



■ **Stephan Schwarzingler, Felix Brauer and Paul Rösch**

Research Center for Bio-Macromolecules, University Bayreuth, ALNuMed GmbH

■ **Bernd Kämpf**

FoodQS GmbH, Markt Erlbach

Honey, what else?

Rapid testing of variety, geographic origin, and absence of adulteration by NMR-profiling

Honey – for thousands of years it has been the only source of sweet taste, and it still is the prototype of an all-natural, healthy food. In particular with the growing trends for organic food and a healthy life-style honey has enjoyed steadily increasing popularity. Unfortunately, while demand is on the rise, supply is short. The reasons for this are complex and interwoven and have their origin in bee diseases, climate change as well as agro-industrial production methods. As a consequence, an increasing number of honeys mixed with non-natural sweet syrups has been detected in the international trade. Such economically motivated adulteration is seen also in other foods such as fruit juice, olive oil or wine, and it is most reliably exposed by *NMR-profiling*. This technology is based on the comparison of hundreds of spectral features of authentic honeys with the sample to be tested.

Owed to its special role as sweet food in the development of mankind, honey today enjoys special protection by the law¹. However, serious issues in honey production complement the rise in the demand for honey. A complex combination of factors, including the *Varroa* mite putting stress on bee colonies by transferring other bee pathogens and the intensification of agricultural production with an escalating use of fertilizers, pesticides, and insecticides, have led to a phenomenon called colony collapse disorder (CCD). CCD resulted in losses of more than

80% of the hives in certain cases in the past years². In addition, the changing climate impacts honey production, as observed, e.g., in 2014, when long periods of rain in the flowering season resulted in a complete loss of production in some European countries.

Economically motivated adulteration on the rise

Production shortages lead to price increases, which in turn give rise to a growing number of adulterated honeys in the market. In the particular

case of honey the authentic product is diluted with various sugar syrups, which are produced at industrial scales from, e.g., corn, rice, or wheat at a fraction of the price of honey. Mixed products, or products with the pollen being removed are strictly forbidden in the European market, but are accepted elsewhere and thus participate in global trade. Economically motivated adulteration not only includes mixing of honey with cheap syrups, but extends to disguising the geographic origin of a particular honey, a type of fraud known as ‘honey laundry’. Honey laundry became public recently in the ‘Honeygate’ scandal in the USA, where honey from China was wrongly declared on a large scale to obfuscate its country of origin. Lost excise duties were rumoured to be as high as USD 180 million³. To further obscure the product’s geographical origin – typically tested by analysing the pollen spectrum – pollen is now increasingly being filtered out of the honey, which – on the other hand – is simple to verify.

Counterfeiting honey with plant-derived sugar syrups, on the contrary, is analytically very demanding to prove. In essence, honey consisting mainly of the two mono-saccharides glucose and fructose is adulterated with its main constituents. Syrups from so-called C4-plants, such as corn or cane, exhibit a different ratio of the stable isotopes of carbon, ¹²C and ¹³C, which can be proven by stable isotope ratio mass spectrometry (IRMS), a method routinely used in honey analysis⁴. However, addition of syrups originating from wheat, rice, and other C3-plants cannot be detected by the same method. To ensure the authenticity of a honey, typically a combination of several analytical methods is applied today that includes IRMS, detection of non-honey oligo-saccharides, enzymes used in industrial syrup production, as well as small molecule markers for foreign syrups. In addition, dedicated substances can be used to test for authenticity requiring a set of different analytical techniques. All this takes a lot of time and is costly; more complex issues such as those involving a food’s provenance, however, cannot be answered at all by only quantifying a limited set of substances.

NMR fingerprints prove quality, authenticity and unveil adulteration in just one measurement

Remedy is provided by nuclear magnetic resonance (NMR) spectroscopy, a technique that is also applied in medical imaging (MRT). In contrast to other techniques, which typically focus on the detection of specific, pre-selected markers (‘targeted analysis’), an entire spectrum of the ingredients of the sample is recorded. Hence, high-resolution NMR spectroscopy permits the ‘non-targeted’ detection and – at the same time – quantification of typically several dozens of substances within a

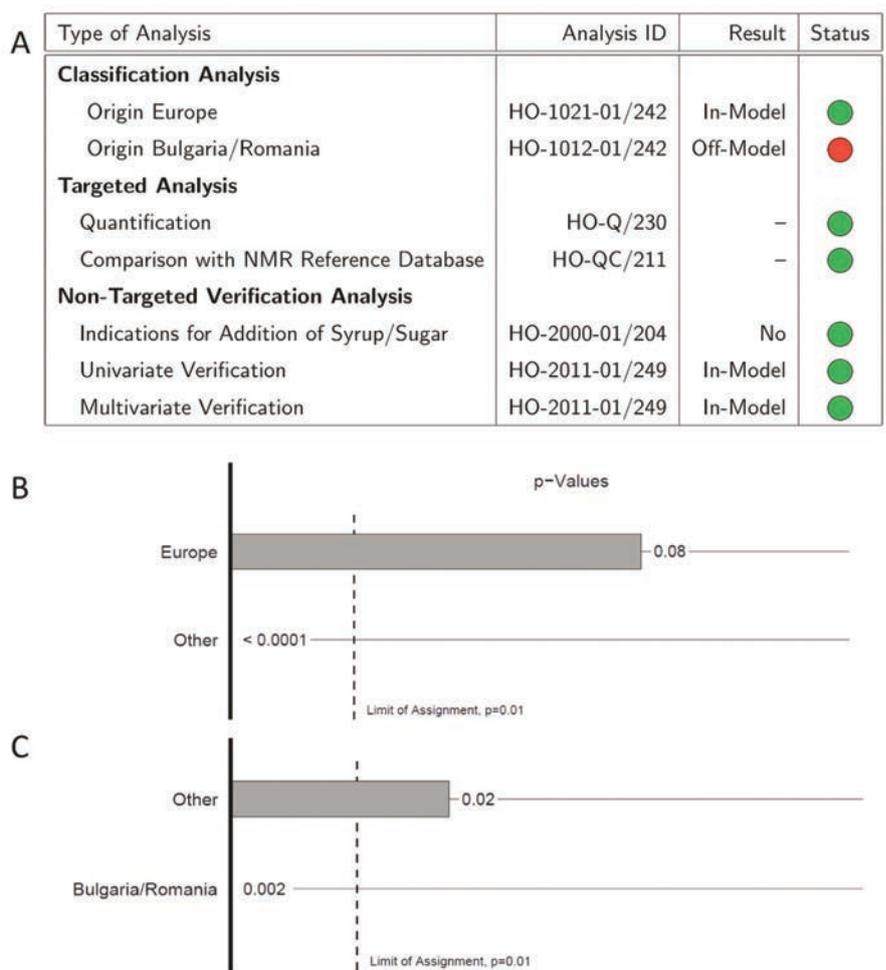


Figure 1: Excerpt from *Honey-Profiling™* report for a honey with falsely declared geographic origin. A) Traffic-light summary of results of the analysis. B) p-value for the statistical classification of the region of origin, which is Europe in this case. C) p-value for the classification of the country of origin. In this case, the threshold limit is not reached, which means that the origin claimed cannot be confirmed

“The NMR-spectrum of an unknown sample can be compared with spectra from authentic reference samples by automatic methods”

single measurement. This takes only a few minutes. Accordingly, the method is predestined for the efficient analysis of complex substance mixtures as found in food: unlike methods using, e.g., chromatography, no time-consuming separation of the components in a mixture is required. At the same time, the sample can also be measured without extensive chemical preparation (e.g. extraction, derivatisation). Moreover, due to its unique dynamic range the reproducible retrieval of signals arising from substances at very low concentrations (ppm range) in the presence of highly concentrated compounds (% range) is possible. We might consider a Riesling wine, 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 products (metabolites). Yet variations in climate and soil quality will lead to relative differences in concentration.

Accordingly, the systematic differences in concentration of a range of metabolites can be aggregated into a pattern, which can be used as a marker for geographical origin or the absence of unlawful adulterations. This way, the NMR-spectrum of an unknown sample can be compared with spectra from authentic reference samples by automatic methods. These mathematical procedures can then be used to classify new and unknown samples by comparison with the reference database.

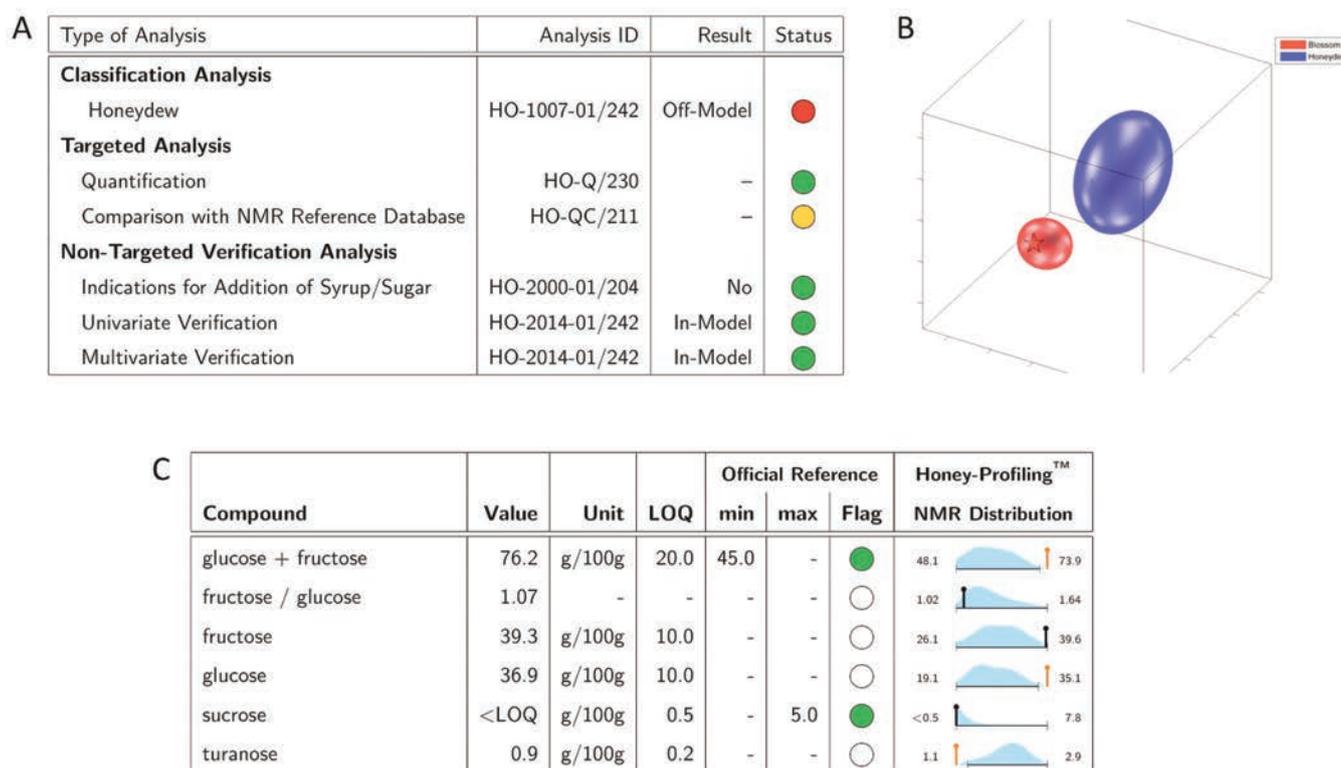


Figure 2: *Honey-Profiling™* analysis of variety of a honey. A) Traffic-light summary immediately indicates that the honey investigated is not a more costly honeydew honey and the deviations in the targeted analysis occur in comparison the NMR reference distribution. B) Graphical representation of statistical classification result: sample falls into the cloud representing blossom honeys. C) Example for deviation of targeted analysis from NMR-reference distribution: Although all parameters are in line with quality regulations for honey in general clear deviation from the NMR-reference distribution for honeydew honey is detected. In total, six out of 38 parameters deviate from the reference distribution indicating this is not honeydew honey

The procedure is already routinely used in the *FoodScreener™* (*Bruker BioSpin*, Rheinstetten, Germany) for fruit juice (*SGF-Profiling™*) and wine (*Wine-Profiling™*), where a significant number of quantitative quality parameters required by regulatory bodies are obtained⁵. In addition, many novel parameters characteristic for, e.g., variety, origin, vintage, degradation, and processing can be recorded in full automation during a measurement time of just 15 minutes. Also in the case of honey NMR spectroscopy offers a versatile multi-parameter screening tool to test for quality, authenticity, and adulteration with minimal time effort and cost.

Key to success – the reference database

Precondition for such analysis is the establishment of a sufficiently large reference spectra database of material with proven authenticity. A consortium of several analytical laboratories specialised in honey analytics and the NMR-based instrument maker and solution provider *Bruker BioSpin* worked together to develop this database as major part of the new the *Honey-Profiling™* which works on the base of the well established *FoodScreener™* platform. Conventional analytics had been used to support conformity of data base entries. Hence, methods included mellisopalynology (pollen analysis) to verify botanical variety and geographical origin, classical quality parameters such as glucose, fructose, HMF, moisture, pH, conductivity, enzyme activity, organic acids, ethanol, as well as the classical methods for detection of adulteration as above. A total of up to 18 different parameters were determined for each authentic sample.

Central database and ISO 17025 accreditation

To ensure creation of a sufficiently large database covering many floral

varieties as well as worldwide origins the *Honey-Profiling* consortium had to undertake an unparalleled collaborative effort in the honey business. However, to establish statistical models for discriminating adulterated from authentic samples a suitably large number of adulterated samples is required. Accordingly, a large number of samples were mixed with syrups and bee-feed at defined levels. To this end, the *Honey-Profiling™* Consortium has measured several thousand honeys, including comprehensive supplementary conventional analysis. This way a previously unmatched coverage of the natural variation of ingredients of authentic honey can be defined, which is indispensable to define the ingredient profile of authentic honey. The acquired knowledge builds the foundation for establishing a positive definition of how authentic honey should look like. Any deviation from the patterns found in the database that does not match any variety or geographical origin might be an indication for an adulteration. Inclusion of samples from different years of vintage secures that the statistical models generated are generally applicable. The large scatter in geographic origins further ensures that a large variety of poly-floral patterns are represented in the database. Thus, even annual changes in floral composition in a honey are covered. Larger changes, which may be due to climate change or changes in agriculture, are surveyed by annual updates of the database by the members of the *Honey-Profiling™* consortium, at the same time leading to an increase of coverage by the database. For *Honey-Profiling™* a remote data analysis concept was established located and maintained by *Bruker BioSpin*. This approach, where the NMR spectrum of the samples is recorded at the user's site and remotely evaluated has proven to be successful already in the case of fruit juice and wine testing: To ensure comparable and robust analytical results, both the modules for

A

Type of Analysis	Analysis ID	Result	Status
Targeted Analysis			
Quantification	HO-Q/230	–	●
Comparison with NMR Reference Database	HO-QC/211	–	●
Non-Targeted Verification Analysis			
Indications for Addition of Syrup/Sugar	HO-2000-01/204	Yes	●
Univariate Verification	HO-2019-01/242	Off-Model	●
Multivariate Verification	HO-2019-01/242	In-Model	●

B Non-Targeted Verification Analysis

Indications for Addition of Syrup/Sugar

(Analysis-ID: HO-2000-01/204)

There are indications for an addition of syrup or sugar.

Please note that botanical variety and geographical origin of the sample have to be regarded before deducing final conclusions.

Univariate Verification

(Analysis-ID: HO-2019-01/242)

Applied Model: AllTypes

Result: Deviating signals were found at following chemical shifts:

3.286^{high}

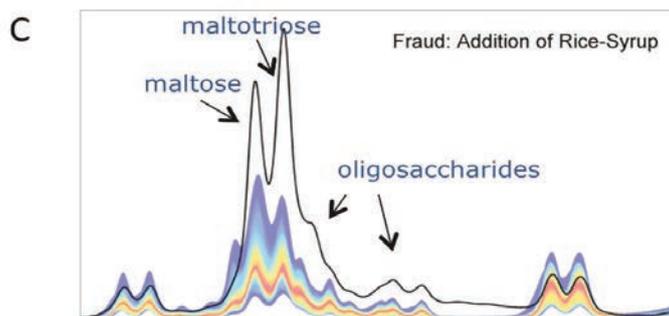


Figure 3: Analysis of honeys adulterated with syrup. A) Traffic-light summary indicating addition of syrup. B) For the summary shown characteristic concentration ration (compiled into indices) for authentic honeys are violated resulting in a remark highlighting the possible presence of syrup. In addition, the deviation detected in the NMR spectrum in the course of univariate testing are reported, in this case indicating a higher than typical maltose/maltotriose concentrations. C) Addition of rice syrup typically manifests in clear deviation (black line) from the naturally occurring variation in authentic honeys (colored area)

statistical testing as well as for targeted direct quantification of substances are also carried out as server-based services by *Bruker BioSpin*. This procedure, which results in a straightforward interpretation of the data for the user, also allows fast implementation of new statistical models and targeted parameters as these become available. These central modules for quantification as well as the untargeted statistical testing for origin, variety, and adulteration were accredited as apart of the *Bruker BioSpin* laboratory’s flexible accreditation early in 2015 according to the ISO 17025 standard for fruit juices, wine, and also honey and are available to *FoodScreener™* platform users.

“It is of pivotal importance to judge a honey by several criteria simultaneously”

standardized *FoodScreener™* platform from *Bruker BioSpin*, operating at 400MHz. The system is operated in a push-button mode and automatically takes over all necessary steps for obtaining reproducible high-quality spectra, including tuning, matching temperature equilibration, pulse width calibration, as well as spectral processing. This way, operator specific influences are removed. The system is under full control of a LIMS system which controls operation of the NMR spectrometer and communicates with the server at *Bruker BioSpin*, where the automated spectral processing, data evaluation, and report generation is performed.

**Push-button operation:
Automation indispensable for accurate results**

For comparison of NMR spectra generated by third-party laboratories reproducibility of the spectra is of utmost importance as well as ease of operation. Honey is dissolved and samples were mixed with *Bruker BioSpin Wine-Profiling* buffer, and the pH was precisely adjusted utilizing an automated *Bruker pH titration* unit. NMR spectra are obtained by a

Practical application proves: NMR fingerprints characteristic for variety and geographic origin of honey and adulteration

It is of pivotal importance to judge a honey by several criteria simultaneously, which *Honey-Profiling™* provides from a single measurement and in an automatically generated report interpretable by the food specialist. This shall be demonstrated in three examples.

First, we demonstrate the power of *Honey-Profiling™* to verify a given country of origin. This is of growing importance as the country of origin is increasingly used in marketing and declared on the honey jar label. In many countries – if declared on the label – the honey must exclusively originate from this named country, which often justifies a higher price. E.g., when a non-EU honey is used in a blend this honey must not be labelled as originating from EU countries. **Figure 1** (page 2) depicts an excerpt of the report for a honey, where the supplier has claimed Bulgaria as country of origin. *Honey-Profiling™* proceeds stepwise, first testing for the region of origin (here Europe). Second, the honey's NMR profile is compared with authentic samples from the claimed country of origin. It can clearly be seen in the traffic-light summary of the report (**Figure 1A**, page 2) that the country of origin claimed does not match the reference database. Although the region of origin, Europe, could be confirmed (**Figure 1B**, page 2), the country of origin is not Bulgaria (**Figure 1C**, page 2). Experience shows that falsely declared origin often is accompanied by additional manipulations, which are not readily detectable.

Honey-Profiling™ also allows testing for the variety without the need of pollen analysis. For instance, a blossom honey labelled as honeydew can easily be detected (**Figure 2**, page 3). Not only statistical classification immediately identifies the fraud (**Figure 2B**, page 3), *Honey-Profiling™* also provides numerous quantitative parameters strengthening the overall analysis. The comparison with the distribution of a parameter being looked at in comparison with the reference database has proven particularly useful. It can clearly be seen that concentrations for glucose and the sum of glucose and fructose are too high in comparison with all other honeydew honeys, while turanose concentration is too low when compared to the reference distribution for honeydew honey (**Figure 2C**, page 3). At present *Honey-Profiling™* can classify honeydew and poly-floral honey as well as mono-floral varieties, e.g., Manuka. For the latter, which due to its anti-bacterial activity reached high prices in the market, specific marker compounds correlating with the value of this honey (MGO, DHA, phenyllactic acid) are determined allowing rapid analysis of this expensive honey.

Testing for adulteration can also be done without this information as shown in **Figure 3** (page 4). The poly-floral honey tested was supplied without further information about its possible origin. However, the traffic-light summary of the *Honey-Profiling™* report immediately signals a possible addition of syrup. By systematic comparison of the ratio of a large number of NMR signals in authentic and proven adulterated honeys, a set of indices was derived that indicates adulteration. If violated, a corresponding remark is made in the report. In case additional signals in the sample, which are absent in all authentic honeys (or *vice versa*), are detected in the course of univariate analysis, the corresponding region in the spectrum is reported (**Figure 3B**, page 4). In most cases of addition of syrup clear indications can be found in the spectra, such as the presence of elevated concentrations of oligosaccharides or maltose or maltotriose (**Figure 3C**, page 4). Of note, the deviation depicted in **Figure 3C** (page 4) originates from the addition of rice syrup, which cannot be detected by carbon IRMS.

In summary, using a highly automated NMR approach in combination with a carefully assembled centralised database of authentic as well as known adulterated samples it is possible by means of ISO 17025 accredited untargeted methods to establish quantitative molecular fingerprints characteristic for natural honeys from different varieties and

different origins. An important advantage of this approach is the fact that even yet unknown adulterations can be detected by untargeted uni- or multivariate tests. In addition, as NMR is quantitative for all signals detected above a threshold intensity, it also permits a targeted approach with a multitude of parameters simultaneously. Since NMR covers the analytical statements of several methods conventionally used in quality and authenticity testing of honey it can contribute to saving time and costs as well as producing so far unavailable information on authenticity and adulteration.

The authors thank Dr. Manfred Spraul and Dr. Birk Schütz (*Brucker BioSpin*), as well as Mrs. Gudrun Beckh and Dr. Arne Dübecke (*QSI*, Bremen) for many insightful discussions and Britta Zimmermann (*ALNuMed*) for expert technical help.

About the Authors

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.

Bernd Kämpf studied food chemistry at the FH Isny. Since 1999 he is head of the laboratory and head of quality assurance at Breitsamer and Ulrich. 2014 he was appointed executive manager at FoodQS. His interests lie in the field of honey analytics, in particular residue analytics.

Felix Brauer completed his graduate studies in biochemistry and a master's in biochemistry and molecular biochemistry at the University of Bayreuth. He has worked as a research assistant at ALNuMed GmbH and doctorand at RC BIOmac since 2013. His research interests include NMR-based food analytics and the integration of methods used in metabolomics and spectroscopic profiling.

Paul Rösch studied physics at the universities of Karlsruhe and Heidelberg before receiving his doctorate at the Max Planck Institute for Medical Research in Heidelberg. This was followed by a post-doc at the University of Pennsylvania Medical School in the USA, and a post as research assistant at the Max Planck Institute for Medical Research in Heidelberg. In 1989, he completed his habilitation in biophysics at the University of Heidelberg. He has been head of the Department of Biopolymers since 1990 and Executive Director of the Research Centre for Bio-Macromolecules (RC BIOmac) at the University of Bayreuth since 2007. His key areas of research are NMR-based biomedical structural research, focusing in particular on the molecular basis of food allergies, and research into bacterial transcription as a target for new antibiotics.

References

1. Council Directive 2001/110/EC of 20 December 2001
2. http://en.wikipedia.org/wiki/Varroa_destructor. Genersch, E. et al. (2010) The German bee monitoring project: a long term study to understand periodically high winter losses of honey bee colonies, *Apidologie* 41, 332–352. Sgolastra, F. et al. (2012) Effects of neonicotinoid dust from maize seed-dressing on honey bees. *Bull. of Insect.* 65, 273–280
3. <http://www.foodsafetynews.com/2013/02/honeygate-sting-leads-to-charges-for-illegal-chinese-honey-importation/#.U-H6lVY4ywc>
4. Elflein, L., Raezke, K.-P. (2008) Improved detection of honey adulteration by measuring differences between $^{13}\text{C}/^{12}\text{C}$ stable carbon isotope ratios of protein and sugar compounds with a combination of elemental analyzer — isotope ratio mass spectrometry and liquid chromatography — isotope ratio mass spectrometry ($\delta^{13}\text{C}$ -EA/LC-IRMS), *Apidologie*, 39, 574–587
5. Spraul, M. et al. (2009) NMR-based multi parametric quality control of fruit juices: SGF profiling, *Nutrients* 1, 148–155. Godelmann, R. et al. (2013) Targeted and nontargeted wine analysis by ^1H NMR spectroscopy combined with multivariate statistical analysis. Differentiation of important parameters: grape variety, geographical origin, year of vintage, *J. Agric. Food Chem.* 61, 5610–5619

Authenticity & Quality

FoodScreener™
Honey



- Simultaneous identification and absolute quantification of a multitude of relevant parameters with reference to NMR distribution
- Development of statistical models for authenticity and geographical origin
- Detection of frauds like addition of rice syrup or other types of sugar
- Detection of unexpected and even unknown frauds

Contact us for more details: www.bruker.com/honey



NMR