Challenges in Nuclear Magnetic Resonance Spectroscopy Based Non-Targeted Analysis

Freddy Thomas, Dr. Eric Jamin – Eurofins Analytics France
28 – 29 November 2016, Berlin
Eurofins’ experience in non-targeted testing using 1H NMR

The advantages of using 1H NMR to ensure food authenticity

What are the challenges to the successful standardisation of non-targeted 1H NMR

Some solutions: what has already been achieved and what could be done in the future
Key figures
Eurofins group

✔ 1.95 billion € of TO
✔ 25,000 staff
✔ 39 countries

✔ 250 laboratories
✔ 130,000 analytical methods
✔ > 150 million assays performed each year
Specialised in food authenticity testing

Eurofins Analytics France laboratory based in Nantes (France) is the group Competence Centre for authenticity of food products.

Nantes, France
Our vision of market needs

Analyses must help to enforce regulations & protect brands but:

- Need for a high number of parameters
- Often limited time, and
- Always a limited budget

- Targeted analysis efficiency is often limited
- A large part of adulterations are still undetectable

➔ Profiling 1H-NMR is a complementary tool
Eurofins experience in NMR non-targeted approach

• 4 NMR spectrometers in Nantes (2 for SNIFNMR, 2 for Profiling)
  BRUKER 400MHz with autosampler, BCU and BOSSIII
  2 qualified instruments for non-targeted approach
  2 BBI probes

• a dedicated Production Unit Profiling NMR (since 2012)

• > 5000 routine analyses in 2015
  fruit juices@, wines@, honey@, coffee@, soft drinks@, milk, spices....

@ ISO-17025 accreditation for quantification (for the moment…)
(scope available under http://www.cofrac.fr/Annexes/Sect1/1-0287.pdf)
Two approaches using the same experiment

Targeted

Non-targeted
Features of 1H-NMR Profiling

- Complex signal with multiple resonances for a single compound
- Primary method for quantification no need to calibrate each compound
- High reproducibility, even inter-laboratory
- Non-targeted detection of all protons
- 1H-NMR profile can be regarded as unique fingerprint of the sample
- Long-term build of reference databases possible
- Retrospective analysis possible also quantification of further compounds

Example: preparation and acquisition in 3 different labs (wine sample)
Eurofins routine applications for **non-targeted 1H NMR**

- **Fruit juice**
  - Fruit type
  - Concentrate v. NFC
  - Adulteration
  - Origin

- **Coffee**
  - Arabica v. Robusta
  - Other varieties
  - Adulteration

- **Wine**
  - Grape variety
  - Adulteration

- **Honey**
  - Adulteration
  - Origin

And more...
The challenges to the standardisation of non-targeted $^1$H NMR

**Sample preparation:** differences at this stage can lead to different NMR spectra for the same sample.

$^1$H NMR **measurement:** a clear protocol is required (field strength, pulse type, acquisition time, etc.) to produce repeatable/reproducible data.

**Data processing and analysis:** different statistical approaches can lead to different evaluations.

**Interpretation of product authenticity:** relies on the existence of a comprehensive database.
The challenges to the standardisation of non-targeted 1H NMR

What can be done to ensure standardized sample preparation and $^1$H NMR measurement?

**Need:**
- Include internal quality control measures
- Carry out regular comparison of NMR instruments (internally and in peer to peer comparison)
- Validation, qualification

- SOP preparation
- SOP acquisition
- Quality criteria
Collaborative analytical tools: 
**FoodScreener™ - Platform-Concept**

Large databases for widely spread single-ingredient commodities

- **SGF-Profiling™ Module**
  - >16,000 samples

- **Wine-Profiling™ Module**
  - >19,000 samples

- **Honey-Profiling™ Module**
  - Now: >4,000 samples
  - Soon: >8,000 samples

Standardized Platform FoodScreener™
- Quantification
- Classification models
- Verification models
- Non-targeted
Internal Reference Material, two per session
Daily Quality Control

<table>
<thead>
<tr>
<th>QC1</th>
<th>EX1</th>
<th>EX2</th>
<th>EX 3</th>
<th>EX 4</th>
<th>EX 5</th>
<th>EX 6</th>
<th>EX 7</th>
<th>EX 8</th>
<th>QC2</th>
</tr>
</thead>
</table>

Black => QC of the day
Blue and Green => upper and lower limits based on 3 weeks characterisation (more than 20 experiments)
Comparison between our 2 instruments - Qualification

Red -> instrument A
Black -> instrument B

coffee

honey
Inter-laboratory process validation: Peer to Peer comparison Bruker - Eurofins

Lab Comparison

1.1 White Wine
1.1.1 Spectroscopic View

Methods: Wine-Profiling

Lab 1: Bruker BioSpin GmbH, Rheinstetten
Lab 2: Eurofins, Nantes
Date: March 2013

Following figure shows the spectroscopical comparison of the white wine samples prepared in both labs (4 samples in each lab, phase 3). There is no evidence for a significant difference between the preparation and measurement of both labs besides the normal achievable variation.

The signals of tartaric acid are all between 4.5820 ppm and 4.6120 ppm which indicates an appropriate sample preparation (including pH-adjustment, refer to following figure). The actual deviation between both labs coincides with the known achievable variation.

1.1.2 Quantitative Comparison

Following table shows the comparison of some quantified parameters of the white wine samples which were prepared in both labs (4 samples in each lab, phase 3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>( x_1 \text{ Lab1} )</th>
<th>( x_2 \text{ Lab2} )</th>
<th>rel. comparison</th>
<th>rel. std. Lab2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3-butanediol</td>
<td>382</td>
<td>385</td>
<td>100.6</td>
<td>5.9</td>
</tr>
<tr>
<td>3-methyl-butanol</td>
<td>213</td>
<td>206</td>
<td>96.1</td>
<td>0.7</td>
</tr>
<tr>
<td>acetic acid</td>
<td>355</td>
<td>343</td>
<td>96.8</td>
<td>1.4</td>
</tr>
<tr>
<td>alanine</td>
<td>27</td>
<td>28</td>
<td>101.7</td>
<td>5.0</td>
</tr>
<tr>
<td>ethanol</td>
<td>9344</td>
<td>9557</td>
<td>101.7</td>
<td>0.6</td>
</tr>
<tr>
<td>fructose</td>
<td>2673</td>
<td>2660</td>
<td>99.5</td>
<td>1.9</td>
</tr>
<tr>
<td>glucose</td>
<td>792</td>
<td>725</td>
<td>95.1</td>
<td>12.6</td>
</tr>
<tr>
<td>glycerol</td>
<td>4952</td>
<td>4912</td>
<td>99.2</td>
<td>1.3</td>
</tr>
<tr>
<td>lactic acid</td>
<td>294</td>
<td>295</td>
<td>100.4</td>
<td>8.2</td>
</tr>
<tr>
<td>malic acid</td>
<td>1919</td>
<td>1880</td>
<td>96.9</td>
<td>1.5</td>
</tr>
<tr>
<td>succinic acid</td>
<td>617</td>
<td>594</td>
<td>96.3</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Result:

There is no significant deviation between Lab1 and Lab2 (mean values). The standard deviation of Lab2 is for high-concentrated compounds less than 2% (e.g., ethanol, malic acid) which indicates an appropriate sample preparation.
Periodic comparison: wine, juice, honey

Results Summary

<table>
<thead>
<tr>
<th>Type of Analysis</th>
<th>Analysis ID</th>
<th>Result</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classification Analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White Wine Variety</td>
<td>Wi 1104-01/0001</td>
<td>In-Model</td>
<td></td>
</tr>
<tr>
<td>Targeted Analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantification</td>
<td>Wi 01001</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Comparison by NMR Reference Database</td>
<td>Wi-QC-0707</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Untargeted Verification Analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Univariate Verification</td>
<td>Wi 2002-02/05</td>
<td>In-Model</td>
<td></td>
</tr>
<tr>
<td>Multivariate Verification</td>
<td>Wi 2002-02/05</td>
<td>In-Model</td>
<td></td>
</tr>
<tr>
<td>Wine Content Analysis</td>
<td>Wi 2002-02/05</td>
<td>In-Model</td>
<td></td>
</tr>
</tbody>
</table>

Untargeted Verification Analysis

Applied Model: Pinot Gris/Gris

Univariate Verification

Result: No deviation was detected in univariate verification (In-Model).

Multivariate Verification

Result: No deviation was detected in multivariate verification (In-Model).

Wine Content Analysis

Result: Based on the comparison with the reference database, there is no indication for an addition of water.
The challenges to the standardisation of non-targeted 1H NMR

Data processing and analysis

- What are the effects of processing and how can discrepancies be overcome?

=> automatic process is the safest solution! (Matlab routines, FoodScreeneer...)
Collaborative study Example: Wheat: organised by a PT organisator (only quantification)

Validation of NMR fingerprinting methods: effects of processing on measure reproducibility and laboratory performance assessment

Table 2

<table>
<thead>
<tr>
<th>Registered Participants</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Available NMR spectrometers</td>
<td>46</td>
</tr>
<tr>
<td>Delivered set of samples</td>
<td>46</td>
</tr>
<tr>
<td>Spectrometers producing results</td>
<td>39</td>
</tr>
</tbody>
</table>

Magnetic Field (Larmor frequency)

<table>
<thead>
<tr>
<th>Magnetic Field</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.4 T (400 MHz)</td>
<td>16</td>
</tr>
<tr>
<td>11.7 T (500 MHz)</td>
<td>7</td>
</tr>
<tr>
<td>14.1 T (500 MHz)</td>
<td>14</td>
</tr>
<tr>
<td>16.4 T (700 MHz)</td>
<td>2</td>
</tr>
</tbody>
</table>

Spectrometer manufacturers

<table>
<thead>
<tr>
<th>Spectrometer manufacturers</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bruker</td>
<td>35</td>
</tr>
<tr>
<td>Varian</td>
<td>4</td>
</tr>
</tbody>
</table>

Innovative Solutions S.r.l., zona H 150/B, 70015 Noci (BA), Italy
Contact person: Prof. Vito Gallo
Phone: +39 0805963607
Email: direzione@innovative-solutions.it
Table 3

<table>
<thead>
<tr>
<th>Session</th>
<th>Number of operators</th>
<th>Processing procedure</th>
<th>Software</th>
<th>Integration mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>ILC1</td>
<td>many</td>
<td>phase and baseline correction according to operator expertise</td>
<td>no limitation</td>
<td>no limitation</td>
</tr>
<tr>
<td>ILC2</td>
<td>one</td>
<td>manual phase correction and automatic baseline correction</td>
<td>TOPMIX &amp; AMIX</td>
<td>integral</td>
</tr>
<tr>
<td>ILC3</td>
<td>one (different from session ILC2)</td>
<td>manual phase correction and automatic baseline correction</td>
<td>NMR spectra</td>
<td>run</td>
</tr>
<tr>
<td>ILC4</td>
<td>one (the same as session ILC3)</td>
<td>manual phase correction and automatic baseline correction</td>
<td>NMR spectra</td>
<td>peak</td>
</tr>
</tbody>
</table>

*Integration method is named differently according to the different software.

It is important to point out that laboratory performance assessment is strongly dependent on the operator. It was found that laboratories obtaining unacceptable |z-scores| (>10) in the first elaboration, ILC1, gained better results in the new elaborations ILC2-4 carried out by a single operator and in many cases their performance were satisfactory. No substantial effects of the software and of the integration procedure were found.

In line with the results reported in the previous volume, the new data elaboration indicate that the NMR experiment (1D iH NOESY) proposed for the fingerprinting of wheat and flour aqueous extracts is a robust experiment. In fact, the majority of the participants produced NMR spectra that can be considered “statistically equivalent”. Thus, the main goal of this inter-laboratory comparison, the validation of the 1D iH NOESY experiment, was achieved.
The challenges to the standardisation of non-targeted 1H NMR

How to avoid errors in the interpretation of product authenticity

=>a unique validated database is the key!
Validation Files
- Classification Models -

- Validation by Monte-Carlo/Cross-Validation
- Analysis of Confounders
- Wrong prediction rate < 3%

### Overview of classes

<table>
<thead>
<tr>
<th>Class</th>
<th>Samples</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-Acacia</td>
<td>2753</td>
<td>96.3</td>
</tr>
<tr>
<td>Acacia</td>
<td>107</td>
<td>3.7</td>
</tr>
<tr>
<td>Total</td>
<td>2860</td>
<td>100.0</td>
</tr>
</tbody>
</table>

#### 3.2.1 PLIMIT = 0.01

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>Amb.OK</th>
<th>None</th>
<th>Wrong</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: non-Acacia</td>
<td>96.2</td>
<td>2.3</td>
<td>0.0</td>
<td>1.5</td>
<td>2.3</td>
</tr>
<tr>
<td>2: Acacia</td>
<td>97.2</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
<td>2.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>Double</th>
<th>Triple</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: non-Acacia</td>
<td></td>
<td></td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2: Acacia</td>
<td></td>
<td></td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

- Correct Prediction Rate Unique: 96.2%
- Correct Prediction Rate Ambiguous: 0.0%
- Correct Prediction Rate Total: 96.2%
- False Prediction Rate: 2.2%
- Prediction Rate 'None': 1.6%
Larger Collaborative studies already made

Method validation

Peer to peer

Collaborative Study
1-Juice : In the frame of BIPEA PTS

5 instruments routine conditions (=PTS)

4. Results from SGF-ProFiling™

In our 2015 inter-laboratory Comparison all cooperation laboratories using the SGF-ProFiling™ in their routine work participated with this proton NMR technique, as it was done in 2014.

Type of Fruit “Apple” was assigned.

Following clusters are available:


E: Cranberry, E: Black Currant, E: Sour Cherry, E: Blueberry, E: Raspberry, E: Blueberry, E: Blackberry, E: Green Apple, E: Jamaican Guava

Figure 1: Assignment of sample to Apple juice

The results provided from these laboratories are quite impressive since all of them identified the samples as apple juice and as 100% juice from concentrate.

Figure 2: Assignment of product type: from concentrate

All the participating laboratories reported that the sample fits in the multivariate verification model:

Figure 3: Sample fitting in the multivariate verification model

As for the analysed parameters all the laboratories gave similar values, and moreover, the obtained values are very much in line with the target values. In Table 4, the values obtained by the NMR can be seen.
Quantitation of Compounds in Wine Using $^1$H NMR Spectroscopy: Description of the Method and Collaborative Study

Anlage 1: Verzeichnis der Teilnehmer

15 instruments
Manual integration

8 instruments
Automatic integration

Part of the collaborative study included the integration of signals and data evaluation in automatic mode with WineScreener™ (Bruker BioSpin). This indicates that the elimination of personal effects/influences during the spectral evaluation did have a decisive influence on the reproducibility of the results, notwithstanding uniform instrumental equipment and measurement settings.
Next challenge

Method validation

Peer to peer

Collaborative Study

Proficiency Test
Our suggestion: from FIT-PTS towards Profiling-PTS

- **FIT-PTS:**

  Initiated in 1994 => more than 20 years of experience!

  Dedicated to Food analysis using Isotopic Techniques (IRMS, SNIFNMR)

  Complies with the ISO/IUPAC/AOAC International Harmonised Protocol for Proficiency Testing of analytical laboratories

  70 participants (worldwide)

  Recognized by accreditation bodies
Our vision of future NMR profiling proficiency testing

- **Profiling PTS**
  Initial project: matrices shared with isotopic PTS (Wine, Juice, Honey)

  1 sample per trimester

  Targeted: calculation of z-scores
  Non-targeted: Classification scores? ....

  Spectra evaluation and quantifications?

  A need for accreditation and commercial acceptance

  First Round will be in 2017, in parallel with the FITPTS distribution,

  Eurofins will take care of the organisation (preparation, parcels, results...)
Accreditation process

- Accreditation: Formal recognition of laboratory’s competence to carry out a specific test.
- Proficiency Testing: Performance assessment of participating laboratory.
- Quality Control: Continuous verification of analytical system to ensure consistent quality.
- Validation: Fitness for purpose of analytical system (method protocol, type of matrix, concentration range of analyte).

ISO 17025: inter-laboratory accreditation

**Preparation**
- standardized operating procedure (SOP)

**Acquisition**
- Criteria to have spectra validated (SOP)

**Analysis and Reporting**
- reference database and analysis routines (chemometrics, models) (SOP)

Data Analysis Server at Bruker

Ok with ISO 17025
( = subcontract of the last part)

Work in progress
Thank you for your attention!

Roundtable Discussion after Jana’s speech...