EDULAB FOR INSTRUCTORS

Something’s Brewing in NMR

Differentiation of Beer and Cider Samples using NMR-based Chemometrics

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Experiment Hashtag:

#Something’sBrewing

Keywords:

Beer analysis, Cider analysis, Chemometrics, Principal component analysis (PCA), Food Chemistry, Mixture Analysis

Target group:

Undergraduate, General Chemistry, Analytical Chemistry

Objectives:

- Gain hands-on experience using the Fourier 80 NMR spectrometer.
- Understand the use of NMR for the analysis of real-world samples.
- Learn how chemometrics can be used to tell us if, and why, categories of complex samples, such as beer and cider, are compositionally different.
- Understand how, by excluding major components such as water and ethanol from the statistical analysis, differences caused by low-concentration species in the flavor profiles can be revealed.
- Learn how to differentiate between various types of food and beverage samples using PC1-PC2 score plots.
- Understand how loadings plots can be used to explain what (in chemical terms) differentiates the groups of samples from each other.

Background of the Experiment:

Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful spectroscopic approach that has various applications in chemistry, including food science. Applications include the identification and quantification of metabolites in food and drink samples such as vegetable oil, tea, coffee, beer, cider, fish, and chicken, for example.[1–6] This EduLab will focus on the NMR analysis of two groups of alcoholic beverages – beer and cider.

Studies show that alcoholic beverages such as beer and wine were produced and consumed as far back as 7000-8000 years ago.[7] Alcoholic beverages are still widely consumed today, accounting for approximately 64% of the world's total beverage expenditure.[7] Increased globalization and trade have also led to alcoholic beverages being consumed in countries outside of where they were historically brewed. Recent studies have shown declining consumption rates of beer in traditionally beer-consuming countries as wine is becoming increasingly popular, and vice versa for traditionally wine-consuming countries where beer is growing in popularity.[8] Consumers can now choose from a wide range of alcoholic beverages such as wines, beers, ciders, whiskeys, vodkas, gins, and many more.

While each of the above drinks contains alcohol, there are notable differences in the taste and strength of each beverage. This is because different alcoholic drinks are made using different recipes and brewing techniques, thus each beverage has a characteristic flavor profile.[9] But what makes beer "beer" or cider "cider"? This EduLab will help you tackle this question and understand how we can use NMR spectroscopy in combination with chemometrics to distinguish between beers and ciders and explain which chemical components are responsible for these differences. These beverages were specifically chosen since they are made from different sources (cider from apples and beer from barley/hops).

In this EduLab we will explore how chemometrics can be used to analyze NMR data. Chemometrics is defined as the science of extracting information from chemical systems by data-driven means. For this experiment specifically, a statistical technique called Principal Component Analysis (PCA) is used to determine if the NMR spectra of beer and cider are statistically different from each other. Here Cider and Beer are used as they are readily available around the world. However, in principle, the same framework could be used to differentiate other groups of samples, such as cosmetics, wines, teas, juices, etc.

Preparation and Prerequisite:

To perform this experiment, a properly installed and adjusted Fourier 80 system with TopSpin Software is required. Additionally, a sonicator (optional) and a 1000 µL micropipette should be available for use during the experiment.

This experiment should take approximately eleven hours to perform (including, all data collection), with an additional two hours for report writing given that students have some prior understanding of 1D NMR and the basics of spectral interpretation. Due to the spectrometer time required, this experiment may need to be split between two weeks of experimentation depending on how much laboratory time is allotted to students per week. Alternatively, samples can be run overnight if an autosampler is available. The experiments are designed to be completed in groups of 3-6 students. This investigation aims to demonstrate several key NMR concepts, such as the collection of 1D ^{1}H NMR spectra, using PCA to identify different components, understanding PC1-PC2 scores plots, and identifying the compounds responsible for class differentiation via spectral loadings.

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

Chemometrics: The science of extracting information from chemical systems by data-driven means

PCA: A statistical analysis tool that aims to simplify complex datasets by reducing the number of variables to sets of principal components (e.g. PC1, PC2, etc.) to show only the most relevant variations in the data

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

Experimental Setup:

- 11 beers (any brands of choice, unflavoured)
- 11 ciders (any brands of choice, unflavoured)
- 5 mm NMR tubes with caps (22)
- 1000 μL micropipette
- Pulse programs: zg, zgcppr

Sample Preparation:

1. Degas the beer and cider samples prior to use. This can be done by pouring about 20 mL of each sample into separate 50 mL beakers and allowing the samples to stand in the fridge overnight. Any remaining carbonation can be shaken out on the day of the experiment. This is to remove gas bubbles which can induce susceptibility distortions and broaden the line shape in NMR spectra.

2. Alternatively, instead of shaking the samples by hand, they can be sonicated for 2-3 hours if a sonicator is available.

3. Transfer ~0.6 mL (600 μL) of each sample into clean 5 mm NMR tubes using a 1000 μL micropipette.

4. Seal all the NMR tubes with caps.

Experimental Procedure:

1. Place the first NMR tube inside the spectrometer and obtain a 1D ^{1}H NMR spectrum using the **zg** pulse program. The water (solvent) peak from this spectrum will be used to determine the frequency of the proton channel (O1P) for the subsequent **zgcppr** experiment. The O1P value should be identified as accurately as possible as this is essential for achieving effective solvent suppression. The O1P should be set to the apex of the water peak.

2. Using the same sample, obtain a 1D ^{1}H NMR spectrum with water suppression. The pulse program **zgcppr** is recommended but other programs may also be used. The chosen experiment should reduce the water signal, however as there are peaks close to the base of the water, the suppression is mainly to narrow the water signal rather than completely suppress it. The chosen experiment should suppress the water signal but should not be too aggressive as there are peaks of interest close to the water base. A B_1 field of 10 Hz (or less) is recommended for presaturation. This can be calculated in "edprosol" and corresponds to a ~67 dB attenuation on the standard Fourier 80. If instructors are not confident with water suppression it would be possible to perform the experiment without it. However, this introduces a limitation as it will result in a slightly wider water signal, meaning some peaks close to the base of the water may be lost. Unlike high-field NMR, with the Fourier 80 there is no gain in signal-to noise-when a receiver gain > 1 is used. This is because the receiver is already optimized for S/N and dynamic range with the minimum receiver setting. Therefore, the dynamic range will not be impacted if water suppression is not used.

3. If using the **zgcppr**, it is recommended the parameters D1=3, DS=8, and NS=256 are used. Fewer scans could be used if instrument time is restricted.

4. Repeat these steps for the other 21 samples. For the spectra to be comparative, all parameters should be kept the same except for the O1P which is specific to each sample.

Abbreviations

NMR: Nuclear Magnetic Resonance

1D: One dimensional

PCA: Principal Component Analysis

PC1: Principal Component 1

PC2: Principal Component 2

O1P: O1 (or O1P for the value in ppm) is the carrier frequency used for the hard pulses. Or in simpler terms, the middle of the spectrum. When doing water suppression, the O1P is often set to the chemical shift of water.

D1: Relaxation delay

DS: Dummy Scans

NS: Number of Scans

Data Processing:

- Process all data following standard protocols, including properly phasing each spectrum and correcting the baselines. An exponential apodization corresponding to a line broadening of 0.1 Hz is recommended. Depending on the sample type 0.1 Hz at 80 MHz is a reasonable line broadening that retains sharp signals, while improving signal-to-noise ratios. If the samples selected are much broader, for example, viscous honey, then increasing the line broadening may be justified.
- Calibrate the ppm axis of each spectrum using the center of the ethanol triplet. The center should be at 1.186 ppm. If the sample does not contain ethanol pick another spectral component using the literature for reference. The key is to pick a peak that is well resolved in all spectra so they can easily be aligned for statistical analysis. In MNova and AMIX there are built-in procedures to automatically align spectra across a series. In AMIX see "advanced calibration" in Mnova see "advanced > align spectra".
- Download the data from TopSpin to a computer and use any chemometrics package available (e.g. MNova, Amix) to run a PCA of the data. There are hundreds of paid and free packages for the analysis of NMR data. For a decent starting point, see: Open NMR Project[10]. The authors recommend MNova as it is simple to use yet very powerful in its features. Here MNova is used. The "sum" option for normalization of PCA is recommended, with a bucket width of 0.01 ppm. Obtain the PC1-PC2 plots for the data. Distinguish the beer and cider data points by color to make them easier to visually differentiate in the plots. In MNova data points can be color-coded and put into classes by editing the "scores table".
- Obtain the PC1-PC2 plots with water and ethanol, without water, without ethanol, and finally without water and ethanol. This is to explore how the PCA differentiation can improve when the largest peaks are excluded from the spectra. This is because large peaks have a strong weighting on the statistical results. However, when water and ethanol are removed, the smaller peaks that differentiate the beer and cider are given greater weighting. In MNova regions are easily excluded interactively using the "cuts" function before the bucket table is calculated. In other software, the excluded regions are input as chemical shift ranges during the creation of the bucket table.

Case Study:

The main samples analyzed in this EduLab are two groups of alcoholic beverages – beer and cider. When conducting this experiment yourself, you can either choose to analyze alcoholic beverages as was done in this EduLab, or you could also analyze any other food or beverage samples of your choice. This case study is provided to give you an idea of why PCA is such an important tool as it helps extract class-defining information about samples from their NMR spectra.

Notes

Analysis of Raw NMR Data

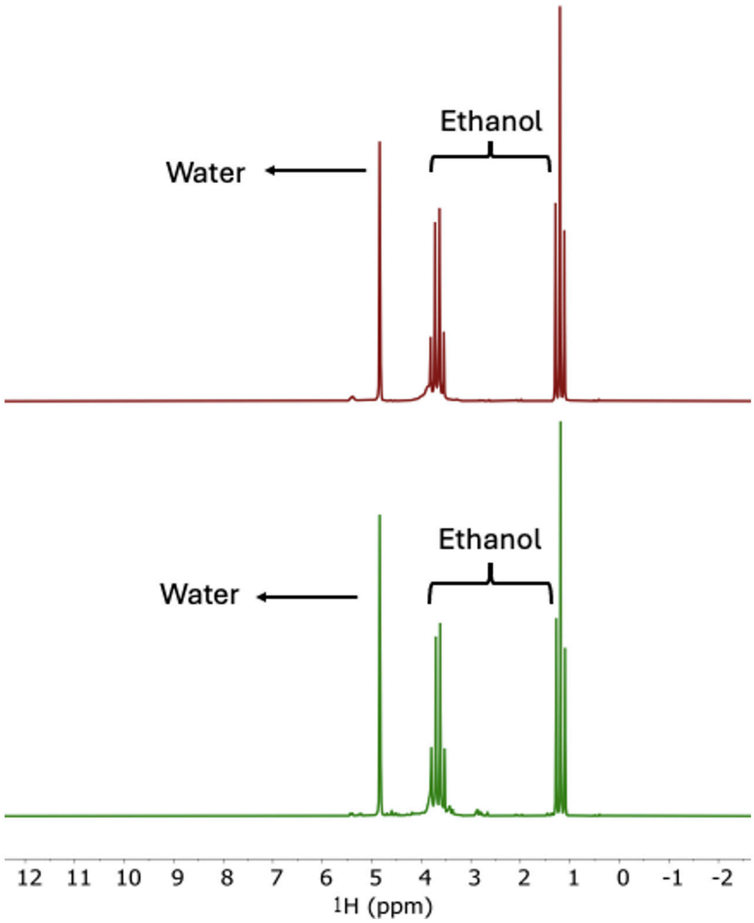

Figure 1 Comparison of beer (red) and cider (green) NMR spectra

Looking at the above figure, both the beer and cider spectra are dominated by water (partially suppressed) and ethanol. Some small peaks can be observed near the baseline, but they are hard to distinguish without vertical expansion of the spectra. If not considered carefully the above spectra could give a false impression that beer and cider samples are identical. In reality, we know that this is not the case since beer and cider are fermented using different ingredients (beer from barley and hops; cider from apples).

Notes

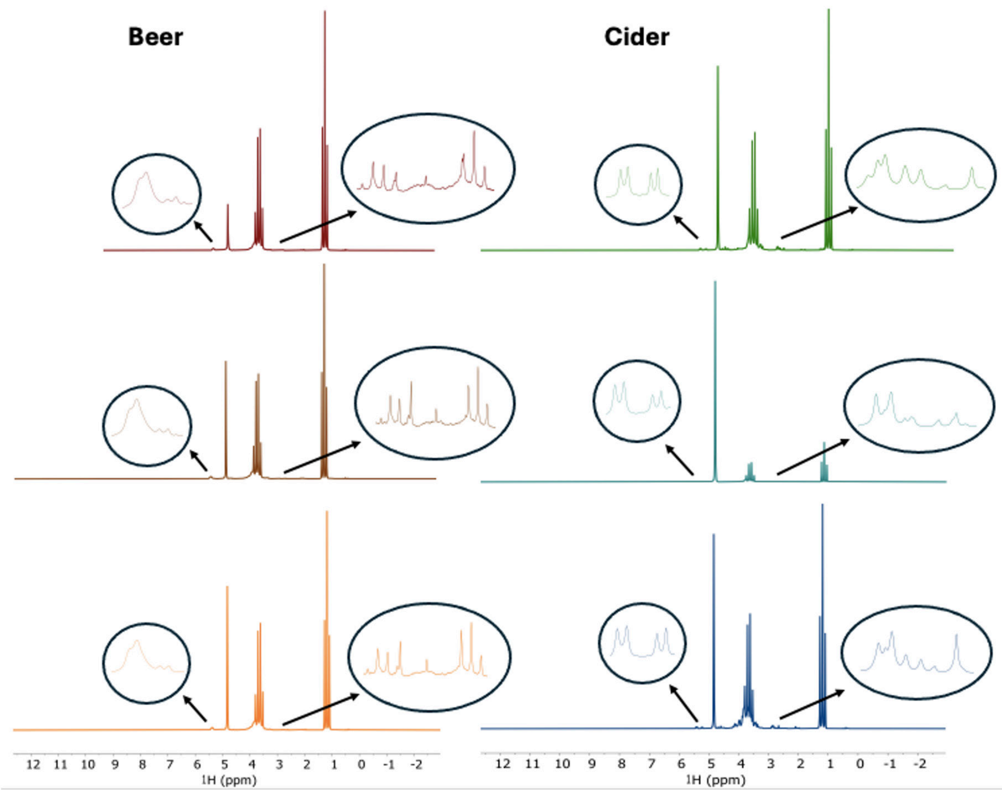

Figure 2 NMR spectra of beer (left) and cider (right) samples with zoomed-in regions showing less concentrated compounds that are much smaller than the water and ethanol peaks.

One approach could be to search amongst the smaller peaks (see zoomed-in regions on example spectra in Figure 2), however, across a large number of samples, this becomes tedious. Even after compiling a list of different peaks that vary between sample types, it is hard to be sure which differences are statistically significant and which only apply to those sub-spectra that were selected for detailed manual inspection. Luckily, chemometrics in the form of PCA makes such analysis of large groups of data much easier.

Taking a Closer Look Using PCA

Principle Component Analysis provides a robust approach to determine if groups of samples are different. This is called an "unsupervised method" which in this case, means we do not tell the software which samples are beers and which are ciders. Instead, all the data is provided as is and the software produces statistical groupings based on the NMR spectra. In this section, you will see how excluding the water and/or ethanol peaks from the spectrum leads to better discrimination between beer and cider samples, as it allows the more dilute components to be better represented.

Notes

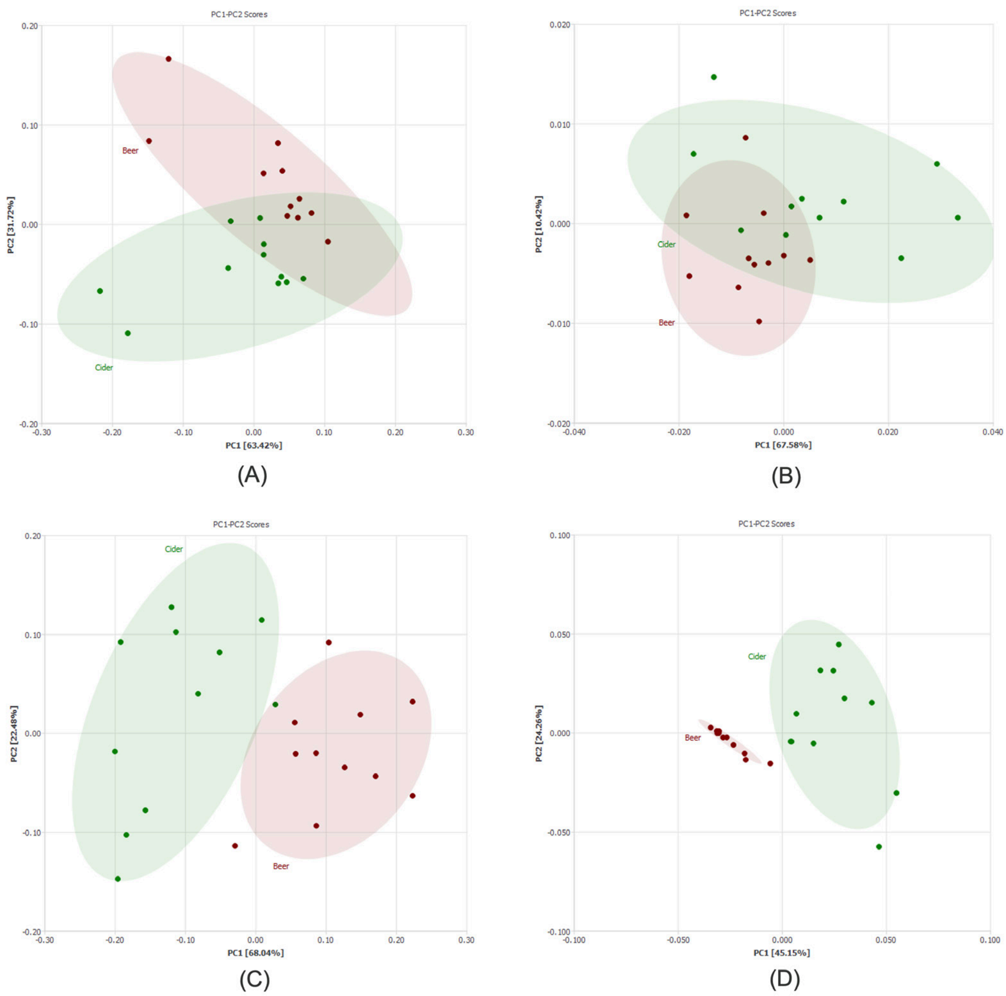

Figure 3 PCA results (**A**) including water and ethanol, (**B**) excluding water only, (**C**) excluding ethanol only, and (**D**) excluding both water and ethanol. Ellipses set at a 90% confidence threshold.

Figure 3A shows the PCA results when the water and ethanol peaks are included in the analysis. The separation of beer and cider data points is not very distinct, and the confidence ellipses overlap. The same can be seen in **Figure 3B** when the water peak is excluded from the analysis. **Figure 3C** shows an improved separation between the beer and cider groupings when the ethanol peaks are excluded from the analysis, with the confidence ellipses barely overlapping. This suggests that the relatively high ethanol contents in the beers and ciders were strongly influencing the statistical results reducing the influence of the less concentrated "flavor profiles". Finally, **Figure 3D** shows the PCA results when both water and ethanol are removed. This results in the cleanest separation of data points: there is no overlap between the confidence ellipses, and a clear difference can be seen between the beers and ciders used in the experiment.

An important point to note is that the beer data is much more tightly grouped than the cider data. This could be because the beer used in this study was from major brands that likely have carefully controlled production methods and recipes that produce broadly similar products. On the other hand, the ciders available were local brands from craft breweries, that likely have very different brewing procedures and may have used different types and quantities of apples and yeast giving a more diverse range of products. The results from Figure 3 help us to see that low-field NMR in combination with PCA can be used to tell us that beers and ciders **are** different. However, an arguably more important question is **why** are they different?

Notes

Identifying Key Components in Beer and Cider

In the previous section, we determined that PCA can be used to tell us that the spectra are different. In this section, you will learn how to identify which spectral features contribute to the differences between beer and cider. This can be done using spectral loadings. In simple terms, loadings tell us which spectral features (i.e. NMR signals) were important to the separation and which were not. Each NMR signal is given a value (i.e. loading) during the PCA analysis. In MNova these can be displayed in a table, as well as in the spectral features, with the greatest loadings highlighted by bands on top of the spectra. This is shown in Figure 4

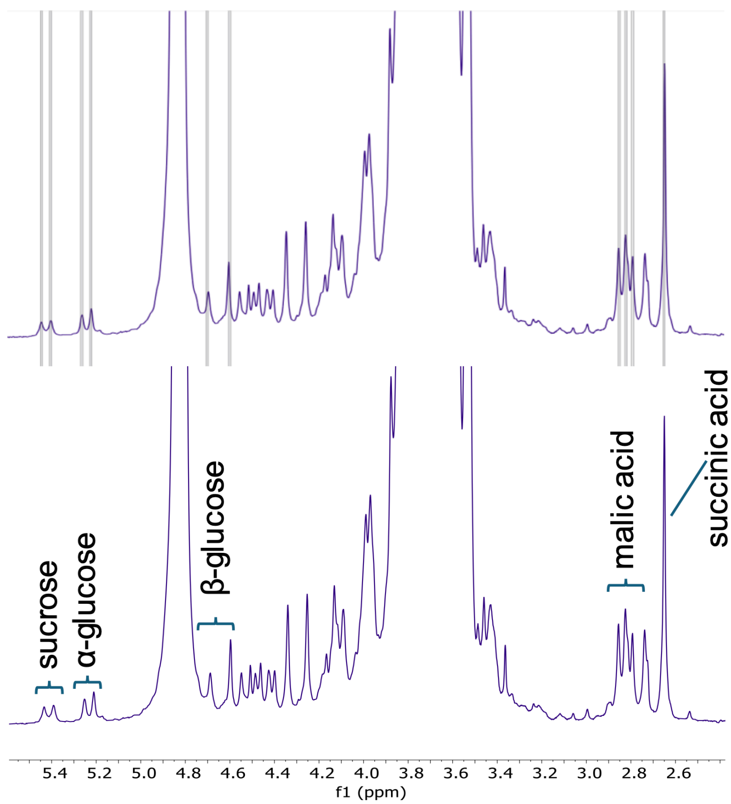

Figure 4 ^{1}H spectra of an example cider. (**A**) The dark bands demonstrate the signals that have strong loadings in PC1. I.E. these are the signals that cause separation along the PC1 axis (X-axis in Figure 3D) and in turn cause the spectra from beer and cider to group differently. (**B**) Assignments of the compounds identified by the loadings.

Figure 4 shows the spectral features that had the highest loadings (PC1) and therefore caused separation along the X axis in Figure 3D. The PCA tells us "these are the signals that matter", or put another way "these are the signals that make cider different from beer". Instead of assigning all the peaks in the mixture, all we have to do is assign the signals that the PCA has identified as important. Here we used the Bruker Bioreference Databases to search for peak matches. However, other databases such as The Human Metabolome Database[11] can be searched freely or even a literature search of the NMR of cider quickly identifies these peaks as sucrose, α-glucose, β-glucose, malic acid, and succinic acid. Ciders are generally sweeter than beers therefore high concentrations of free sugars are not unexpected. Therefore, it is highly logical that these components are important to the compositional profile that defines cider. While many organic acids are derived from apples, both malic and succinic acids have been reported as common and key acids in ciders.[12]

Notes

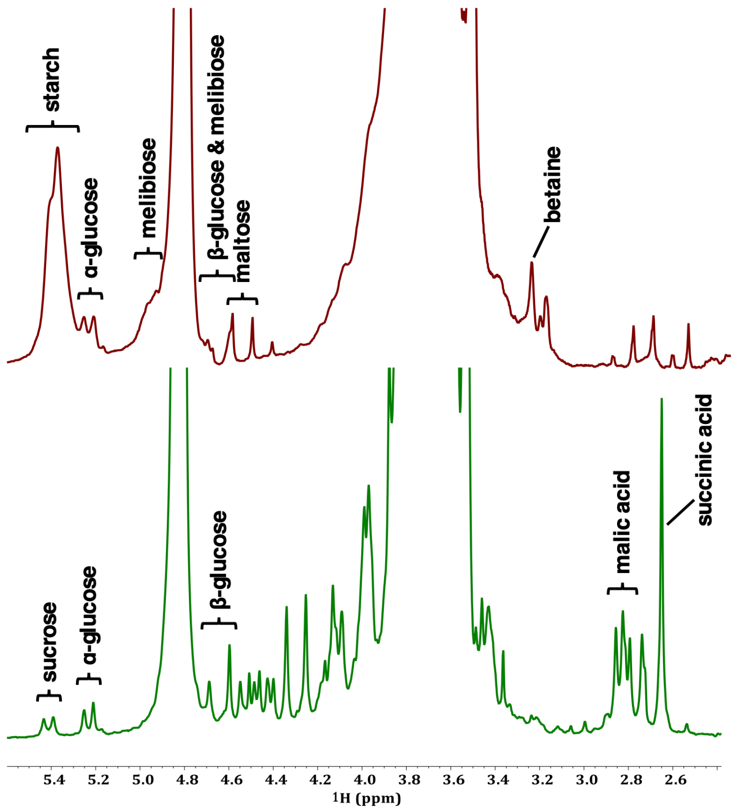

Figure 5 Comparing the [1]H spectra of an example beer (red) and cider (green) sample. Assignments are shown to highlight some of the compounds that are present in the different beverages.

Notes

For comparison, Figure 5 highlights a few of the compounds that can be used to distinguish between beer and cider. For example, betaine, a naturally occurring compound in some beers,[13] was identified in the beer but is not present in ciders. Additionally, beers contain a notable contribution from starch, a fermentable material that is necessary to produce beer, but that is not present in ciders.[14] Signals from maltose, the sugar produced during the fermentation of starch,[15] were also identified and are similarly unique to beers. In the example shown here, melibiose was also identified. The presence of this compound not only differentiates this beer from the cider samples but can be used to differentiate between different types of beer. This is because melibiose is formed as an intermediate in the degradation of raffinose, which is a sugar that is found in lagers but not ales.[16]

Assignment:

To complete the exercise, perform the tasks and answer the following questions:

1. Process your NMR data, and provide an example NMR spectrum, with and without water suppression. Referring to the NMR literature, can key peaks be identified? If it is not possible explain why? If possible, please label relevant peaks and include literature references. The answer will depend here on the types of samples that were chosen and the amount of NMR literature available. Samples such as honey, wine, and juices have a broad base of literature to cite.

2. Carefully reference a peak in your sample across all spectra. This is so all peaks in all samples align as best possible. Do all the sample peaks align perfectly? If not, can you think of a reason why they may not (remember NMR peaks can shift due to pH, temperature, concentration, viscosity)? Note: While the goal of this project is to introduce NMR-based chemometrics on intact samples, often in larger studies looking for human disease in blood

and urine, buffers and internal standards are added to help normalize the samples for better spectral alignment. This question is to get the students to think about sample preparation, before running large statistical analyses.

3. Using the Chemometrics package recommended by your instructor perform PCA on the data. Plot the scores plot for PC1 vs PC2.

4. Look at your PC1-PC2 plots for all the PCA tests conducted. Compare and contrast how the plots change as the regions containing the large peaks are excluded. Can you see a clear separation of your data points according to their type? Explain which PC1-PC2 plot you think gives the most useful results. The answer will depend on the type of sample studied.

5. Look at the PC1 vs PC2 loadings. In some software, this can be a table, in others a spectral-like plot. If you do get separation in the PCA use the loadings to try to identify the compounds responsible for the discrimination between the groups. If you do not get discrimination between the groups in PCA, the loadings can still tell you the most important spectral features that influenced the PCA results. For example, if you were to compare 11 strawberry candies, and 11 raspberry candies, your loading plots might tell you that carbohydrate signals influence the PCA distribution. This would explain that the sugar content across the candies is much more statistically significant than the tiny amount of flavoring that is added. Perhaps this would not be the result you had expected, but it is an important result, nonetheless. As chemometric packages discuss and display loadings differently, the instructor will need to read about the specific package they intend to use. The point is that loadings help explain why the spectra are different, which is a very powerful tool for non-targeted analysis.

6. Short Essay: Think of some other areas in which low-field NMR and PCA can be useful within food science. Discuss the potential of NMR and PCA for understanding components in frequently consumed foods and drinks. Discuss some industries that you think could benefit from a greater use of NMR combined with Chemometrics. This question gets the students to review the literature. An instructor could ask for a minimum page requirement and assign significant marks to this question as it is likely to separate the class nicely in terms of effort and the depth of their literature review.

Notes

Key Take Home Messages:

- NMR is a powerful analytical tool that can be applied to many real-world samples, including beverages, foods, and much more.
- The presence of large peaks like water and ethanol can hide characteristic components of a sample. Data processing tools allow for these large peaks to be eliminated, thus allowing for the visualization of key components in alcohols.
- Each type of alcohol (beer/cider) has its own range of compounds that can be distinguished by the PCA of NMR spectra. This shows the strength of NMR in non-targeted analyses. I.E. asking big questions like "What makes beer and cider different?"

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