Research Article

Magnetic Resonance in Chemistry

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Mixture analysis by NMR as applied to fruit juice quality control

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Nuclear magnetic resonance (NMR) spectroscopy is rapidly gaining importance in mixture analysis, originally driven by the pharmaceutical and nowadays also by clinical applications within metabonomics. Quality control of food-related material has very similar requirements, as it also deals with mixtures, and many of the compounds found in body fluids are analyzed as well. NMR allows analysis in two ways within one experiment: namely, targeted and untargeted. Targeted stands for the safe identification and consequent quantification of individual compounds, whereas untargeted means the detection of all deviations visible by NMR using statistical analysis based on normality models. Very important is the stability and reproducibility of the NMR instrumentation used, and this means inherent minimized system internal variance. NMR is especially suited for such requirements, as it allows detection of the smallest concentration changes of many metabolites simultaneously. High-throughput flow-injection NMR as the basis for fruit juice screening allows low cost per sample and delivers substantially more relevant information than any other method and is probably the only method to produce such results.

Keywords: NMR; mixture analysis; fruit juices; quantification; statistical analysis

Introduction

Traditionally, NMR has been perceived as a tool for structure verification, elucidation and purity analysis. However, driven by the needs of metabonomics/metabolomics, NMR has rapidly expanded into the field of mixture analysis and multiple screening applications during the past years.[1–5] Emphasis was especially on the pharmaceutical application of drug toxicity screening using rodent urines.[6–13] This was enabled by high-throughput sample changing technology (both tube- and flow-injection[14] based), integrated sample preparation and improved quality of digital spectrometers in terms of stability, reproducibility and receiver dynamics. Besides flow-injection NMR, also the hyphenated methods of liquid chromatography (LC-NMR) and LC-NMR/MS in combination with post-column solid-phase extraction have especially enabled mixture analysis by isolating and identifying individual compounds.[15–19]

Today, NMR is a major tool in a wide range of metabonomics-related applications including drug toxicity and efficacy screening on animal models, clinical diagnostics in lipoprotein subclass analysis[20,21] as well as general health status screening in the context of epidemiological research,[22] nutrition and functional food effects,[23,24] to name a few examples. In such studies, hundreds of samples have to be screened per day, looking for identity and concentration of selected metabolites as well as for between-sample comparison of spectral line patterns using multivariate statistics, e.g. in order to obtain classification and discrimination information. NMR is an especially suited *detector* for analysis of fluids of biological origin, food materials or drinks.[25–29] It combines truly quantitative and structural information with high throughput (a 1D spectrum can be measured in a few minutes) and excellent reproducibility, mostly relying on minimum sample preparation without derivatization needs (typically just buffer addition).

In this article, it will be shown that the principles established in metabonomics NMR have been successfully transferred to yet another but closely related field of mixture analysis, i.e. quality control of beverages. A method for fruit juice analysis developed in a joined effort by Bruker BioSpin GmbH and SGF International e.V. has been introduced under the name SGF-profiling recently, where SGF stands for spin generated fingerprint.[30] The system is fully automated with respect to sample transfer, measurement, data analysis and reporting and is setup on a 400-MHz flow-injection NMR spectrometer. For each juice sample, it simultaneously evaluates a multitude of quality- and authenticity-related parameters from a single dataset acquired within a few minutes combining targeted and untargeted analysis. With this multi-marker/multi-aspect screening approach, which involves low cost per sample, NMR becomes highly competitive to conventional and targeted juice quality control based on, e.g. enzymatic tests, GC or LC.

Initially, the new NMR-based method was developed as a cost- and time-efficient pre-screening tool to identify suspicious samples likely to have a quality/authenticity issue and hence to be sent for conventional analysis. However, after having established a spectral database, currently containing spectra of more than 3000 reference juices of *ca* 1000 fully authentic samples taken by inspectors on site, one can clearly see that the potential goes far beyond this.

Figure 1 shows a comparison of different juice ^{1}H spectra at 400 MHz: lemon, grapefruit, black currant, peach and pineapple.

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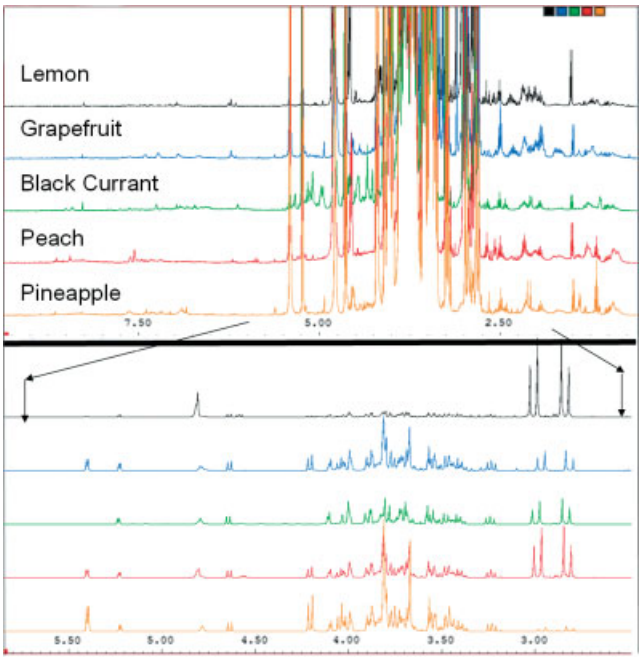

Figure 1. NMR spectra of different types of fruit; upper part: unscaled spectra overview, lower part: sugar/citric acid region scaled to the largest signal.

The lower part shows the spectra scaled to the largest signal in the sugar/citric acid region, and the upper part shows Y-expansions of the whole spectra. Spectra even of different juice types often have very high similarity, and differentiation by visual inspection is difficult if not impossible. Despite this fact, NMR can deliver a number of parameters using, especially, statistical analysis.

Instrumental Considerations and Acquisition Details for Fruit Juice Quality Control

Fruit juice profiling for cost reasons is optimally based on a 400-MHz NMR spectrometer with especially shielded magnet technology, which in the case of a 400-MHz system ensures that the five gauss line of the magnet stays within the magnet can. The system most efficiently operates with flow-injection NMR having a 4-mm o.d. flow probe with Z-axis gradient using water as transfer medium. A liquid handler is used for sample preparation, intermediate storage and transfer. Preparation consists of buffer addition and consequent mixing to adjust to a pH of ~3. Juices with solid particles, such as pineapple or orange juice, have to be centrifuged in the first preparation step.

D_2O is part of the buffer addition to adjust a juice-to-D_2O ratio of 90 : 10. This allows locking the spectrometer on deuterium for long-term spectral quality. To minimize handling errors, the samples are provided in barcoded cryovials or wellplates, and cooling racks keep the samples at low temperature (about 4 °C) prior to injection. A heated sample transfer line from the liquid handler to the probe and a heated capillary inside the probe preheat the sample during transfer such that the sample would have attained the correct measurement temperature (300 K) when it arrives in the measurement cell. In this way, no extra temperature equilibration time inside the measurement cell is needed prior to measurement, thereby minimizing the turnover time. The overall NMR procedure has to consist of (i) automated tuning and matching of the probe, (ii) automated gradient shimming, (iii) pulse calibration, (iv) measurement of a gradient profile for filling status assessment, (v) acquisition and processing of a 1D version of the 2D NOESY sequence[31] with water presaturation and pulsed field gradients, (vi) acquisition and processing of a fast J-resolved 2D spectrum (see Fig. 2 and caption) and (vii) a second gradient profile to ensure correct sample positioning not only after transfer but during the complete measurement procedure. Data analysis and reporting is automatically performed to complete the overall process. Due to the quality of preparation and measurement, all steps described are possible completely unattended, thus cutting the analysis cost substantially. Cost efficiency is vital in this analysis, as NMR is entering a field where expensive machinery is typically not used; however, the higher initial cost can be counteracted by low cost per sample in a high-throughput screening mode. Figure 2 shows a Y-scaled superposition of 40 replicates of an apple juice that have been prepared, transferred to the NMR flow cell, measured and processed under full automation. This type of reproducibility is unique for NMR and shows the very low system internal variance that is needed for meaningful statistical data evaluation.

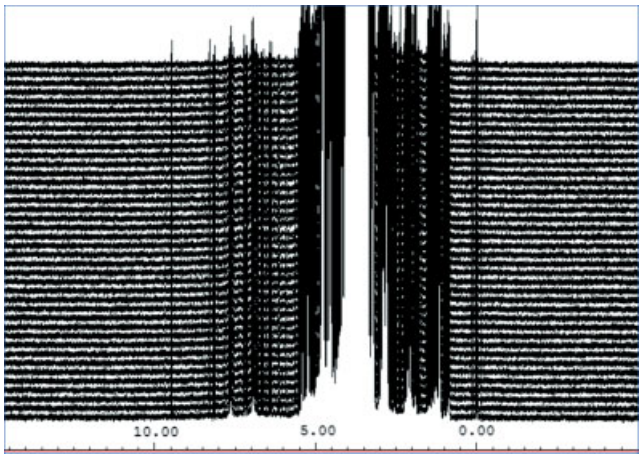

Figure 2. Automatic preparation, transfer, measurement and processing of an apple juice, repeated 40 times on a flow-injection 400-MHz NMR spectrometer. No manual interaction or baseline correction is needed, and phasing is done fully automatically.

Automatic Data Analysis

A typical batch size of samples in fruit juice screening is between 50 and 120 in one measurement session, so automatic data analysis and report generation are mandatory in fruit juice profiling. Several compounds have to be quantified (targeted approach), and a cascade of statistical tests and classification and discrimination steps has to be applied to the spectra in order to investigate multiple quality aspects (untargeted approach).

Identification and quantification of selected compounds

Specific deviations in concentrations of single compounds or combinations thereof may indicate characteristic quality problems as found, for example, in the addition of sugar. Therefore, concentration information is of primary interest for the food chemist in the classical juice assessment procedure. Here, NMR spectroscopy has a clear advantage, being able to quantify

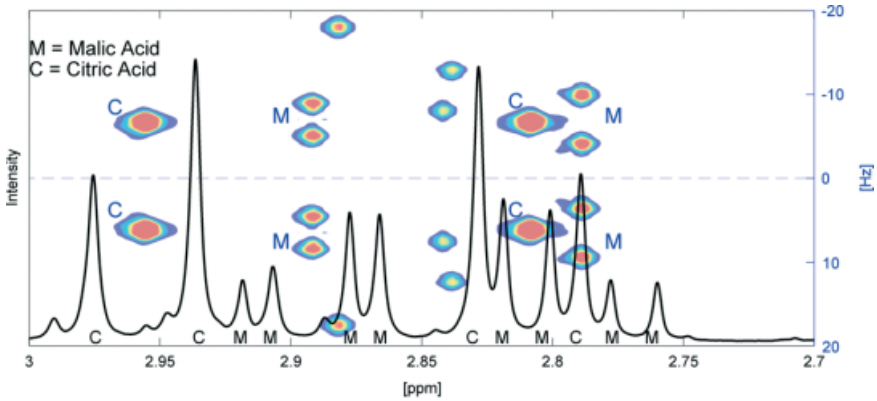

Figure 3. NMR experiments in screening: 1D NOESY with fast J-resolution. The 2D J-RES is helpful for identifying compounds during the quantification process (the figure shows the overlap of citric acid (C) and malic acid (M) in peach puree). Unlabeled peak groups stem from impurities and higher order artefacts.

multiple compounds from just one measurement in addition to the statistical untargeted evaluations explained in the following. At the moment, in the SGF profiling process, concentrations of more than 20 different compounds are determined (depending on the type of juice): i.e. malic acid, citric acid, lactic acid, fumaric acid, quinic acid, succinic acid, citramalic acid, formic acid, benzoic acid, sucrose, glucose, fructose, proline, alanine, 5-hydroxy-methylfurfural (HMF), phlorin, ethanol, methanol, acetic acid and galacturonic acid. Furthermore, meaningful internal relations of these values are calculated, such as the ratio of glucose and fructose or the ratio of sucrose *versus* total sugar.

In Europe, a Code of Practice has been established by the fruit juice industry, which contains concentration ranges of several indicative compounds for different types of juices. The automation established in the SGF profiling compares the values found by NMR and uses a traffic light approach to indicate if a concentration determined is within the range defined by the Code of Practice. With NMR, however, there is constant addition of concentration information for new indicative compounds not listed in the Code of Practice.

Before a concentration can be determined, the corresponding compound has to be identified securely, and this is often not possible by only using 1D NMR. Therefore, a rapid 2D J-resolved spectrum[32] is executed for each sample, in addition to the 1D spectrum, and typical experiment time for this is about 2–3 min, acquiring 40 increments and applying linear prediction in F1.

Figure 3 shows the use of the 2D in the example of peach puree with overlapping signals of citric and malic acid in 1D, which are clearly separated in the J-resolved spectrum.

After peak-picking in the 2D spectrum and recreating a 1D spectrum from the peak-picking list, one has the information on which signals have to be integrated together to produce the correct concentration values for each of the compounds involved using a deconvolution approach. The process described is executed under full automation after the measurement and processing step is finished.

Further parameters to be looked at for correct quantification are the T1 times of the relevant compounds. Since the measurement procedure described runs under high-throughput conditions, it is not possible to wait for full relaxation for all compounds. Therefore, a correction factor is needed to compensate for signal reduction due to T1. T1 values have to be measured once for every juice type and stored into a knowledge base.

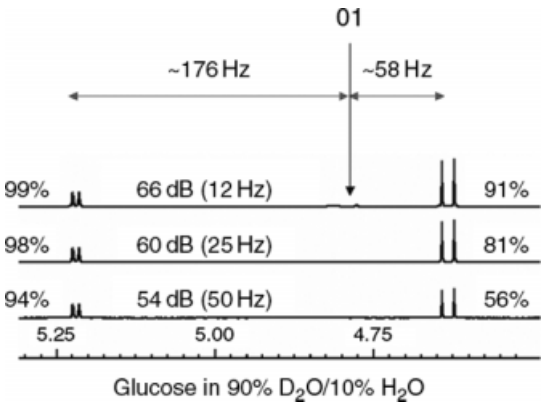

Figure 4. effect of presaturation strength on anomeric signals of alpha- and beta-D-anomeric protons of D-glucose at 400 MHz (alpha left, beta right side of spectrum).

When quantifying glucose directly, it is also necessary to calibrate the presaturation power used for water suppression. Even with a 25-Hz field at 400 MHz, the beta-D-glucose anomeric proton at 4.6 ppm is still reduced in intensity, while the alpha anomeric signal is affected only very slightly. Since NMR concentration determination of D-glucose needs the addition of both anomeric signals (these are used because of minimized overlap), the reduction factor for the beta anomeric signal also has to be taken into account. Figure 4 shows the effect of changes in presaturation power on the two anomeric signals of D-glucose. It is obvious that without calibration the results can be incorrect and comparison of results from different instruments would also not be possible.

Absolute concentrations of compounds on modern spectrometers with digital receiver units can be directly calculated after calibration has been done once with a known compound and known amount. With this property of digital receivers, it is not necessary to add artificial signals like the ERETIC signal[33,34] into the signal pathway during measurement any more. For visualization purposes alone, it is now sufficient to add an artificial signal after the measurement. After calibration, this signal, of course, can also be used for quantification. Even so, the receiver gain of the system is held at a fixed value in the fruit juice measurement, and it is possible to run on different receiver gain values and use a correction factor that is always constant between two distinct receiver gain values.

Magnetic Resonance in Chemistry

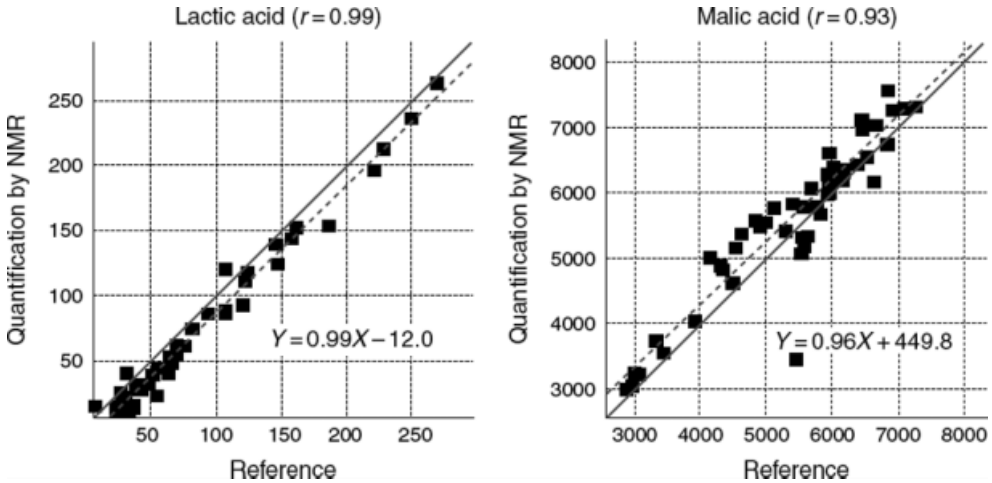

Figure 5. Correlation between NMR and conventionally determined concentrations of lactic acid (left enzymatic) and malic acid (right, HPLC) in milligrams per liter.

Extensive testing of NMR quantification results in comparison to conventional analysis has shown a very high correlation between the two ways of getting quantitative information.

Figure 5 shows the correlation for lactic acid (against enzymatic method) and malic acid (against HPLC method), reaching correlation values of 0.96 and 0.93, respectively.

As described so far, NMR-based concentrations have been obtained by directly integrating the corresponding signals in the spectrum by deconvolution. However, there is another approach using ridge regression analysis.[35] Linear regression analysis looks for a linear correlation between the measured (NMR) data A and a variable b by solving the equation $Ax = b$. This can be done by solving $x = (A^TA)^{-1}A^Tb$. In many cases $(A^TA)^{-1}$ does not exist or the matrix A is ill conditioned. Therefore the ridge regression introduces a regularization term λI (where I is the identity matrix, $\lambda > 0$): $x = (A^TA + \lambda I)^{-1}A^Tb$. This regularization improves the conditioning of the problem, thus enabling a numerical solution.

In ridge regression, for a certain juice type, like direct apple juice or apple juice made from concentrate for example, glucose can be determined as well if conventionally obtained values are available for a large number of juice spectra. After training the system with the conventional values, the concentrations can be calculated for new spectra. Figure 6 shows the result obtained for glucose using ridge regression analysis on apple juice spectra at 400 MHz. A very high correlation factor of $r = 0.96$ is achieved in this case.

It is of interest to use ridge regression not only to quantify individual compounds but also to access quality criteria based on multiple compounds. A good example in this case is the glucose/fructose ratio. Fructose concentration assessment by NMR deconvolution is difficult, as fructose consists of five isomers with signals in a heavily overlapped spectral region. A possible solution is to assume a constant ratio between the isomers and to quantify the main isomer only and use a correction factor to scale to the total fructose content.

It is obvious that the concentrations obtained in this way will not be very precise. Another solution is to determine the glucose/fructose ratio by ridge regression; having a precise glucose value from deconvolution allows the calculation of a good fructose concentration value. Figure 7 shows the ridge regression-based correlation for the glucose/fructose ratio.

In a similar way, total acidity of the juice can be determined indirectly from the NMR, even though there is a whole set of molecules involved. Even more interesting is the possibility to apply ridge regression on 400-MHz proton NMR spectra to measure concentration of potassium and magnesium. This might be considered impossible in first place; however, if one knows that the concentration of these ions influence ^{1}H chemical shift of many compounds in aqueous solution, it can be understood how this works. Increase or decrease of concentration levels leads to upfield or downfield shifts in the proton NMR spectrum always in the same way. Figure 8 shows the results obtained for potassium and magnesium in milligrams per liter for an orange juice made from concentrate.

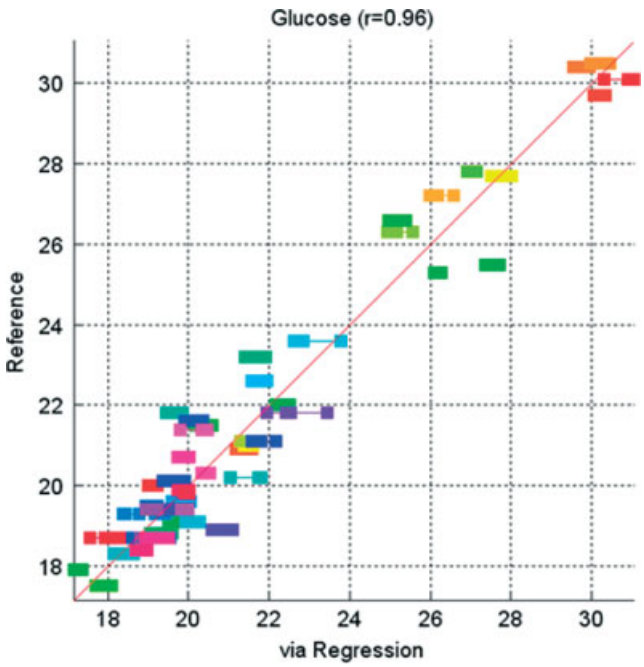

Figure 6. NMR concentrations of D-glucose in apple juice determined by ridge regression based on 400 MHz spectra.

Again, it has to be stated that all values described above are obtained from one single NMR run for each juice. In addition, there are further quality-related parameters available from the same spectra used in quantification and ridge regression. These are described in the subsequent part.

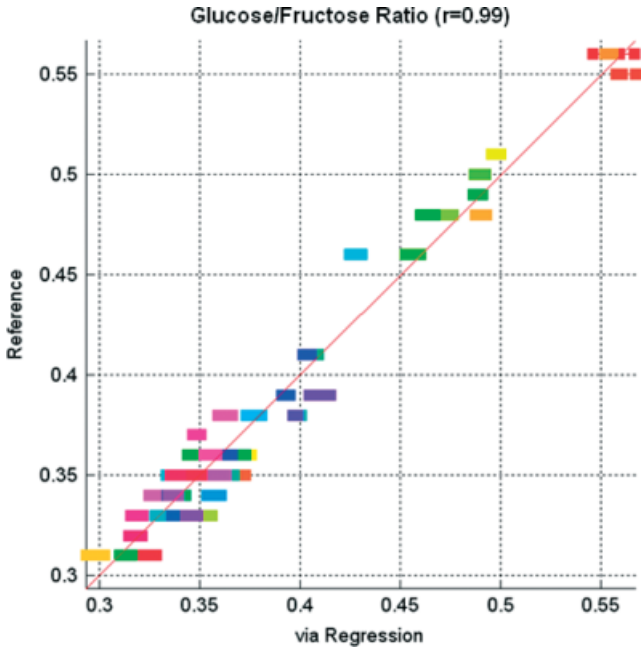

Figure 7. Glucose/fructose ratio determined via regression using 400 MHz for apple juice from concentrate.

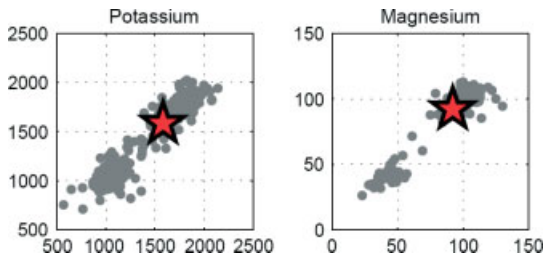

Figure 8. Ridge regression used to determine the potassium and magnesium content in orange juice made from concentrate based on 400-MHz ^{1}H-NMR data.

Statistical analysis

Besides the quantification, an exhaustive statistical analysis has been developed for fruit juice analysis. Like in metabonomics, the classification and/or verification of samples is the major purpose.

In preparation for the statistical analysis, the amount of data of the spectra has to be first reduced (32 K data points per spectrum at a basis of about 1000 spectra can obviously yield overfitting). This is done via bucketing: the resolution of the spectra is reduced by the integration of equally sized regions (the so-called buckets). This data is now called a *bucket table*. Typical bucket widths are between 0.04 and 0.005 ppm. Further statistical analysis is always done on the basis of these bucket tables and not directly on the spectra.

The statistical approach taken here is to first exactly identify the sample by cascading classification models and then validating the sample with respect to its most qualificatory group (e.g. rediluted orange juice concentrate from Brazil). This reduces the variance of the validation models and therefore increases their power. The basis of the statistical analyses is a large reference NMR spectral database of more than 3000 samples with more than 30 different types of fruit from more than 50 countries. About 1000 of the samples used are fully authentic, meaning that the juice has been collected by an inspector from running production on site and sent off for analysis together with detailed technology information.

The cascading statistical process starts in this case by estimating the type of fruit. This so-called 'global model' can differentiate at the current stage between apple, orange/mandarin, sour cherry, pineapple, black currant, passion fruit, lemon, grapefruit, banana and grape. Of course, this information usually comes with the metadata and there is rarely a reason for complaints except for undeclared mixing of apple with pear juice or orange juice (*Citrus sinensis*) with mandarin juice (*Citrus reticulata*). The latter is often cheaper and therefore a few companies are adding it to orange juice without declaration (which is not allowed in Europe). Also, mandarin addition in small amounts produces a more intense and sweeter taste. Even with conventional analysis, an addition of mandarin juice is hard to detect. With the NMR technology and models developed, one can at least detect an addition of 10%.

Just to stay with orange juice, the more specialized models in the second step of the cascade can distinguish between direct juice and juice from concentrate and can detect the origin of the fruit as well as the exact type of citrus fruit (orange, mandarin and blood orange). Figure 9 shows the result of the estimation of the exact type of variety (orange, mandarin, blood orange) as well as the geographical origin (orange juice): the available groups are Spain, Greece, Brazil, Belize/Mexico/Costa Rica, Cuba and USA. A 3D projection of the discrimination model space is shown on the left with a projected sample of interest (red star). The similarities on the right are calculated in the complete discrimination space and declare the result of estimation (juice is from Brazil).

When looking to the models in a 3D projection, one should be aware that, like in the origin case, this is a projection of a 6D into a 3D space. So what might look like partly overlap in three dimensions typically is completely separated in the original space. Up to now, detailed classification models for orange juice, as shown, apple juice (origin, concentrate/direct juice), sour cherry and pineapple have been developed. The underlying statistical method is a combination of principal component analysis (PCA) and discriminant analysis.[36] The accuracy is permanently checked via cross-validation and Monte Carlo analyses.[37] It has to be stated that 3000 samples looks like a large dataset; however, one has to keep in mind that the vast majority in this collection are orange and apple juices. Those have to be separated into additional groups according to geographical origin and direct juice or rediluted concentrate. For rare juices, it will take more time to develop such models beyond the general fruit type, as at least 30–40 samples of each subclass are needed to start building models.

After the determination of the most accurate group, the sample is verified in two steps: the univariate analysis compares each spectral region with the reference dataset and therefore detects deviations of compound quantities. In Fig. 10 the upper part (left) shows the spectrum in front of the quantiles of the reference spectra building the model set (here rediluted apple juice concentrate from China) and unusual amounts can be easily detected. The second approach is a multivariate analysis to detect deviations which are invisible in the univariate analysis. This is done by an extended soft independent modeling of class analogy (SIMCA) modeling[38] (shown in Fig. 10 upper part, right). If both methods have a positive result, the sample seems 'representative' and has successfully passed the NMR prescreening. There is no need to further focus on this sample with expensive conventional analyses for the authenticity aspects checked here. Of course, several relevant contaminants in low

Magnetic Resonance in Chemistry

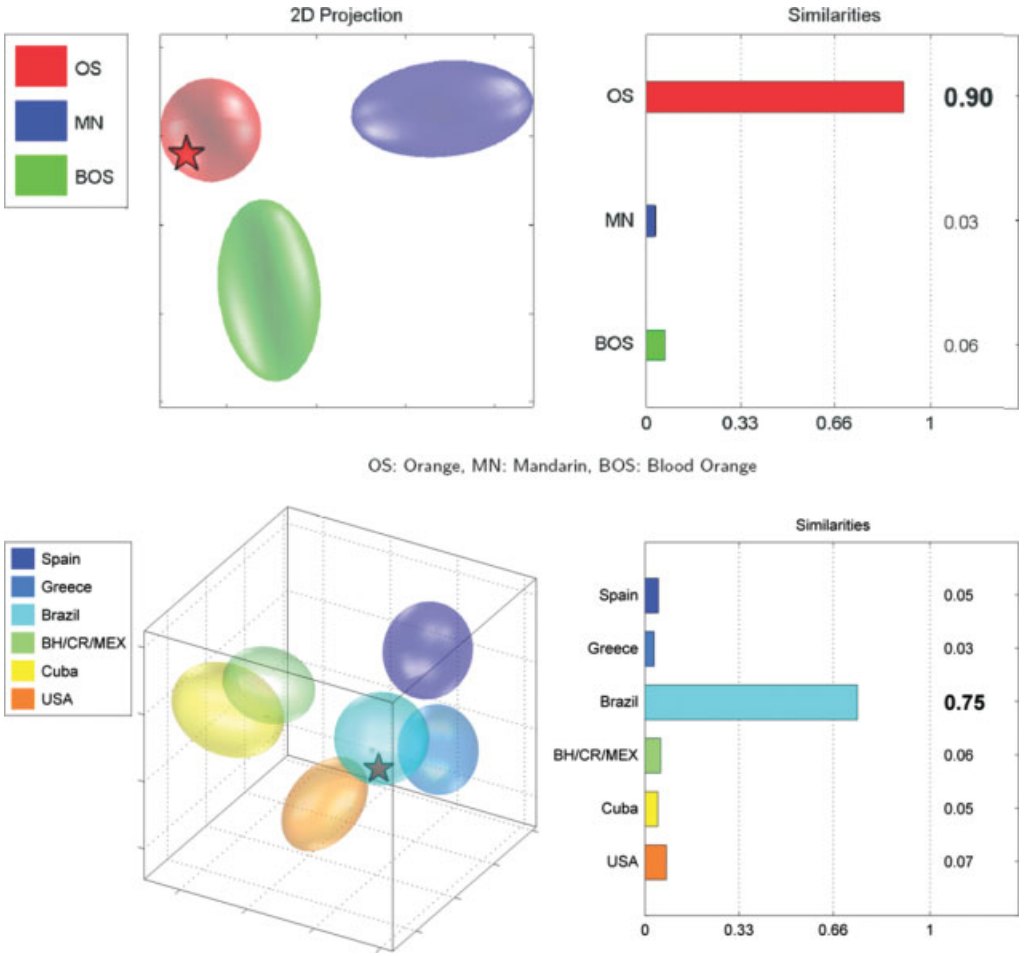

Figure 9. Estimation of the exact variety of citrus fruit (upper part) and origin (lower part) of an orange juice. The left plot shows a 3D projection of the model space with the ellipsoids of possible groups. The star symbolizes the actual sample.

concentration, such as heavy metals, mycotoxins or pesticides, are not covered by the SGF profiling. It is clear that this approach works untargeted, as this is not related to individual compounds in the mixture; this means also that a new type of fraud will be detected automatically. However, in such a case additional work needs to be done to identify the compounds giving rise to a change. Furthermore, the method is able to detect nonpermitted production techniques such as excessive pressure during citrus juicing or production of apple juice concentrate from pomace only. In the lower part of Fig. 10, a sample is shown that was recognized as an outlier, one of the reasons for this being the doublet falling outside of the model quantiles. Thus, 5-hydroxymethylfurfural was identified as the reason for the deviation from normality. This compound shows up in apple juice if the juice is overheated during processing or if sugar couleur is added to the juice.

Another important method for verification especially of market samples is the estimation of the fruit content of the juice. Conventionally, this is done by quantifying selected organic compounds and minerals and comparing these amounts with the reference distributions. In NMR screening, one is dealing with hundreds of variables on basis of one spectrum and hence a regression analysis for the estimation of the fruit content can be used. It can be shown that this analysis yields results with an accuracy of about 10% (for more than 95% of the samples).

Conclusion and Outlook

Fruit juice profiling by NMR has been introduced as a method for authentication and verification in the quality control of fruit juices. Besides the quantification of several compounds, this fully automated screening approach uses statistical models for the estimation of fruit content or the type and origin of the juice. Based on the properties of NMR, a general filter has been obtained that can show known and unknown deviations from normality. Currently, routines are under development to identify unknown deviations by building signal patterns which can be compared to reference compound spectral databases for biofluids and food materials automatically to try and identify signals that cause deviations from normality.

Due to the constant update of the reference juice database, further statistical models will be available in the near future for fruit type, fruit origin or other discrimination purposes. At the same time, the quality of the currently existing models will constantly be improved as well. Thus, the predictive power of this type of profiling will be refined and increased over time.

The application introduced of screening fruit juices can also be seen as proof-of-principle study for other upcoming applications. The same workflow (preparation, measurement, processing, reporting) and underlying mathematical methods can be easily transferred to other food quality control applications, such as screening milk, wine or beer. Beyond that, all future metabonomics

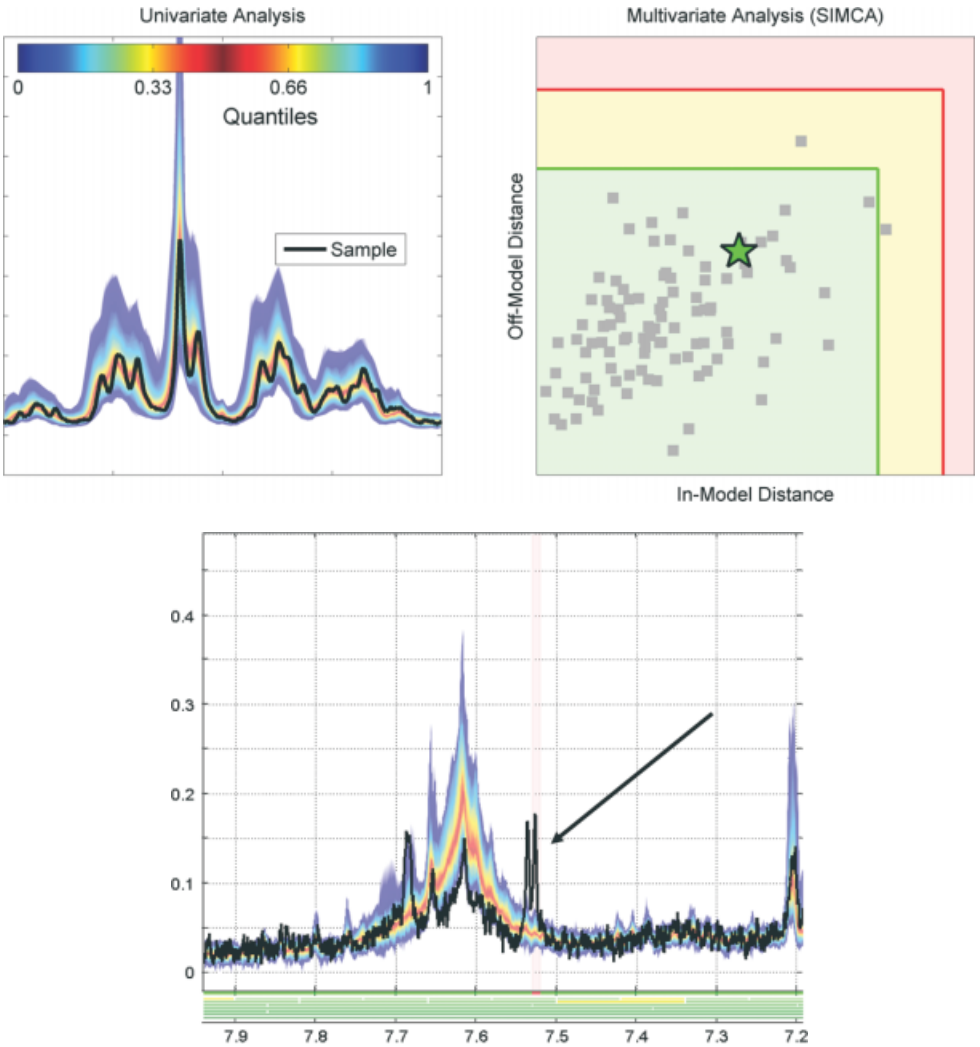

Figure 10. (Upper part) Verification of a sample: the left figure shows the spectrum in front of the quantiles of the model spectra set (univariate analysis of apple juice at 2 ppm). The right figure shows an influence plot of multivariate SIMCA analysis (green: sample ok, red: sample not representative) (Lower part) Identification of a deviation in an apple juice spectrum at 7.52 ppm, which could be identified as 5-hydroxymethylfurfural.

screening including clinical diagnosis on any kind of body fluids (urine, plasma, cerebrospinal fluid, etc.), like for example inborn error screening, will benefit from the experience obtained with the fruit juice application. Again, it has to be stated that this approach combines targeted and untargeted analysis and therefore can recognize situations unknown so far. It is also obvious that it needs the reproducibility of the NMR to be able to detect multiple, simultaneous changes in the spectra.

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