Authentic food

Why a single analysis parameter is not enough

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Authentic food is growing in popularity with consumers. In a heavily industrialized market, a regional, single-source and/or specially manufactured product is increasingly becoming a guarantor of greater value. In the premium segment in particular, economically motivated "food fraud" can reap huge profits. Products affected include wines, honey and olive oil, where designations of origin and quality considerably increase value, and continue to be counterfeited, even to the present day. Honey, a product whose name has been synonymous with an all-natural, healthy food for thousands of years, is particularly affected: honey traded on the global market is adulterated with industrial sugar syrup – thus short-changing packers, distributors and consumers alike.

The only effective countermeasure is a reliable testing method. The "authenticity" of a food is an extremely complex state of affairs, however, whose regulation has not been standardized to date. Assessing the safety of foods in terms of their potential to contain residues of antibiotics, for example, is a considerably less complex matter, as will be briefly illustrated in the example below. Substances that are suspected of presenting a health risk are subjected to an appropriate evaluation. If the substance (or substance class) does indeed present a health risk, then legislation is passed that stipulates an appropriate maximum permitted level (cf. EU Regulation on Maximum Residue Levels). For analysis work, this means that a sufficiently sensitive method must be developed, but the substance (class) to be detected is of course known. Typically, pure reference substances are used to optimize the method and matrix effects are determined. If the antibiotic chloramphenicol is detected in a sample of honey, for example, this may no longer be marketed in the EU (zero tolerance; (EU) 37/2010).

				Official Reference			Honey-Profiling™
Compound	Value	Unit	LOQ	min	max	Flag	NMR Distribution
glucose + fructose	75.5	g/100g	20.0	60.0	-	●	59.2 80.0
fructose / glucose	1.22	-	-	-	-	○	0.97 1.55
fructose	41.4	g/100g	10.0	-	-	○	32.3 44.2
glucose	34.1	g/100g	10.0	-	-	○	23.7 39.1
sucrose	<LOQ	g/100g	0.5	-	5.0	●	<0.5 2.8
turanose	1.5	g/100g	0.2	-	-	○	0.6 2.5
maltose	1.3	g/100g	0.5	-	-	○	<0.5 6.3
melezitose	1.0	g/100g	1.0	-	-	○	<1.0 3.1
~							~
citric acid	93	mg/kg	50	-	-	○	<50 564
malic acid	160	mg/kg	100	-	-	○	<100 721
~							~
5-hydroxymethylfurfural	10	mg/kg	5	-	40	●	<5 52
acetic acid	18	mg/kg	10	-	-	○	<10 132
acetoin	<LOQ	mg/kg	20	-	-	○	<20 mg/kg in reference dataset
ethanol	45	mg/kg	5	-	-	○	9 1420
~							~
3-phenyllactic acid	526	mg/kg	300	-	-	○	<300 1202
dihydroxyacetone	870	mg/kg	20	-	-	○	<20 1934
kynurenic acid	<LOQ	mg/kg	60	-	-	○	<60 mg/kg in reference dataset
methylglyoxal	346	mg/kg	30	-	-	○	<30 1486
shikimic acid	<LOQ	mg/kg	80	-	-	○	<80 114

Fig. 1 Excerpt from the targeted multi-parameter quantification performed by *Honey Profiling*™ for a high-quality authentic mānuka honey. For each parameter, the unit displays the concentration measured (if above the limit of quantification, LOQ), the corresponding unit, the limit of quantification and, if available, lower and/or upper limits, supplemented with a corresponding "traffic light" indicator. The right-hand column presents a comparison of the measured values to the distribution in a reference database, in this case one holding data on authentic mānuka and kānuka honeys. The compounds 3-phenyllactic acid, dihydroxyacetone and methylglyoxal (MGO) are particularly characteristic for mānuka honeys. The MGO content is a value marker for honeys from the New Zealand mānuka bush (*Leptospermum scoparium*) and the Australian jelly bush (*Leptospermum polygalifolium*).

With authenticity, the facts of the matter are much more complex. For a food to be "authentic", its type, its geographical origin and its method of manufacture (conventional, organic, etc.) must be known, and the food product itself must not have suffered any adulterations such as being commingled with unrelated or substandard products. The consequence for analysis work is that, in contrast to the analysis of residue levels, a negative evaluation cannot reference merely a single parameter. Instead, an authentic product must, at the very least, meet all of the legal quality requirements stipulated for this foodstuff. For a blossom honey this means, in general terms (excerpt from the German Honey Regulation, HonigV [1]):

- Glucose and fructose content not less than 60 g/100 g
- Maximum sucrose content of 5 g/100 g
- Maximum water content of 20 %
- Conductivity not greater than 0.8 mS/cm
- Free acidity not greater than 50 mequ/kg
- Enzyme activity (diastase number, Schade assay) not less than 8
- HMF (hydroxymethylfurfural) not more than 40 mg/kg

If a blossom honey does not meet these requirements – which require no less than six separate analysis methods and a considerable investment of time – then it cannot be marketed. Neither the floral origin of the honey nor its geographical origin can be determined by using these parameters. Nor can recent types of adulteration such as blending with industrial fructose glucose syrup be detected.

Conventional analysis targeting honey adulteration requires many separate methods

The arsenal of analytical methods available to answer these questions is rather limited. As one example, the test method often used for detecting the addition of foreign sugars, namely stable isotope-ratio mass spectrometry (IRMS), can only detect the addition of syrups from "C4" plants such as maize or sugar cane [2]. To detect other syrups, such as from rice, sugar beet or cereal, other detection and identification methods must be used. Furthermore, to safely exclude adulteration to the greatest possible extent, three to four separate analysis methods must be applied as a minimum [3]. To identify the floral source, modern techniques analyze physicochemical parameters, organoleptic properties as well as the pollen profile, which additionally provides information about the

geographical origin. Then again, filtration is often used to remove pollen from honey, so as to avoid paying high import duties, for example [4]. In this case, or after the addition of foreign pollen to the honey, even this analysis is unable to supply any information.

Using NMR for the rapid screening of ingredient profiles

This situation can be remedied by using nuclear magnetic resonance (NMR) spectroscopy, which has been deployed in chemical analysis and medical imaging (as MRI – Magnetic Resonance Imaging) for many years. For several years now, this technology has also been successfully used for the authenticity testing of fruit juices and wines, where it has already resulted in improvements to consumer safety [5]. Properties such as reproducibility and a dynamic bandwidth, i.e. the capability to simultaneously collect data on substances at very high (e.g. 500 g/kg) and very low (e.g. 5 mg/kg) concentrations in a single measurement, make NMR spectroscopy uniquely suited to analysis work. As one example, Bruker's *FoodScreener*™, a standardized 400 MHz NMR spectrometer, facilitates the fully-automated recording of quantitative ingredient profiles for foodstuffs in just 20 min. Here, a single measurement generally collects quantitative data on 100 to 250 ingredients (depending on the foodstuff); it also proceeds beyond the detection of many known ingredients to include many substances not further characterized. With *Wine Profiling*™, for example, as many as 53 separate parameters can be quantified from a single measurement.

38 separate parameters for honey from a single measurement

With *Honey Profiling*™, a 20-minute measurement of a honey sample yields a molecular fingerprint that currently comprises 38 targeted quantitative analyses [6]. These not only include traditional chemical quality parameters such as the concentrations of fructose and glucose, their total and ratio, sucrose, maltose, melicitose, turanose, proline, HMF and ethanol, but, significantly, a range of other parameters that the literature has already defined as quality characteristics for honey (Fig. 1). As one example, the concentrations of a range of organic acids (citric acid, malic acid, lactic acid) can point to the addition of invert sugar syrup. If levels of acetic acid, formic acid and fumaric acid are too high, this may indicate fermentation. Amino acids such as alanine, leucine, phenylalanine and/or tyrosine can be investigated to enable the identification of certain floral sources, such as lavender or thyme [7]. For establishing the honey's floral source, chemical markers are essential – especially if the pollen has been removed from the honey. The marker used may be a single, individual substance or a specific combination of separate substances that correlates to a specific botanical variety. For the substances acetoin and kynurenic acid, for example, it is known that high concentrations of these compounds are characteristically found in eucalyptus honey and chestnut honey [8]. Our studies also show that both shikimic acid and succinic acid, which are two key metabolic end products in plants, can be found in high concentrations in honeydew honeys (Fig. 2).

One recent example of the need to combine multiple substances into a single marker is mānuka honey from New Zealand, a honey with medical properties [9]. The honey's antibacterial activity is thought to stem primarily from the compound MGO (methylglyoxal). Accordingly, the honey's market value is proportional to the concentration of MGO in the product. Since MGO is readily available and can be artificially added to the honey, an authenticity assay must therefore investigate a range of ingredients. In the case of mānuka honey,

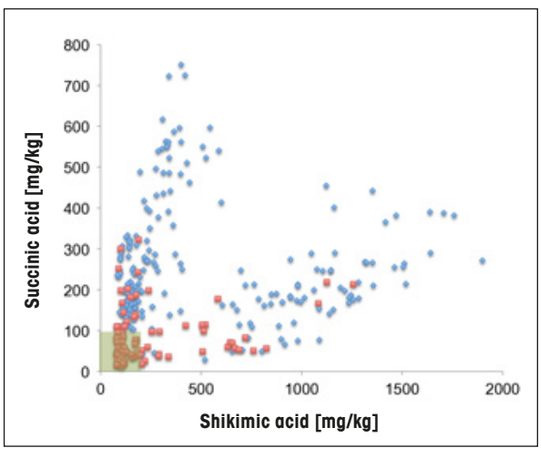

Fig. 2 Shikimic and succinic acid concentrations in honeydew honeys (blue diamonds) and blossom honeys (red squares). Of over 3,400 samples investigated, honeys are only shown for which quantification of both substances was possible. In honeydew honeys, higher levels were measured in both cases: of 298 honeydew honeys analyzed, shikimic and succinic acid concentrations of > 100 mg/kg were found in 69 % and 77 % of samples, respectively, contrasted with 2% and 5% (of over 3000 samples), respectively, for blossom honeys. If both substances are considered together (shikimic acid > 150 mg/kg and succinic acid > 100 mg/kg, exclusion area shown in bright green), honeydew honeys and blossom honeys can be correctly differentiated to an accuracy of over 98%. Combining additional parameters results in a comprehensive chemical definition of honeydew honeys.

Honey Profiling™ is also able to quantitatively determine the presence of dihydroxyacetone, whose maturation process gives rise to MGO, as well as phenyllactic acid [10]. The concentrations of these and all other ingredients measured, plus their relative ratios to one another, can then be combined to generate a characteristic fingerprint. If a substance is merely added to the honey, this disrupts the concentration ratios and makes the counterfeiting process detectable.

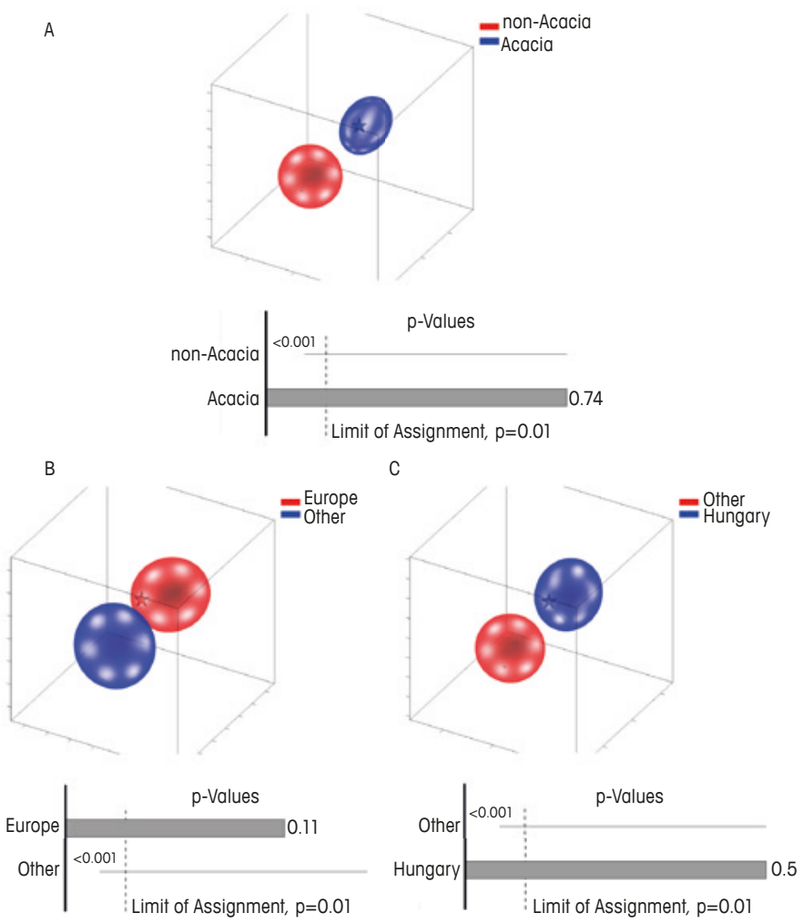

Fig. 3 Presentation of statistical analyses from *Honey Profiling*™ in relation to the floral source and geographical origin of honeys for a Hungarian acacia honey. The statistical models used are based on the quantitative ingredient profiles of several thousand honeys verified as authentic by means of conventional analysis methods. To perform the test, the operator must input the floral source and origin. The machine then tests the sample to see whether it matches the specified target model for source/origin or whether it is classified as a general honey. For country of origin testing, the sample is first tested against supraregional parameters (continent or major geographical region). The result is then shown as a statistical figure (p-value) and as a high-level graphic. If the p-value is higher than the level of assignment then the honey is classified as a member of the corresponding group. Verification of geographic origin is a key element of authenticity testing and can itself offer important indications that adulteration has taken place.

Reference database with several thousand authentic honeys

A key factor in the assessment of ingredient profiles is knowledge of the natural variance of the individual ingredients within honey. For this purpose, several thousand honeys of known source and origin were first investigated using a conventional analysis of up to 50 parameters, so as to confirm that these samples had not been adulterated by adding syrup or in some other way. The analysis results for these honeys were used to establish NMR ingredient profiles that, taken together, represent a positive definition for authentic honeys. If we now compare a new, unidentified sample with this reference database of authentic honeys and discover there is one signal too many (or too few) in the ingredient profile, although this signal is present (or absent) in thousands of other honeys, this may indicate that the honey in question has been illegally tampered with. With the aid of modern statistical procedures, this approach can use a non-targeted analysis to test honey for adulteration. Since any addition of a substance – such as a syrup – disrupts the relative concentrations of ingredients, NMR profiling can also identify unexpected or previously unknown counterfeiting techniques. This considerably expands the analytical methods portfolio, not least because conventional, targeted methods can only ever discover the type of counterfeiting being searched for. Although relevant for IRMS, as we have seen, the botanical origin of the syrup (C3 or C4 plant) is irrelevant for *Honey Profiling*™: accordingly, adulteration with rice-, wheat- or sugar beet-based syrups can all be detected [6].

Another important aspect of a comprehensive reference database is the possibility to construct ingredient profiles that are typical for a floral or geographic origin. This substantially augments the targeted analysis approach, based on the quantification of markers. *Honey Profiling*™ already enables the differentiation of important types of honey independently of the pollen profile and other conventional parameters (e.g. multifloral honey, honeydew honey, acacia honey, mānuka honey) [11]. Geographic origin is also represented in the ingredient profile: the ingredient profiles for several thousand multifloral honeys can be differentiated to permit their assignment to geographical regions and countries (Fig. 3). This can be used as an effective counter to practices such as "*honey laundering*", i.e. the deliberate misdeclaration of the geographical origin of honey while simultaneously filtering out pollen or adding foreign pollen.

Comprehensive, accredited multi-parameter testing

As we have seen, honey can now also be comprehensively screened to an extent not possible before with *Honey Profiling*™, which combines targeted quantifications of numerous parameters with modern, non-targeted statistical tests [6]. Since all of the data can be acquired with one, short measurement, this translates to considerable time and cost savings. For samples without abnormalities, this means many conventional analyses can be omitted. Abnormal parameters detected in rapid screening can be confirmed by the targeted use of appropriate conventional methods, translating to a reduction in the analysis work needed even for anomalous samples. Conversely, however, the fact that *Honey Profiling*™ tests each sample against the full range of parameters available to NMR profiling massively increases the meaningfulness of the analysis results.

For analysis work to be rapid and reproducible, it also requires a simple and robust application. This is safeguarded firstly by a uniform, optimized standard operating procedure (SOP), which specifies sample preparation in detail. By using Mettler-Toledo Quantos™ dosing balances to partially automate the initial weighing of honey samples, compatible results are obtained while considerably reducing sample preparation effort [12; Schwarzinger et al., unpublished results]. Secondly, the standardized Bruker *FoodScreener*™ is used to perform a fully-automated NMR measurement as a "push-button" procedure. The spectra measured are evaluated by a centralized service provided by Bruker BioSpin [6]. This approach, which is combined with an additional independent analysis of spectra quality, has been applied with great success for several years now to the authenticity and quality screening of fruit juices and wines, and provides users around the world with access to datasets that are always up-to-date. In this context, it should be noted that in 2015, both the central analysis module for targeted quantification work and the module for uni-/multivariate statistical analysis were accredited in accordance with the ISO/DIN/EN Standard 17025 for testing and calibration laboratories [6].

NMR profiling for honey: outlook

Options for expanding NMR-based honey analysis have been highlighted by current research work. The steadily-growing database of reference honeys forms the basis for expanding statistical tests in the future to floral sources and origin (currently available for 10 important exporting countries). By analyzing additional syrups and characterizing honeys adulterated with these syrups in the lab, we can look forward to the availability of more accurate detection patterns and markers for the addition of foreign sugars to honeys. Supplementing the 38 parameters already covered by *Honey Profiling*™, the quantification module can be expanded to include further ingredients. The focus here is on compounds that have been made available from ongoing development work or from the

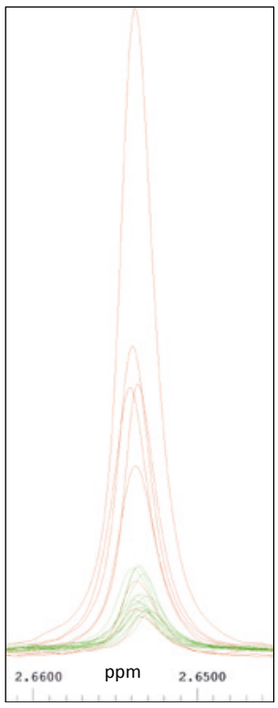

Fig. 4 Succinic acid as a fermentation parameter in ripe (green) and unripe (red) honey of identical botanical origin (region from the NMR spectrum). Ripe honey with a low water content (<20%) contains significantly less succinic acid than unripe honey with a higher water content (>20%). Apart from succinic acid, this is also true of other fermentation parameters such as ethanol, acetic acid and lactic acid. Combining these parameters means very meaningful statements can be obtained.

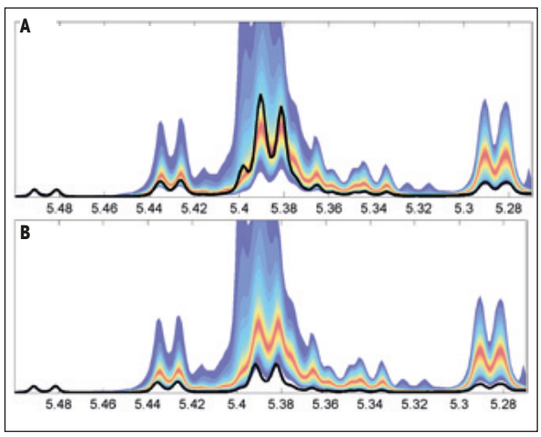

Fig. 5 Quantile plot of the sugar region in the NMR spectrum (5.28 ppm to 5.48 ppm chemical shift) from authentic honeys in the *Honey Profiling*™ reference database with two honeys of identical botanical origin (black lines). In this graph, which has been calibrated on total sugar content, the differences between various spectra can be identified independently of sample water content. Ripe honey (A) exhibits considerably higher intensities than unripe honey (B). This finding can be applied to differentiate between ripe and unripe honeys; if then combined with other parameters from the NMR measurement (cf. Fig. 4), this will further enhance the reliability of the analysis.

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literature, so as to make further improvements to the authentication of mānuka honey, for example. A fundamental advantage of NMR spectroscopy is the fact that parameters can be retrospectively detected in the spectra of thousands of authentic honeys held in the reference database as they become available, without needing to measure the samples again. This means the validation of new markers with authentic samples can be performed immediately.

One current area of research is unripe honey. An "unripe" honey is one that has been harvested before achieving the necessary level of maturity and whose water content is therefore too high, which can promote fermentation.

In some Asian countries, including China, it is customary practice to churn honey before the honeycomb has been capped with wax by the bees. Before capping, the water content of the nectar brought to the hive is reduced by the

bees themselves. The honeycomb is capped only once the water content has fallen to an appropriately low level. During the handling of the honey by the bees, a range of enzymatic reactions also takes place, which forms part of the honey maturation process. As there are key legal distinctions between using unripe honey in production and the deliberate adulteration of honey, these practices need to be distinguished.

Unripe, watery honey is often heavily processed. Volatile fermentation products and excess water are both removed using vacuum treatment, although EU law (Directive 2001/110/EC) in fact proscribes such treatment.

Preliminary results confirm that NMR data permit conclusions to be drawn about the ripeness of the honey. One approach here is to evaluate a range of fermentation parameters such as succinic acid (Fig. 4), acetic acid, lactic acid and ethanol. In water-rich (>20 %), unripe honey, fermentation leads to a considerable rise in the levels of these substances in comparison to ripe honeys with a low water content (<20 %). Other deviations can be detected by means of the proline content and in parts of the sugar region of the NMR spectrum (Fig. 5). By calibrating on total sugar content, differences between the NMR spectra can be detected without these being influenced by the deviations in water content between the samples [13]. In this context, unripe honeys exhibit lower intensities than comparable ripe honeys from the same botanical origin. The ultimate aim is to integrate these observations into the statistical models, thereby enabling these models to distinguish between ripe and unripe honey, and between unripe and adulterated honey.

Furthermore, comprehensive preliminary experiments have already shown that NMR spectra can even be correlated with parameters such as water content, acid values, conductivity and other parameters as listed in the Honey Directive (Fig. 6).

Honey Profiling™ offers a new and comprehensive rapid analysis system for honey, which reduces costs on the one hand while also being capable of increasing the reliability of the analytical verdict, since many parameters can be considered simultaneously. Current research leads us to expect that NMR-based authenticity and quality screening for honey can be made even more sensitive and comprehensive in the future. This will establish further barriers against adulteration or misdeclaration, thereby further strengthening customer confidence in honey as a natural product.

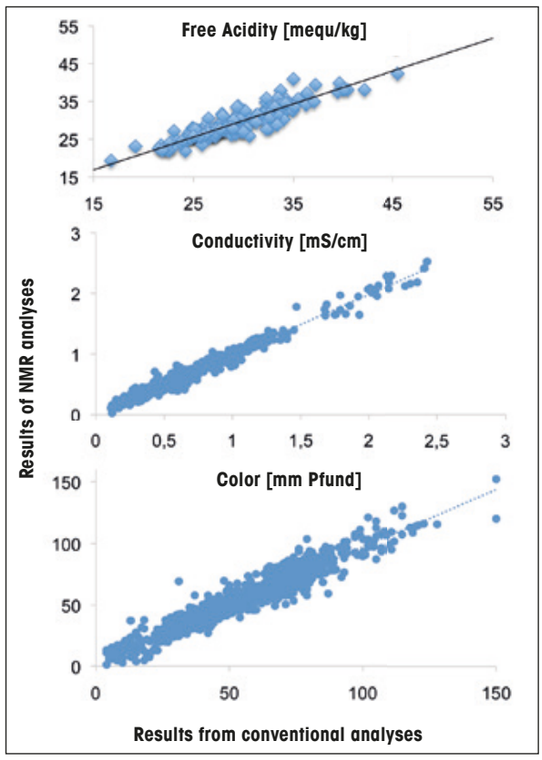

Fig. 6 Correlation of NMR data with data from other conventional analyses. Since NMR profiling can be used to collect data on a great many ingredients, such as acids or compounds responsible for color, correlations with other parameters can also be found. This involves using statistical methods (such as PLS regression) to correlate conventional and NMR analyses to produce statistical prediction models that can then be applied to analyze new and unknown samples. In an initial investigation of the acid value (max. 50 mequ/kg) from over 140 honeys, for example, a correlation coefficient R^2 of 0.83 was found between NMR data and conventionally titrated results (Köberle, Dudenhöfer, Schwarzinger et al., unpublished results). A further study involving over 1,400 honeys has demonstrated very good correlations between NMR spectra and a honey's conductivity (R^2=0.98) and color (R^2=0.93). Correlations with additional parameters such as water content and enzyme activity have also been the focus of other studies (Brauer, Kämpf, Schwarzinger *et al.*, unpublished results).

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