

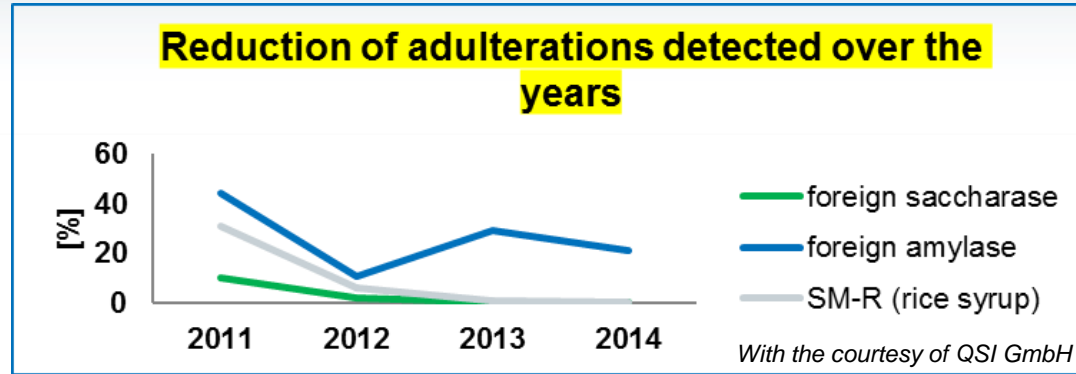


Honey Authenticity Seminar 13.11.2019

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The Need for Multi-Marker and Non-Targeted Methods to tackle Dynamic Food Fraud



- Standard targeted methods more and more fail to detect fraud, as the fraudsters become more sophisticated and learn quickly how to deceive these tests.
- Significant reduction of the positive findings by such techniques after app 2 years.
- Costs of looking for specific markers of adulteration and the inability to keep up with the fraudsters has resulted in the amount of adulteration soaring tremendously in the last decade.

Example Canadian Food Inspection Agency



- 240 honey samples collected (including importers, packers, retailers...).
- The samples were tested with: Stable Isotope Ratio Analysis and NMR.
 - 52 samples were unsatisfactory by both methods: 21.7%
 - 16 samples were unsatisfactory for SIRA testing: 6.37%
 - 44 samples were unsatisfactory for NMR testing: 18.3%
- As of January 2019, CFIA had taken enforcement actions that prevented an estimated 12,762 kg of adulterated honey valued at \$76,758 CAD from entering the Canadian market.
- CFIA intends to use the results of this surveillance to improve the targeting of future sampling and inspection activities, inform analytical needs, and refine program design.
- CFIA also plans to apply lessons learned to other commodities at risk.

Example Famille Michaud:

NMR vs. conventional methods for detection of sugar syrup in Honey



SAMPLES NUMBER	DETECTED ADULTERATED USING C3/C4	DETECTED ADULTERATED BY NMR
5035	80	508

SAMPLES NUMBER	DETECTED ADULTERATED USING FOREIGN OLIGOSACCHARIDES ELSD	DETECTED ADULTERATED BY NMR
2659	50	515

With the courtesy of *Famille Michaud*
APICULTEURS DEPUIS 1920

Example Famille Michaud:

NMR vs. conventional methods for detection of sugar syrup in Honey



DATA-RESULTS-19 GEOGRAPHICAL ORIGIN-112 BOTANICAL ORIGIN

When no adulteration is detected by conventional methods, NMR can still detect exogenous sugars

SAMPLES NUMBER	DETECTED COMPLIANT USING ALL TYPES OF CONVENTIONAL METHODS	DETECTED ADULTERATED BY NMR
2115	2115	421

20% of adulterated samples pass all conventional methods, but are detected with NMR

With the courtesy of *Famille Michaud*
APICULTEURS DEPUIS 1920

1H NMR overview



- The **signal patterns** (number of peaks, relative intensity of peaks, distance between peaks) are unique for each compound and can be used to **identify** them amongst other signals in a mixture.
- The **integral values** can be used to **quantify** the compounds, as long as their chemical structures and signal assignment is known.

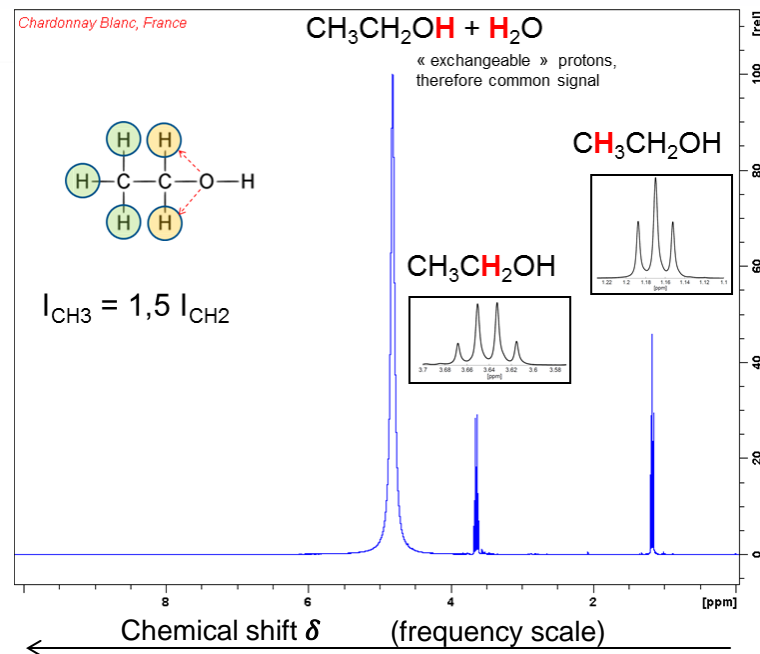
$$I_x = k_s \cdot N_x$$

I_x = Integral value

N_x = number of protons contributing to I_x

k_s = "spectrometer constant"

Example: ethanol in wine



1H NMR overview



- The integral values can be used to quantify the compounds, as long as their chemical structures and signal assignment is known.

$$I_x = k_s \cdot N_x$$

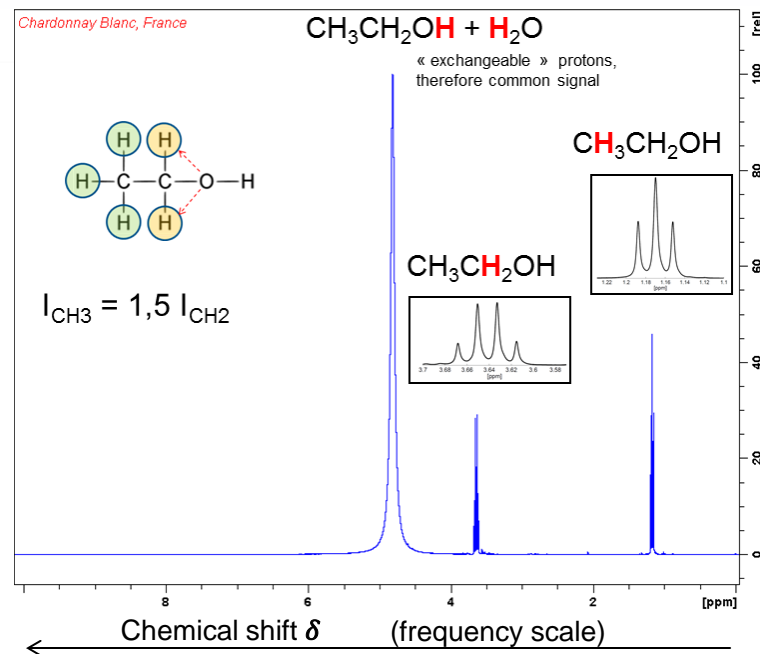
I_x = Integral value

N_x = number of protons contributing to I_x

k_s = "spectrometer constant"

- NMR is quantitative by nature**
- No need for compound-specific calibrations
- External calibration with a reference compound allows absolute quantification of compounds

Example: ethanol in wine



Reproducibility

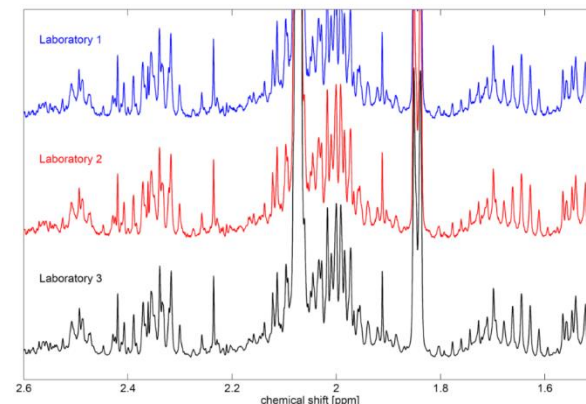


While working under identical conditions of sample preparation and measurement, a sample analyzed in different laboratories gives the same result.



Advantages:

- ❖ Cooperation between laboratories to create a common database and common data analysis methods
- ❖ Creation and maintenance of long-lasting databases
- ❖ Reliable statistical analysis
- ❖ Reliable automated quantification of compounds
- ❖ Retrospective analysis



Example of a wine

Pre-requisite: usage of ***common SOPs***

^1H -NMR Food Screening applications



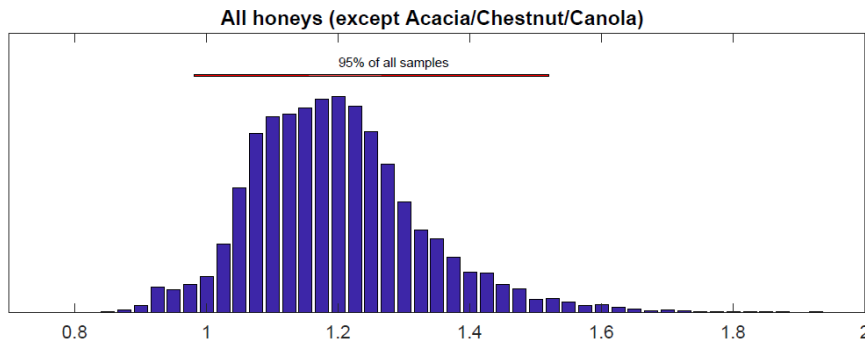
Targeted and non-targeted analysis

-Compound quantification, fingerprinting and detection of deviations in one run

- Purity of the product
- Authenticity of declared origin
- Authenticity of declared variety
- Typicality of the product
- Quantification of ingredients or markers

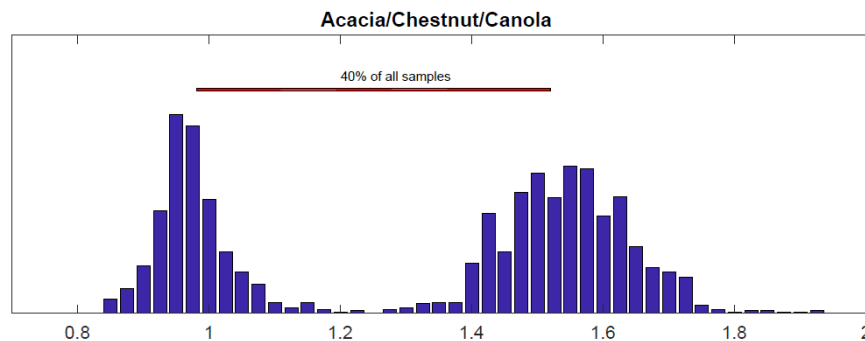
The importance of the database:

Validation of markers and related thresholds



- Independently of the analytical technique used (NMR, MS, HPLC-UV...) and of the parameter measured (unknown compound or identified molecule like e.g. mannose):

To define what is "normal" it is mandatory to have a set of representative samples, to make sure markers chosen are not naturally present in a specific type of honey.



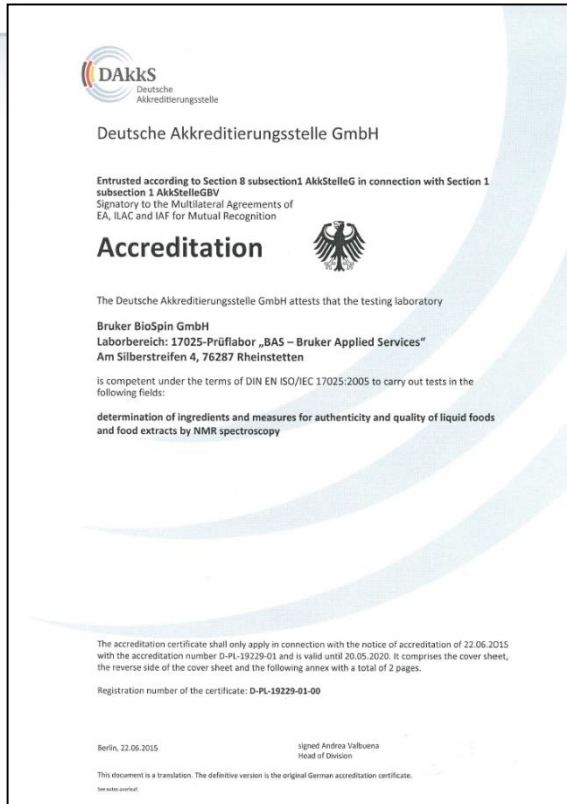
➤ **AVOID THE RISK OF FALSE POSITIVE**

(False positive = authentic sample detected as being adulterated)

- In NMR Honey-Profiling, the markers are all in the spectral regions related to sugars, which is less sensitive to origin.

Example of Fructose/Glucose ratio

ISO 17025 accreditation



Flexible-scope accreditation

- ISO-17025 accredited for liquid food and food extracts
- present accreditation includes the matrices fruit juice, wine, honey, and coffee extracts
 - quantification of components
 - statistical analysis - authenticity and quality



Database: example for honey



- 16100 authentic samples / 1900 adulterated samples
- > 50 country of origin & 100 botanical varieties.
- Monofloral and polyfloral honeys from a single country source.
- Blends of honeys from different countries.
- 2.000 honey dew
- 1.500 industrial honeys (or "baker honey")

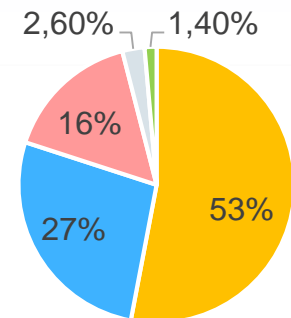
Samples per country in the Data Base:

> 100: USA, Guatemala, Austria, El Salvador, Serbia, Tanzania...

> 200: New Zealand, Brazil, Vietnam, Thailand, India, Turkey...

> 500: Spain, Germany, Mexico, Cuba, Romania, Chile...

> 1.000: China, Ukraine, Argentina, France...



■ Europe

■ America

■ Asia

■ Australia-Oceania

■ Africa

Detection of Sugar Syrups

NMR Honey-Profiling

- **Quantitative analysis** of many parameters: up to 60 parameters per sample
- The values are compared to the reference thresholds
- Reference thresholds have been determined and validated thanks to a large database of authentic and known adulterated honeys from various countries and botanical varieties

Detection of Sugar Syrups

(Analysis-ID: HO-2000-02/0167)

Following tests have been applied in order to detect sugar syrups:

Nr	Type	Description	Result	Value	Limit	Out
1	Intensity /Ratio	3.263 (absolute quantitative)	passed	475	<1279	-
2	Intensity /Ratio	5.077 (absolute quantitative)	passed	154	>39	-
3	Intensity /Ratio	3.636 (absolute quantitative)	passed	2604	<4674	-
4	Intensity /Ratio	4.262 (absolute quantitative)	passed	74	>29	-
5	Intensity /Ratio	4.195 (absolute quantitative)	passed	128	<1200	-
6	Intensity /Ratio	5.271 (absolute quantitative)	passed	30.6	>5.6	-
7	Intensity /Ratio	4.280 (absolute quantitative)	passed	58	>20	-
8	Intensity /Ratio	5.113/(3.270-3.310)	passed	0.005	<0.036	-
9	Intensity /Ratio	4.496/(3.270-3.310)	passed	0.039	>0.012	-
10	Intensity /Ratio	5.334/(5.270-5.300)	passed	0.08	<0.13	-
11	Intensity /Ratio	3.546/(5.270-5.300)	passed	1.15	>0.62	-
12	Intensity /Ratio	3.740/(5.270-5.300)	passed	3.2	>1.2	-
13	Intensity /Ratio	3.857/(5.200-5.260)	passed	0.0145	>0.0037	-
14	Intensity /Ratio	4.150 (absolute quantitative)	passed	258	>115	-
15	Intensity /Ratio	5.181 (absolute quantitative)	passed	50	>24	-
16	Intensity /Ratio	4.055/(5.030-5.070)	passed	3	<46	-
17	Intensity /Ratio	1.809/(5.030-5.070)	passed	0.2	<1.0	-
18	Intensity /Ratio	3.708/(5.030-5.070)	passed	312	<872	-
19	Intensity /Ratio	6.765/(5.250-5.270)	passed	0.009	<0.046	-
20	Intensity /Ratio	2.200/(5.305-5.315)	passed	0.236	>0.019	-
21	Intensity /Ratio	3.326/(3.270-3.310)	passed	0.415	>0.034	-
22	Intensity /Ratio	4.037/(3.270-3.310)	passed	1.56	>0.73	-
23	Intensity /Ratio	4.006/(5.270-5.300)	passed	1.10	>0.70	-
24	Intensity /Ratio	3.564/(5.270-5.300)	passed	20.2	>10.0	-
25	Intensity /Ratio	5.388/(5.370-5.400)	passed	0.19	>0.13	-
26	Intensity /Ratio	3.524/(4.075-4.110)	passed	0.064	<0.070	-
27	Intensity /Ratio	3.182/(4.075-4.110)	passed	0.0024	<0.0045	-
28	Intensity /Ratio	3.785/(4.075-4.110)	passed	0.060	>0.036	-
29	Intensity /Ratio	3.857/(4.075-4.110)	passed	0.0093	>0.0021	-
30	Intensity /Ratio	4.267/(4.970-4.990)	passed	1.5	<4.7	-
31	Intensity /Ratio	4.276/(4.970-4.990)	passed	0.3	<5.4	-
32	Intensity /Ratio	4.204/(5.090-5.110)	passed	1.1	<5.7	-
49	Quantification	Fructose/Glucose	passed	1.12	>0.85 and <1.95	-
50	Quantification	Fructose+Glucose	passed	69.4	>40	-
51	Quantification	Turanose	passed	1.43	>0.3	-
52	Quantification	DHA(D) and Mannose(M)	passed	3 / 0.000	D<30 or M<0.05	-
53	Quantification	Sucrose	passed	0.2	<15	-

False declaration of origin



- Hiding real country of origin or variety.
- Intend: Avoid higher tariff rules, avoid further testing, reach higher market value
- Transshipment = sending the honey to another country where it is relabeled before being exported.
- **Pollen grains manipulation** to deceive detection by pollen analysis:

Filtration of endogeneous pollen grains and addition of pollen grains from another country

Floral source (e.g. Manuka)

Riddle of how 1,700 tons of manuka honey are made... but 10,000 are sold



Geo. origin (e.g. Transshipment)

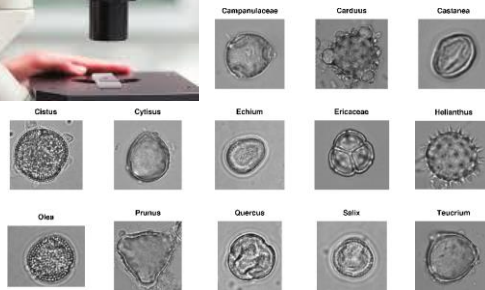
China to US, via quick-change ports

US: FOOD LADDER: re-labeled re-embarked honey transshipments from China through third countries to dodge US customs



Detection of false declaration of origin

Pollen analysis



- Identification and counting pollen grains by microscopy
- Requires a trained expert and is time consuming
- Not working on pollen-filtered honeys.
- Not able to differentiate the real pollens from exogeneous pollen grains.

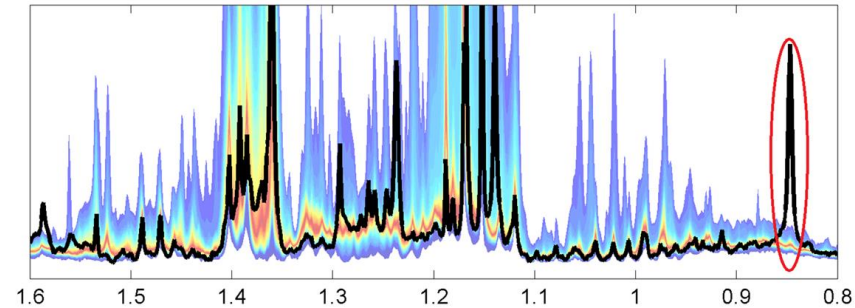
Detection of false declaration of origin

Marker compounds



- Compounds commonly used as markers can often be synthetically produced and added into the honey.
- E.g. DHA and MGO are cheap chemicals
- The non-compliance of marker compound(s) can be used to detect a fraud
- However, the **compliance of 1 or few markers is not sufficient to prove authenticity**

Mixture of 47% Manuka honey with 53% Thyme honey



Result in detail:

6 elevated signals observed, not typical for Manuka
(0.846high 0.850high 6.359high 6.363high 6.400high 6.404high)

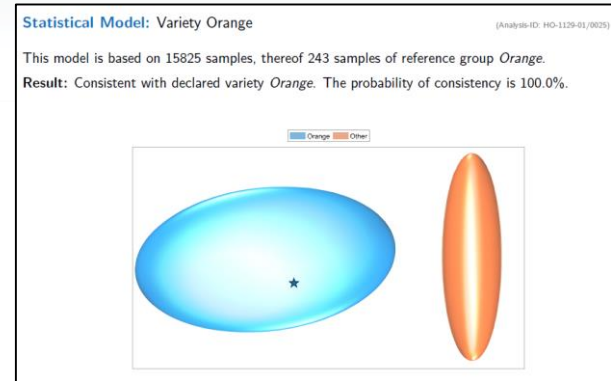
With the courtesy of QSI GmbH

Detection of false declaration of origin

NMR Honey-Profiling

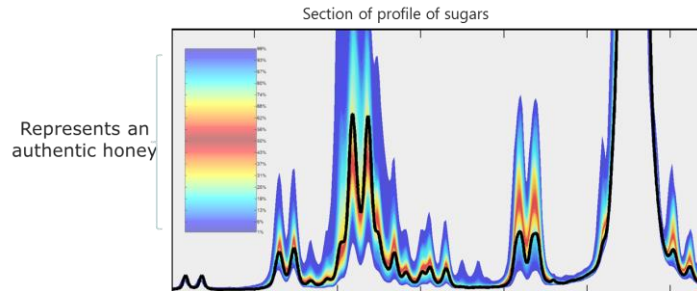


- Based on the **complete chemical composition of the honey** observed by 1H-NMR
- Statistical analysis of the NMR spectrum: untargeted buckets/variables.
- Differentiation of the supposed/declared origin or variety with all other ones present in the Database (50 countries / 100 varieties).
- Statistical models have been validated by Monte Carlo cross validation (Criteria: TP > 98%)
 - ***Very difficult/ impossible to deceive***
 - ***No need for an expert and fast***
 - ***Applicable to pollen-filtered honey***



Detection of atypical samples

- Statistical comparison of the NMR profile with “normal” profiles from the same floral source
- Potential to detect new frauds at early stage.



Non-Targeted Verification Analysis

Univariate Verification

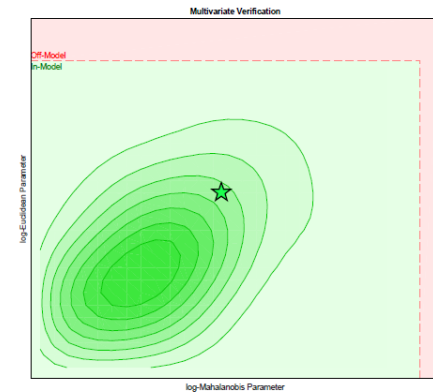
Applied Model: Eucalyptus

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

Multivariate Verification

Applied Model: Eucalyptus

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



Quantitative analysis

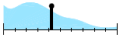




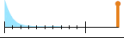


Quantitative Analysis

(Analysis-ID: HO-Q/1363)

In the following table the results of the quantitative analysis are given. The concentrations are obtained by direct quantification. Parameters labelled with * are calculated parameters. The reference range is derived from the *Eucalyptus* samples in the Honey-Profiling Database. The reference range bases on 158 samples.

- Absolute quantification
- Sugars, organic acids, amino acids, freshness and quality criteria
- Comparison to reference values (according to Database samples)

Compound	Value	Unit	LOQ	Reference Range	Flag
alanine	22	mg/kg	5	<5  46	●
aspartic acid	<LOQ	mg/kg	150	<150 mg/kg in reference dataset	●
glutamine	<LOQ	mg/kg	200	<200  295	●
leucine	<LOQ	mg/kg	40	<40 mg/kg in reference dataset	●
proline	618	mg/kg	200	288  837	●
valine	13	mg/kg	10	<10  23	●
tyrosine	276	mg/kg	50	<50  246	●
phenylalanine	776	mg/kg	100	<100  581	●

Regulated parameters in EU-directive / Codex Alimentarius

- Absolute quantification
- HMF, glucose+fructose, sucrose
- Conclusion according to directive.

Codex Alimentarius and EU-Directive 2001/110/EC:

Following parameters are required according to Codex Alimentarius and EU-Directive 2001/110/EC. The concentrations are obtained by direct quantification. Parameters labelled with * are calculated parameters.

Compound	Value	Unit	LOQ	Official Reference		
				min	max	Flag
glucose + fructose *	69.4	g/100g	20.0	45	-	●
sucrose	<LOQ	g/100g	0.5	-	10	●
5-hydroxymethylfurfural (HMF)	<LOQ	mg/kg	5	-	80	●

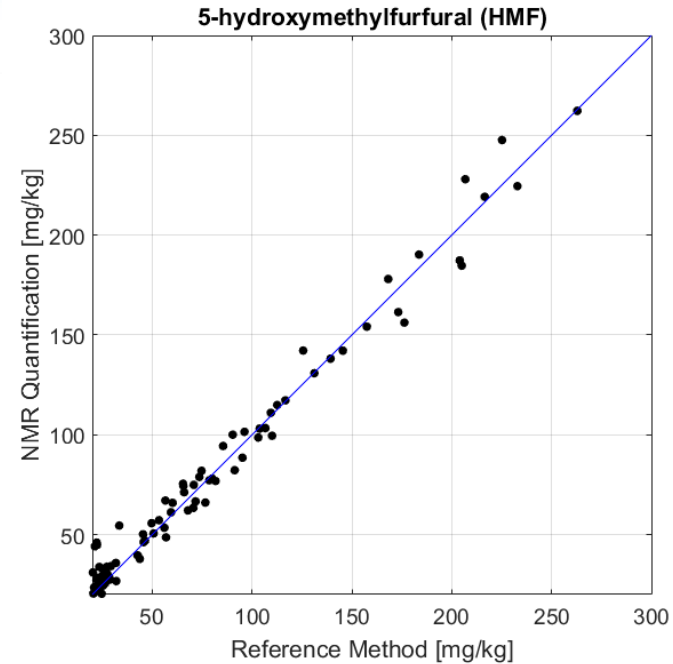
Following flags are used according to Codex Alimentarius and EU-Directive 2001/110/EC:

Compound	Flag	Concentration	Declaration	Interpretation
glucose + fructose	●	< 45 g/100g	All	Not compliant
		< 60 g/100g	Blossom	Not compliant for blossom honey
	●	≥ 60 g/100g	All	Compliant
		≥ 45 g/100g	Honeydew	Compliant for honeydew honey
	●	≥ 45 g/100g, < 60 g/100g	Unknown	Compliant for honeydew honey and blends of honeydew honey with blossom honey. Not compliant for blossom honey.

Validation



- Daily quality controls
- Recovery experiments for each single compound (> 600 spikings done)
- Monte-Carlo/Cross-Validation of statistical methods
- Comparison with other analytical methods
- Inter-laboratory tests (collaborative trials)
- Participation in international ring tests
- Validation with external datasets



NMR and Food-Profiling

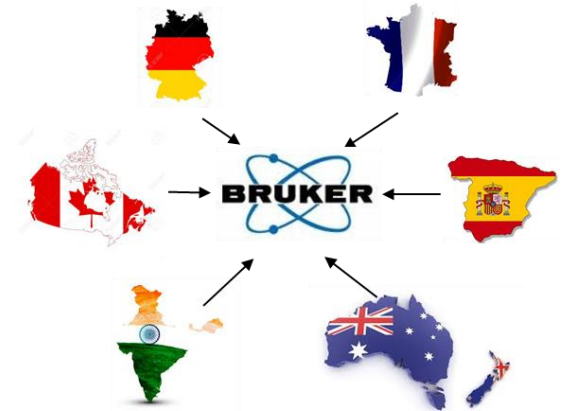


- An **all-in-one method**, comprehensive authenticity test:
 - Detection of foreign compounds (e.g. sugar syrups in honey)
 - Verification of labelling/declaration of origin (country and botanical source/variety)
 - Detection of atypical samples
 - Quality control (composition, freshness, regulated parameters)
- **deeply validated methods**, relying on huge Databases of well-characterized samples
- Several labs (including Bruker BAS lab) accredited **ISO17025** for Food-Profiling
- no NMR expertise required. Easy to operate, fully automated, no regular instrument cleaning
- Fast: 20 – 25 min / sample
- Difficult to deceive
- Wide and worldwide network of partners and users

NMR and Food-Profiling



- **Standardized protocols** for sample preparation and measurement to ensure data reproducibility and the usage of common and centralized data analysis tools.
- **Centralized Database** with worldwide network including governmental laboratories.
- **Transparency** about parameters used and related thresholds (purity criteria)
- A method which is **continuously expanded** and updated
- **Round tables** have been kicked-off to specifically discuss such NMR **purity criteria** amongst honey experts



Wide & Worldwide Adoption of NMR for Food Analysis

Adoption of NMR by Industry



Famille Michaud
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Gov Agencies	
Commercial Testing Labs	
Food Research	
CPGs (Consumer Packaged Goods)	
High Value Suppliers	
Large Retailers	

Adoption of NMR by Commercial Service Providers



Gov Agencies	
Commercial Testing Labs	
Food Research	
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High Value Suppliers	
Large Retailers	

Adoption of NMR by Governmental Laboratories



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Australian Government
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Institute

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