CHEM 4590 – 2017

Quantitative Site-Specific Natural Isotope Fractionation Nuclear Magnetic Resonance (SNIF-NMR)
**Stable isotope analytical methods**

Powerful to ensure food and beverage authenticity.

Based on the principle that C, H, O, N of organic matter exist in their naturally occurring isotopic forms - $^{13}\text{C}/^{12}\text{C}$, $^{2}\text{H}/^{1}\text{H}$, $^{18}\text{O}/^{16}\text{O}$, $^{15}\text{N}/^{14}\text{N}$.

Isotopic distribution is influenced by physical, chemical and biochemical factors.

These methods offer a means of verifying botanical, synthetic and geographical origin of a product.

- Isotope-ratio mass spectrometry (IRMS)
- Site-Specific Natural Isotope Fractionation-NMR
The SNIF-NMR® CONCEPT

- Pioneered by Professor G.J. Martin of the University of Nantes
- Further developed by Eurofins Inc., a spinoff company created by Martin
- Uses $^2$H NMR spectroscopy to measure the distribution of deuterium in different sites of a given molecule.
- Official method of the OIV (International Wine Office) and of the European Commission to control wine chaptalization.
- Now an official AOAC (Association of Official Analytical-Chemists) method for detecting sugar addition to fruit juice and for authenticating the natural origin of vanillin.

http://www.eurofins.com
Stable isotopes

<table>
<thead>
<tr>
<th>Element</th>
<th>Isotope</th>
<th>Relative abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen</td>
<td>$^1\text{H}$</td>
<td>99.9855</td>
</tr>
<tr>
<td></td>
<td>$^2\text{H} (\text{D})$</td>
<td>0.0145</td>
</tr>
<tr>
<td>Carbon</td>
<td>$^{12}\text{C}$</td>
<td>98.892</td>
</tr>
<tr>
<td></td>
<td>$^{13}\text{C}$</td>
<td>1.108</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>$^{14}\text{N}$</td>
<td>99.6337</td>
</tr>
<tr>
<td></td>
<td>$^{15}\text{N}$</td>
<td>0.3663</td>
</tr>
<tr>
<td>Oxygen</td>
<td>$^{16}\text{O}$</td>
<td>99.7586</td>
</tr>
<tr>
<td></td>
<td>$^{17}\text{O}$</td>
<td>0.0375</td>
</tr>
<tr>
<td></td>
<td>$^{18}\text{O}$</td>
<td>0.2039</td>
</tr>
</tbody>
</table>

The measurements of the ratios ($X_{\text{heavy}} / X_{\text{common}}$) deliver information about the botanical animal or geographic origin of the products.

Isotopic analyses are very useful for many scientific studies:

- Biomedical studies
- Environmental research
- Food authentication
- Fight against frauds
HDO vs. H₂O

- D/H in water: from ~90 ppm (South Pole) to ~160 ppm (equator).
- H₂O is slightly more volatile than HDO: when water evaporates the remaining liquid phase contains more HDO than the gas phase.
- Transpiration of water from plants favours lighter isotopes (H₂O). There is more D in plants from warmer climates.
• Water in plants is used in the photosynthesis of different compounds, e.g. the production of glucose.

• The D, H, $^{16}$O, $^{18}$O content of water is transferred to glucose and other compounds synthesized in the plant.

• Both the metabolism and physiology of the plant influence the final D content of the sugars.
### Application in food authentication

<table>
<thead>
<tr>
<th>Product</th>
<th>Adulteration</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruit juices</td>
<td>Watering</td>
<td>(D/H) and $^{18}$O-IRMS</td>
</tr>
<tr>
<td>Fruit juices</td>
<td>Sweetening</td>
<td>$^{13}$C-IRMS and SNIF-NMR</td>
</tr>
<tr>
<td>Honey</td>
<td>Addition of inverted and cane sugars</td>
<td>$^{13}$C-IRMS</td>
</tr>
<tr>
<td>Aromas, flavours (vanillin, raspberry ketone, ...)</td>
<td>Mislabelling (artificial ↔ natural)</td>
<td>$^{13}$C-IRMS, $^{18}$O-IRMS, SNIF-NMR</td>
</tr>
<tr>
<td>Alcoholic beverages</td>
<td>Mislabelling (geographical and botanical origins)</td>
<td>$^{13}$C-IRMS, $^{18}$O-IRMS, SNIF-NMR, $^2$H- and $^{18}$O-pyr-IRMS</td>
</tr>
<tr>
<td>Wines</td>
<td>Watering</td>
<td>(D/H) and $^{18}$O IRMS</td>
</tr>
<tr>
<td>Wines</td>
<td><em>Chaptalisation</em> (addition of sugars)</td>
<td>$^{13}$C-IRMS, SNIF-NMR</td>
</tr>
<tr>
<td>Olive oils</td>
<td>Addition of cheaper oils</td>
<td>$^{18}$O-IRMS</td>
</tr>
<tr>
<td>Dairy products</td>
<td>Addition of undeclared milk, mislabelling (geographical origin)</td>
<td>$^{13}$C- and $^{15}$N-IRMS</td>
</tr>
<tr>
<td>Meat (beef, lamb, ...)</td>
<td>Mislabelling (geographical origin) and feeding diet</td>
<td>$^{18}$O-IRMS and $^1$H, $^2$H and $^{13}$C-NMR</td>
</tr>
<tr>
<td>Fish meat</td>
<td>Mislabelling (wild ↔ farmed)</td>
<td>Multi-isotope profiling</td>
</tr>
</tbody>
</table>
Deuterium NMR

- Deuterium has a spin of 1.
- The NMR signal produced by D nuclei is ~1000 times weaker than for H nuclei signal.
- The natural abundance is typically D/H = 0.0145%.
- Overall the NMR D signal is at least $10^5$ times weaker than the H signal.
- D usually yields broad signals with line widths typically varying between a few Hz and a few kHz.
Deuterium NMR

• The spectrum has the same narrow chemical shift range as for $^1$H but low resolution and lower sensitivity make D NMR a poor alternative.

• D-D couplings are about 40 times less important than H-H couplings and are therefore not observed.

• In partially deuterated molecules, D-H couplings can be observed.

• The main uses of D-NMR spectra are for determining the effectiveness of chemical deuteration and for determining the relative amount of D in molecules.
Comparison H-NMR and D-NMR

Amphetamine sulfate-\(d_3\)

In TFA-\(d\)

The methyl is deuterated and there is some deuteration of the \(\text{NH}_2\text{CD}_3\)

http://chem.ch.huji.ac.il/nmr/techniques/1d/row1/h.html
Food sugar vs. ethanol

- Sugars are difficult to study by deuterium NMR.
- Instead, it is possible to detect the D content in CH$_3$CH$_2$ of ethanol produced from the fermentation of sugars.
- Ethanol has D/H ratios representative of original sugars.
- The D content can be determined for each non magnetically equivalent site.
- In ethanol, it is possible to determine the D content of the methyl group (CH$_2$D) and of the methylene group (CHD(OH)).
- Example: NMR can be used quantitatively to determine if ethanol in wine or liquor is from natural grape sugars.
Glycolysis: from glucose to pyruvate
What happens to pyruvate? It depends on the cell and the conditions.

Glc

no $O_2$

2 Pyr

no $O_2$

no $O_2$

2 Ethanol + 2CO$_2$

yeast

2 Lactate

$muscle$

$microbes$

2 Acetyl CoA + 2CO$_2$

TCA

O$_2$

4CO$_2$ + 4H$_2$O

$animals$, $plants$, $microbes$
From pyruvate to ethanol

Again, just enough NAD⁺ is made to replenish glycolysis.

Yeast makes ethanol from pyruvate.
D-NMR spectrum of alcohol from Bacardi rum
Ethanol from wine (grape) ↔ ethanol from beet

From fermentation medium

\[ \text{CH}_3-\text{CHDHOH} \]

\[ (\text{D/H})_\text{II} \]

\[ \text{CH}_3-\text{CH}_2\text{OD} \]

From sugar

\[ \text{CH}_2\text{D-CH}_2\text{OH} \]

\[ (\text{D/H})_\text{I} \]
Since 1991: more than 1,500 authentic wines per year

**SNIF NMR method**

**Quality Control**

Certified Reference Material
Wine: multi-component and multi-isotopic analysis
Role of IR-MS

• Other isotopic indicators ($^{18}$O in wine and $^{13}$C in ethanol) can be used to detect other types of fraud, such as watering down the wine or false declarations of geographic origin.

• These parameters are usually measured by pyrolysis or combustion IR-MS.

• IR-MS does not provide the site-specific information given by deuterium NMR.
The relative deuterium concentration and specific deuterium-site locations in a molecule can be determined using Site-Specific Natural Isotope Fractionation-Nuclear Magnetic Resonance (SNIF-NMR). For a given compound (e.g., ethanol) SNIF-NMR can provide information about the chemical pathway of formation and, in some cases, information about the geographic origin of a sample can also be discerned. SNIF-NMR has been applied to the analysis of wines and other alcoholic beverages. In this work, data were collected on samples of apple brandy, tequila, rum, potato vodka, cognac, and synthetic ethanol. Signal-to-noise considerations limit the samples that can be studied without preconcentration to those with relatively high alcohol contents.
Experimental

- $^2$H$^1$H NMR spectra were recorded on a Bruker DRX-400 FT-NMR spectrometer at 61.38 MHz using a 5-mm broadband tunable probe.

- Spectra were acquired using a 90° pulse (13 s), WALTZ decoupling, and a relaxation delay of 5 s.

- All samples were run unlocked as neat liquids in a 5-mm-diameter coaxial tube at 297 K. A reference standard of 1 μL benzene-D6 in 2 mL of benzene was contained in the sealed, inner coaxial tube.

- The probe was tuned and the spectrometer was shimmed on the proton FID prior to each experiment. Data from 600 pulses were collected in each experiment. The acquisition time for a single experiment was approximately 2 hours.
Three D sites:

\[
\begin{align*}
\text{HOCH}_2\text{CH}_2\text{D} & \quad \text{HOCHDCH}_3 & \quad \text{DOCH}_2\text{CH}_3 \\
(\text{I}) & \quad (\text{II}) & \quad (\text{III})
\end{align*}
\]

As D NMR signals for HDO (from solvent) the -OD group of ethanol often overlap, site (III) should be ignored.
- Water in the fermentation medium affects the D content of methylene (II)

- Sugar determines the D content of methyl (I)

$R$ describes the relative D distribution in the ethyl portion of the molecule.

II = methylene  \[ R = \frac{3(\text{II})}{(\text{I})} \]

I = methyl

- With no isotope fractionation (even distribution of D), $R = 2$.

- Experimentally, $R$ values observed are always $\geq 2$.

- $R \gg 2$ indicates D enrichment on methylene (cold climate)

- $R > 2$ indicates D enrichment on methyl (warm climate)
The R value alone is adequate for discerning some, but not all sugar sources. The relative deuterium concentration, \( C \), can provide additional information about samples.

\[
C = \frac{(I) + (II)}{(s)\kappa}
\]

(I) and (II) are the peak heights of the methyl and methylene sites

(s) is the peak height of the external standard.

To account for different ethanol concentrations, a correction factor, \( \kappa \), is employed.
Results

- Ethanol samples from the same source (i.e., sugar cane grown at one location) should have similar R and C values.

- Ethanol from different sources (e.g., sugar cane vs. potato) or different locations should have R and C values that can be discerned from one another.

- Synthetic ethanol?
**TABLE 1.** Statistical data for the experimentally determined relative deuterium concentration in the alcohol fermented from various sugar sources.

<table>
<thead>
<tr>
<th>Sample Source</th>
<th>No. of Expmnts</th>
<th>Value</th>
<th>Average</th>
<th>Standard Deviation</th>
<th>Standard Deviation of the Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic</td>
<td>15</td>
<td>R</td>
<td>2.262</td>
<td>0.027</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>1.464</td>
<td>0.010</td>
<td>0.003</td>
</tr>
<tr>
<td>Apple</td>
<td>20</td>
<td>R</td>
<td>2.552</td>
<td>0.032</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>1.140</td>
<td>0.016</td>
<td>0.004</td>
</tr>
<tr>
<td>Grape</td>
<td>10</td>
<td>R</td>
<td>2.528</td>
<td>0.023</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>1.173</td>
<td>0.012</td>
<td>0.004</td>
</tr>
<tr>
<td>Potato</td>
<td>15</td>
<td>R</td>
<td>2.697</td>
<td>0.043</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>1.083</td>
<td>0.014</td>
<td>0.004</td>
</tr>
<tr>
<td>Sugar Cane</td>
<td>11</td>
<td>R</td>
<td>2.312</td>
<td>0.019</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>1.232</td>
<td>0.012</td>
<td>0.004</td>
</tr>
<tr>
<td>Agave</td>
<td>20</td>
<td>R</td>
<td>2.235</td>
<td>0.027</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>1.203</td>
<td>0.007</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Industrial preparation of ethanol

Before the reaction occurs, ethene is adsorbed onto the catalyst surface.

After the reaction, ethanol is desorbed from the surface.

Hydrogen ions are obtained from, and lost to, the catalyst surface.
FIGURE 2. Deuterium in Ethanol
To sum up:

Detection of wine chaptalisation

\[ \text{SNIF-NMR}^\circ \text{ is the official method for the detection of over enrichment or chaptalisation of wines (EC 2676/1990).} \]

The method can also be used to control sugar addition in sweet wines. This application has been used by the sweet wine producers of the Anjou region in France to guarantee the authenticity of their produce.

Other applications include: botanical origin of alcohol in spirits, agricultural origin of bioethanol.