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Fast and global authenticity screening of honey using ^{1}H-NMR profiling

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ABSTRACT

An innovative analytical approach was developed to tackle the most common adulterations and quality deviations in honey. Using proton-NMR profiling coupled to suitable quantification procedures and statistical models, analytical criteria were defined to check the authenticity of both mono- and multi-floral honey. The reference data set used was a worldwide collection of more than 800 honeys, covering most of the economically significant botanical and geographical origins. Typical plant nectar markers can be used to check monofloral honey labeling. Spectral patterns and natural variability were established for multifloral honeys, and marker signals for sugar syrups were identified by statistical comparison with a commercial dataset of *ca.* 200 honeys. Although the results are qualitative, spiking experiments have confirmed the ability of the method to detect sugar addition down to 10% levels in favorable cases. Within the same NMR experiments, quantification of glucose, fructose, sucrose and 5-HMF (regulated parameters) was performed. Finally markers showing the onset of fermentation are described.

1. Introduction

The Codex Alimentarius ("Codex Alimentarius, Revised Codex Standard for Honey (Rev. 2, 2001)."") defines honey as "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." According to this standard "Honey consists essentially of different sugars, predominantly fructose and glucose as well as other substances such as organic acids, enzymes and solid particles derived from honey collection" and " shall not have added to it any food ingredient, including food additives, nor shall any other additions be made other than honey. Honey shall not have any objectionable matter, flavor, aroma, or taint absorbed from foreign matter during its processing and storage. The honey shall not have begun to ferment or effervesce." Some analytical criteria regarding the composition are listed: moisture, sugar content, water-insoluble solids, free acidity, diastase activity, hydroxymethylfurfural (HMF, a sugar degradation product linked to thermal treatment), and electrical conductivity (linked to mineral content).

The EU directive for honey ("Council directive 2001/110/EC of 20 December 2001 relating to honey," 2002) has a similar definition, adding the condition that honey is produced by *Apis mellifera* bees and listing similar analytical criteria. The authors of a recent article (Strayer, Everstine, & Kennedy, 2014) are asking for a similar FDA (Food and Drug Administration) standard which would help to control the USA domestic market. The same article provides an up-to-date review of the main adulteration issues encountered in honey and their economic motivation. The most frequent is the presence of exogenous sugar, either from a deliberate addition of a cheaper sugar source to honey, or from the use of sugar for bee feeding during the production season (which is prohibited). Bee feeding is authorized during winter to maintain bee colonies, but should be discontinued during the production season, when

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natural feeding sources are widely available. A longitudinal feeding study demonstrated that due to the fast metabolic turn-over of the sugar feed, there is no influence of the winter feeding on honey production and therefore only unauthorized practices are analytically detectable (Simkova & Kolar, 2012).

The analytical methods used to control honey quality traditionally include the above physico-chemical parameters and microscopic pollen examination. While they enable an overall quality check and a fairly precise classification of honeys according to botanical and even geographical origin, these traditional methods can only detect the presence of sugar adulterants that have a very different chemical composition to that of honey (e.g. a high amount of sucrose or maltotriose). On the other hand cheaper sugar sources exist on the market that can perfectly mimic the composition of the main sugars of honey. When C4-plants (cane or maize) are used, they can efficiently be detected by the AOAC Carbon 13-IRMS method (AOAC International, 2010). However sugars from C3-plants (rice, wheat, beet, etc.) are not detectable in this way because they have a similar isotope fingerprint to honey. SNIF–NMR (Giraudon, Danzart, & Merle, 2000) can detect beet sugar addition in specific monofloral honeys such as *Citrus* or acacia, but even then it is not sensitive enough to detect sugar syrups derived from C3 cereals. More recently, a multi-component approach using LC–IRMS has been proposed (Cabañero, Recio, & Rupérez, 2006; Elflein & Raezke, 2008), but in practice this can easily be circumvented by using sugar syrups from C3 plants showing honey-like isotopic characteristics. Similarly the efficiency of methods based on specific trace chemical markers (Xue et al., 2013) or residual foreign enzyme activities is limited to favorable cases showing these properties. Last but not least, using a combination of the methods described above, as required due to their limited performances, can be extremely costly and time-consuming, which is not compatible with today's honey trading conditions.

Therefore there is a need both for more efficient adulteration detection and for screening methods which can help reduce the cost and technical time of honey analysis. Some attempts have been made to use infrared spectroscopy (Sivakesava & Irudayaraj, 2001), and more recently Nuclear Magnetic Resonance has been successfully applied in model studies to evaluate several botanical origins (Donarski, Jones, Harrison, Driffield, & Charlton, 2010; Ohmenhaeuser, Monakhova, Kuballa, & Lachenmeier, 2013) and some sugar additions in Italian honeys (Bertelli et al., 2010). This article presents a large and systematic NMR profiling study of authentic honeys representative of a worldwide range of botanical and geographical origins, in order to establish a general methodology for the targeted and non-targeted analysis of honey authenticity.

2. Materials and methods

2.1. Samples

A total of 816 reference samples of monofloral and multifloral honeys, of different colors (white, amber, brown), in different physical states (liquid, creamy), from more than 60 different botanical origins and from more than 35 different countries (see Table 1) were collected over more than a decade from local markets, small shops, and directly from bee keepers. The reliability of declarations regarding origins and floral types has been cross-checked by performing microscope pollen examinations.

205 market samples were also bought from supermarkets, for separate analysis and comparison with our database set.

Finally several industrial sugar syrups of various types and sources (beet and cane full invert sugars, glucose syrups from wheat and rice, High Fructose Corn Syrup, and High Fructose Syrup from Inulin), including solutions used by bee-keepers for winter feeding, were tested as potential adulterants and used in spiking experiments.

2.2. Chemicals

Reagents and standards used for quantification of the major compounds were all of high analytical purity. The following standards were all purchased from Sigma–Aldrich, acetic acid (purity > 99.8%), alanine (purity > 98%), citric acid trisodium salt dihydrate (purity > 99%), 1,3-dihydroxyacetone (DHA, purity > 97%), erlose (purity > 97%), glucose (purity > 99%), isomaltose (purity 98%), 1-kestose (purity > 98%), sodium lactate (purity > 99%), maltose monohydrate (purity > 99.0%), maltotriose hydrate (purity > 99.0%), melezitose hydrate (purity > 99.0%), succinic acid (purity > 99.0%), sucrose (purity > 99.5%), trehalose dihydrate (purity > 99%), and turanose (purity > 98%). 5-HMF (5-hydroxymethylfurfural, Alfa-Aesar, purity > 98%), absolute ethanol (AnalaR Normapur), and fructose (Fluka, purity > 99%) were also purchased. The NMR buffer was prepared by dissolving 10.21 g of KH_2PO_4 (Sigma–Aldrich, purity > 99.5%) and 9.8 mg of sodium azide (NaN_3, Sigma–Aldrich, purity > 99%) in 50 mL of pure water and then by adjusting the pH to 4.5 with H_3PO_4 85% (Sigma–Aldrich) or NaOH 5 M (prepared by dissolving 200 g of NaOH pellets Riedel-de Haën in 1 L of pure water). The lock solution is prepared by dissolving 50 mg of trimethylsilyl propionate (TSP, Sigma–Aldrich, purity > 98%) in 50 mL of D_2O (Sigma–Aldrich, purity > 99.8%).

2.3. Sample preparation and calibration

The procedure was adapted from (Ohmenhaeuser et al., 2013). Honey must be liquid and homogeneous before preparation. If this was not the case, the sample was placed in an oven at 50 °C for 2 h, until all crystals were dissolved; then the honey was cooled down by placing samples in a water bath at 20 °C. The moisture content was obtained for each honey according to the method "Determination of moisture, refractometric method" (Bogdanov, Martin, & Lullmann, 2002). Exactly 200 mg moisture-free honey (corresponding to about 240 mg of a honey sample with about 20% water) was weighed and combined with 300 μL of NMR buffer (see above), 700 μL of distilled water and 100 μL of the NMR lock solution (see above). A standard solution was prepared by dissolving 5 g of glucose, 5 g of fructose, 500 mg of sucrose, 100 mg of 5-HMF and 8.5 mL water. This reference solution was then treated exactly as a honey sample: addition of the same volume of buffer, lock solution and water.

2.4. ^{1}H-NMR measurements at 400 MHz

All NMR measurements were performed on a Bruker Avance 400 Ultrashield spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 5 mm BBI probe with *Z*-gradient coils, using a SampleXPress autosampler (Bruker BioSpin, Rheinstetten, Germany). 1H NMR spectra were acquired at 301.8 K without sample spinning. 64 scans of 65 k points with 4 prior dummy scans were acquired with a spectral width of 20 ppm, a receiver gain of 32, and an acquisition time of 4.096 s. Recycling delay and mixing time were respectively of 8 s and 0.01 s. Water suppression was achieved using the NOESY-presaturation pulse sequence (Bruker 1D noesygppr1d pulse sequence) with irradiation at the water frequency (1881.2 Hz) during the recycle and mixing time delays. The data were acquired automatically under the control of ICON-NMR (Bruker BioSpin, Rheinstetten, Germany), requiring about 20 min per sample (including 5 min for temperature equilibration once the tube is inside the magnet, before NMR acquisition). Tuning