

Harmonisation of testing methods to determine exogenous sugars in honey (**HarmHoney**)

Update

CDG on animal production– Focus on beekeeping and
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Objectives

- To assist the optimisation and the harmonisation of appropriate analytical methods to detect adulteration of honey with exogenous sugars.
- To quantify performance criteria of selected methods and their diagnostic capability.
- To write recommendations towards standardisation by Standard Development Organisations (such as CEN, ISO or AOAC International).

Chemical Markers

- Unique (?) to adulterants (sugar syrups)
 - Starchy plants (e.g. maize, rice, wheat, ...) & other plants (e.g. sugar cane, sugar beet, ...)
 - Use of foreign enzymes to obtain sugar syrups
 - Oligo-/polysaccharides as by-products in case of starchy plants
 - But various purified or bioprocessed sugar syrups and difficulty to get samples

Chemical Markers (cont'd)

- Unique (?) to genuine honey
 - Require a compositional database to know the natural variability of a marker in honeys and to decide on thresholds.
 - Must includes atypical honeys and appropriate sampling.
 - But many influencing factors on the chemical composition of honeys exist impacting the sampling.

Chemical Markers - Mannose

- In blossom honey, if > 0.04 g mannose / 100 g honey then indicate presence of starch based syrups or treatment with ion exchange resins.
 - However in some plants mannose could be naturally present in higher amounts*: jujube (87%), eucalyptus (7%), vitex (2%), lime ($< 1\%$), or polyflorals (1%).
 - It is suggested to check also the Dihydroxyacetone (DHA) as indicator of exogenous sugars or industrial processing practices. But DHA is known to be present naturally in *Leptospermum* (*Myrtaceae*) and a recent study (2023) determined it also in two other species of the same family. Threshold used by Bruker is 5 mg/kg (If DHA exceeds this value then the sample is suspicious).

* Estimated from a dataset provided by a private company.

Chemical Markers – Mannose (cont'd)

- In honeydew honey originating from some trees/plants, mannose could be also naturally present in higher amounts*: chestnut (15%), eucalyptus (11%), or polyfloral (6%).
- In blends of blossom and honeydew honeys, mannose could be naturally present in higher amounts*: chestnut (20%), eucalyptus (14%), or polyfloral (3%).
- The electric conductivity can be used to differentiate blossom honey from honeydew honey and their blends.

* Estimated from a dataset provided by a private company.

Chemical Markers - Proline

- Dominant amino acid in honey and indicator of protein amount in honey.
- No legislative limit but a min. threshold of 0.018 g proline / 100 g honey is proposed.
- Less than 1% of compliant honeys were reported* below the threshold, mainly false acacia, rapeseed and some polyfloral honeys*. Such observation was also reported in the literature.
- All the compliant honeydews reported in the dataset provided by a private company were above the threshold.

* Estimated from a dataset provided by a private company.

Chemical Markers

On top of mannose, proline, **2-acetylfuran-3-glucoopyranoside**, and **difructose anhydride**, the following markers were considered so far:

- **Psicose**: epimer of fructose; it can be produced from fructose, glucose, sucrose, and starch in enzymatic or microbial fermentation. It was detected in jujube honeys and also several sugar syrups and reported in nature in few species like wheat, cane molasses, *Itea*. A psicose content above 0.1 g/100g was proposed to indicate the presence of sugar syrups.

Chemical Markers

- **Ectoine**: a role of macromolecule protectant of proteins (e.g. glucose isomerase) from degradation. Also detected in high amounts in authentic South American bee feeds based on soybean or jatobá (*Fabaceae*) or in small amounts in a German feed dough including soy.
- **Betaine** (Trimethylglycine): identified as a marker for browned beet sugar (adding molasses to refined beet sugar).
- **3-Methoxytyramine**: reported as the Special Marker for Beet sugar (SMB) is a natural pigment of betaxanthins (yellow water-soluble pigments); confined in 13 families of Caryophyllales including *Amaranthaceae* (e.g. *Amaranthus*, *Beta*, *Chenopodium*).

Analytical Methods – EA/LC-IRMS

- **EA-IRMS** (AOAC official method 998.12) to detect sugar syrups made from C4 plants.
- Recent studies from Bangladesh & Philippine reported that fraudsters are still using this adulteration depending the vulnerability of a food market.
- Limitations with some honey origins (e.g. Manuka, kamahi), with honeys having naturally low protein content (e.g. acacia, lavender) or having high yeast content; with mesquite honey (CAM plant in desert), etc.
- **Multi-isotopic analysis** (incl. $\delta^2\text{H}$) could support the determination of the botanical origin of the sugar syrups.

Analytical Methods – EA/LC-IRMS

- **LC-IRMS**: Due to the natural variability of honeys and commercial honey blends, the stated limit values represent a compromise in terms of detection sensitivity and prevention of false positives.
- ⇒ Honeys from various countries differ (e.g. acacia from China are outliers).

Analytical Methods – LC-HRMS

- **Multi-analytes approach** with a potential to develop a metabolomics approach.
- A cross-validation-study of EA/LC-IRMS, $^1\text{H-NMR}$ profiling and LC-HRMS was performed on 1000 samples and reported that less than 0.7% of the adulterated samples were not detectable by LC-HRMS whereas nearly 13% were not detectable by the other two methods.
- As the concentration of the markers varies in the different sugar syrups, there is no analytical standard to quantify the amount of sugar syrups exactly.

Sampling collection

- In order to check recommendations made by experts and the relevance of new potential markers, JRC prepared guidance to the collectors and sampling templates.
- JRC is in contact with various stakeholders of the honey supply chain that is providing samples.
- So far, 550 honey samples from ES, EL, FR, and 3rd countries and 15 sugars/sugar syrups/bee feeding products were collected.

Method optimisation – LC-IRMS

- The method should be released soon by CEN.
- In 2024, JRC proposed an estimated critical difference using the precision of the $\Delta\delta^{13}\text{C}$ values for comparing a test result with a reference value according to ISO 5725-6:1994.
- JRC is working on the improvement of the system of calibration using carbohydrate based reference materials and the optimisation of the peak intensities.
- JRC analysed 182 additional honey samples from which 11% were suspicious.

Method optimisation – LC-HRMS

- Integration of additional potential markers into the previously established LC-HRMS **qualitative** method.
- New batches of honey and syrup samples were analysed as well as a re-evaluation of the prior sample measurements:
- **Proline** was present exclusively in honey samples, despite some authors reported that proline could be detected in sugar syrups (e.g. glucose-fructose or maltose corn starch syrups).
- **Psicose** was identified in two polyfloral samples, two agave syrups, and two sugar cane syrups.

Method optimisation – LC-HRMS (cont'd)

- **Betaine** was detectable in nearly all analysed honey samples and in some of the syrups and bee feeding products derived from sources as corn, rice, spelt, sugar beet, sugarcane.
- **3-Methoxytyramine** and **ectoine** were so far not present in any of the samples tested

Conclusions & Outlooks

- New chemical markers (5) were integrated into the established LC-HRMS qualitative method.
- The fact that a marker can be present naturally in honeys and in sugar syrups implies to consider an appropriate sampling of the existing variability in the composition of honeys and adulterants.
- It will be necessary to define thresholds for taking decisions and therefore quantification of markers is the next step of the optimisation of the LC-HRMS.
- In parallel, JRC is pursuing testing new potential markers appearing in the scientific literature.
- JRC will investigate on the application of measuring $\delta^2\text{H}$ in sugar syrups to identify the botanical origin when used as adulterants in honey.

Thank you

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