EDULAB FOR INSTRUCTORS: FOURIER 80

Milky Way to NMR

NMR Analysis of Milk

Authors & Affiliation:

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Experiment Hashtag #: #milkywaytoNMR #educate2resonate

Keywords:

Compound identification, NOESY, solvent suppression

Target group:

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

Objectives:

NMR has proven to be an extremely powerful and versatile tool for the analysis of food. In this exercise, the students will learn what information can be extracted from milk samples using NMR. The goal of this exercise is to teach the students how to measure the fat content and detect the presence of lactose from ^{1}H 1D NMR spectra of milk. In addition, the students will learn how to run 1D-NOESY-presat experiments, which provide effective suppression of the solvent peak when other solvent suppression methods (such as the ones that rely simply on presaturation) do not provide satisfactory results.

Background of the Experiment:

Milk is a common part of our diets, especially in western countries, as it is rich in nutrients. Humans consume mostly milk produced by cattle (which contributes to more than 80% of the global milk production), followed by buffalo, goats, sheep, and camels.

Milk is a complex, multi-phasic colloidal suspension whose chemical composition is influenced by the type of milk, as well as by the specific source (e.g., age, genetics, stage of lactation, nutrition, and health status of the animal). In general, it is composed mostly of water, carbohydrates, fats, proteins, and minerals, and it contains traces of vitamins and phospholipids. The nutritional importance of milk mostly lies in the fact that it is an important source of essential amino acids, as well as of minerals (such as calcium and magnesium) and vitamins (B5 and B12 for example). The carbohydrates are predominately present in the form of lactose, a disaccharide made of galactose and glucose subunits. Lactose requires the lactase enzyme to be metabolized, and the enzyme's absence (or low level) is the main reason behind lactose intolerance.

The hundreds of millions of tons of milk produced globally every year are either consumed raw or processed into a variety of dairy products. Either way, the quality standards and regulations behind these products require accurate chemical analysis of the milk composition and properties. Starting from its very first application for the analysis of milk in 1950, NMR has emerged as a robust and powerful technique to study and analyze milk, and is still today routinely used along with other analytical techniques such as FT-IR. Thanks to its versatility, NMR can be used to extract a variety of information about the milk, ranging from a qualitative and quantitative analysis of milk composition to studies on conformational and aggregation states of milk proteins.

The acquisition of a standard ^{1}H 1D NMR experiment for milk yields little information due to the presence of a large, overwhelming water signal. To obtain more information, it is necessary to acquire an experiment with suppression of the water peak. Typically, solvent-suppressed datasets can be acquired by adding a long, low-power radio frequency pulse (presaturation) which saturates the solvent and nearly suppresses the peak (Pyridine [37], of version 001 Fourier EduLab Students Guide, which is provided on a USB stick along with the Fourier 80). This, however, is not the case for our milk samples. Here the amount of water is very large with respect to the compounds of interest, and this simple method of solvent suppression techniques that rely only on presaturation do not yield satisfactory signal cancellation. We use instead the 1D-NOESY-presat pulse sequence, which is often implemented for water-rich samples as it gives a good balance between suppression performance and challenges in the optimization/implementation.

The 1D-NOESY-presat pulse scheme is shown in the figure below. It starts with a long, low-power pulse during the recycle delay that saturates the water signal. Then, the magnetization is inverted by the application of two 90-degree pulses, the system evolves for a specific delay (called mixing) during which a second saturation pulse is applied, and finally a last 90-degree pulse is applied followed by the acquisition of the FID. The extra saturation during the mixing time serves as an additional filter and suppresses water that is on the edges of the NMR coil, where the homogeneity of the radio frequency pulses is not as good as in in the center of the NMR coil. The mixing time should be set such that the second saturation pulse is as close as possible to a 90-degree pulse (which can be achieved either by changing the power level, as we will do here, or the pulse length). The phase cycling used allows good suppression of the solvent peak.

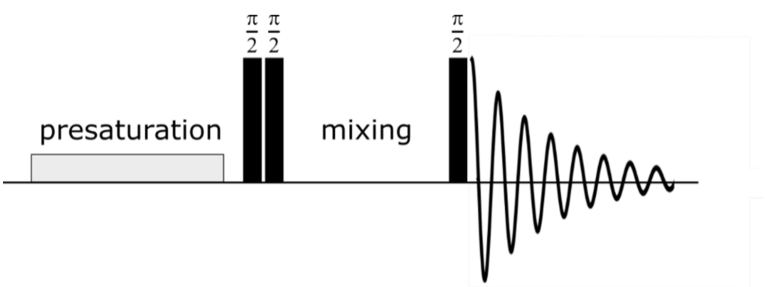

Figure 1 1D NOESY-presat Pulse Scheme

Glossary

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

Quantitation: NMR is quantitative by nature because the intensity of the signal is directly proportional to the concentration of the molecule that produces the signal. Quantitation is the process of measuring the intensity of a NMR signal and calculating the concentration of the molecule that produces the signal.

Water Suppression: A technique used in NMR to minimize the usually strong signal deriving from water. This is important because the water signal can interfere with the signal from other molecules you are interested in investigating, resulting in information loss.

Colloidal suspension: a mixture of usually two materials in which one is microscopically dispersed in, but not chemically bound to the other.

FT-IR: Fourier Transform Infrared Spectroscopy uses infrared light to analyze the chemical composition of materials by measuring the absorption and transmission of infrared radiation.

FID: Degradation of the magnetization detectable in magnetic resonance after an excitation pulse.

The 1D NOESY-presat spectra provide important information about the milk composition. The much better spectral quality obtained by suppressing the water peak allows us to detect and characterize a higher number of compounds compared to simple 1D experiments. In particular, for this exercise we are interested in identifying lactose.

To make milk drinkable for lactose-intolerant people, lactose-free milks are produced. These products are processed in such a way that the lactose molecules are converted into glucose and galactose (analogously to what the lactase enzyme does). NMR is a perfect technique to detect residual amounts of lactose. As shown in the figure below, the 1D NOESY-presat spectra of milk samples acquired at 400 MHz show clear fingerprints for the milk containing lactose (in green in the figure) and ones where the lactose was converted into glucose and galactose (in blue in the figure).

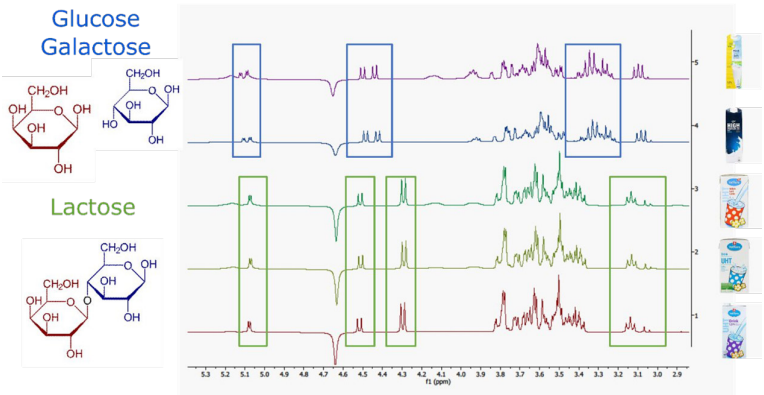

Figure 2 1D NOESY-presat NMR Spectra of Milk Samples Acquired at 400 MHz

In this exercise, we will use NMR to analyze milk properties. We will acquire ^{1}H 1D NMR spectra of different types of milk commercially available and use them to quantify the fat content, as well as to qualitatively detect the presence of lactose.

Preparation and Prerequisite:

The main objective of this investigation is to illustrate key NMR concepts based on lactose-free milk versus normal milk. To ensure efficient completion of the experiments, it is recommended to form groups with a maximum of 3-4 students.

The experiments are expected to take around 3-4 hours to perform. After completing the experiments, an additional 2-3 hours will be needed to process and interpret the data and write a report. It is assumed that students have already covered introductory concepts of 1D NMR and have a basic understanding of spectral interpretation.

For comprehensive information on these key NMR concepts, students can refer to the version 001 Fourier EduLab Students Guide, which is provided on a USB stick along with the Fourier 80. Additionally, it is important for students to have a tutorial on how to use processing software like MestreNova.

Instructors are strongly advised to set up the experimental templates in advance, as this exercise focuses on fundamental acquisition, processing, and data analysis skills.

To perform this experiment, a properly installed and adjusted Fourier 80 system with TopSpin software is required. In addition, pipettes should be available.

Experimental Setup:

Materials:

- Samples of milk – take at least two or three samples with different fat percentages, and
- one lactose-free sample.
- 5 mm NMR tubes and caps.

Glossary

T1: After the radio frequency pulse, the nuclear spins realign themselves along the external magnetic field, releasing energy in the form of heat to the environment. This process of realignment, or more precisely, of rebuilding the longitudinal magnetization, is called T1.

D1: The time it takes for the spins to fully return to equilibrium. It is recommended to take 5-7 times T1, as D1.

1D NOESY-presat: Pulse scheme that combines NOESY with presaturation pulses to selectively suppress the signal from solvent molecules and enhance the observation of nuclear spin interactions in a one-dimensional spectrum.

Abbreviations

NMR: Nuclear Magnetic Resonance.

FT-IR: Fourier-transform infrared spectroscopy.

O1P: O1 (or O1P for the value in ppm) is the carrier frequency used for the hard pulses.

ppm: parts per million.

FID: free-induction decay.

NOESY: Nuclear Overhauser Enhancement Spectroscopy .

plw9: power level of the saturation pulse

Parameter sets

- ^{1}H 1D NMR spectrum
- 1D-NOESY-presat spectrum
- T1_f
- Suppression_f

Sample Preparation:

1. Prepare your NMR milk samples by pouring ~0.6 ml of milk in a an NMR tube

2. Close the tube with a cap (purple cap)

Experimental Procedure:

1. Place one of the samples in the spectrometer and acquire a ^{1}H 1D NMR spectrum. You will use the ^{1}H 1D NMR experiments to quantify the fat content, so be sure these spectra are acquired in quantitative conditions. To ensure that quantitative conditions are met, measure T1 (parameters set T1_f) and set the recycling delay D1 long enough to ensure that the magnetization fully returns to equilibrium (at least 5 times T1).

2. Acquire a 1D-NOESY-presat spectrum. To set up this experiment, create a new dataset and choose Suppression_f as parameter set. Before running a new experiment, calibrate the pulses (pulsecal). The radio frequency pulse needs to have the same frequency as the solvent peak, so be sure to change the frequency of the proton channel (parameter o1p) to match the frequency of the water signal (that you can determine from the ^{1}H 1D NMR experiment without water suppression (pps), be as precise as possible, e.g., 4.786 ppm). Finally, to obtain a good solvent suppression, you need to optimize the saturation pulse, which can be done by changing the power level of the saturation pulse (plw9). Acquire several 1D-NOESY-presat experiments with different plw9 values until you reach good suppression of the water peak, i.e., signals of interest are not overlapped by the water signal anymore. Note that it is important not to use values exceeding 0.1 W to prevent damaging the instrument.

3. Acquire the ^{1}H 1D NMR and 1D-NOESY-presat spectra of the other milk samples. Use the same parameters (D1, PLW9) as the experiments before, but change the frequency of the proton channel (o1p) to match the water signal in each of the samples.

Data Processing:

To process the 1D experiments, use the standard procedure (xaup) (be sure to use the same processing parameters for all spectra). Then reference the water peak in the ^{1}H 1D NMR spectrum to 4.7 ppm (the expected value at 25 °C and pH 7), and then copy the spectrum reference frequency (SR) to the ^{1}H 1D-NOESY-presat dataset of the same sample. Do this for all spectra for referencing.

From ^{1}H 1D NMR experiments without water suppression it is possible to quantify the fat content of milk. Most of the protons contained in the fatty acids will give a signal around 1 ppm (To calculate the integral, enter .int and select the appropriate area for the integral). The ratio between the integral of the water signal and the fat signal gives an estimation of the fat content. By comparing the spectra of the milk with different fat content identify the peak given by fat acids in the spectra and quantify the fat content for all your samples.

Notes

Results:

- ^{1}H 1D NMR spectrum of a lactose-free 3.5% fat milk. The spectrum was obtained using the zg sequence with 1 scan and a recycle delay D1 of 15 s.

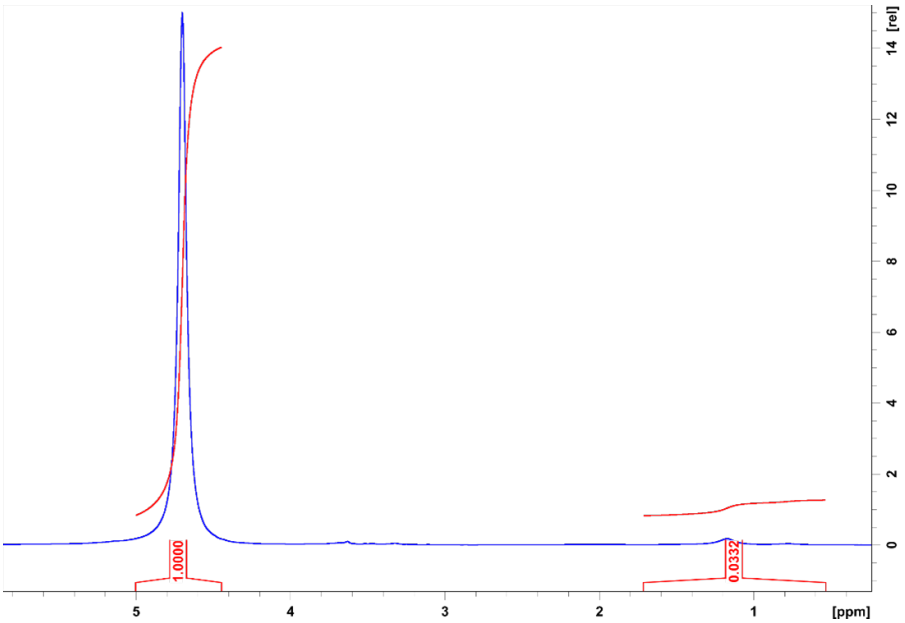

Figure 3 ^{1}H 1D NMR Spectrum of a Lactose-Free 3.5% Fat Milk

The figure above shows an example of a typical milk spectrum. Given the high-water concentration, the spectrum is characterized by the presence of an overwhelming water peak (centered around 4.7 ppm) and all the other compounds are hardly visible. The relatively high fats concentration can be quantified using the signals of the protons on the fatty acids chains around 1.5 ppm. All the ^{1}H 1D NMR spectra of the milk sample look similar to the one of in the figure above, and for this reason we do not show them here as standalone figures, but we provide the datasets as supplementary datasets.

- Zoom of ^{1}H 1D NMR spectra of milk. The water signal (around 4.7 ppm) is cut to highlight the other relevant features of the spectra. The spectra were obtained using the zg sequence with 1 scan and a recycle delay D1 of 15 s. The label 'LF' is used for the lactose free milk sample.

Notes

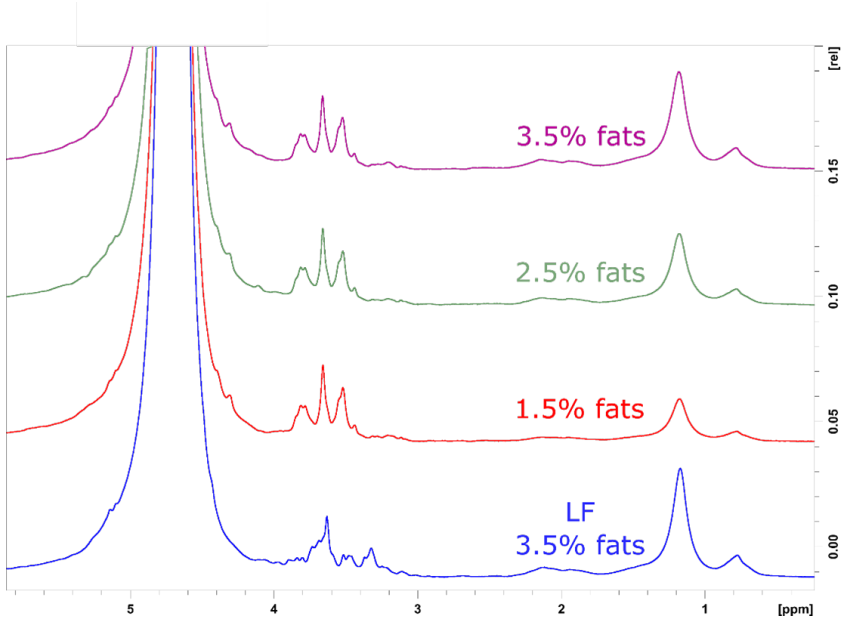

Figure 4 Zoom of ^{1}H 1D NMR Spectra of Milk

- ^{1}H 1D-NOESY-presat NMR spectra of milk. The spectra were obtained using the NOESYPR1D sequence with 8 scans and a recycle delay D1 of 15 s. The power level of the pre-saturation (plw9) was set to match a bandwidth of 4 Hz, and the mixing time D8 was 50ms. The green and blue dashed lines show the signals given by the lactose (green) and the mixture of glucose and galactose (blue). The label "LF" is used for the lactose-free milk sample.

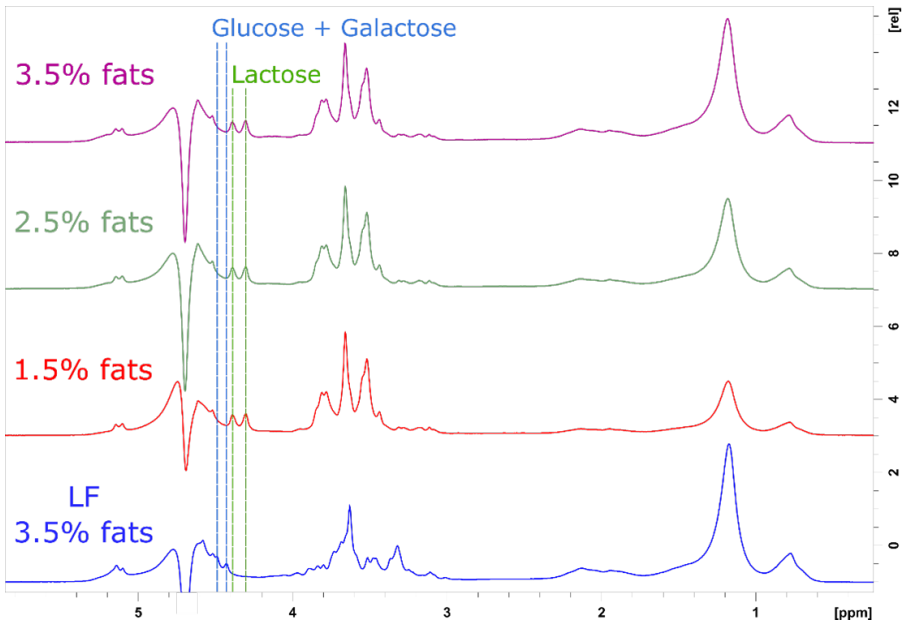

Figure 5 ^{1}H 1D-NOESY-presat NMR Spectra of Milk

Notes

- Efficiency of the solvent suppression in ^{1}H 1D-NOESY-presat NMR spectra as a function of the presaturation power level. The sample was the lactose-free 3.5% fat milk. The spectra were obtained using the noesypr1d sequence with 8 scans and a recycle delay D1 of 15 s. The power level of the presaturation (plw9) was set to various effective bandwidths given in the labels (from 1 to 50 Hz), and the mixing time D8 was 50 ms.

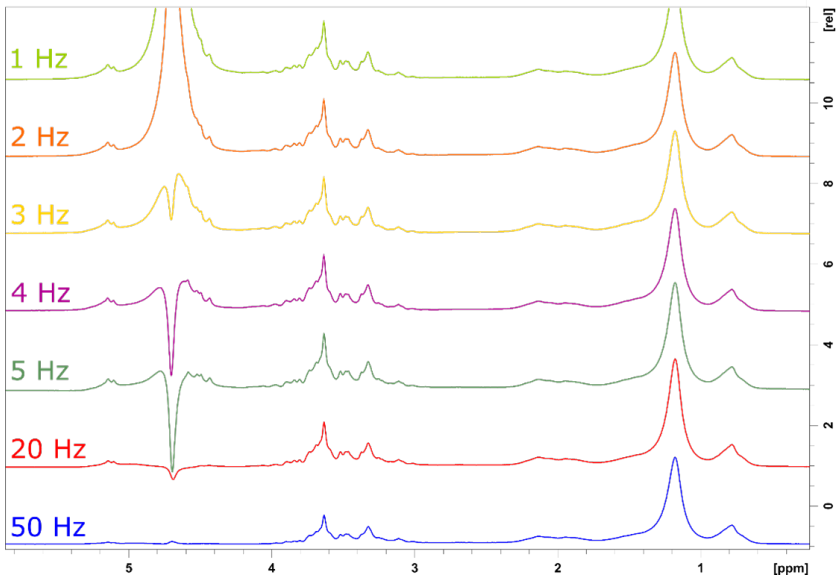

Figure 6 Efficiency of the Solvent Suppression in ^{1}H 1D-NOESY-presat NMR Spectra

The figure above shows the efficiency of the solvent suppression in 1D-NOESY-presat experiments as a function of the presaturation power level (plw9). Using power levels that correspond to larger bandwidth (e.g. 50 Hz) leads to full suppression of the solvent peak, as well as of the adjacent signals. Decreasing this level decreases the suppression of the solvent peak, but at the same time allows one to observe signals near the suppressed region. Note that the quality of the suppression is very sensitive to small changes in the plw9 value. In our case, the best solvent suppression was achieved using plw9 corresponding to a bandwidth of 3 Hz (yellow in the figure). With higher values the suppression is overpowered, which leads to negative peaks for small overpowering (which might still allow observing signals adjacent to the solvent, but typically leads to problems with automatic phase correction). With lower values the water signal is not fully suppressed. Correctly calibrating the plw9 value is an important step in this exercise.

The best results are typically obtained when the second presaturation pulse is as close as possible to a 90-degree pulse. Note that for a correct optimization of the sequence, also the length of the presaturation pulse during the mixing time (parameter D8) should be optimized. This is due to the fact that the presaturation power level plw9 affects both the first and the second presaturation pulse. This is then a way to quickly find parameters that overall provide a good suppression, but to improve the results further a fine tuning of the length of the second presaturation pulse is needed. However, the goal of this exercise is to teach students that it is important to optimize the parameters for some sequences, and to give them an example of the effects on the spectral quality of a parameter mis-setting. A proper optimization would require longer time and a deeper understanding of NMR, and it is perhaps out of the scope of this exercise.

To calculate the power level corresponding to a given bandwidth value x, use the pulse command in the command line. By typing pulse x Hz (where x is the desired bandwidth value), the command will output the power level to use (in db) to match that bandwidth value. Note that this requires to have correct probe parameters, so be sure to execute getprosol beforehand.

Notes

Discussion:

1. How does the measured fat content correlate with the declared one? If they are different, could you give a reason?

   The measured fat content correlates quite well with the declared one. We obtain the following measured for our samples:

   - Declared 1.5% fat, measured 1.55%
   - Declared 2.5% fat, measured 2.42%.
   - Declared 3.5% fat, measured 3.32%.
   - Declared 3.5% fat (lactose-free sample), measured 3.32%.

Differences might arise due to an incomplete integration of the water or fats signals. Note also that using this method we are quantifying the fatty acids by comparing the intensities of the proton signals of H_2O and the CH_2 of the fatty acid chains. While this method provides quite a good estimate of the fat content, it is not elaborate enough to provide accurate quantification of the milk components (e.g. it does not take into account the difference in weight between H_2O and CH_2, as well as it does not consider the components of the fatty acids that do not give ^1H NMR signals or the ones that have signals at different chemical shift values).

2. Discuss the fingerprint-regions of lactose. Do you think it would be feasible to determine lactose content of milk using NMR? Why? Would you use an 80 MHz or a 400 MHz spectrometer for this?

Figures 2 (400 MHz) and 5 (80 MHz) show the comparison of the ^1H 1D-NOESY-presat spectra of milk containing and not containing lactose. These two types of milk show differences in the region between 3 and 4 ppm that could be used to determine the presence of lactose. Additionally, the milk samples containing lactose show signals around 4.3 ppm (in green in the figure) while the lactose-free ones show a signal at slightly higher ppm (in blue in the figure). This difference allows the use of ^1H NMR spectra to determine the presence of lactose in a milk sample. In principle, if those peaks are resolved, it is possible to use ^1H 1D NMR to quantify the lactose content.

This is not the case, however, for the 80 MHz spectra. Indeed, while the signals in the 1D-NOESY-presat spectra can be used to quantitively determine the presence of lactose, glucose, and galactose, they are not resolved enough for quantification purposes. The 400 MHz 1D-NOESY-presat spectra (refer to the student manual), on the other hand, have fully resolved lactose, glucose, and galactose signals, and thus can be used for quantification.

Key Take Home Messages:

- NMR is a robust analytical tool that can be applied to real world samples without alteration
- Interpret the NMR spectra of milk samples and learn to quantify fats and detect lactose in ^1H NMR spectra.
- Learn to run a ^1H 1D NOESY-presat experiment to suppress the water peak effectively.

Notes

References:

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