was then removed, covered in aluminum foil and placed in a dessicator to cool. The KHP was weighed using an analytical balance, transferred to a 5 mL volumetric flask and diluted with to the mark with D_2O to prepare a stock solution.

Mass of weighing paper = 0.2219 g

Mass of weighing paper + KHP = 0.3533 g

To measure the T_1 relaxation times of the KHP protons, a 600 µL aliquot of this stock solution was transferred to an NMR tube. A series of inversion recovery spectra were acquired as a function of the variable delay between the 180^o and 90^o pulses. The spectrometer frequency was 599.923 MHz. This experiment used an acquisition time of 2 sec, and an additional relaxation delay of 35 sec. 8 FIDs were coadded for each of the following spectra. Download and analyze these spectra to determine the T_1 relaxation times of the KHP protons.

To download a file click the file name and once the text window opens go to File and Save As to save the file as a text file. To process the downloaded file using NUTS, open the wxNUTS program and under the File menu click Import and then select the file to process.

Variable delay	JCAMP File
0.005 (s)	T1-measurement-KHP-051708_0s.dx
2	T1-measurement-KHP-051708_2s.dx
2.5	T1-measurement-KHP-051708_2p5s.dx
3	T1-measurement-KHP-051708_3s.dx
3.5	T1-measurement-KHP-051708_3p5s.dx
4	T1-measurement-KHP-051708_4s.dx
6	T1-measurement-KHP-051708_6s.dx
10	T1-measurement-KHP-051708_10s.dx
15	T1-measurement-KHP-051708_15s.dx
20	T1-measurement-KHP-051708_20s.dx

B. Determination of the malic acid concentration in a D_2O stock solution

To test our ability to quantitatively measure the malic acid concentration in an unknown apple juice sample using KHP as an internal standard, a solution containing a known malic acid concentration was prepared by transferring a known mass of malic acid to a 5 mL volumetric flask and diluting to the mark with D_2O.

Mass of paper = 0.1897 g

Mass of paper + Malic acid = 0.3324 g

The solution for Q-NMR was prepared by combining 1.00 mL of the KHP stock solution and 1.00 mL of the malic acid stock solution. The solution was mixed well and a 600 µL aliquot transferred to an NMR tube for analysis. The frequency of the spectrometer was 599.923 MHz. The spectrum was measured using a 2 s acquisition time and an additional 35 s relaxation delay. 64 FIDs were coadded.

The FID below was acquired for the quantitative analysis of the malic acid standard solution using KHP as an internal standard. Download and analyze this spectrum to determine the concentration of the malic acid in this stock solution.

Q-NMR-Malic-JHP-061108_Run2.dx

C. Determination of the malic acid concentration in a fruit juice solution

The KHP stock solution was diluted by mixing 1.00 mL of the stock solution prepared in part A with 1.00 mL of D_2O. After mixing, a pipettor was used to add 100 µL of the diluted KHP solution to 900 µL of apple juice obtained from the grocery store (note that better accuracy and precision would have been achieved if a 1 mL glass pipette were used instead of a pipettor). The pH of the solution was adjusted to approximately 1.35 using HCl. A 600 µL aliquot of this solution was transferred to an NMR tube and the spectrum recorded at a frequency of 599.923 MHz. The spectrum was measured using a 2 s acquisition time and an additional 35 s relaxation delay. The solvent resonance was suppressed by saturation during the relaxation delay. 480 FIDs were coadded.

The FID below was acquired for the quantitative analysis of the malic acid in apple juice using KHP as an internal standard. Download and analyze this spectrum to determine the concentration of the malic acid in the apple juice sample.

Q-NMR-Apple-JHP-061108A_Run1.dx

Dry Lab Report:

1. Using the mass and volume used to make the stock solution in part A, calculate the KHP concentration.
2. Using the inversion-recovery data, determine the T_1 relaxation times of the two KHP resonances.
3. Using the mass and volume used to make the stock solution in part B, calculate the malic acid concentration.
4. What is the concentration of malic acid determined from the Q-NMR experiment using KHP as an internal standard?
5. What is the concentration of the malic acid in the apple juice?

4.4: Wet Lab

Wet Lab Procedure:

Part A. Preparation of standard solutions

1. Prepare a solution of the reference standard by weighing dry KHP and dissolving in an appropriate volume of deionized water or D_2O (Your instructor will indicate which solvent should be used in your experiments). Exact knowledge of the mass and volume of the KHP solution is important because the concentration of this solution controls the accuracy of the analysis.
2. Prepare a stock solution of malic acid by weighing dry malic acid and dissolving in an appropriate volume of deionized water or D_2O (Your instructor will indicate which solvent should be used in your experiments). Adjust these stock solutions to around pH 1 with HCl

   Note: if deionized water is used as the solvent you will need to add some D_2O (usually around 10%) as a lock solvent. You also may want to consider using a chemical shift reference standard.

Part B. NMR parameters and quantitative analysis of standard solutions

1. Combine aliquots of the malic acid and KHP solutions you made in part A.
2. Determine and record the values for the each parameter listed below. Be prepared to justify your choice in each case. Obtain an NMR spectrum of the solutions you prepared in part B1.

   Acquisition time =

   Relaxation delay* =

   Pulse width =

   Spectral width =

   Receiver gain =

   Temperature =

   Number of scans =

   * The appropriate value of the relaxation delay can be determined from an inversion recovery experiment. If this is not feasible, your instructor may help you decide on the appropriate value of this parameter.
3. After the experiment is completed, process the spectra using appropriate line broadening, zero filling, phasing, and baseline correction.
4. Assign the KHP and malic acid resonances.
5. Calculate the S/N for this measurement by dividing the integrals measured for resolved malic acid resonances by the rms (root mean square) noise over a region of the spectrum that free of resonances and has a flat baseline.
6. Determine the concentrations of malic acid in your stock solution relative to KHP. How does the concentration you determined by Q-NMR compare with the value you would calculate from the mass and volume used in the preparation of the stock solutions?

Part C. Determination of the malic acid content of unknown fruit juice samples

1. If necessary clarify the juice sample by centrifugation. Prepare a 5-fold dilution of the juice sample with deionized water, adjusting the pH to around 1 with HCl.
2. Prepare at least 3 replicate solutions for quantitative analysis using KHP as an internal standard.
3. Acquire NMR spectra for the juice solutions starting from the optimized parameters used for the standard solutions in part B.
4. Process the spectra and calculate the average concentration of malic acid in the fruit juice. Determine the relative standard deviation of your measurements.

Wet Lab Report:

1. Report the amount of malic acid in your sample along with the relative standard deviation. How does the amount of malic acid you determined in the juice sample compare with the amount reported in the table in the background section of this lab experiment?
2. Using the S/N values calculated for the standard malic acid solution, estimate the limit of quantitation and detection.

3. Is the splitting pattern for any of the resonances of the compounds studied different than what you might predict from the simple rules you learned in organic chemistry? To what do you attribute these differences?

Reference

del Campo, G.; Berregi, I.; Caracena, R.; Santos, J. I. *Analytica Chimica Acta*. **2006**, *556*, 462-468.

CHAPTER OVERVIEW

5: Q-NMR Applications

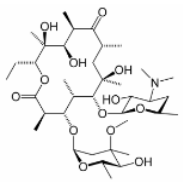
This section presents summaries of several common Q-NMR applications. Q-NMR is widely used for both purity and impurity analyses. Q-NMR finds extensive use in the food and beverage industry, where it is used to detect adulteration and to follow the progression of processes such as fermentation. A relatively recent application of Q-NMR is in the area of metabonomics, which follows relative changes in the level of metabolites in biofluids like urine or plasma, or in tissue biopsies to shed insight into complex biological processes including the effect of genetic variations, disease progression, drug efficacy and the effect of toxicants.

Topic hierarchy

Template:HideTOC

5.1: Q-NMR for purity determination of macrolide antibiotic reference standards- Comparison with the mass balance method

Macrolide antibiotics are derived from microbial fermentations. As a class the macrolide antibiotics contain a 14- or 16-membered macrocyclic lactone, containing amino groups and deoxy sugars. The antibiotic activity of these compounds results from the inhibition of bacterial protein synthesis. Because macrolides accumulate within leukocytes, they are transported to the site of infection. Some macrolides also have immunomodulatory effects that can reduce inflammation. One of the best known macrolide antibiotics is erythromycin, the structure of which is shown here.



This application examines the use of quantitative ^{1}H NMR measurements for determining the purity of macrolide antibiotic reference standards. Samples of clarithrmycin, roxithromycin, azithrimycin, dirithromycin and midecamycin were tested. The Q-NMR experiments were performed at 500 MHz using 1,4-dinitrobenzene as an internal standard. Rather than determining T_1 relaxation times, the relaxation delay (64 s) was selected by comparing the peak area for the internal standard using d_1 delays of 1, 5,10, 20, 32, 64 and 256 s.

The Q-NMR results were compared with the more conventional approach of determining mass balance, shown in Table 1 below. In mass balance determinations, the content is calculated as shown in Equation 5.1.1.

$$\text{Content } \% = (1 - \text{impurity}\%)(1 - \text{water}\% - \text{volatile material } \% - \text{sulfated ash}\%) \times 100 \qquad (5.1.1)$$

In this determination the % impurity is determined by HPLC-UV. Water content is determined by Karl Fischer titration and residual solvents are measured using gas chromatography. The amount of sulfated ash (primarily a European designation) corresponds to the amount of residue remaining after ignition of the sample. One of the drawbacks of HPLC-UV for these measurements is the lack of chromophore with a characteristic absorption wavelength. In addition, a general problem with this approach is that the UV absorption coefficient of each impurity is different, and in many cases unknown.

Purity determinations using NMR rely on the comparison of the integrals measured for analyte's resonances (I_x) with the integral of a quantitation standard (I_{std}). In this case an internal standard was used. Because the masses of the sample (m_x) and the internal standard (m_{std}) are known, the content of the analyte can be determined. Note that the underlying assumption in this method is that resonances of impurities do not overlap with the resonances used to measure the analyte content. The purity of the analyte, Px, is calculated as shown in Equation 5.1.2:

$$P_x = \frac{I_x}{I_{std}} \frac{N_{std}}{N_x} \frac{M_x}{M_{std}} \frac{m_{std}}{m_x} P_{std} \qquad (5.1.2)$$

where M_x and M_{std} are the molar masses of the analyte and the standard, respectively, P_{std} is the purity of the standard, and N_{std} and N_x are the number of spins responsible for the integrated standard and analyte signals, respectively. The content results, summarized in Table 1, for the macrolide antibiotics obtained with ^{1}H NMR and by the mass balance method are in good agreement. The main source of uncertainty in the Q-NMR method was in the sample weight, about 15 mg in these experiments. Use of a larger mass would decrease the uncertainty in the results but would consume larger amounts of deuterated solvents. An informative feature of this report is the detailed error analysis it contains.

Table 1. Results of the Q-NMR and mass balance methods (adapted from Liu and Hu, Anal. Chim. Acta **2007**, *602*, 114-121)

	^{1}H Q-NMR method (%)			Mass balance method (%)				
	Average Content[a]	RSD	$U_{expanded}$	Impurity/u_1	Water/u_2 [b]	Residual Solvents/u_3 [b]	Sulfated ash/u_4 [b]	Content/ $U_{expanded}$
Clarithromycin	96.3	0.49	1.89	3.35/0.0299	1.4/0.0148	<0.001/<8.4 x 10^{-6}	0/0	95.3/2.64

	^{1}H Q-NMR method (%)			Mass balance method (%)				
Roxithromycin	95.7	0.44	1.82	2.50/0.0223	2.2/0.0233	<0.001/<8.4 x 10^{-6}	0/0	95.4/2.64
Azithromycin	94.3	0.50	1.36	1.59/0.0142	4.5/0.0486	<0.001/<8.4 x 10^{-6}	0.02/0.0198	94.0/2.75
Dirithromycin	96.9	0	1.81	3.30/0.0295	0.6/0.00636	<0.001/<8.4 x 10^{-6}	0/0	96.1/2.67
Midecamycin	97.1	2.0	1.96	3.94/0.0352	0.16/0.000035[c]		0/0	95.9/1.71

a. Calculated using 4 NMR signals
b. u_1 is the uncertainty in the impurity, u_2 is the uncertainty in water, u_3 is the uncertainty in residual solvents and u_4 is the uncertainty in the sulfated ash
c. The content of water and residual solvents were not determined for midecamycin. Total volatile materials of midecamycin were determined by the method of loss on drying

Reference

Liu, S.-Y.; Hu, C.-Q. “A comparative uncertainty study of the calibration of macrolide antibiotic reference standards using quantitative nuclear magnetic resonance and mass balance methods” Anal. Chim. Acta 2007, 602, 114-121.

5.2: Determining Enantiomeric or Isomeric Purity of Active Pharmaceutical Ingredients

This document contains two separate applications that discuss the use of quantitative NMR measurements for the analysis of enantiomeric or isomeric purity of pharmaceutical compounds.

Application 1. Q-NMR for Determination of the Enantiomeric Purity of Pharmaceutical Ingredients

Enantiomeric purity (EP) is important in the development of active pharmaceutical ingredients (API) by the pharmaceutical industry. For some drugs, API enantiomers can produce dramatically different pharmacological responses. Enantiomeric purity is commonly determined by effecting a chiral separation however, chiral separations can be time consuming and typically involve the use of expensive chiral columns. Although it is not possible to distinguish enantiomers directly with NMR, derivatization to form diastereomers produces molecules with distinct NMR spectra.[1] Determination of EP by NMR using chiral solvating agents (CSAs) alleviates the need for chemical derivatization or standards. CSAs interact with the enantiomers in solution, in effect forming transient diastereomers. The use of CSAs in NMR was first reported in 1966 by Pirkle.[2] The CSA used in this study was 1,1'–binaphthol shown as compound 1 in Figure 1. This compound is known to resolve chiral amines such as the compounds 2-5 shown below. Some of the chiral compounds in Figure 1 are prescribed clinically to treat such disorders as depression and anxiety (e.g., 2, Zoloft® and 3, Paxil®). Compound 5, fenfluramine, was a component of the anti-obesity drug Fen-Phen, which was withdrawn from the US market after reports linked it to heart damage.

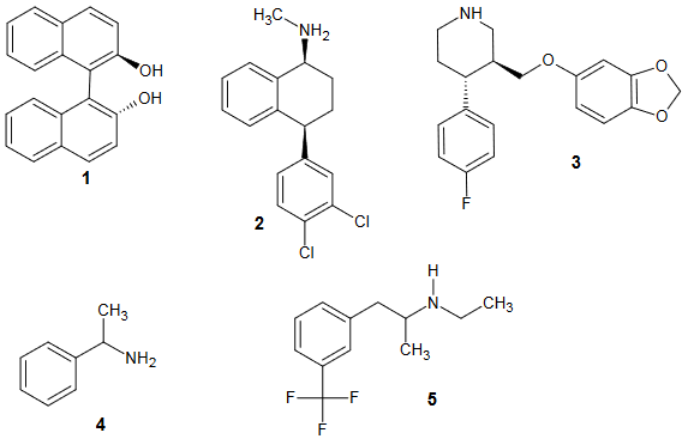


Figure 1. Structures of (1) the chiral solvating agent (R)-1,1'-bi-2-naphthol, (2) (+)-sertraline HCl, (3) (-)-paroxetine HCl, (4) racemic methylbenzylamine, and (5) racemic fenfluramine HCl.

Enantiomeric separation by NMR is based on the intrinsic differences in the diastereomeric complexes formed and/or differences in the association kinetics of the equilibria below:

$$\mathrm{E} + \mathrm{S} \rightleftharpoons \mathrm{ES} \tag{5.2.1}$$

$$\mathrm{E}' + \mathrm{S} \rightleftharpoons \mathrm{E}'\mathrm{S} \tag{5.2.2}$$

where S represents the CSA molecule while E and E' represent the different solute enantiomers.[3]

In experiments by Salsbury et al. to determine enantiomeric purity of the APIs, fenfluramine, sertaline and paroxetine, and the model compound, methylbenzylamine (MBA, compound 4 in Figure 1), the analytes were dissolved in $CDCl_3$ and chemical shifts referenced to tetramethylsilane (TMS).[1] The ^1H NMR spectra used to determine the limits of detection and quantitation were measured with 64 transients, tip angle of 30°, relaxation delay of 1s and line broadening of 0.3 Hz. Proton chemical shift assignments were confirmed using COSY. Standards were weighed (1-4 mg) and $CDCl_3$ solutions containing an appropriate molar ratio of the analyte and 1,1'–binaphthol were prepared. The ability of the CSA interactions to resolve the mixtures of enantiomers was evaluated using MBA mixtures. Using standards of methylbenzylamine at different concentrations to obtain a calibration curve, the limit of quantitation was determined to be below 1% of the minor component. Analysis of racemic fenfluramine revealed that it contained 50.2 ± 0.4% the S-enantiomer. Although chiral HPLC could not be performed for fenfluramine or sertraline

without derivatization, the analysis of paroxetine enantiomers was carried out by both NMR and HPLC yielding results of 7.5 ± 0.3% and 8.5%, respectively.

References

1. Salsbury, J. S. and Isbester, P. K. *Magn. Reson. Chem.* **2005**, *43*, 910-917
2. Pirkle, W. H. *J. Am. Chem. Soc.* **1966**, *88*, 1837
3. Pirkle, W. H. and Hoover, D. J. *Top. Sterochem.* **1982**, *13*, 263

Application 2. Q-NMR for the Quantitation of the E/Z Isomer content of Fluvoxamine

Fluvoxamine is an antidepressant with two possible isomeric structures as shown in Figure 1 below. The activity of fluvoxamine resides in the E-isomer (Figure 1A). However, the Z-isomer (Figure 1B) occurs in all the synthesis pathways. Transport proteins can discriminate between the E- and Z-isomers. The British Pharmacopeia limits the content of Z-isomer to 0.5%. The QNMR method described measures the Z-isomer to the 0.2% level in 15 mg of the drug substance.

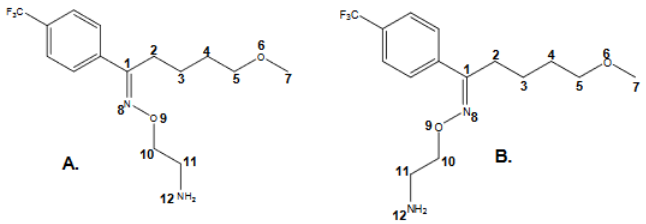


Figure 1. Structures of (A) (*E*)-fluvoxamine and (B) (*Z*)-fluvoxamine. The pharmaceutical formulation is available as the maleate salt of fluvoxamine. The numbering of the atoms correlates with the NMR spectrum reported in the reference.

An advantage of Q-NMR for determining the Z-isomer content is the minimum sample preparation required. In this example, 15 mg of material was dissolved in deuterated methanol which was also used as the ^{1}H (3.31 ppm) chemical shift reference.[1] ^{1}H NMR spectra were acquired by coaddition of 128 transients over a spectral width of 4595 Hz. FIDs were apodized by multiplication with an exponential function equivalent to 0.3 Hz line broadening. For quantitation, the C-2 proton resonances of the Z- (2.62 ppm) and E- (2.90 ppm) fluvoxamine isomers were manually integrated and the values compared.

Before performing quantitative measurements, it was necessary to determine the limits of quantification and detection for each isomer. Although the pure E-isomer was commercially available, the pure Z-isomer was not. Instead the authors had access only to a 1:1 (E/Z) mixture. Therefore a stock solution containing 5.13% (Z) fluvoxamine was prepared by mixing appropriate amounts of the pure E and E/Z mixture. Serial dilutions were made from this stock solution for NMR analysis. With each dilution, the concentrations of the E- and Z-isomers decreased, but the %Z content remained at 5.13%. For the spectrum measured at each concentration, manual integration of the C-2(Z) and C-2(E) proton resonances was performed three times. The difference between calculated and determined values of the Z-isomer content was less than 5% at concentrations down to 0.07 mg/L. Greater deviation from calculated values was observed at lower Z-isomer concentrations. Based on these experiments, the limits of quantitation and detection were determined to be 0.07 mg/L and 0.018 mg/L, respectively. To determine linearity, a set of mixtures containing 0-10% Z-isomer were measured in triplicate. The correlation coefficient of the calibration plot was found to be 0.9999 with a coefficient slope of 0.9923. Since the British Pharmacopeia tests requires the content of Z to be less than 0.5%, solutions were prepared containing 0.15-1.01% of the Z-isomer. NMR spectra were measured in triplicate and each spectrum integrated three times. Linear regression analysis yielded a correlation coefficient of 0.994 with a slope of 1.042, indicating that the Q-NMR assay is linear over this concentration. The Q-NMR method was found to be an accurate, sensitive, and timesaving method for the determination of Z- fluvoxamine content.

Reference

1. Deubner, R. and Holzgrabe, U. *Magn. Reson. Chem.* **2002**, *40*, 762-766

5.3: Q-NMR for Analysis and Characterization in Vaccine Preparations

PedvaxHIB is a vaccine that is made by chemically conjugating the capsular polysaccharide of *Haemophilius influenzae type b* (Hib) to an outer membrane protein from *Neisseria meningitidis* to form a protein-conjugated vaccine that is very effective in preventing invasive Hib infection in infants and young children. This example shows the utility of NMR for both the characterization of the derivatized polysaccharide and its quantitative analysis. The advantages of NMR include its nondestructive nature and its ability to detect molecules that do not contain a UV-visible chromophore.

To obtain an accurate determination of the solution temperature, a linear calibration of the HDO chemical shift was carried out. Shimming of the magnet was performed using the DMSO peak and the proton 90° pulse was calibrated for each solution to compensate for differences in solution ionic strength. The DMSO was also used as the internal reference. The spectral width was 10.5 ppm with a digital resolution of 0.3 Hz. Spectra were measured with 16 transients with a total recycle time of 60 s. The total data acquisition time was 20 min.

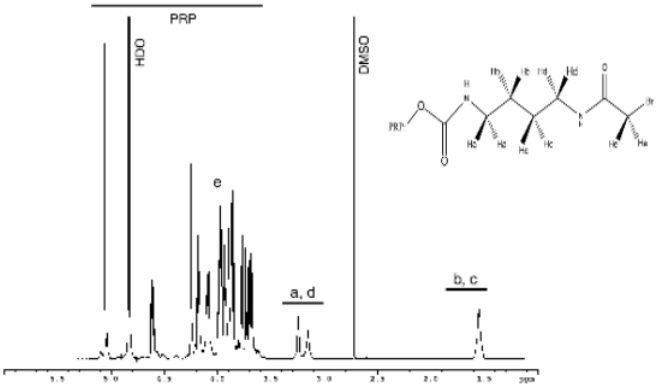


Figure 1. ^{1}H NMR spectrum of the derivatized capsular polysaccharide. Inset is the sidechain structure of the PRP dervativzed with butanediamine bromoacetyl chloride (PRPBuA2). Reprinted from Anal. Biochem. 337, Q. Xu, J. Klees, J. Teyral, R. Capen, M. Huang, A. W. Sturgess, J. P. Hennessey, Jr., M. Washabaugh, R. Sitrin, C. Abegunawardana, Quantitative nuclear magnetic resonance analysis and characterization of the derivatized *Haemophilus influenzae* type b polysaccharide intermediate for PedvaxHIB, 234-245, Copyright (2005), with permission from Elsevier.

Figure 1 shows the ^{1}H NMR spectrum of an intermediate in the synthesis of the capsular polyribosylribitol phosphate (PRP) – outer membrane protein complex. PRP is first activated with 1,1'=carbonyldiimidazole and then reacted with an excess of butanediamine. The resonances of the derivatized PRP are well resolved in this spectrum. Quantitation was performed using an internal reference because it alleviates the need for a standard calibration curve. In determining the percentage of the various forms, neither the molecular weight of the polysaccharide nor the degree of polymerization was needed for calculation. The Q-NMR assay developed in this paper can find application in product release or process monitoring in the pharmaceutical industry and can potentially replace tedious chromatographic and colorimetric methods.

Reference

Xu, Q.; Klees, J.; Teyral, J.; Capen, R.; Huang, M.; Sturgess, A. W.; Hennessy, J.P.; Washabaugh, M.; Sitrin, R.; Abeygunawardana, C. *Anal. Biochem.* **2005**, *337*, 235-245

5.4: Q-NMR-Based Metabonomics of Blood Samples

Cyclosporine A (CsA) is a potent calcineurin inhibitor used in transplantation medicine. It is potentially nephrotoxic. The use of CsA in combination with other immunosuppresants, such as sirolimus (SRL) or everolimus (RAD), has been reported to produce a beneficial synergistic effect. In this study, blood samples were collected from rats treated with CsA (10 mg/kg), CsA(10 mg/kg) + SRL(3 mg/kg), and CsA(10 mg/kg) + RAD (3mg/kg). Whole blood samples were collected and processed by dual chloroform/methanol extraction to yield water- and lipidsoluble extracts. The extracts were analyzed by Q-NMR to predict the metabolic toxicity, and to identify and quantify metabolic biomarkers. A 600 MHz NMR spectrometer was used and the proton NMR spectra obtained by coaddition of 40 transients, using a relaxation delay of 12 s and a tip angle of 90°. The water resonance was suppressed by selective saturation. A solution of trimethylsilylpropionic acid-d_4 (TMSP-d_4, 0.00 ppm) was placed in a capillary and inserted into the NMR tube for use as an external standard. All spectra were normalized to the intensity of the TMSP-d_4 singlet resonance.

To obtain accurate and meaningful information, NMR spectral data must be carefully processed prior to attempting statistical analysis of the results. In this experiment, Fourier transformation, phase correction and baseline correction were performed. For multivariate statistical analysis, all spectra were normalized to the TSP-d_4 intensity and the full spectrum was bucketed into 0.04 ppm intervals, except the regions containing the solvent resonances of water and methanol which were excluded from the statistical analysis. Principal component analysis (PCA) was performed using the AMIX 3.1 software to classify the NMR spectra obtained from animals subjected to different experimental treatments. For metabolite quantification, each ^{1}H peak of identified metabolites was integrated. Absolute concentrations of the identified metabolites were calculated using the equation shown below:

$$C_x = \frac{\frac{I_x}{N_x} \times C_{TMSP}}{\frac{I_{TMSP}}{N_{TMSP}}} \times \frac{V}{M} \tag{5.4.1}$$

where C_x = metabolite concentration; I_x = integral of the metabolite ^{1}H NMR resonance; N_x = number of protons giving rise to the metabolite ^{1}H peak (from CH, CH_2, CH_3, etc); C_{TMSP} = TMSP concentration; I_{TMSP} = integral of TMSP ^{1}H resonance at 0 ppm; N_{TMSP} = 9 because this resonance is produced by the 9 equivalent protons of the 3 methyl groups; V = volume of the extract; and M = volume of the blood sample.

In the results obtained by PCA from the PC1 vs PC2 scores plot, spectral results for all 5 placebo-treated control animals clustered together and were overlapped with the samples for the CsA+RAD treated animals. The results for the CsA treated animals were well-separated from the control animals. The spectral results that were most different from the controls were those of the CSA+SRL treated animals, which clustered as their own group distinct from the CsA-only treated animals. The PCA loadings plot indicated that the intensities of the following metabolites differed among the CsA+SRL, CsA, and placebo groups: hydroxybutyrate, lactate, total glutathione, creatine + creatinine, trimethylamine-N-oxide (TMAO), and glucose. The only metabolite that increased in all 3 treatment groups was cholesterol. QNMR measurements of individual metabolite levels showed that CsA administration significantly increased the blood concentrations of glucose, hydroxybutyrate and creatine + creatinine. However the levels of glutathione dropped in both CsA and CsA+SRL treated animals. The blood levels of these metabolites were not significantly different for the CsA+NAD treated animals and the placebotreated controls.

The increase in blood glucose and hydroxybutyrate confirmed the ability of CS to induce hyperglycemia and hyperketonemia. The decreased levels of glutathione were thought to be related to CS-induced oxidative stress. The increased concentrations of metabolites such as creatine and creatinine could reflect decreased renal clearance of these substances. While coadministration with SRL enhanced the metabolic changes indicative of toxicity, combination treatment with RAD partially alleviated these effects. This example illustrates the utility of metabolic profiling by Q-NMR and the need to monitor the toxicodynamic effects of immunosuppressant combinations.

Reference

Serkova, N. J. and Christians, U. *Ther. Drug. Monit.* **2005**, *27*, 733-737

5.5: Q-NMR for Time Course Evolution of Malic and Lactic Acid

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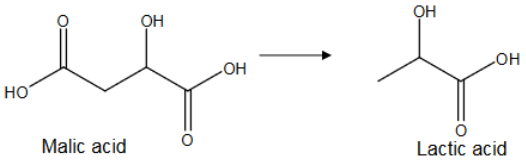


Figure 1. Structures of malic acid and lactic acid. Conversion of malic acid to lactic acid can take place during malolactic or alcoholic fermentation.

Wines consist of various components present at different concentrations. The major components are ethanol, water, glycerol, sugars and organic acids such as malic and lactic acid shown in the figure above. Low levels of malic acid (0.4-0.5 g/L) are a prerequisite for the commercial production of some red wines. In addition, the regulation of this acid is essential in the elaboration of other types of wines such as white and rosé. The levels of malic acid can be controlled by allowing the spontaneous growth of lactic acid bacteria which carry out the malolactic fermentation as shown in Figure 1 above. Q-NMR is useful in evaluating wine quality with respect to age, origin, and effects of adulteration. Control of the fermentation process is essential in determining the desired wine quality. This example illustrates the use of Q-NMR to monitor the fermentation process by measuring levels of malic and lactic acid over the concentration range of 1-3.2 mM. The effectiveness of Q-NMR analysis is compared with the results of enzymatic measurements.

In this experiment, wine samples were collected from various tanks containing grapes of different varietals. Samples were collected directly and preserved at -25 °C. Prior to recording NMR spectra, sample pH was adjusted to 3.0. Succinic acid was used as an external standard. Matrix effects were evaluated by spiking the wine with malic and lactic acid. NMR spectra were obtained by coaddition of 128 transients using a 90° pulse, a spectral width of 10 ppm and a relaxation delay of 60 s. The water resonance was suppressed by irradiation with a selective presaturation pulse.

In order to evaluate the effectiveness of Q-NMR for this analysis, the results were compared with those obtained from an enzymatic assay which consumes malate and produces NADH (Boehringer test). In this test the UV absorption of NADH at 340 nm is used for quantitation. The results of the enzymatic assay were in good agreement with the corresponding measurements by Q-NMR. The major advantages of Q-NMR in this example include minimal sample preparation and rapid analysis.

Reference

Avenoza, A.; Busto, J. H.; Canal, N.; Peregrina, *J. M. J. Agric. Food Chem.* **2006**, *54*, 4715-4720

CHAPTER OVERVIEW

6: Instructor's Guide

This guide is intended to assist instructors in the utilization of the q-NMR learning module. While the module is designed as a stand-alone resource to accommodate self-learners, it can also be used as an active learning resource. This instructor's guide is designed to help.

Topic hierarchy

Template:HideTOC

6.1: Basic Theory Concept Questions

The questions below are also listed on the webpage that links to the Basic Theory section. These questions can be handed out in class or given as a homework assignment. Students should be able to answer these questions using the Basic Theory section of this module as an instructional resource. I would also recommend assigning the excellent web resource created by Joe Hornak, since it contains embedded animations that help clarify many difficult to understand processes in NMR. This site can be accessed at http://www.cis.rit.edu/htbooks/nmr/.

- What is spin?
- How does absorption of energy generate an NMR spectrum?
- Why is NMR less sensitive than UV-visible spectroscopy?
- What is chemical shift and how does it relate to resonance frequency?
- What is precession?
- How does precession produce the macroscopic magnetization (Mo)?
- How can the nuclear spins be manipulated to generate the NMR spectrum?
- What is the tip angle?
- What is a Free Induction Decay?
- How do T_1 and T_2 relaxation affect NMR spectra?

6.2: Answers to Questions in the Basic Theory section

In addition to the conceptual questions, the Basic Theory section also contains a series of simple quantitative questions, the answers of which are provided below.

Question 1

How many spin states would you predict for ^{2}H?

Solution

Deuterium has a spin of 1. Therefore there should be 3 possible spin states: +1, 0 and -1.

Question 2

Given the same magnetic field and temperature, how would the difference in population for ^{1}H and ^{31}P compare?

Solution

For this problem we will use the following equation:

$$\frac{N_{upper}}{N_{lower}} = e^{\frac{-\Delta E}{kT}} \tag{6.2.1}$$

The difference in population for ^{1}H and ^{31}P will be related to the differences in their ΔE values. Since $\Delta E=\gamma h B_o/2\pi$, for a fixed magnetic field the only differences between ^{1}H and ^{31}P is in their magnetogyric ratios.

$$\frac{\Delta E(^{1}H)}{\Delta E(^{31}P)} = \frac{26.752}{10.84} = 2.468 \tag{6.2.2}$$

The ratio of the N_{upper}/N_{lower} for ^{1}H is $e^{2.468}$ or =11.80 times larger than the ratio of N_{upper}/N_{lower} for ^{31}P.

Question 3

Calculate the wavelength of electromagnetic radiation corresponding to a frequency of 500 MHz.

Solution

The wavelength of electromagnetic radiation corresponding to a frequency of 500 MHz is 0.6 m.

Question 4

What range of frequencies would be excited by a 10 μs rf pulse?

Solution

A 10 μs rf pulse would excite a range of frequencies covering 100,000 Hz.

Question 5

What are the resonance line widths of nuclei that have apparent T_2 relaxation times (i.e.T_2* values) of 1 and 2 sec.

Solution

$$w_{\frac{1}{2}} = \frac{1}{\pi T_2^*} \tag{6.2.3}$$

Therefore, the two resonances have line widths of 0.32 and 0.16 Hz.

6.3: Practical Aspects Concept Questions

The questions below are also listed on the webpage that links to the Practical Aspects section. These questions can be handed out in class or given as a homework assignment. Students should be able to answer these questions using the Practical Aspects section of this module as an instructional resource.

- How do I choose a reference standard for my Q-NMR analysis?
- How is the internal standard used to quantify the concentration of my analyte?
- What sample considerations are important in Q-NMR analysis?
- How do I choose the right acquisition parameters for a quantitative NMR measurement?
- What data processing considerations are important for obtaining accurate and precise results?

6.4: Answers to Questions in the Practical Aspects section

Question 1

A quantitative NMR experiment is performed to quantify the amount of isopropyl alcohol in a D_2O solution. Sodium maleate (0.01021 M) is used as an internal standard. The integral obtained for the maleate resonance is 46.978. The isopropanol doublet at 1.45 ppm produces an integral of 104.43. What would you predict for the integral of the isopropanol CH resonance as 3.99 ppm. What is the concentration of isopropanol in this solution?

Solution

The isopropanol CH resonance is produced by a single proton whereas the doublet is produced by the 6 methyl protons. Therefore, the CH integral should be 1/6th that of the methyl doublet, or 17.405.

To find the isopropanol concentration we first have to calculate normalized areas for isopropanol and our standard, maleate. The isopropanol(IP) doublet is comprised of 6 protons due to the two equivalent methyl groups of this compound.

$$\text{Normalized Area (IP)} = \frac{104.43}{6} = 17.405 \tag{6.4.1}$$

Similarly, the normalized area for maleate (MA) is:

$$\text{Normalized Area (MA)} = \frac{46.978}{2} = 23.489 \tag{6.4.2}$$

The concentration of the isopropanol can be calculated using the known the maleate concentration.

$$[\text{IP}] = \frac{[\text{MA}] \times \text{Normalized Area (IP)}}{\text{Normalized Area (MA)}} \tag{6.4.3}$$

$$[\text{IP}] = \frac{0.01021\ \text{M} \times 17.405}{23.489} = 0.007565\ \text{M} \tag{6.4.4}$$

Because the accuracy of the determination depends on how well the maleate concentration is known, the standard solution should be prepared with care, using dried sodium maleate of high purity, weighing carefully a mass that is known to an appropriate number of significant figures (in this case 4), transferring the maleate quantitatively to a volumetric flask and finally dilution to the mark. Again, an appropriate solution volume must be selected to produce the desired number of significant figures given the manufacturer specifications for the glassware used.

Question 2

A solution prepared for quantitative analysis using NMR was acquired by coaddition of 8 FIDs produces a spectrum with an S/N of 62.5 for the analyte signals. How many FIDs would have to be coadded to produce a spectrum with an S/N of 250?

Solution

S/N increases in NMR experiments as the square root of the number of scans coadded.

$$\text{S/N} \propto (\text{n})^{0.5} \tag{6.4.5}$$

To increase the S/N from 62.5 to 250 (a factor of 4 increase in S/N) would require coaddition of 16 times as many FIDs as was used to produce a spectrum with S/N of 62.5. The answer is that coaddition of 128 FIDs (8 x 16) would be required to achieve an S/N of 250.

Question 3

A ^{1}H NMR spectrum was measured using a 400.0 MHz instrument by acquisition of 8192 total data points (8192 real points) and a spectral width of 12.00 ppm. What was the acquisition time? Calculate the digital resolution of the resulting spectrum? Is this digital resolution sufficient to accurately define a peak with a width at half height of 0.5 Hz?

Solution

We can calculate the acquisition time knowing the spectral width and the total number of data points.

$$\mathrm{AT} = \frac{\mathrm{NP}}{2\ \mathrm{SW}} = \frac{16384}{2 \times 400 \times 12} = 1.707\ \mathrm{sec} \tag{6.4.6}$$

$$\mathrm{DR} = \frac{\mathrm{SW}}{\mathrm{NP(real)}} = \frac{2 \times 400 \times 12}{8192} = 1.172\ \mathrm{Hz/pt} \tag{6.4.7}$$

This would not be adequate digital resolution to accurately define a peak with a 0.5 Hz width at half height. A longer acquisition time would allow for collection of more points. Also, zero-filling could also be used to help increase the digital resolution.

6.5: Q-NMR Drylab

KHP T_1 relaxation times

There are two ways of getting the T_1 relaxation times for the KHP resonances. The simplest way is to have students estimate the null times for the two resonances in the inversion-recovery spectra provided. Selected spectra from the dry lab data set were used to make the figure in the Practical Aspects section of the module. Alternatively, students can process the spectra and measure resonance integrals for each peak. These can be plotted vs. the relaxation delay and fit to determine T_1. The integrals we obtained are summarized in the Table below. The fits obtained using Origin 7.5 are also provided. We obtained T_1 values of 4.79s and 3.11s for the KHP resonances at 7.75 and 7.61 ppm, respectively.

The concentration of KHP in the stock solution is determined from the mass of KHP.

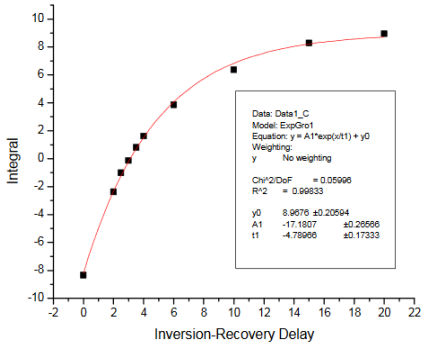
$$\text{Mass KHP} = 0.3533\ \text{g} - 0.2219\ \text{g} = 0.1314\ \text{g} \tag{6.5.1}$$

$$[\text{KHP}] = \frac{0.1314\ \text{g}}{204.22\ \text{g/mol}} \times \frac{1}{0.005\ \text{L}} = 0.1287\ \text{M} \tag{6.5.2}$$

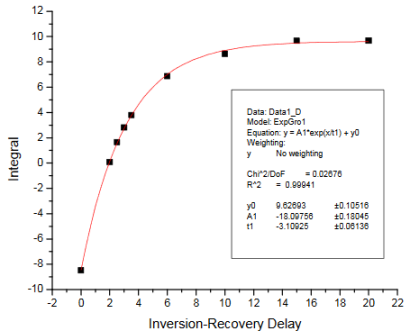
Table 1. Inversion-recovery data for KHP

Delay	Integral R1 (7.75 ppm)	Integral R2 (7.61 ppm)
0 s	-8.34	-8.48
2	-2.38	0.07
2.5	-1.01	1.65
3	-0.14	2.81
3.5	0.8	3.78
4	1.62	4.57
6	3.87	6.86
10	6.37	8.63
15	8.28	9.67
20	8.95	9.65

Inversion-recovery plot for R_1



Inversion-recovery plot for R_2



Malic Acid Standard Solution Determination

From the mass of malic acid weighed and the solution volume, we can calculate the concentration of this standard.

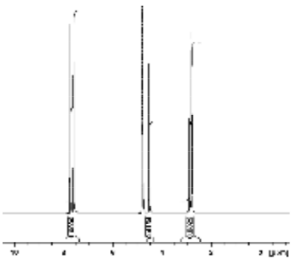
$$\text{Mass of MA} = 0.3324\ \text{g} - 0.1897\ \text{g} = 0.1427\ \text{g} \tag{6.5.3}$$

$$[\mathrm{MA}] = \frac{0.1427\ \mathrm{g}}{134.09\ \mathrm{g/mol}} \times \frac{1}{0.005\ \mathrm{L}} = 0.2128\ \mathrm{M} \tag{6.5.4}$$

Since equal volumes of the KHP and malic acid were mixed to prepare this solution, the dilution factor can be neglected and the concentration of malic acid calculated as shown below. Here we used the integral of the resonances at 2.82 and 2.89 ppm corresponding to the inequivalent malic acid CH_2 protons.

$$[\mathrm{MA}] = [\mathrm{KHP}] \times \frac{\mathrm{Int_{MA}}}{\mathrm{N_{MA}}} \times \frac{\mathrm{N_{KHP}}}{\mathrm{Int_{KHP}}} = 0.1287\ \mathrm{M} \times \frac{0.8322}{2} \times \frac{4}{1.000} = 0.2142\ \mathrm{M} \tag{6.5.5}$$

Spectrum of malic acid standard solution containing KHP



Determination of Malic Acid in Apple Juice

The concentration of malic acid can similarly be calculated in an apple juice sample. Here though we will need to take into account the dilutions performed.

The KHP was diluted twice in preparing this solution. First it was diluted by half with D_2O, and subsequently 100 µL of was further diluted by addition to 900 µL of apple juice for a total volume of 1 mL. The KHP concentration in the apple juice sample can be calculated as shown below. Note that the volume added in making the pH adjustment to 1.35 is not important since both the KHP and the malic acid will be diluted by the same amount. This pH adjustment is necessary to resolve the malic acid resonances from those of the other apple juice components. Because of the simplicity of the malic acid standard spectrum, pH adjustment is not needed.

$$[\mathrm{KHP}]_{\mathrm{juice}} = [\mathrm{KHP}]_{\mathrm{stock}} \times \frac{1}{2} \times \frac{100}{1000} = 0.1287\ \mathrm{M} \times \frac{1}{2} \times \frac{100}{1000} = 0.00643\ \mathrm{M} \tag{6.5.6}$$

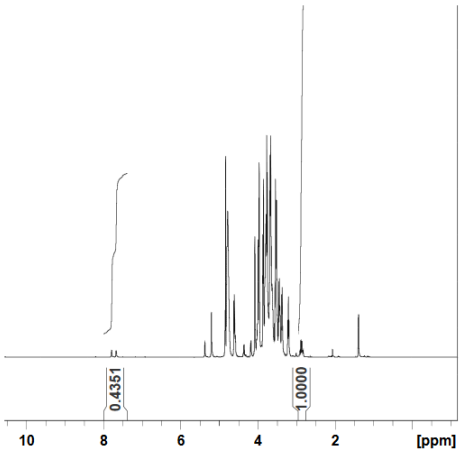
The malic acid concentration can then be calculated as before, providing that the dilution resulting from addition of the KHP solution is included in the calculation.

$$[\mathrm{MA}]_{\mathrm{juice}} = [\mathrm{KHP}]_{\mathrm{juice}} \times \frac{\mathrm{Int_{MA}}}{\mathrm{N_{MA}}} \times \frac{\mathrm{N_{KHP}}}{\mathrm{Int_{KHP}}} \times \frac{1000}{900} = 0.00643\ \mathrm{M} \times \frac{1.000}{2} \times \frac{4}{0.4351} \times \frac{1000}{900} = 0.0328\ \mathrm{M} \tag{6.5.7}$$

For purposes of comparison with the table provided in the background section of this laboratory, we can convert this concentration to g/L using the molecular weight of malic acid.

$$0.0.328\ \mathrm{M} \times 134.09\ \mathrm{g/mol} = 4.40\ \mathrm{g/L\ malic\ acid} \tag{6.5.8}$$

Spectrum of apple juice sample containing KHP



Index

Advanced Theory

The discussion presented in this module was intended to introduce NMR at an introductory level with a focus on its application for quantitative measurements. Students interested in a deeper treatment of NMR theory are encouraged to review the resources below:

Web Resources

- Fundamental's of NMR by Tom James
- Angel de Dios' Graduate NMR Course
- Introduction to 2D NMR
- James Keeler's Lectures on NMR

Books

- *Understanding NMR Spectroscopy* by James Keeler, Wiley (2005).
- *High-Resolution NMR Techniques in Organic Chemistry* by Timothy Claridge, Elsevier (1999).
- *Spin Choreography: Basic Steps in High Resolution NMR* by Ray Freeman, Oxford University Press (1999).
- "Modern NMR Spectroscopy: A Guide for Chemists", 2nd Edition, by Jeremy K. M. Sanders and Brian K. Hunter, Oxford University Press, 1993.

In addition to the additional references in the Advanced NMR Theory section, the resources listed below provide additional information on quantitative NMR measurements.

Web Resources

- The qNMR Portal

Selected Q-NMR Papers

- L. Orfi, C. K. Larive, D. Jayawickrama, L. Orfi "Quantitative Analysis of Peptides with NMR Spectroscopy" Appl. Spectrosc. (1997) 51:1531-1536.
- D.A. Jayawickrama, C.K. Larive "Analysis of the Trimethylsilylpropionic Acid -β (12-28) Peptide Binding Equilibrium with NMR Spectroscopy" Anal. Chem. (1999) 71:2117-2112.
- G. F. Pauli, B. U. Jaki, D. C. Lankin "A Routine Experimental Protocol for qHNMR Illustrated with Taxol" J. Nat. Prod. (2007)70:589-595.

Books

D. L. Rabenstein and D. A. Keire "Quantitative Chemical Analysis by NMR" in *Modem NMR Techniques and their Application in Chemistry* by A. I. Popov and K. Hallenga, Practical Spectroscopy Series, Vol. 11, Marcel Dekker (1990) ISBN 0824783328

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    - 1.11: Where should I look to learn more about NMR? - *CC BY-NC-SA 2.5*
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    - 2.5: Effects of Tip Angle in Quantitative NMR Experiments - *CC BY-NC-SA 2.5*
    - 2.6: What data processing considerations are important for obtaining accurate and precise results? - *CC BY-NC-SA 2.5*
    - 2.7: References - *CC BY-NC-SA 2.5*
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    - 3.1: Virtual Laboratory - *CC BY-NC-SA 2.5*
  - 4: Q-NMR Experiment - *CC BY-NC-SA 2.5*
    - 4.1: Prelab Exercises - *CC BY-NC-SA 2.5*
    - 4.2: Background - *CC BY-NC-SA 2.5*
    - 4.3: Dry Lab - *CC BY-NC-SA 2.5*
    - 4.4: Wet Lab - *CC BY-NC-SA 2.5*
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