More than honey?

Rapid authenticity testing for honey with NMR spectroscopy and consequences for sample preparation

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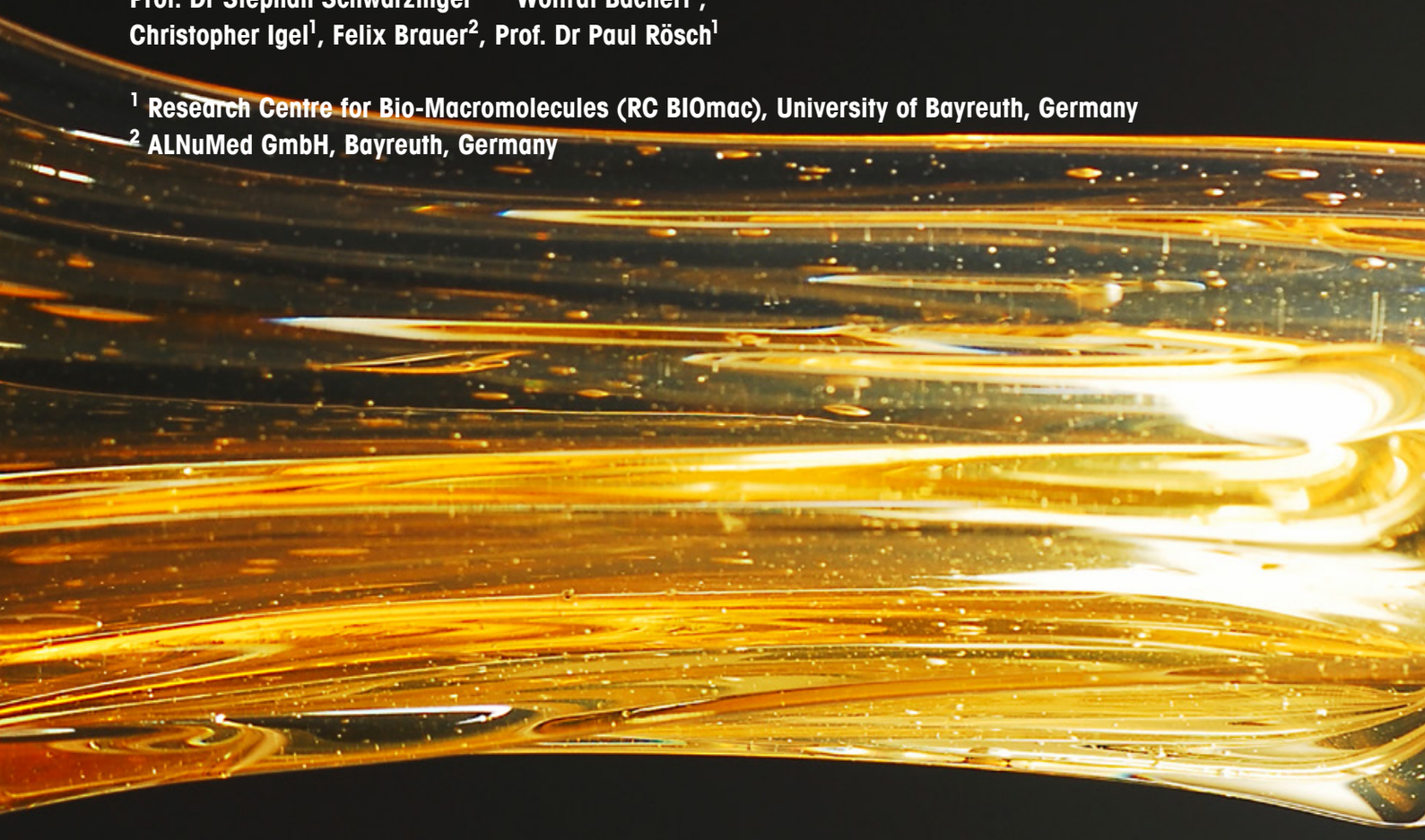

For thousands of years, the word "honey" has been synonymous with an all-natural, healthy food. Unsurprisingly, honey has also enjoyed unwavering popularity with consumers – and especially in times when organic food and a healthy lifestyle are more in vogue than ever before. While demand is rising, however, the availability of the product is finite, not least as a result of honey bee pests and diseases. As a consequence, international trade is increasingly seeing honey "padded out" with sugar syrups – a practice that can be exposed only with intricate and time-consuming analysis.

The only remedy here is a reliable rapid test that enables high-throughput testing. One such method is nuclear magnetic resonance (NMR) spectroscopy, a technique that is already being deployed for routine authenticity testing – whether targeted or non-targeted – of fruit juices and wines. In this article, we show how NMR spectroscopy in conjunction with appropriately efficient sample preparation produces a highly promising method for the rapid analysis of honey authenticity.

According to Annex I of the German Statutory Order on Honey (Honigverordnung, HonigV), honey is the "naturally sweet substance produced by the honey bee." On account of its significant role in the history of humankind and its perpetual popularity with consumers, honey is subject to a very strict set of regulations within both Germany and the EU. Annex 2 of the Order continues by stating: "No other substances except honey itself may be added to honey... nor, equally, may any constituent particular to honey be removed from honey..."

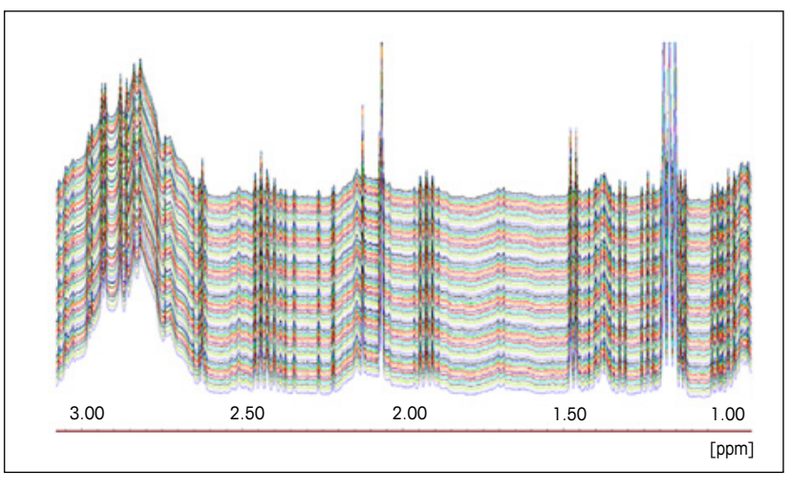

Fig. 1 Absolute reproducibility is a precondition for food profiling: detail (approx. 20 %) from 72 NMR spectra of a fruit juice. Even the smallest peaks are reproducibly retrieved. Relative differences in concentration exhibited by ingredients, which need not initially be further identified, can then form the basis for defining authenticity testing markers.

In great demand and in short supply

The rising demand for honey conflicts with serious problems in honey production, however. An issue of prime concern here is the stress placed on bee colonies by the *Varroa* mite, which in recent decades has caused major colony loss for beekeepers located throughout the world [1]. The rising demand for food around the world goes hand in hand with a rapid intensification of the use of agricultural land, associated with the extensive spread of monocultures and the large-scale use of insecticides and pesticides. It is now considered probable that the combination of *Varroa*, other pathogens and chemicals is responsible for the dramatic hibernation losses of in some cases more than 80 % of hives, as observed in recent years in many of the world's regions [2]. The consequence is a supply shortage for honey of a high quality, as appreciated particularly by consumers in Germany. With per capita consumption of over a kilogramme every year, Germans are among the world's leading consumers of honey [3]. Since domestic production is capable of meeting only about a quarter of this demand, however, Germany is also one of the world's largest honey importers [4].

To ensure that German honey continues to enjoy its solid reputation, it is subject to stringent quality controls. These involve determining the honey's origin and its purity, for example.

Each of these quality parameters is now increasingly plagued by attempts at adulteration. One such example is the "Honeygate" scandal that went public in the USA last year: this involved honey from China being misdeclared on a grand scale to obfuscate its country of origin. Lost excise duties were rumoured to run to as much as USD 180 million [5]. To make it harder to pin down the product's geographical origin – a test typically utilising the pollen spectrum – pollen is now increasingly being filtered out of the honey. Even more problematic is counterfeiting involving various plant-derived sugar

syrups, since the sugars glucose and fructose make up as much as 70 % of honey itself. While some sugar syrups – such as those with a high proportion of sucrose, for example – are comparatively easy to detect in honey, glucose-fructose syrups (GFS) on the other hand, composed of the primary constituents of honey itself, have proven very hard to detect to date, such as by using the technique of mass spectrometry to calculate isotope ratios, for example [6]. GFS are large-scale industrial products for the food and beverages industry, and are available for a fraction of the price of honey. This is therefore a highly lucrative form of adulteration. As a countermeasure to these developments, a new, robust rapid test procedure is required, to ensure consumers worldwide can continue to rely on the authenticity of their honey.

Rapid authenticity testing with NMR spectroscopy

One form of authenticity testing utilises suitable markers, for example. Typically, these markers designate a substance whose presence (or absence) correlates exclusively with the parameter under investigation. As one example, an increased proportion of certain organic acids can be used as an indicator for adulteration with invert sugar syrup. More complex issues, such as those involving a foodstuff's provenance, cannot be answered merely by looking at a single substance, however. We might consider a Riesling, for example, cultivated at two different sites. Since the plants are genetically identical, the same ingredients are to be expected in both cases, in the form of the plants' metabolic end products (metabolites). Yet variations in climate and soil quality will lead to relative differences in concentration, however. Accordingly, the systematic differences in concentration of a range of metabolites can be aggregated into a marker – such as a marker for geographical origin, for example.

This kind of analysis is amenable to spectroscopic methods, and NMR spectroscopy in particular – a technique that, in a similar form (magnetic resonance imaging, MRI) is also used in medicine. With NMR spectroscopy, a magnetically active atomic species, e.g. ^{1}H or ^{13}C, is placed in a strong external magnetic field and its response to a radio frequency pulse is measured. The signals produced permit conclusions to be drawn about the chemical environment of these atoms and hence the identity of the substance under analysis. Indeed, high-resolution NMR spectroscopy permits the detection – and in many cases the quantification – of multiple substances within a single measurement. Accordingly, the method is predestined for the efficient analysis of complex substance mixtures, such as are found in food: unlike methods using chromatography (for example), no time-consuming separation of the components in a mixture is required. At the same time, the sample can also be measured without any chemical preparation: the risk of losing or misplacing substances during separation is therefore minimal or even excluded entirely. One characteristic that is unique to NMR spectroscopy is the fact that signals can be quantified

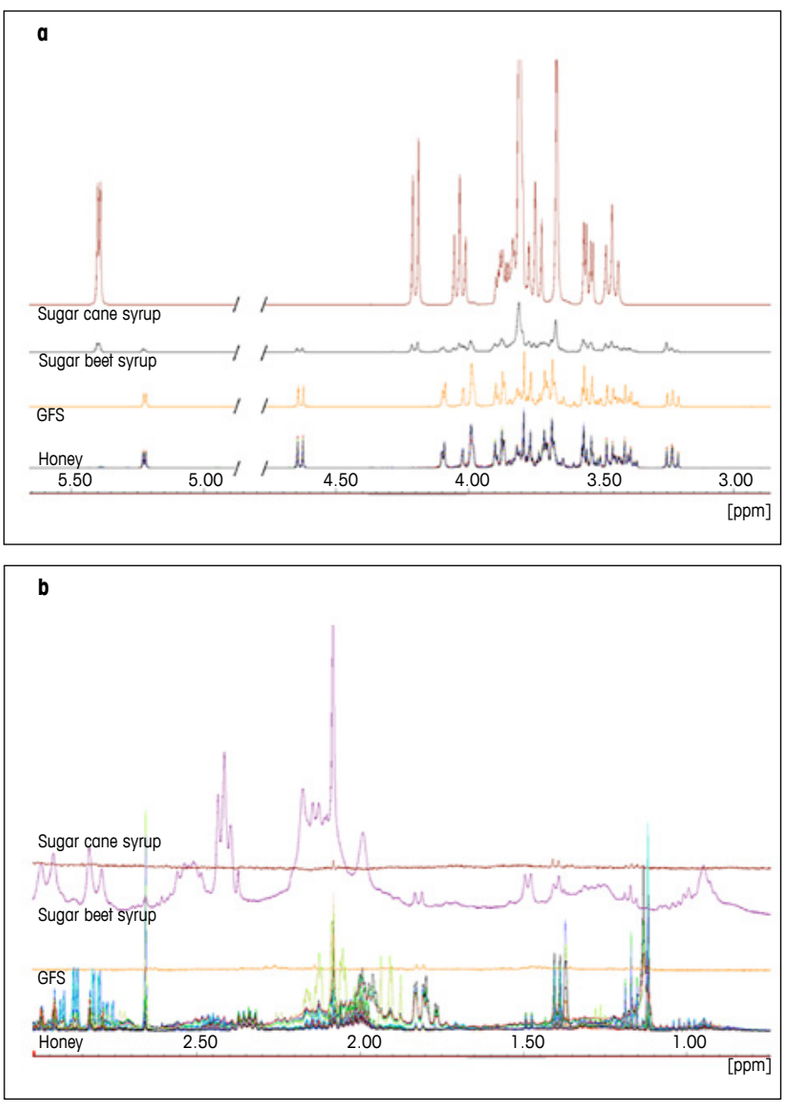

Fig. 2 NMR spectra of a GFS (orange-coloured), a sugar beet syrup (violet), a sugar cane syrup (red) and of 46 pure types of honey. Figure **2a** shows the region of the sugar peaks (chemical shift of 3.0–5.5 ppm; excluding the residual water peak region at 4.8 ppm). Significant differences between honey and sugar beet/sugar cane syrup can be seen, as can the similarity of GFS to honey. Figure **2b** is an enlargement of the 0.8–3.0 ppm chemical shift region, which shows less highly concentrated substances in honey and in sugar beet syrup. While sugar beet syrup also contains many substances in addition to its sugars, GFS and sugar cane syrup are largely free of other substances.

Stephan Schwarzinger studied technical chemistry in combination with business administration at the University of Linz, completing his doctorate there in 1999. This was followed by a period of post-doc work at The Scripps Research Institute, La Jolla, CA, before joining the Department of Biopolymers at the University of Bayreuth in 2000, where he completed his habilitation in 2006 in biophysical chemistry. In 2008 he served as interim head of the Department of Biochemistry at the University of Bayreuth. A member of the BIOmac research centre since 2010, he has been adjunct professor there since 2013. He is also CEO of ALNuMed GmbH. His research interests include NMR methods for the characterisation of flexible proteins, NMR-based food analytics, and the application of combined analysis methods.

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over a dynamic range of more than five orders of magnitude, coupled with very high reproducibility for the method (fig. 1). As a result, a measurement lasting just a few minutes enables the reproducible retrieval of signals arising from substances at very low concentrations (ppm range) in the presence of highly concentrated compounds (% range).

All of which means NMR spectroscopy is currently unique among analysis techniques in its ability to create quantitative ingredient profiles for foods, and to leverage statistical procedures to correlate these with authenticity and quality parameters in a targeted and non-targeted manner. The first step in the process involves creating a spectra database from known samples. Statistical procedures can then be used to classify new and unknown samples by comparing these samples to this reference database. The procedure is already routinely used for fruit juice and wine profiling: here, authenticity parameters such as variety, origin, vintage and processing can be recorded quantitatively and fully automatically alongside up to 50 other quality parameters during a measurement time of just 15 mins [7]. Recently, we used the example of meat to demonstrate that NMR is also suitable for the rapid authenticity analysis of solid foodstuffs, by the preparation of a corresponding extract [8]. And, although its formulation makes it difficult to investigate, honey can also be subjected to a detailed analysis with NMR spectroscopy. Among other findings, we have shown that, as well as a number of sugars and degradation products such as 5-Hydroxymethylfurfural (HMF), even the water content can be determined in the same measurement [9].

Rapid, exact measurements require rapid, exact sample preparation

With measurement times of about 15 minutes, around 20,000 samples can be analysed annually by a single NMR spectrometer. Due to the high degree of reproducibility for measurements – which is the essential foundation for authenticity testing using a classification based on statistical methods – effort is also higher for lab-based sample preparation. The consistency of the sample makes considerable demands on effort, as does the degree of precision required: any imprecision when handling the sample indirectly reduces the measurement accuracy, one important effect of which is to cause signals from substances at low concentrations to be lost in background noise.

This applies to honey in particular. In order to receive reproducible spectra within the available dynamic range, gram quantities of honey measured down to the last milligram must be dissolved in a precisely defined volume. Theoretically, this is quick to do with a volumetric flask. Yet in practice, honey has a viscous, paste-like consistency that makes weighing out difficult and time-consuming, especially for small quantities. Automation of these processes is therefore essential, for two key reasons: first, this enables processes to be accelerated; second, processes become reproducible and more reliable – such as by eliminating individual

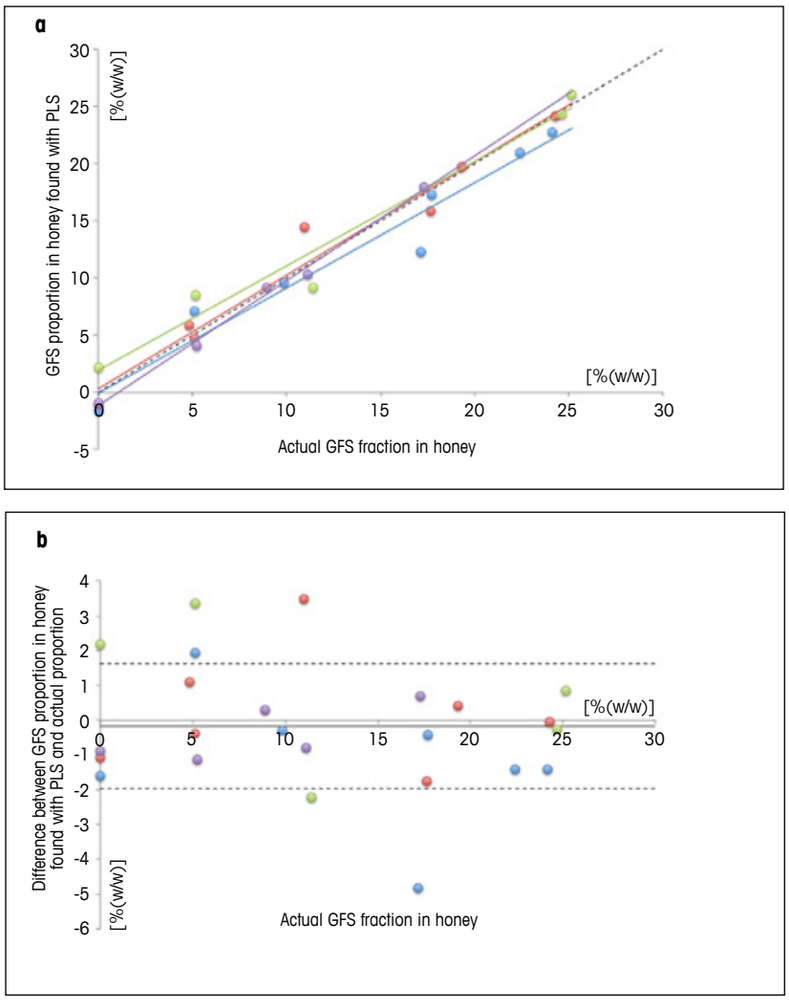


Fig. 3 Test of PLS analyses for honey adulterated with GFS (in % w/w). Four independent tests were carried out in total, each shown here in a different colour. Figure **3a** shows the application of the actual values versus the values found by PLS. The coefficients of determination (R^2) for the correlations found lie between 0.95 and 0.99. The trend lines for the individual tests are shown as coloured lines, the diagonal as a dashed black line. Figure **3b** shows the deviations (in % w/w) of the GFS concentrations determined using PLS from the true values. The average value of the deviations from 24 PLS analyses is –0.18 % (black line), while the root mean square error of the PLS prediction (RMSEP; dashed line) amounts to 1.8 %.

variance in manual pipetting operations, for example, or by avoiding errors due to mix-ups. Specifically targeting the acceleration of the weighing-out process, ALNuMed GmbH has produced a semi-automated sample preparation technique based on the Quantos^{tm} system from Mettler-Toledo. Instead of preparing a quantity of honey defined to milligram precision in a volumetric flask, a quantity approximate to the target is weighed out in a sealable flask. The Quantos system gives feedback via a display, confirming that a specified minimum quantity of the original sample is present, to ensure the required volume is met for the later NMR analysis. The original sample is now determined with up to five digits of precision. By factoring in the physical/chemical properties of honey and the solvent used, the quantity of solvent necessary to produce a corresponding volumetric solution is calculated and then dosed fully automatically using a gravimetrically controlled dosing mechanism.
This technique cuts the time required for sample preparation in half. This automatic process is especially advantageous for high sample throughput. Samples are first identified with the aid of a barcode reader. System parameters such as date, temperature, etc. are stored alongside weighing parameters in the lab information system by the same automated process. This systematically reduces sources of error such as sample mix-ups and transposed digits. The integration of the Quantos weighing system therefore helps to maximise NMR spectrometer utilisation and generally improve process quality while simultaneously cutting costs.

Table 1 PLS analysis test results for honey types that were adulterated with various syrups. A total of 24 samples with up to 25 % w/w syrup content were available, n is the number of samples for which predictions were made. 24-n samples were used for the generation of the PLS models (calibration). For tests 1 to 4, the root mean square error of calibration (RMSEC, in % w/w) and the root mean square error of prediction (RMSEP, in % w/w) are each given. For the cross-validation with 10% of the data, the corresponding RMESP-CV (in % w/w) is given.

	GFS	Sugar beet	Sugar cane
Test 1	n = 7 RMSEC: 0.41 % RMSEP: 1.59 %	n = 6 RMSEC: 0.32 % RMSEP: 1.63 %	n = 6 RMSEC: 0.19 % RMSEP: 3.32 %
Test 2	n = 7 RMSEC: 0.41 % RMSEP: 2.20 %	n = 6 RMSEC: 0.38 % RMSEP: 0.63 %	n = 6 RMSEC: 0.14 % RMSEP: 1.88 %
Test 3	n = 5 RMSEC: 0.51 % RMSEP: 0.82 %	n = 6 RMSEC: 0.34 % RMSEP: 0.92 %	n = 6 RMSEC: 0.09 % RMSEP: 2.54 %
Test 4	n = 5 RMSEC: 0.35 % RMSEP: 2.08 %	n = 6 RMSEC: 0.29 % RMSEP: 0.83 %	n = 6 RMSEC: 0.24 % RMSEP: 4.36 %
Cross-validation 10 %	RMSEP-CV: 2.74 %	RMSEP-CV: 1.61 %	RMSEP-CV: 4.84 %

Detecting sugar syrups in honey

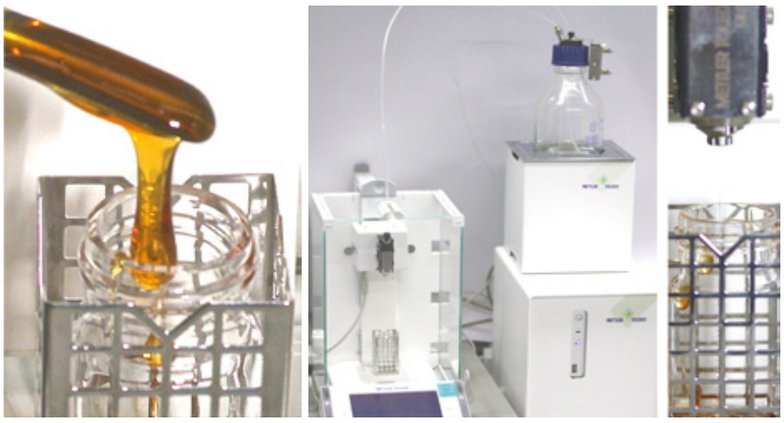
We set up a feasibility analysis whereby we adulterated five different types of honey (three types of honeydew honey, one acacia honey and one multifloral honey) each with three kinds of sugar syrup (a GFS, a sugar beet syrup and a sugar cane syrup), and with each syrup at four separate concentrations up to 25 % (w/w). The samples were then prepared automatically with the Quantos weighing system, dissolved in water, screened using a Bruker 400 MHz Food Screener and analysed using the partial least squares (PLS) method implemented in the AMIX software package. Figure 2 shows the NMR spectra of the pure sugar syrups as well as the spectra of the pure honey types. As can be seen from this figure, the sugar syrups clearly exhibit different similarities to the honey types. While sugar cane syrup (consisting almost entirely of sucrose) and sugar beet syrup (exhibiting many extra signals due to its production process) possess comparatively clear distinctions to the honey spectra, the large-scale industrial product FGS essentially contains nothing except fructose and glucose, and thus has almost no features distinguishing it from honey.

In this situation, the use of the PLS method offers real benefits. With this method, known samples are used to create mathematical regression models, with which the properties of unknown samples can then be predicted. For this study, a dedicated model was first created for each of the syrups utilised. To test the quality of the models so created, the available samples with a known degree of adulteration (24 per syrup) were subdivided into two groups. The first group, comprising 70 % to 80 % of the samples, was used for the creation of the statistical models. The remaining samples were then predicted using this model, and the deviation between the prediction and the true value was then calculated. This test was carried out four times for each syrup. As an example, Figure 3 shows the results of the PLS analyses for GFS: these involved a random selection of five to seven samples, for which predictions were then made using the PLS models created with the remaining 19 or 17 samples. The high quality of the models can be clearly seen: even samples with only 5 % glucose-fructose syrup added were identified correctly.

Table 1 summarises the results for the four individual tests and for a cross-validation performed with 10 % of the data. For each syrup used, it was possible to reliably identify a mere 5 % adulteration with the syrup. The present study leads us to expect that, once scaled-up

appropriately, the method will be capable of routinely identifying adulterations of at least 10 %.

NMR spectra contain a wealth of information about ingredients and their relative concentration ratios, and these ultimately permit us to expose the adulteration of honey with syrups. Accordingly, NMR spectroscopy is a highly suitable candidate for a corresponding rapid test. One especially relevant aspect of this approach is that NMR permits the quantification of quality-relevant ingredients in the same measurement, thus potentially leading to cost savings. Automation – by using the Quantos system, for example – also plays its part in designing a reproducible process for sample preparation and thus lowering costs.



Rapid preparation of honey samples for NMR analysis: instead of weighing-out out honey to mg precision, an approximate preparation is made. Quantos weighs the original sample precisely and adds the corresponding solvent dose automatically. Samples can be identified via barcodes. The method is quick, simple and reproducible: it cuts costs and improves the quality of the analyses.

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