EDULAB FOR INSTRUCTORS: FOURIER 80

The Caffeine Kick

NMR of Coffee

Authors & Affiliation:

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Experiment Hashtag #: #caffeinekick, #Educate2Resonate

Keywords:

Compound identification, food analysis, authenticity, fraud

Target group:

Advanced Undergraduate or Graduate, General Chemistry, Analytical Chemistry, Food Chemistry, Food Safety and Control Laboratory, General Life Sciences

Objectives:

NMR has been 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 coffee samples using NMR. The goal of this exercise is to teach the students how to find out the type of coffee under analysis from the ^{1}H 1D NMR spectrum, and how to determine the presence of important compounds such as caffeine, kahweol, cafestol, and 16-O-Methylcafestol (16-OMC). In addition, the students will learn how to run experiments and use the ERETIC2 module available in TopSpin to quantify a compound in the sample (in this case, caffeine).

Background of the Experiment:

The earliest evidence of coffee drinking in the form of the modern beverage appears in southern Arabia in the middle of the 15th century. From that time on, coffee consumption has continued to increase worldwide, reaching approximately 2.25 billion cups consumed daily. There are dozens of varieties of coffee beans that can be used to make your cup of coffee, but only two kinds are typically cultivated for drinking: Arabica and Robusta.

These two coffee species are quite different from each other. Arabica tends to have a sweeter taste, while Robusta has a stronger and more bitter one. Robusta beans typically contain more caffeine than the Arabica ones and fewer lipids and sugars. Robusta is much easier to grow and gives a higher yield than the Arabica species. Thanks to their differences, these two kinds of coffee are often blended together, so that Robusta can add depth of flavor to the sweet Arabica (and it also helps to create a perfect crema, if you are an espresso lover).

To regulate the coffee market, analytical methods had to be developed to evaluate the properties and the quality of coffee. Not surprisingly, NMR has been proven to be able to provide information about many of the properties of coffee. It is possible to quantify compounds such as caffeine for example, as well as perform more complicated tasks such as addressing the authenticity and traceability of products (using a combination of NMR and statistical methods).
There are a few markers used to determine whether a coffee species is Robusta or Arabica.

Robusta coffee is characterized by the presence of 16-O-Methylcafestol (16-OMC) in its lipid fraction, which gives a peak around 3.2 ppm in ^{1}H NMR spectra. This peak should not overlap with any other resonances, and it is often used as a marker to detect Robusta. Arabica coffee, on the other hand, contains a diterpenoid named kahweol, which is not present in Robusta. This compound can be detected in ^{1}H NMR spectra as it shows a few peaks between ~5.8 and ~6.5 ppm. Note that in this region there is also a signal corresponding to cafestol, a diterpenoid structurally similar to kahweol, which is present both in Robusta and Arabica coffees.

In this exercise, we will analyze coffee using NMR. We will acquire the spectra of an Arabica and a Robusta coffee, as well as the spectrum of a caffeine-free coffee. We will qualitatively find the differences between them, and then quantify the caffeine in the three samples. The caffeine concentration in coffee beans is reported to be 1-2 % w/w.

For the quantification of caffeine, we will use the ERETIC2 module. This is a tool based on PULCON, a method that correlates the absolute intensities of two different spectra. Providing that the concentration of one of the samples is known (in this case, the concentration of pure caffeine), it is possible to quantify the concentration in the unknown sample using

$$C_{unk} = kC_{ref}\frac{A_{unk}T_{unk}\theta_{unk}NS_{ref}}{A_{ref}T_{ref}\theta_{ref}NS_{unk}}$$

where A is the integral value, C is the concentration, T is the temperature, θ is the pulse length, NS is the number of scans used for the experiment and k is a correction factor which takes into account incomplete relaxation.

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

Cafestol and kahweol: Natural diterpenes extracted from coffee beans, which mainly present as fatty esters in unfiltered coffee

Diterpenoids: Chemical compounds containing 20 carbon atoms and belong to the terpenoid class

ERETIC2: qNMR experimental technique to measure analytes based on the signal of the reference compound without additional hardware equipment

PULCON: A method that correlates the absolute intensities of two different spectra

Inversion recovery experiment: Measures the recovery of magnetization over time using an initial 180-degree pulse, followed by a variable recovery time and a 90-degree pulse to determine the longitudinal relaxation time (T1)

Preparation and Prerequisite:

The main objective of this investigation is to illustrate key NMR concepts. This includes measuring T1 using NMR, acquiring and interpreting 1D ^{1}H NMR spectra of coffee extracts, as well as differentiating and quantifying various coffee varieties. To ensure efficient completion of the experiments, it is recommended to form groups with a maximum of 3-4 students. The estimated time for sample preparation is approximately 1 hour. The coffee experiments are expected to take around 2-3 hours to perform. Specifically, the T1 experiment will require 2-3 hours, and the coffee experiments will take approximately 2 hours in total (including 3 samples and 1 standard solution). After completing the experiments, an additional 1-1.5 hours will be needed to 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 these experiments, a properly installed and adjusted Fourier 80 is required. In addition, the Program TopSpin, either a vortex or an ultrasonic bath, syringe filter with pore size 0.45 µm, a balance with 3 decimal places and pipettes should be available.

Experimental Setup:

Materials:
- Samples of powdered coffee – take at least one sample of Robusta, one of Arabica and one that is caffeine-free
- Powdered caffeine
- $CDCl_3$
- 5 ml volumetric flask
- 2 ml vials for extraction
- 5 mm NMR tubes and caps

Parameter sets
- ^{1}H 1D NMR spectrum
- PROTON_f
- T1_f

Sample Preparation:
Prepare NMR samples of your coffees by extraction in $CDCl_3$

1. For each sample, insert 200 mg of the powdered coffee sample in a vial (take note of the exact weight of your sample), and add 1.5 ml of $CDCl_3$.

2. Shake or ultrasonicate the sample for ~20 minutes.

3. Filter using a syringe filter with pore size 0.45 µm, place 0.6 ml of the filtered solution in an NMR tube and close the tube with the cap.

Prepare a sample of caffeine with known concentration

1. Place the caffeine powder in a 5 ml volumetric flask (calculate the amount of powder to use in order to obtain an approximate concentration of 50-100 mM), and then bring to volume (fill up to the mark) with $CDCl_3$.

2. Calculate the exact concentration of your solution, write it down, and place 0.6 ml of the solution in an NMR tube.

3. Close the tube with the cap (purple cap)

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

Abbreviations

NMR:
Nuclear Magnetic Resonance

ppm:
parts per million

ERETIC:
Electronic reference to access in-vivo concentration

PULCON:
PUlse Length-based CONcentration determination

D1:
Relaxation delay

Experimental Procedure:

1. Acquire the ^{1}H 1D NMR spectrum of the caffeine sample using the parameter set PROTON_f. To ensure that this experiment is quantitative, measure the relaxation time T1 in an inversion recovery experiment (parameterset T_1_f) and set a d1 about 5 times larger than the T_1.

2. Acquire the ^{1}H 1D NMR spectra of the coffee samples using the parameter set PROTON_f. Use the same d1 used for the caffeine experiment. To improve the signal-to-noise ratio and allow detection of the compounds present at a lower concentration, use 128 scans (*NS*) for each experiment.

Data Processing:

1. To process the inversion recovery experiment, you can use the command **xf2**. The calculation of the T_1 value then can be done directly in TopSpin. We recommend using the Dynamics center software, which you can open via the 'Dynamics drop-down menu in the Applications tab in TopSpin. Please refer to the instructions within this software to calculate the T_1. If something is unclear, please refer to the "Help" section of the Dynamics Center software or get in contact with us.

2. To process the 1D experiments, use the standard procedure. Be sure to use the same processing parameters for all spectra.

3. To calculate the concentration of an unknown sample using ERETIC2, start by defining one or multiple reference peaks in the spectrum of your reference caffeine sample. First, open the integration window with the Integrate button in the Analyze tab, then manually integrate the peak(s) to use as a reference. Right click on the integrated signal, and choose **Eretic | Define as Eretic Reference**, as shown in Figure 1. You can also use more than one signal as a reference. For this exercise, use all the four caffeine peaks in the spectrum as seen in Figure 2 below.

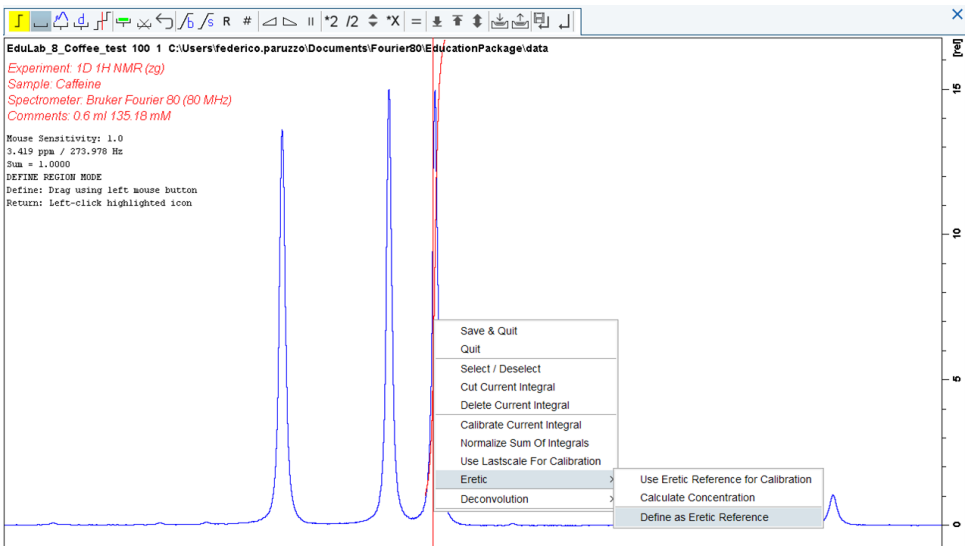

Figure 1 Selection of a Signal for Eretic Calibration

This opens the ERETIC2 calibration window as shown in Figure 2. Define the concentration of the reference sample (mM) and the number of nuclei contributing to each signal. You can optionally also define name, sample volume as well as molar mass.

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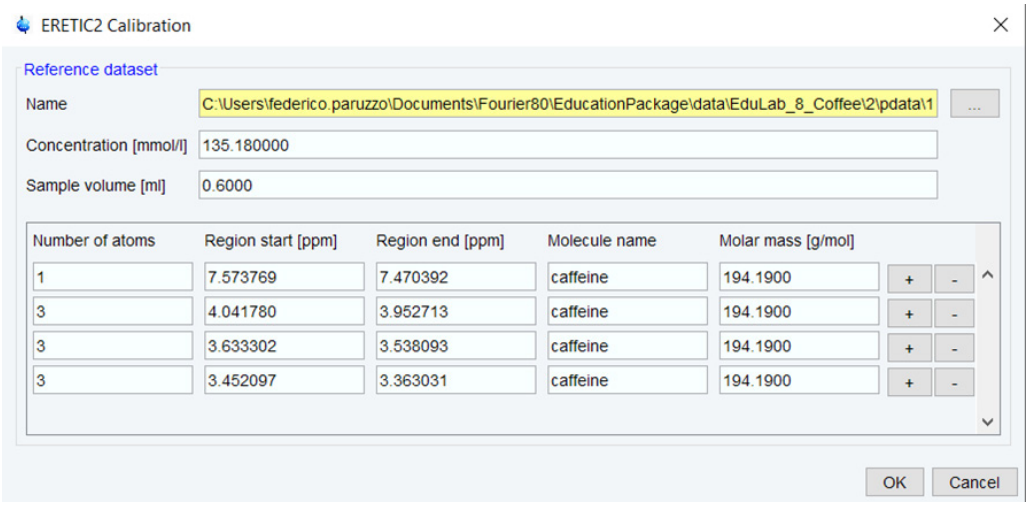

Figure 2 ERETIC2 Calibration Window

Then, open the spectrum you want to quantify and integrate the peaks to use for the quantification. Right-click on an integral and choose Eretic | Calculate concentration. This opens the quantification window as shown in Figure 3. Here be sure that the correct spectrum is selected for 'reference dataset', which is the pure caffeine spectrum you measured. Define the number of nuclei for each signal, as well as (optionally) sample volume, molecule name and molar mass to get the amount of sample in mg in the NMR tube.

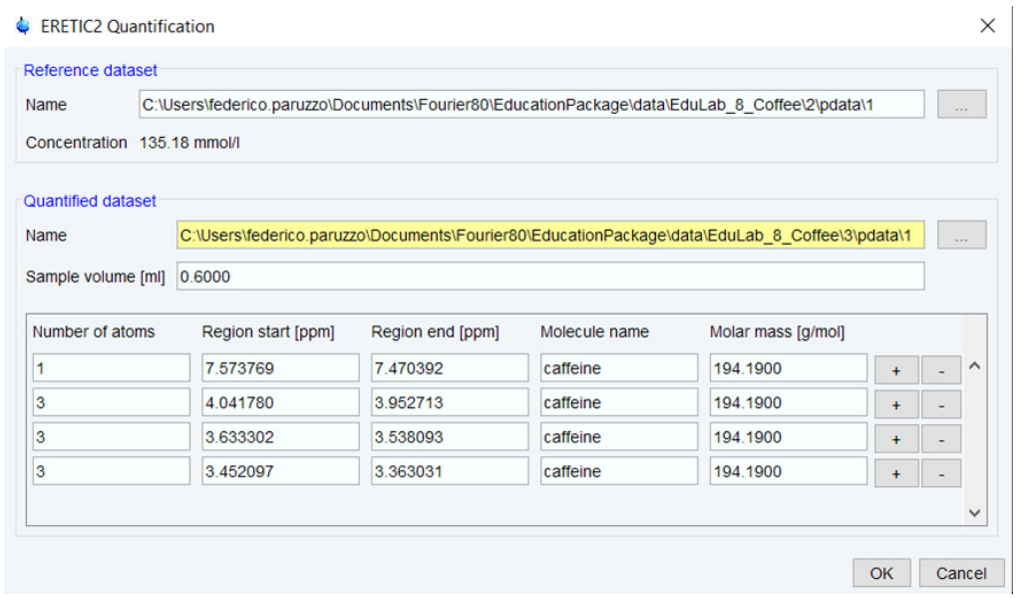

Figure 3 ERETIC2 Quantification Window

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Results & Discussion
Overview Example Spectra

- ^{1}H 1D NMR spectrum of caffeine. The assignment is based on the color scheme given in the 2D structure of caffeine, with each circle representing the protons attached to that carbon atom. The spectrum was obtained using the zg sequence with 4 scans and a recycle delay D1 of 10 s.

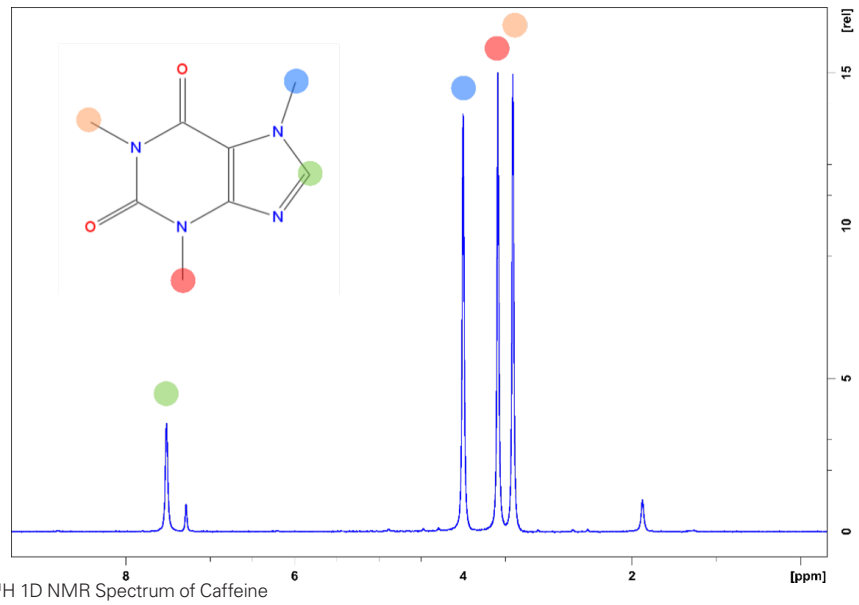

Figure 4 ^{1}H 1D NMR Spectrum of Caffeine

- ^{1}H 1D NMR spectrum of the $CDCl_3$ extract of a blend for lungo leggero coffee. The spectrum was obtained using the zg sequence with 128 scans and a recycle delay D1 of 10 s.

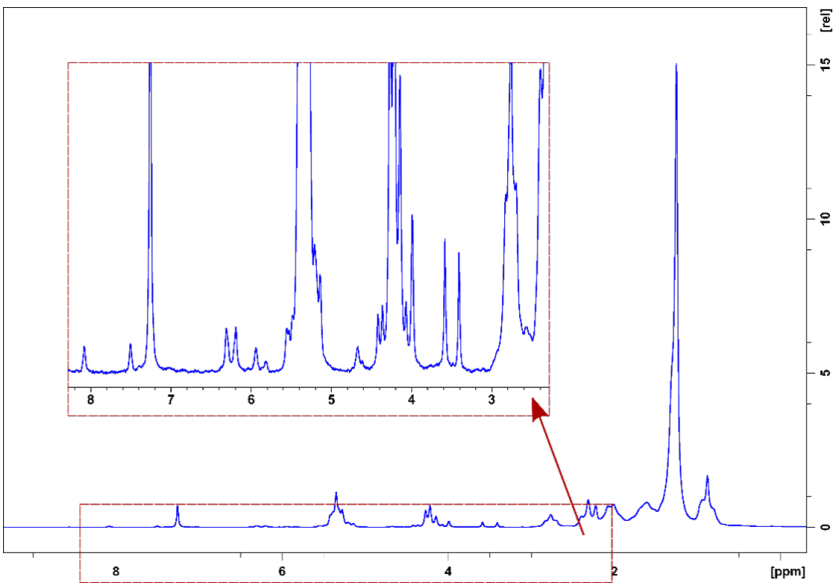

Figure 5 ^{1}H 1D NMR Spectrum of the $CDCl_3$ Extract of a Blend for Lungo Leggero Coffee

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- ^{1}H 1D NMR spectrum of the CDCl3 extract of a blend for espresso forte coffee. The spectrum was obtained using the zg sequence with 128 scans and a recycle delay D1 of 10 s.

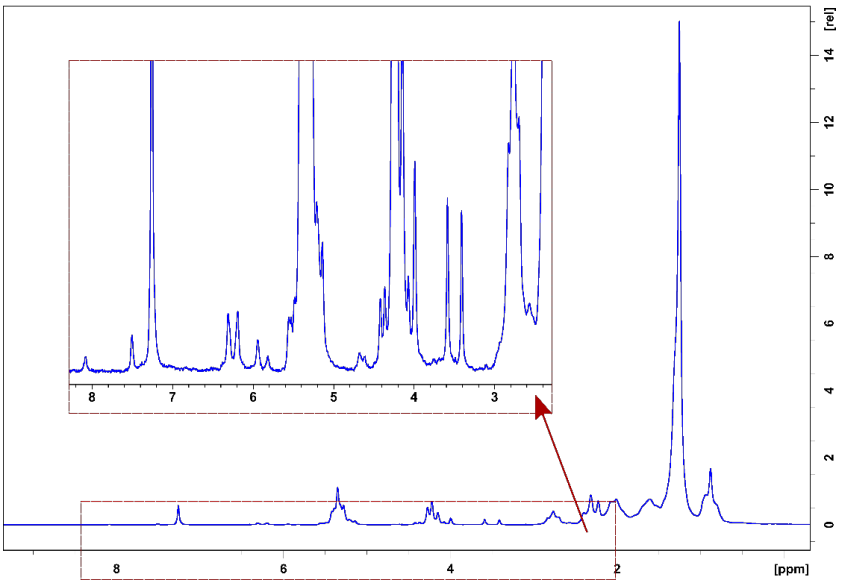

Figure 6 ^{1}H 1D NMR Spectrum of the $CDCl_3$ Extract of a Blend for Espresso Forte Coffee

- ^{1}H 1D NMR spectrum of the $CDCl_3$ extract of a blend for ristretto coffee. The spectrum was obtained using the zg sequence with 128 scans and a recycle delay D1 of 10 s.

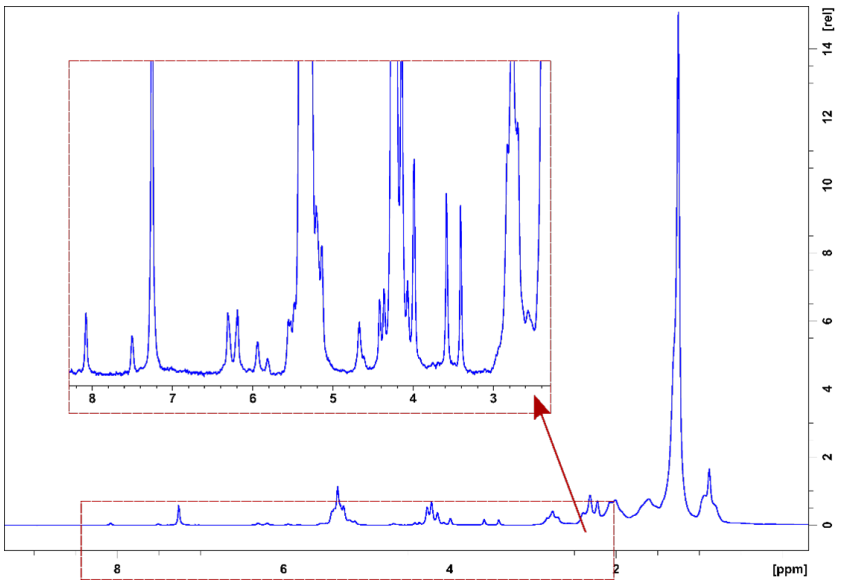

Figure 7 ^{1}H 1D NMR Spectrum of the $CDCl_3$ Extract of a Blend for Ristretto Coffee

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- ^{1}H 1D NMR spectrum of the $CDCl_3$ extract of a blend for a caffeine-free lungo coffee. The spectrum was obtained using the zg sequence with 128 scans and a recycle delay D1 of 10 s.

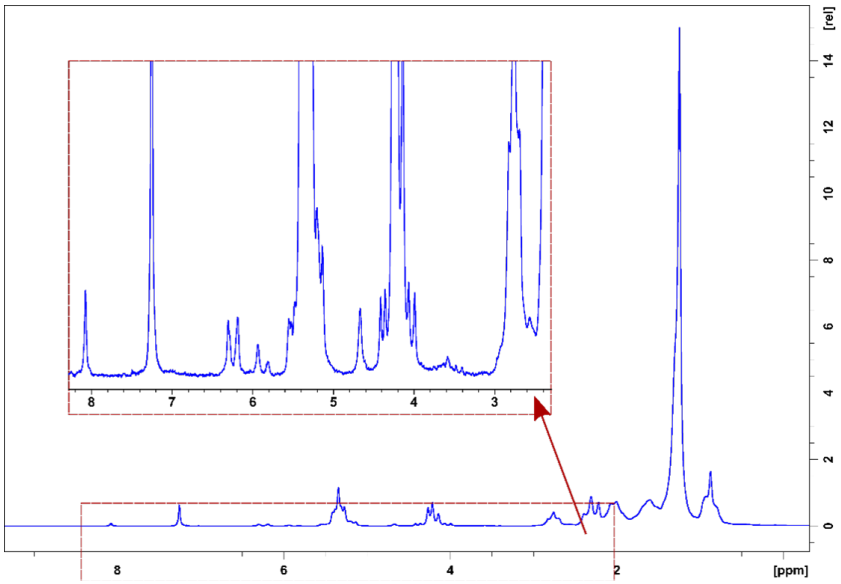

Figure 8 ^{1}H 1D NMR Spectrum of the $CDCl_3$ Extract of a Blend for a Caffeine-Free Lungo Coffee

Questions to be answered by students

1. **Based on the spectra you have acquired, label the spectrum in Figure 7 as Robusta or Arabica, and justify your choice.**

   The coffee is an Arabica variety. The ^{1}H NMR spectrum does not show the peak of 16-OMC at ~3.2 ppm, which is a marker for Robusta coffee. There are also typical peaks of kahweol between 5.8 and 6.3 ppm, which is a marker for Arabica.

2. **Can you determine if the caffeine-free coffee you have analyzed is an Arabica, Robusta or a mix of the two? Do you observe any caffeine signal in the spectrum?**

   The caffeine-free coffee we have analyzed is an Arabica variety, as shown by the spectrum in Figure 8. Indeed, the spectrum shows the kahweol peaks and the 16-OMC signal is missing. We can also confirm the absence of caffeine given that no, or little, caffeine signals are detected.

3. **Print the spectra and label the caffeine, kahweol, cafestol and 16-OMC peaks in all of them.**

   These compounds give signals at the following frequencies:
   Caffeine – 3.4, 3.6, 4.0 and 7.5 ppm
   Cafestol – 6.2 ppm
   Kahweol – 5.8, 5.9, 6.2 and 6.3 ppm
   16-OMC – 3.2 ppm
   Their signals can be visualized in the Figure 9 below.

4. **Quantify caffeine in all your spectra – from this, calculate the caffeine content in the coffee (in mg). How does it compare to what you expect?**

   The ^{1}H spectrum of the espresso blend of Figure 5 gives us a caffeine concentration equal to 1.94 mmol, which corresponds to ~23 mg of caffeine in our sample (corresponds to the amount of caffeine in the NMR tube), and ~0.58 mg extracted from the 200 mg coffee sample (average of the ERETIC quantifications using the four caffeine peaks shown in Figure 4). The ^{1}H spectrum of the espresso forte blend of Figure 6 gives us a caffeine concentration equal to 2.14 mmol, which corresponds to ~0.25 mg of caffeine in our NMR sample

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and ~0.58 mg extracted from the 200 mg coffee sample. Finally, the ^{1}H spectrum of the ristretto blend of Figure 8 gives us a caffeine concentration equal to 2.14 mmol as well, which corresponds to ~0.25 mg of caffeine in our NMR sample and ~0.58 mg extracted from the 200 mg coffee sample.

Those values are a little lower than expected, as caffeine concentration in coffee beans reported to be 1-2 % w/w. The reasons behind this difference might be an incomplete caffeine extraction due to, for example, not enough time in the ultrasound bath for the caffeine extraction. Another reason could be, that caffein in coffee is not only present in free form. It can also be bound to other components; therefore, it is not so easily accessible using the described extraction method.

5. Do you observe differences in the quantification using the different peaks? Why?

In the acquired spectra, the caffeine concentration varies significantly based on the peak that is used for quantification. There are two main factors that contribute to this deviation. The first reason is the overlapping of the caffeine resonances with other peaks. Two of the caffeine resonances (the ones at 3.6 and 4.0 ppm) overlap with other signals. This can be observed by the fact that the relative integrals for those peaks are higher than expected, as well as by the fact that we observe signals at these frequencies in the caffeine-free spectrum (Figure 8). The second reason is that the experimental conditions in which these experiments were run are not fully quantitative. The relaxation time T1 of the resonance at 7.5 ppm is about 3.8 s (while the other resonances have T1 of ~2 s), and so the recycle delay used for these experiments (D1 = 10 s) is not suitable for quantification, since with less than 5 times T1 too short (the caffeine concentration calculated using this peak is indeed always smaller than using the other peaks).

The signal that seems to be the most reliable for the caffeine quantification is the one at 3.4 ppm. Using this signal, we estimate to have extracted 0.48, 0.53, and 0.53 mg of caffeine from the lungo leggero, espresso forte, and ristretto sample respectively.

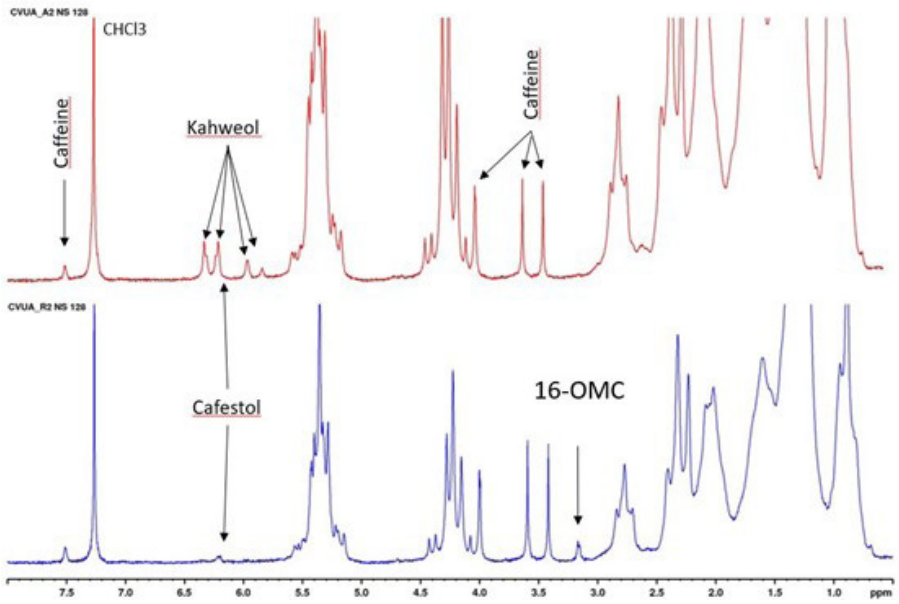

Figure 9 Labelling for the Caffeine, Kahweol, Cafestol and 16-OMC Signals in ^{1}H NMR Spectra of Coffee

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Key Take Home Messages:

- Interpreting the NMR spectra of coffee samples.
- Learning the ^{1}H NMR features typical of the Robusta and Arabica varieties, and to identify some of the compounds typically present in coffee.
- Learning to quantify compounds using ERETIC2.

References:

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