NMR screening in the quality control of food and nutraceuticals

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A B S T R A C T

Modern research in food science and nutrition requires innovative strategies using advanced analytical analysis. Nuclear Magnetic Resonance (NMR) has proven to be a robust analytical tool for the screening and analysis of complex mixtures such as body fluids, food and beverages. The next logical application of NMR based screening methods is for food quality control. Here we show a fully automated quality control analysis of fruit juice and wine. This analysis requires the user to have minimal working knowledge of NMR and delivers reports with results a person untrained in NMR analysis can easily understand. However, to produce reliable results, substantial effort in validation of quantification and statistical analysis is needed initially. These efforts are rewarded with rapid highly reproducible screening capabilities. Further application of the same principles is under investigation for nutraceuticals. Very promising results obtained to date suggest that NMR will provide a needed new analytical tool for product assessment of these highly complex materials.

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1. Introduction

Traditionally NMR is recognized as a fundamental analytical tool in structure verification, elucidation and purity analysis. It has been widely applied in synthetic organic chemistry to investigate the molecular structure and to study kinetics, dynamics, and interactions between molecules and protein conformations (Derome, 1987; Keeler, 2005; Saunders & Hunter, 1993). Resulting from research in the field of Metabonomics (Lindon, Nicholson, Holmes, & Everett, 2000; Nicholson, Lindon, & Holmes, 1999), NMR applications have expanded into new areas that utilize its capabilities in screening and analysis of complex mixtures. Initially established for body fluid investigation, NMR applications in Metabonomics were successfully extended to other complex mixture samples, especially food stuffs (for a recent review see Mannina, Viel, & Sobolev, 2012). Technological and methodological challenges for these new application areas require the highest degree of automation at each step of the process: sample preparation, acquisition, processing, analysis and reporting.

So far, NMR was mainly used in Food Research. In this article, we focus our attention on the next phase of NMR developments in this application field: NMR as a quality control tool for food material and the now popular nutraceuticals. Quality control of food means control of origin, properties, composition, safety, and authenticity and includes all steps of the global supply chain; from the raw material up to the final product sold on the supermarket shelf. Many constituents are present in the crude products whereas other components arise during storage and processing or can be added for preservation or to address a specific nutritional concern. Consequently, food authenticity, composition and traceability are of great concern to consumers, food manufactures, retailer and regulatory bodies and analytical providers. Despite the great concern these products are currently monitored and certified by means of paper documentation, some targeted analysis and sensory properties. Sensory properties are usually tested by human beings and results depend on individual's perception that is influenced by factors like nutritional education, age and current trends. Sensory tests are expensive, time consuming and cannot be applied to food that contains, or could contain, poison or toxins. In principle, several analytical techniques can overcome most of these limitations, but in order to be applied in the "real world" the following requirements must be fulfilled:

– Reproducibility (site to site, country to country, instrument to instrument),
– Sensitivity,
– Targeted AND non-targeted (recognize even unknown deviations),
– Safe compound identification,
– Ability to identify known adulterants,
– Absolute quantification (one calibration for all compounds),
– Rapid and push-button, automatic reports,
– Low cost per sample,
– Low cost per parameter.

Because there is not just one single analytical technique to provide the best evaluation for each of the above-mentioned requirements on a complex mixture, the use of complementary techniques capable of providing a broad range of information is desirable. Despite the lower sensitivity in comparison with other analytical techniques, like MS,

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and the signals' overlapping in the spectra, NMR analysis has a great advantage of being able to give information on all components present in the sample, simultaneously. The single NMR spectrum contains all the signals arising from all the protons of all the molecules present in the sample. This, combined with the fact that the technique is highly reproducible and inherently quantitative, allows the analyst to quickly obtain information regarding the entire chemical composition of the sample.

2. Experimental

For fruit juice sample preparation, NMR analysis and data analysis see Spraul et al. (2009).

For wine sample preparation, NMR analysis and data analysis see Monakhova et al. (2011).

For blueberry leaf sample preparation, NMR analysis and data analysis see Hicks et al. (2012).

For Ginseng sample preparation, NMR analysis and data analysis see Yuk et al. (2013).

3. Results and discussion

3.1. Fruit Juice quality control by NMR

A complex mixture contains many metabolites ranging widely in concentration and chemical properties. For this reason, the ^{1}H NMR spectra can be highly complex due to the large number of signals and overlapping resonances. The analysis of these samples requires appropriate strategies, namely targeted and non-targeted analyses. Specific components are identified and consequently quantified by targeted analysis, whereas non-targeted analysis detects unexpected deviations by applying statistical models to the whole spectrum without the need for interpretation of specific signals.

Spin Generated Fingerprint Profiling (SGF-profiling) (Spraul et al., 2009) is a method for fruit juice analysis developed jointly by Bruker BioSpin GmbH and SGF International e.V. With this example, the application of the principles established for metabonomics NMR as applied to food quality control is demonstrated.

The first analysis step is the targeted analysis to assess the analyzed food matrix on the basis of specific deviations in concentrations of single compounds that may indicate quality problems. A list of compounds can be identified and quantified from a single NMR spectrum. The concentrations of specific components are very important because they allow detecting quality problems like, for example, the addition of sugars. Due to the direct proportionality of the integral of the NMR peaks and the number of observed nuclei, NMR is an inherently quantitative technique and thus it is an efficient method for concentration determination. With appropriate selection of experiments and procedures, the NMR does not need daily prior calibration. The determination of the absolute concentration only requires an external reference compound of known concentration. The absolute intensities of reference and unknown spectra are correlated by the measurement of a precise 360° pulse, direct consequence of the principle of reciprocity (Wider & Dreier, 2006). In addition, the capability of NMR to quantify multiple compounds in a mixture, from just one measurement, is a clear advantage over the targeted standard analytical routines, where prior separation of components is often needed, as it allows detection of the appearance of unexpected ingredients and consequently of unknown frauds. The SGF method is constantly being updated; currently concentrations of more than 30 different compounds are determined (depending on the type of juice). 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.

The second step is the non-targeted analysis. SGF-profiling includes statistical routines to classify and/or verify samples, based on normality models. In this second part of fruit juice analysis, the whole NMR spectrum is used as a fingerprint where every signal is taken into account regardless of whether it was used in the targeted analysis or not. Following a cascade of classification models, each sample is classified and then validated with respect to the most highly probable group. The normality model is a spectral database of more than 3000 samples with more than 30 different types of fruits from more than 50 countries. At least 30–40 samples of each subclass were used to build the models. The samples are fully authentic since they have been collected under the control of an inspector from running production on site.

For the model building, a preparation step is needed in order to reduce the number of data points; this is done via bucketing in which the spectra are divided in equally sized regions (buckets). Then, the applied statistical method is a combination of Principal Component Analysis (PCA) and Discriminant Analysis. The principle of PCA is to get insight into trends (e.g. a separation into groups) in ensembles of spectra, which would not be obvious in the usual spectroscopic space. The variables, which span the spectroscopic space, are the buckets. PCA involves a coordinate transform from the spectroscopic space into a space, which is spanned by new variables, called Principal Components, which in turn are related to directions of largest variances in the ensemble. The first principal component explains most of the variance, the second component the second most, and so on. If interesting features (groups, outliers) are observed in the score plots, the relation between the variables in the new principal component space and original spectroscopic space, through the use of a loadings plot, allows understanding what the reason for this was. The loadings plot shows the buckets, or chemical shift regions of the NMR spectrum, of highest variance. Discriminant Analysis (DA) is then performed, which searches for linear combinations of variables that reflect the largest possible separation between groups of interest. The statistical space built during PCA is the new input matrix for DA, which leads to define a further subspace with optimum group separation. The obtained model is then validated in order to define the predictive capability.

The spectrum obtained from a newly analyzed sample, undergoes the same statistical treatment, and the resulting variables are then compared with the normality model in order to detect similarities or deviations, by using statistical classification routines. The classification tools include the estimation of: 1) the type of fruit (the genus, for example *Citrus*), 2) the variety (for example orange), 3) the product type (discriminating between direct juice and diluted – from concentrate – juice) and 4) the geographical origin. After a sample has been classified as belonging to the most accurate group, it is verified by comparing the spectrum with a reference dataset (univariate analysis) in order to detect deviations of metabolite concentrations and then with multivariate analysis, taking into account also the relation between signals deriving from different molecules and corresponding to the expected ratios between the mixture components, in order to detect eventually deviations that univariate does not show. Moreover, retrospective use of older data in new statistical models or quantification is always possible as all the ^{1}H nuclei are acquired,

Results from both the targeted and non-targeted approaches are obtainable in automation and do not require NMR interpretation from the classical NMR spectrum. Examples of the quality control reports which describe and classify the sample are shown in Figs. 1 and 2. Quantification results are listed in a table showing the concentration of every metabolite identified (Fig. 1a). Where reference values exist for expected concentrations defined by the European Fruit Juice Association (A.I.J.N.) for the same type of juice, the result is also expressed as a traffic light (Flag), green if the obtained value is in the optimal range and red if outside the optimal range. Moreover, the table shows, in the last column, the distribution of metabolite concentration in the authentic set of samples used for statistical model building described previously.

Multivariate classification results are shown as 3D plots where it is possible to see, as ellipsoids, the statistical space occupied by every authentic group, and the position of the new sample being measured