UNIVERSITÄT BAYREUTH

NBNC North Bavarian NMR - Centre

Honey: What is it and how to ensure its authenticity?

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ALNUMED

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What is Honey – Should Know to Prove it‘s Authentic

Biology view
(Bee`s perspective)

Need for energy rich nutrition

Collect nectar/ honey dew (when available) – microbiologically unstable

Preserve it (avoid deterioration)

Deposit nectar/ HD in hive/ cells

Transform (add own compounds & change composition)

Dehydrate (reurgitation, unfolding with mouthpiece, fanning, re-depositioning)

Store in matured state

Human view

Historically:

- Nutrition
- Sweetener

Natural food

Definition

Codex Alimentarius

Chemical view
(Constituents particular to Honey)

Glucose, Fructose and Water

Plant and transformation specific sugars

Organic molecules from plants & bees
- organic acids
- amino acids
- nectar-/ HD-specific
  - kynurenic acid
  - dihydroxy acetone
  - etc.

Minerals and other minor compounds

Compounds from beekeeping, agriculture

CODEX STANDARD FOR HONEY (CODEX STAN 12-1981)

1. SCOPE

1.1 Part One of this Standard applies to all honeys produced by honey bees and covers all styles of honey presentations which are processed and ultimately intended for direct consumption. …

PART ONE

2.1 DEFINITION Honey is the natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, **which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature**. …

3. ESSENTIAL COMPOSITION AND QUALITY FACTORS

3.1 [1]Honey sold as such shall not have added to it any food ingredient, including food additives, nor shall any other additions be made other than honey.

[2]Honey shall not have any objectionable matter, flavour, aroma, or taint absorbed from foreign matter during its processing and storage.

[3]The honey shall not have begun to ferment or effervesce.

[4]No pollen or constituent particular to honey may be removed except where this is unavoidable in the removal of foreign inorganic or organic matter.

Proving Honey‘s Authenticity …

… identify, when a honey is not authentic any more,
i.e., when it does not correspond to the Definition of the Norm

… necessary, because products on the market conflict with this definition AND regulations and guidelines require action to mitigate fraudulent practices.

- ✓ **Identify, where the process/ the product has a vulnerability**
- ✓ **Identify the risks associated**
- ✓ **Identify measures to mitigate fraud**

CODEX STANDARD FOR HONEY (CODEX STAN 12-1981)

Harvest mature and ripe honey from nectar and/ or honey dew, with low moisture (< 20 %), proper sugar composition, condicticvity, acidity, ash, …

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Not allowed: admixture of anything that is not honey

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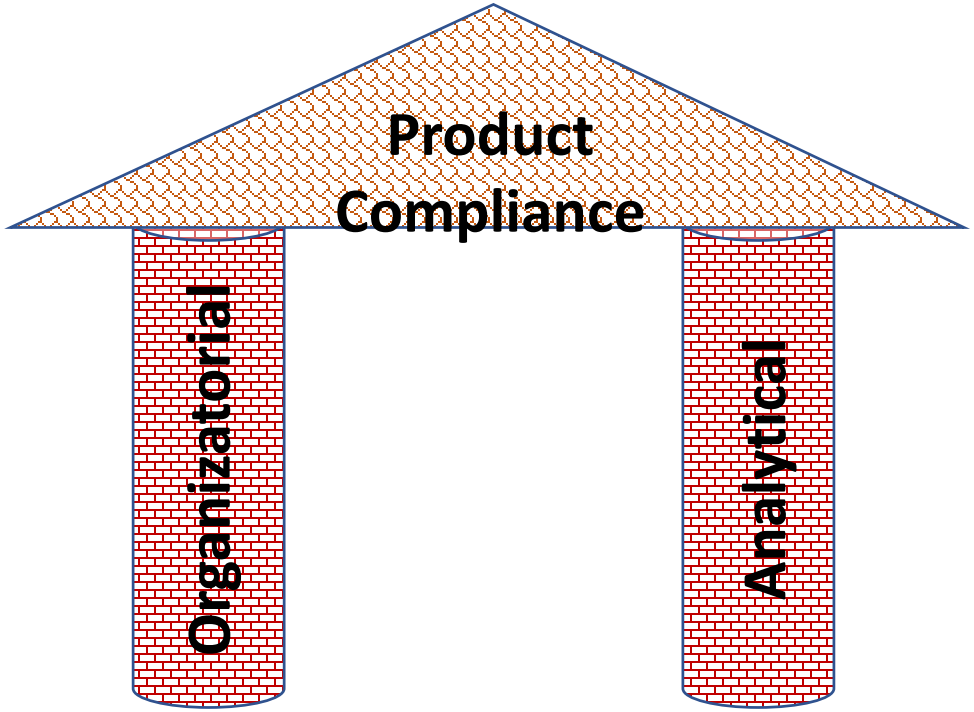
Processing: Cold extraction and filtration so that no pollen or other constituents (including water) are removed

Ensuring Honey‘s Authenticity …

Organizatorial measures:

Custumer - Supplier relations
Audits, own Survaillance
Clear Purchasing Specifications



Analytical measurements:

Compliance of Parameters: typical for natural honey
Absence of substances non-typical for natural honey

How to Establish Analytial Parameters

Analytical measurements:

Compliance of Parameters: typical for natural honey

Analyse large numbers of natural honey samples (= *database*)
one or more parameters
Define typial ranges (ref. Apidoliogy 2004)

glucose+fructose, sucrose,F/G, moisture, sugar profile, conductivity, pH, color, pollen profile, enyzyme activity, sensoric profile, …

Absence of substances non-typical for natural honey

Identify parameter (range) in „non-honey“ product (= database)
Analyse large numbers of natural honey samples
one or more parameters
Define typial ranges, identify exemptions to the rule!

d^{13}C-EA-IRMS, honey-foreign oligo-saccharides, honey-foreign enzymes (ß-FF, a, ß, g, hs, hf-amylase), DNA, syrup markers (rice, beet, mannose, psicose, …), organic acids, trace elements, …

How to Establish Analytial Parameters - PROBLEMS

Analytical measurements:

Problem 1:
Where is the borderline between that is typical in honey *vs*., e.g., a syrup?
How to prove absence of, e.g., a syrup-marker in honey?

- Can only be done when anylsing both groups, honeys and syrups,
- in sufficentily large numbers.

Define typial ranges (ref. Apidology 2004)

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Define typical ranges (ref. Apidology 2004)

Problem 2:

- Diversity of substitution materials available (demand for syrups from various plants)
- And speed in development of new such materials

→Some syrups show certain markers, other show different markers
→Hardly one marker that shows up in all syrups (and not in any honey)

Can Problems be Overcome?

1) Analyse more parameters

Sugar profile
Amino Acids
Organic acids
DNA
Trace elements
Voilatile compounds
Plant metabolites
…

2) Analyse more authentic samples

Authentic: A sample with traceable, known information regarding its properties.

For Honey: known place of hive, surrounding (plants), hive development, treatment of colony, climate conditions, conditions during harvest and processing, storage, shipping, …

Assume, We Would Have Much More Parameters— What Could Possibly be Achieved?

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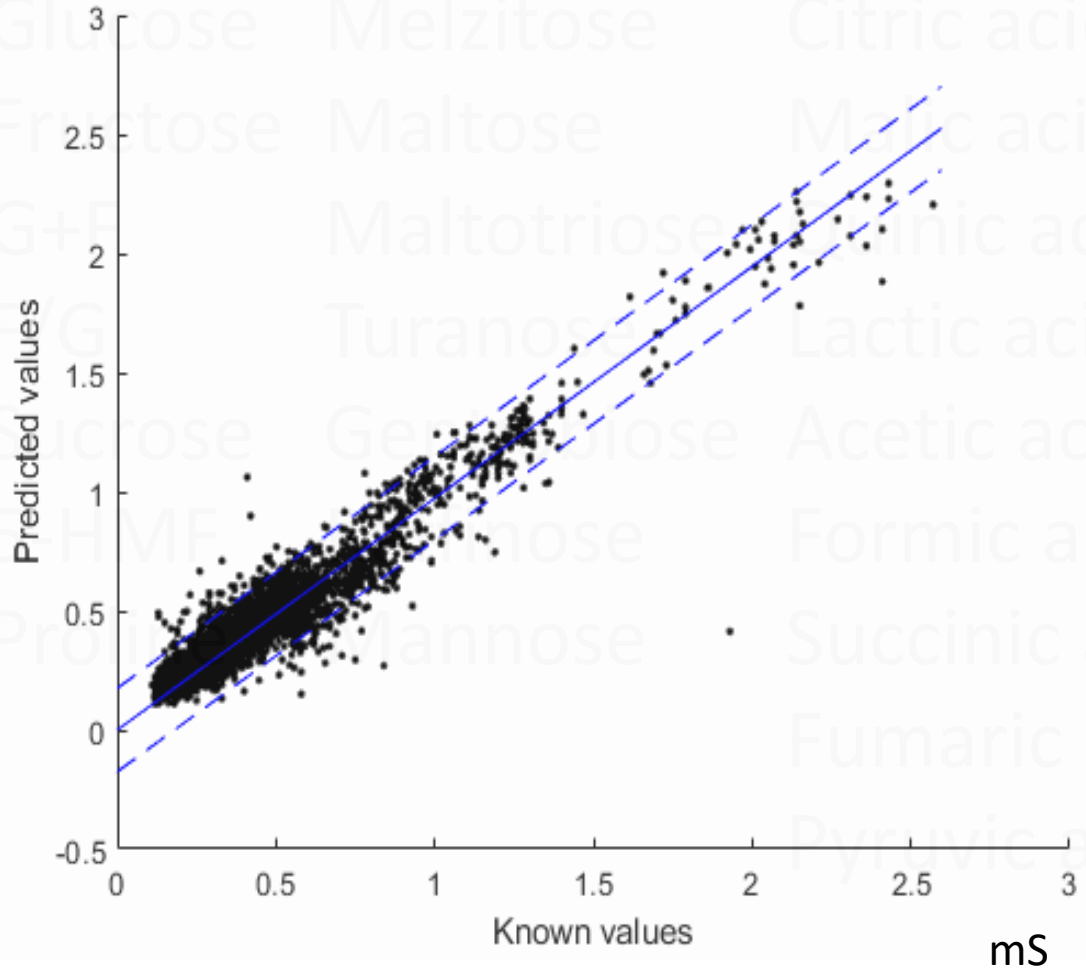
Glucose	Melzitose	Citric acid	Alanine	2,3-Butanediol
Fructose	Maltose	Malic acid	Valine	Acetoin
G+F	Maltotriose	Quinic acid	Leucine	Ethanol
F/G	Turanose	Lactic acid	Aspartic acid	3-Phenyllactic acid
Sucrose	Gentiobiose	Acetic acid	Glutamine	Dihydroxyacetone
5-HMF	Raffinose	Formic acid	Tyrosine	Methylglyoxal
Proline	Mannose	Succinic acid	Phenylalanine	Kynurenic acid
		Fumaric acid		Shikimic acid
		Pyruvic acid		

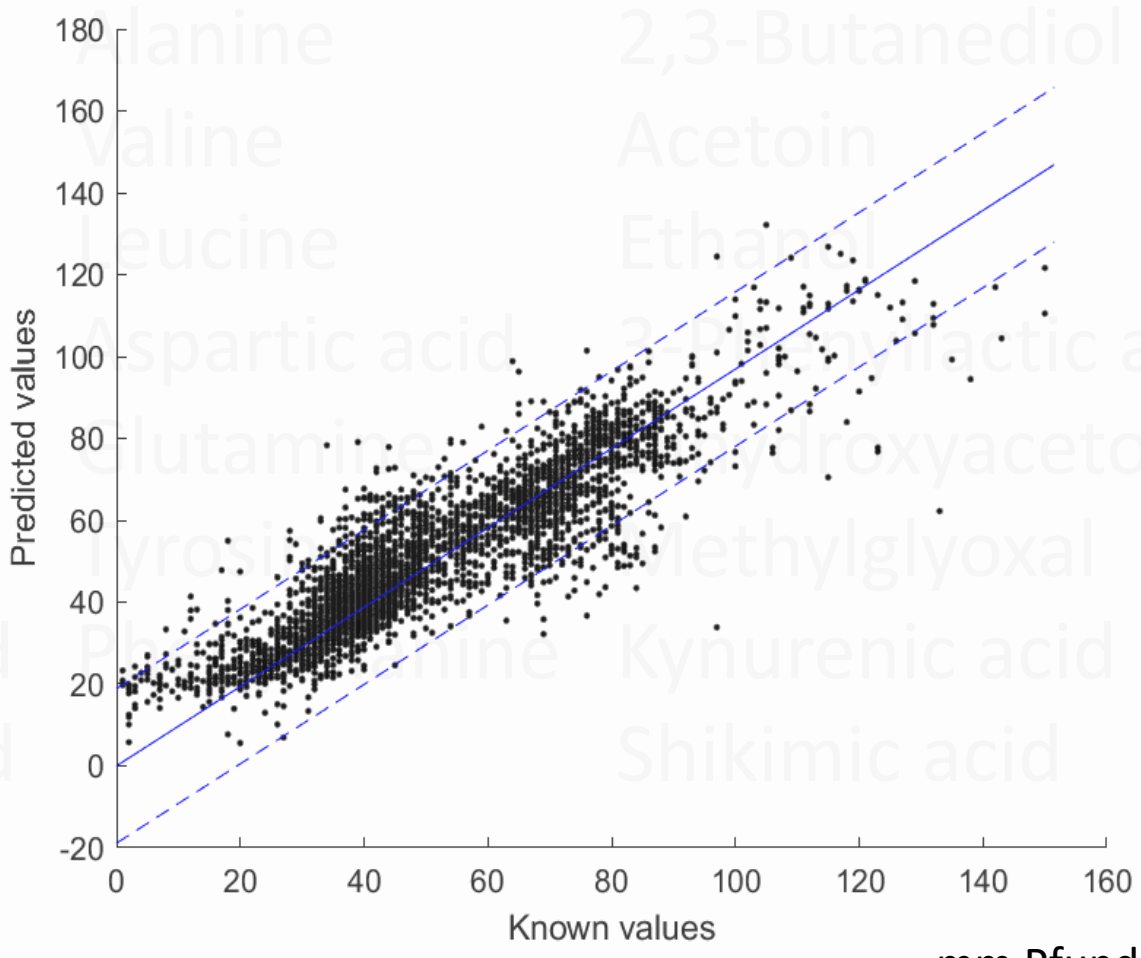
Predict Other Quality Parameters - Get Even More Insight Into the Sample!

Conductivity



mS

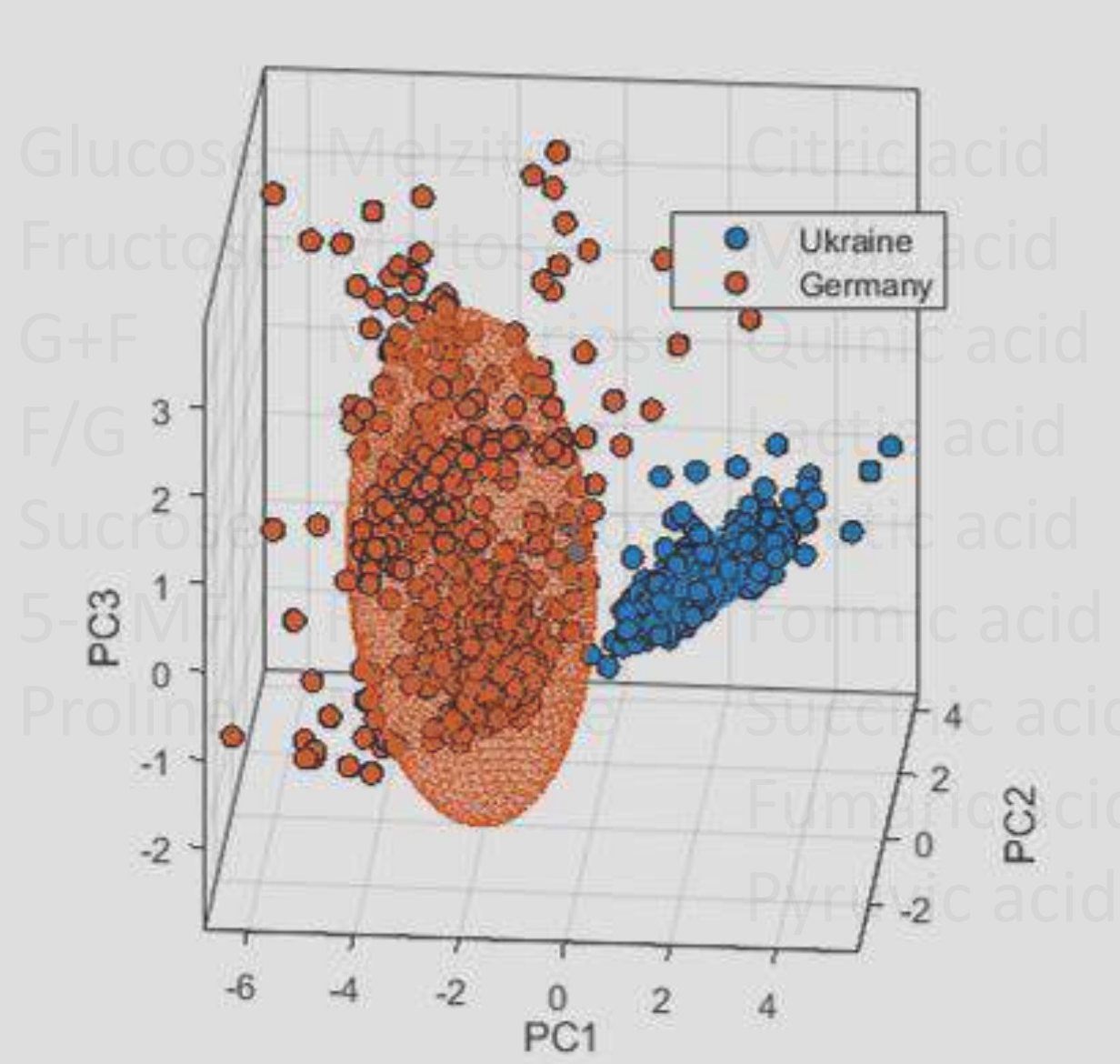
Color



mm Pfund

Moisture, pH, acidity, perhaps even enzyme activity, ….

Differentiate Countries of Origin



- 1000 Samples
- Equal group sizes

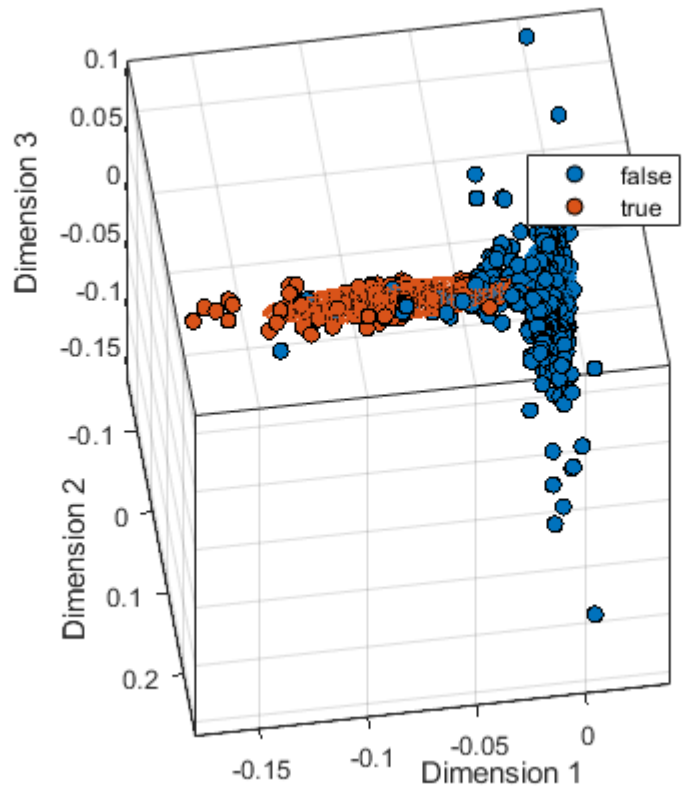
- Only 8 samples misclassified

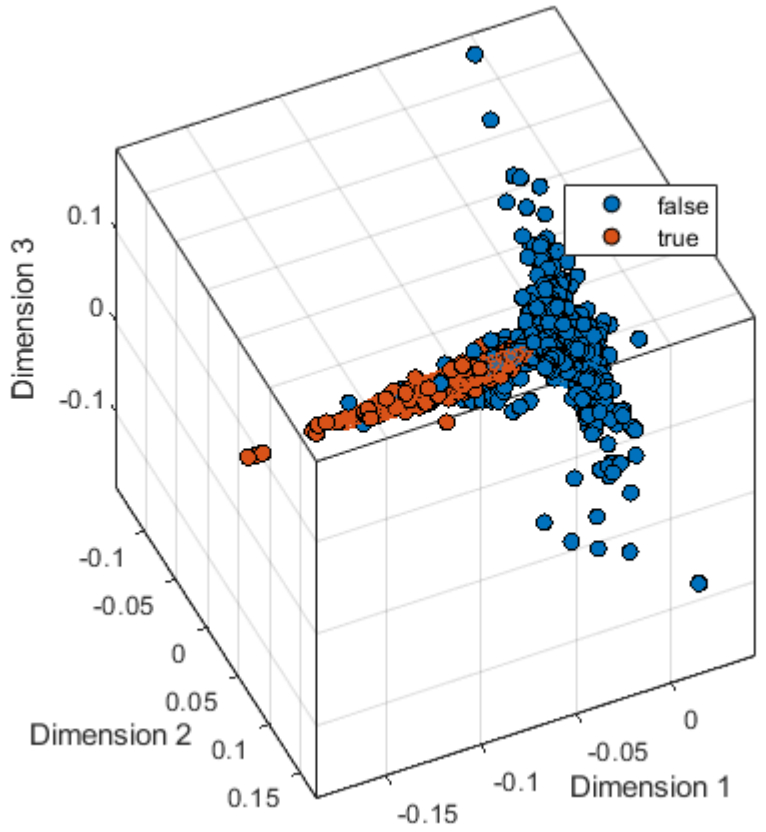
- > 99 % correct classifications

Performed with in-house written MatLab based code – to be released soon!

Differentiate Botanical Varieties

Differentiate Manuka Honey from Honeys all over the World





After eliminating Manuka markers DHA and MGO

So, There Is Potential In Having Many Parameters! How to Obtain in Cost- And Time-Efficient Manner?

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Glucose	Melzitose	Citric acid	Alanine	2,3-Butanediol
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G+F	Maltotriose	Quinic acid	Leucine	Ethanol
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5-HMF	Raffinose	Formic acid	Tyrosine	Methylglyoxal
Proline	Mannose	Succinic acid	Phenylalanine	Kynurenic acid
		Fumaric acid		Shikimic acid
		Pyruvic acid		

Multi-Quantification: Decomposing Food by NMR

What is the food composed off → Quantitative Constituents/ Ingredients Fingerprint

Most powerful NMR of the world @ Universität Bayreuth

Example: 1-GHz NMR-Spectrum of Honey

- **Signal proportional to concentration**
- **Up to 150 – 200 compounds visible**
- **Extremely reproducible**
- **Simultaneous detection of concentrations over 5 order of magnitude** 500 g/kg to 5 mg/kg …

Comparison: Determine size of jelly bean and Burj Khalifa at same time, same precission, same measurment

NMR Honey-Profiling v. 2 (10/2018)

Collaborative Project of Bruker BioSpin, QSI, and ALNuMed (latter in collaboration with FoodQS)
With kind support from Famille Michaud

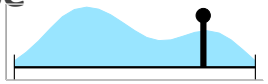
☞ **18.000 samples total in v. 2**
> 50 countries of origin
> 80 botanical varieties
☞ **> 175.000 accompanying conventional analysis**
☞ **> 600.000 NMR quantifications**

ONE ANALYSIS allows ~ 100 targeted parameters!
plus ***untargeted testing!***

Test for: **Codex / EU Directive parameters reported**

Targeted tests: Quantifications (36 compounds)

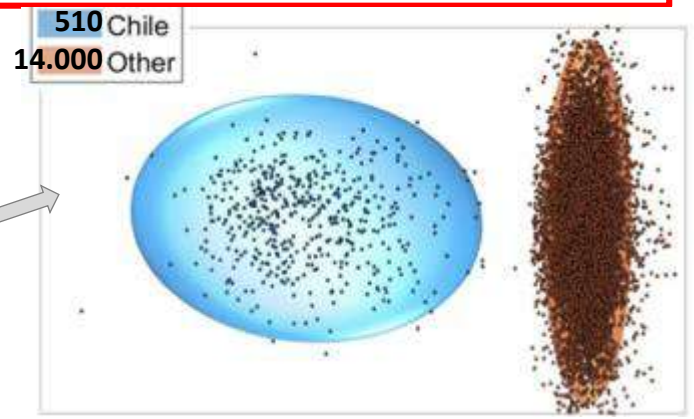
Comparison with NMR Reference Database

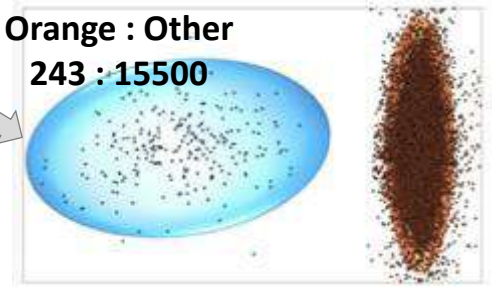
Distribution within Reference Database reported: 59.2  80.0

New: size of reference group used is given

20 countries of origin * (verification of claim)
12 botanical varieties ** (verification of claim)
Adulteration: 60 markers and patterns (targeted)

ADDITIONAL *untargeted* testing (uni-/multivariat)





* Argentina, Brazil, Bulgaria, Chile, China, Cuba, France, Germany, Guatemala, Hungary, India, Mexico, New Zealand, Romania, Spain, Thailand, Turkey, Ukraine, Uruguay, Vietnam

** Acacia, Calluna, Chestnut, Eucalyptus, Lavender, Linden Tree, Manuka, Orange, Pine, Rapeseed, Sunflower, Vitex

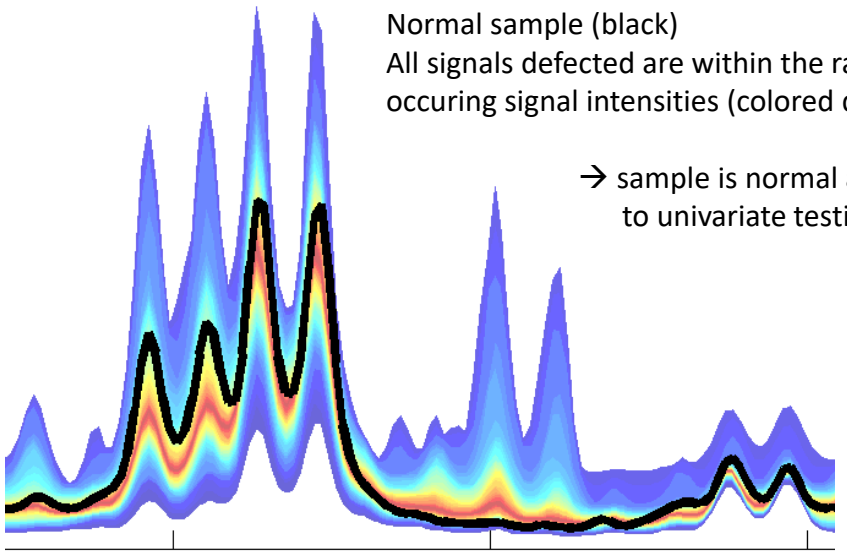
Classification models: linear/quadratic discriminant analysis, extensive MC-cross validation!

Untargeted Analysis: Quantitative Ingredient Fingerprints

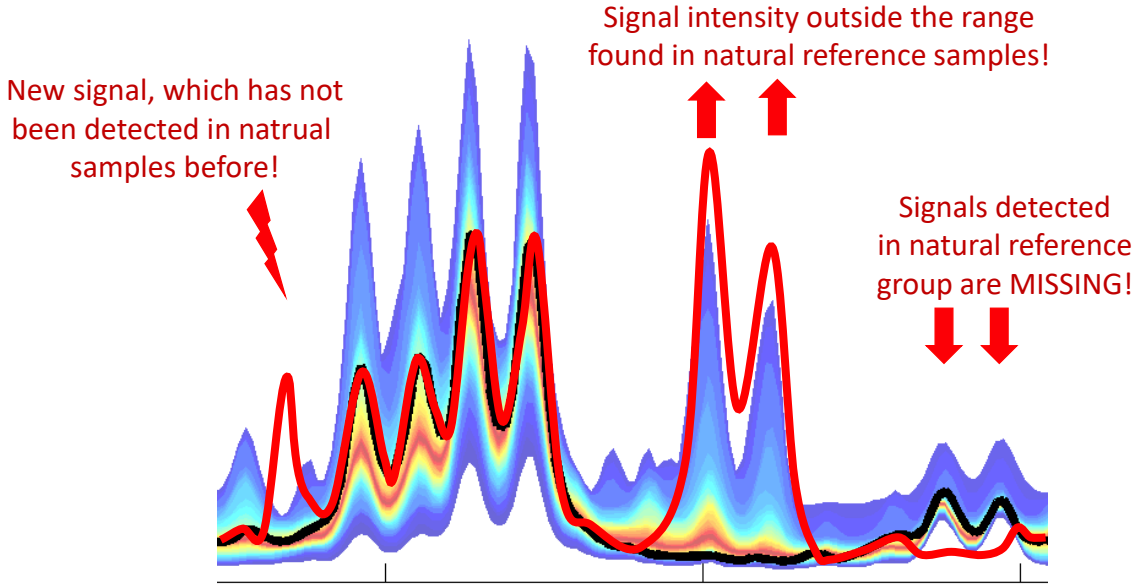
Basic idea: untargeted screening to identify unknown deviations from natural composition

= let us map how natural honey (i.e., the global quantitative ingredient profile) looks like!

- Parameters: e.g. a spectral envelope (NMR, IR-, LCMS, ...)
- Compare new samples with the naturally occuring solution
- “Marker”: not a single compound rather than the concentration distribution (variance) in spectra
  - Deviations: not neccessarily coupled to a single quality parameter!



(Methode/ units of scale are irrelevant for this example!)



Sample with signals deviating from the reference group – univariate suspicous!

EACH deviation found is conspicuousness – EXPERT interpretation required

not every deviation is an adulteration, needs to be clarified if nature is responsible for deviation. **CSI meets Dr. House ;)**

Can We Do Without Databases?

In general: NO.

- **Any development will be the result of a parameter (or range) found in one group but no in another.**
- **Proof, that a parameter is present in one group but no in the other, can only be obtained by analyzing large numbers of samples of both groups.**

Examples from the past:

Sugar cane sugar (sucrose) is high in some natural honeys.
Some enzymes used to produce syups (ß-amylase) can be found in certain plants.
Some markers are predominantly found in syrups, but also in some monofloral honeys (psicose, mannose, BSM)

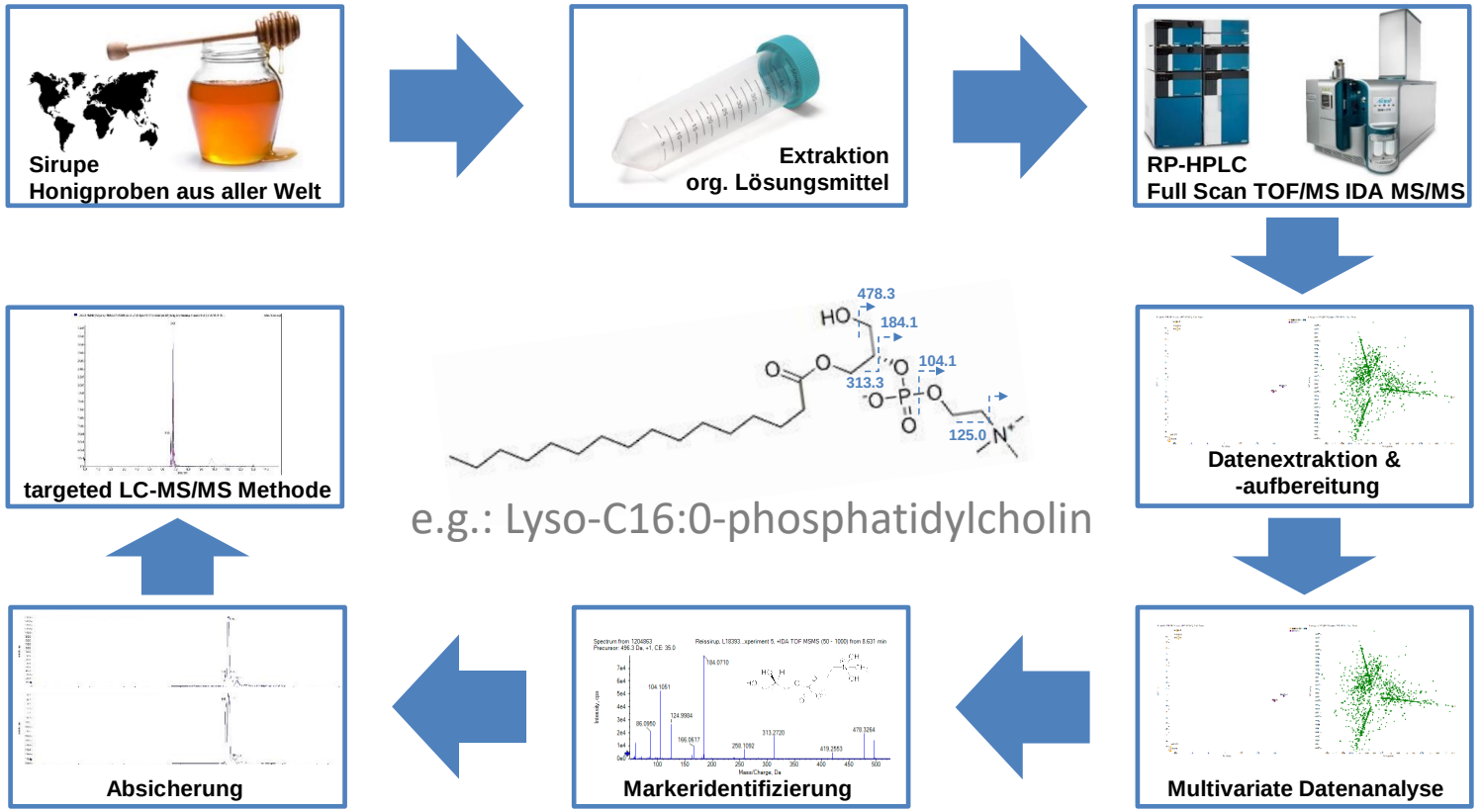
→ It is important to interpret results in the greater context of the sample.

As a matter of fact:
From science theory it is not possible to measure absence of a substance in a sample. Every method has a limit of detection – science is blind to concentrations below that limit.
Likewise, it is not possible to rule out a syrups marker in honey with 100 % certainty, because ist will never be possible to know all honeys of the world.

High Resolution Mass Spectrometry: Similar Approach As NMR, But Different New Markers!

Kindly provided by
J. Haucke & B. Kämpf

Untargeted Metabolomics: Workflow



e.g.: Lyso-C16:0-phosphatidylcholin

Analyse syrups and
(as of now) > 3500 honeys

↓

Determine differing signals
(untargeted search approach)

↓

Identify the compounds
giving rise to these signals

↓

Develop targeted method
for these compounds

Comparison of Methods

	Syrup addition	Mislabeling	Quality	Variety	Country of Origin	targeted	untargeted	comment
Conductivity, Mosture, Ash, Acidity, …		X	X			X		
Pollen Profile		X		X	X	X		not with filtered honey
Sugar Profile (LC)	x	X	X			X		
Enzymes	X		x			X		
RSM (Marker)	X					X		
BSM (Marker)	X					X		
Mannose (Marker)	X					X		
Psicose (Marker)	X					X		
NMR	X	X	X	X	X	X	X	works with processed (filtered) honey
HR-LCMS	X					X	?	aqueous and non-aqueous fractions
DNA	x			?		X		
Voilatiles (GCMS)	x			X		X		
Trace elements	?				?	X		

Subject to change due to ongoing developments …

Which One to Choose?

… that depends on the case, on the risk of that particular sample
(trade partner, flow of commodities, country of origin, variety, …)

Do not rely on a single method!
Vary Parameters looked at!

AOAC 998.12 (^{13}C-EA-IRMS) is often used to white-wash samples!
Reason for failure is known, failure to react may result in fines!

Use Screening Methods, too → **get the overall picture of the sample!**

(Assessment of quality, variety, country of origin, and adulteration)

e.g., NMR Screening plus HR-LCMS, Oligosaccarides, IRMS, …
increase number of tests in case of „grayish“ results, e.g. by enzymes, markers,
Best (recommended): Full Analysis!

Make crystal clear purchase contracts and specifications:
Honey must not contain any syrups!

Summary

Honey is all natural food produced soleyly by bees. Any manupilation other than extraction from combs and filtration is not in line with Codex and other laws (e.g. 2004/110 (EC)). Any addition of syrup is forbidden, as well as removal of water.

Mitigation of food fraud is a matter of organizatorial measures and sufficient chemical analysis.

In this context: Make clear purchase specifications, do not rely on single methods to test for adulteration.

Adulteration is a dynamic field, you will be tricked. IFS and BRC demand proper risk assessment!

Any offer that guarantees to pass a certain test shoud alarm you – use other tests! Do not always use the same parameter - vary!

Selection of parameters for adulteration: no recommendation – always changing, some parameters have short livetimes, but experience shows they cannot be neglected and often show up even long times later.

Screening/ multiparameter methods are recommended – get the greater picture, supply enough information!

Get different interpretations for one sample from different labs: most often for so-called borderline samples.

Talk to the labs – labs have an interest in resolving ambiguities themselves.

Do not forget: When sending samples to the lab – it must be representative for the entire batch.

Developing Methods: Make sure you have suffencently large/ diverse reference and control groups!

e.g.: not sufficient to map a countries honey land scape by sampling some 800+ honeys from said contry but only ~100 from rest of the world!

Make sure methods are harmonized!

Clients: Ask questions about sample-numbers underlying developments!

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