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Recent applications of NMR in food and dietary studies

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Abstract

Over the last decade, a wide variety of new foods have been introduced into the global marketplace, many with health benefits that exceed those of traditional foods. Simultaneously, a wide range of analytical technologies has evolved that allow greater capability for the determination of food composition. Nuclear magnetic resonance (NMR), traditionally a research tool used for structural elucidation, is now being used frequently for metabolomics and chemical fingerprinting. Its stability and inherent ease of quantification have been exploited extensively to identify and quantify bioactive components in foods and dietary supplements. In addition, NMR fingerprints have been used to differentiate cultivars, evaluate sensory properties of food and investigate the influence of growing conditions on food crops. Here we review the latest applications of NMR in food analysis. Published 2016. This article is a U.S. Government work and is in the public domain in the USA.

Keywords: NMR application; food, agriculture and dietary studies; cultivars, growing and processing conditions; sensory and probiotics; other applications

INTRODUCTION

There has been a rapid increase in the development of new foods with potential health benefits exceeding those of traditional foods.[1,2] These foods have found popularity with a newly aware, health-conscious public. The food industry now faces a challenge: how to differentiate nutritionally rich foods from conventional foods and promote their sales.[3] This challenge has been addressed by increased research on nutrition and food composition, at the molecular and the microstructural levels.

Analytically, the characterization of today's food supply is a tremendous challenge. Many different technologies have been applied to this end, each with its strengths and weaknesses. In the past decade alone, there has been an explosive growth in instrumentation, handling of data and statistical methodologies. Today, many investigations on food composition are carried out either with mass spectrometry (MS) or nuclear magnetic resonance (NMR) spectrometry coupled to a variety of liquid (LC), gas (GC), and solid-phase extraction (SPE) techniques, e.g., LC-NMR, LC-SPE-NMR, LC-MS, GC-MS and GC-SPE-MS.[4] LC-NMR has been extensively used in wide array of pharmaceutical applications related to natural product identification, biosynthesis and metabolic studies. Recently, Hiller *et al.* discussed the application of online LC-NMR for polymer analysis.[5] Schlotterbeck and Ceccarelli have discussed the combination of high-end integrated modular systems LC-SPE-NMR-MS for rapid structural elucidation of natural products.[6] A recent review by Vinaixa *et al.* discusses the current status and future prospect of LC-MS and GC-MS based metabolomics databases.[7] Similarly, high-sensitivity mass spectrometry coupled with wide array of separation techniques (LC, GC, CE) are increasingly being used for clinical and metabolomics research.[8]

MS has established itself as the method of choice as it can provide highly resolved and accurate information on the molecular masses and their fragments, permitting detection of a wide range of molecules with speed, sensitivity and accuracy.[9] However, most widely used mass spectral-based approaches face the hurdle of reproducibility due to differences in ionization procedures, mobile phase solvent composition and detection parameters. This is unfortunate since techniques that can provide reproducible data over a long period of time are highly desirable.

NMR is perhaps the only technique that is suitable for the study of food products at both molecular and microscopic scales. NMR has traditionally been the tool of choice to elucidate molecular structures. NMR signals offer the experimentalist a diverse array of measurable parameters such as intensity, frequency (normalized to chemical shift), line shape, line width and relaxation times. These data have been used to determine structure, diffusion rate, viscosity and association constants. Today NMR is being increasingly used for more applied studies in the field of food composition analysis. High-resolution 1D and 2D NMR offer several distinct advantages in the case of metabolomics studies: it is quantitative and highly reproducible. Typically, it is possible to account for as many as 50 metabolites in a crude unfractionated plant extract from a single NMR measurement.

In the last 20 years, NMR technology has undergone vast improvements, with concomitant developments in the field of electronics and computers. Techniques such as solid-state NMR and functional magnetic resonance imaging (fMRI), which were once confined to research labs, have now become commonplace in the food industry. The explosion in NMR-based research in the fields of food composition and metabolomics

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has been previously covered by several excellent reviews focusing on specific areas such as use of NMR in metabolomics and metabolic profiling,[10] two-dimensional NMR for exploring plant metabolome,[11] quantitative NMR of complex samples,[12] NMR of human blood serum,[13,14] recent advances and new strategies in the NMR-based identification of natural products,[15] multidimensional NMR[16] and biomarker characterization.[17] Nevertheless, the sheer number of high-quality research papers being published every year requires periodic appraisal and there is a need for reviews covering broad swathes of research in various areas to aid the typical food scientist and the food biologist to understand the ramifications in their field of research. Herein, we make an attempt to present the state-of-the- art research in the past two years across various sub-disciplines of NMR-based metabolomics research related to agriculture, food and dietary sciences. The application of NMR in specific areas of food science – namely food safety, nutrition, beverages and metabolomics – is extensively reviewed. This review is categorized into the following sections: (i) differentiation of food based on cultivars, growing conditions and processing; (ii) analysis of biological fluids; (iii) sensory NMR; and (iv) probiotics.

CULTIVARS, GROWING AND PROCESSING CONDITIONS

Table grapes

The significance of agronomic practices on the phytochemical composition in commercial table grapes was investigated using three case studies.[18] In the first case study, grapes from Superior Seedless cultivar were grown in two vineyards that were 3 km apart. The second study compared Red Globe grape cultivar produced in six vineyards located in two different areas of Apulia that were 80 km apart. Finally, the third case study compared Italia table grapes located in 18 vineyards, and harvested in two consequent years (2009 and 2010).The results showed that the inter-vineyard variability was found to be greater than intra-vineyard variability. It was also found that the quantities of glucose, fructose, arginine and ethanol were most affected by the farming practices. With Superior Seedless cultivar the sugar content was higher in conventionally grown samples as compared to organic grown samples. Significant differences were reported among the above three cultivars, in their amino acid content. Substantial differences were noted in the ^{1}H NMR spectra between 2.55 and 1.95 ppm. Superior Seedless grapes were also different in having a much higher proline content, compared to negligible amounts in the Red Globe and Italia cultivars. Sugar concentrations differed considerably among the cultivars grown using organic and conventional practices.

Saw palmetto berries

^{1}H NMR spectroscopy was employed as a fingerprinting tool for analyzing extracts of saw palmetto berries used as herbal medicine to treat urological infections. Fifty-seven different products that contain saw palmetto either as single preparation or as a part of multi-component supplements, from nine countries, were analyzed. The samples were dissolved either in deuterated chloroform or methanol. Fatty acid determinations showed a high level of heterogeneity in the total and individual amounts of nine fatty acids. The authors determined that with ^{1}H NMR it was possible to differentiate between saw palmetto products that had been extracted under different conditions using principal component analysis (PCA). PCA assisted in the identification of products based on their metabolomics profile.[19]

Carrot

The variation of primary metabolites and lipids among genetically different carrot species is not known, although secondary metabolites have been extensively studied in this regard. Clausen *et al.* (2014) used a ^{1}H-based metabolomics approach to understand the effect of carrot genotype on metabolite content.[20] After extraction using different solvents, 25 metabolites, including β-carotene, sterols, triacylglycerols and phospholipids, were detected from five genetically distinct cultivars of carrots, namely Bolero, Nipomo, Purple Haze, Mello Yellow and White Satin. Extractions were carried out with both water and chloroform. Variation was based on three principal components during PCA. PC1 distinguished Mellow Yellow/White Satin from Bolero/Nipomo/Purple Haze, whereas PC2 discriminated between Bolero/Mello Yellow and other varieties. PC3 differentiated Nipomo/Bolero from Purple Haze. Major variations were due to the β-carotene and carbohydrate contents among the different carrot types. Interestingly, the classifications based on carbohydrates and on lipids were remarkably alike. Thus ^{1}H NMR spectroscopy coupled with multi-block data analysis was efficiently used to map the carrot metabolome, particularly the differences in carbohydrate, amino acid, nucleotide, fatty acid, sterol and β-carotene contents, and identify genetic differences between varieties.

Black raspberries

Current efforts in translational research have shown that black raspberries (BR) have bioactive compounds with chemopreventive quality against certain types of cancers. Conventional biochemical studies failed to isolate the unique compounds having the anticancer activity because of the large number of secondary metabolites. It was hitherto not possible to identify the exact molecule(s) in the fraction responsible for the bioactivity. A model that compares the ^{1}H spectra of crude BR extracts with the cellular proliferation of HT-29 human colon cancer cells was developed by Paudel *et al.*[21] ^{1}H NMR/multivariate statistics was used to fined the regions in NMR spectra containing signals from bioactive compounds. As an extension of the work, two-dimensional NMR was used to identify the molecules and their structure. Seventy-three different BR extracts were used in the study. Using the bioassay/1D NMR/statistics/2D NMR approach, two anthocyanin compounds – Cyt-3-xylrut and Cyt 3-rut – were identified to have the greatest cytotoxic effects on the HT-29 colon cancer cells. Given the fact that anthocyanins are approximately 10^{2}- to 10^{3}-fold more concentrated in this plant than other phenolic compounds, these compounds could possibly interfere in the kinase signaling cascades that control gene expression. However, a Pearson coefficient analysis using NMR-determined concentrations of these anthocyanins *versus* low/high-dose bioassay data show that the anticancer activity cannot be ascribed to any one compound but a concerted activity of multiple compounds or other unidentified compounds present at trace level.

Oil seeds

There has been little ^{1}H NMR study of the commercially important rapeseed (*Brassica napus* L. subsp. *oleifera*), which is a raw material for edible oils, cattle feed and biofuels. Kortesniemi *et al.* investigated the seeds of the oilseed rape (*Brassica napus*) and turnip rape (*Brassica rapa*) using ^{1}H NMR for differentiation with

respect to species, geographical origin, developmental stage, and quality.[22] Ripened seeds of both species, cultivated in climatically different regions of Finland, were subjected to a facile extraction process using cyclohexane. Some of the samples were redissolved in methanol–chloroform. A small anomeric signal belonging to stachyose, a phytochemical characteristic of brassicaceous seeds, was detected at 5.41 ppm in the ^{1}H NMR spectrum with a J coupling of 3.9 Hz. In the case of developing seed extracts, oil accumulation was evident from the resonances at 0.88, 0.97, 1.25 and 1.30 ppm that arise from acyl moieties. PCA and OPLS-DA of the data showed a clear distinction between cultivars and different geographical regions, but species-level differentiation was poor even with the use of supervised multivariate methods.

Apple

There have been very limited studies on rhamnitol occurring in nature and less so in fruits. Tomita *et al.* used ^{1}H NMR-based metabolic profiling to characterize apples of five cultivars (Fuji, Jazz, Envy, Orin and Jonagold) grown in Japan and New Zealand.[23] Fuji cultivar was collected from both countries. PCA showed distinct separations between the Fuji, Orin and Jonagold classes and the Jazz, Envy classes. This difference was attributed to the sugar signals, namely sucrose, glucose and fructose. Multistep PCA analysis reduced the contribution of major sugars and indicated three minor metabolites, namely aspartic acids, 2-methylmalamate and L-ramnitol. The authors found a relatively intense signal from a doublet occurring at 1.26 ppm with a J coupling of 6.4 Hz, and identified the signal as arising from L-rhamnitol. The contribution of L-rhamnitol to geographic discrimination enables characterization of apples according to various factors, including storage duration, cultivation method and climate. They annotated other metabolites and used targeted PCA and PLS-DA and normalized signal intensities by Z-score transformation. They confirmed that three apple varieties from Japan contained larger amounts of L-rhamnitol than New Zealand apples.

Juniper

The dried cones of *Juniperus communis* (Cupressaceae) are used as a flavoring agent in foods and spirits and also as a herbal medicine in various parts of the world. Falasca *et al.* have reported a systematic ^{1}H NMR analysis of the juniper metabolome.[24] Falasca *et al.* used ^{1}H NMR for the first time to profile the metabolomics of juniper to investigate seasonal variation in *J. communis* berries. PCA and partial least squares discriminant analysis (PLS-DA) of the ^{1}H NMR data showed clear class separation according to ripeness and harvest season, and revealed that the *Juniperus* metabolome is dominated by 26 metabolites including sugars, amino acids, organic acids and diterpenes. In the ^{1}H NMR spectrum the aromatic region was dominated by signals from shikimic acid, whereas signals from the aliphatic region were used to identify quinate. Specific metabolites such as *cis*-communic acid, myrcecommunic acid and imbricatolic acid were identified. The relative levels of the metabolites from summer and autumn berries were similar, whereas the levels were lower in those from spring.

Soybeans

Ionizing radiation has traditionally been used to sterilize crop products. Around the world, 700 000 tons of food materials are sterilized by ionizing radiation each year. There have been efforts to characterize any small changes in the composition of food products induced by gamma irradiation with soybean as the model food. It was expected that the changes to the nutritional content of the food were minimal. In a recent study, a ^{1}H NMR metabonomics model was able to distinguish between gamma-irradiated and non-irradiated soybeans. This model measures ^{1}H NMR spectra of chloroform extracts of soybeans and makes use of PLS-DA to classify the samples and to determine which spectral bins are discriminatory. The model has presented an accuracy of 100% in the face of a real dataset involving 49 samples from diverse cultivars. The gamma-irradiated samples showed an increase in the integrated areas at δ 1.57 ppm (assigned to free fatty acid), whereas non-irradiated samples showed an increase in the integration areas at δ 1.62 ppm (assigned to fatty acids linked to glycerol as esters).[25] A notable result from the above study is that, though a clear separation between irradiated and non-irradiated soybean was possible, it was not possible to observe separation in terms of different doses of radiation.

Olives

The effects of agronomical practices (i.e. organic *vs.* conventional) on oil composition, oil sensory properties and fruit metabolomics were studied in two olive (*Olea europaea* L.) cultivars (Leccino and Frantoio).[26] Studies that differentiate organic olive oil from conventional oil are rare. Fruits and oil samples grown in the same field under same agronomical practices, which differ only in their fertilizer and soil management, were selected for the study, thus keeping the number variables to a minimum. High magic angle spinning (HRMAS) was used to study fresh olive fruits. The use of solid-state NMR in food sciences is still a naïve area, and this study is one of the first using this approach. Leccino and Frantoio cultivars were cultivated in alternate rows and randomly selected fruits were used for extracting oil. ^{1}H and ^{13}C HR-MAS NMR experiments were performed on the fruit pulp. Organic and conventional fruits showed similar carpological traits and there was little difference in acidity and peroxide index, whereas the content of polyphenols was significantly lower in the case of conventionally treated cultivars. The sensory analysis showed increased bitterness (both cultivars) and pungency (*Frantoio*) and reduced sweetness (*Frantoio)* in the organically cultivated olives. Fruit metabolomic analysis revealed significant changes in metabolites, namely glycocholate, fatty acids, NADPH, $NADP^+$, some amino acids, thymidine, trigonelline, nicotinic acid, 5,6-dihydrouracil, hesanal, *cis*-olefin, β-D-glucose, propanal and other unidentified compounds.

Tea

A new approach incorporating the concept of 'terroir', for non-targeted or global analysis, i.e. metabolomics, which is highly reproducible and statistically effective in analyzing a diverse range of compounds, was used to better understand the metabolome of *Camellia sinensis* and determine the influence of environmental factors, including geography, climate and cultural practices, on tea plants.[27] Two hundred and eighty-four samples were collected as representative examples for green, white and oolong teas from China, Japan and South Korea. Extraction of the metabolites was carried out in water. Climatic conditions for the growth of these tea types were obtained from publicly available data of the meteorological agencies of the above countries. Major flavanol compounds such as epicatechin, epicatechin-3-gallate and epigallocatechin-3-gallate were identified from ^{1}H NMR spectra of the samples. The spectra of green tea samples from China, Japan and South Korea were clearly distinguishable by both PLS-DA and OPLS-DA. Interestingly, teas grown in areas as close

as 17 km showed a clear difference in their metabolic profiles. The study revealed a strong correlation between metabolite levels from white and oolong teas and also the tea growing area and tea type. White and oolong teas undergo the least processing among tea types and therefore resemble unprocessed raw tea leaves in their metabolic content. The levels of sucrose and ECG were lower in white tea as compared to oolong tea, whereas the levels of amino acids theanine, alanine, threonine, leucine and valine were higher in the former. This study concluded that the tea quality was dependent on the environmental conditions used for the production of tea. In addition, it provides information on differentiating tea products with consistent quality.

Coffee

The relationship between coffee quality and its chemical constituents was studied by Kwon *et al.*[28] using ^{1}H NMR-based metabolomics analysis. They studied specialty coffee from the species *Coffea arabica* grown in different areas and applied multivariate statistical analysis to NMR data. They obtained green coffee beans from Guatemala, Burundi and Colombia. The coffees selected were based on quality reports that were measured on drink quality and thus the chemical ingredients bias was avoided. Extraction was done in a mixture containing sodium hydroxide and methanol. The whole spectrum was taken for analysis after excluding regions containing residual water. The citrate and malate ^{1}H NMR signals were broadened because of the tendency of these molecules to chelate paramagnetic ions like Mn^{+} and Fe^{+}, present abundantly in coffee. They identified higher levels of sucrose and lower levels of γ-aminobutyric acid (GABA), quinic acid, choline, acetic acid and fatty acids in specialty or high-grade green coffee beans as compared to commercial-grade beans. The authors suggested that these compounds can be used as multiple markers for characterizing specialty green coffee bean. They were also able to observe fatty acids which hitherto had not been observed in green coffee beans extracts because of the use of methanol in their extraction. A positive correlation was found between the levels of acetic and total fatty acids in the extracts. The variations could be a consequence of different environmental conditions, e.g. biotic or abiotic stresses, at growing regions.

BIOLOGICAL FLUIDS AND TISSUES

Biological fluids carry biochemical information that provides information about the physiological conditions of a living being.[29] Metabolomics analysis of biofluids is of prime importance in studying disease conditions such as diabetes and metabolic syndromes.[30] A large variety of biological specimens including blood, urine, bile, cerebrospinal fluid, jejunal aspirate, tissues, cells, milk, semen etc., could be employed to profile metabolites and identify biomarkers associated with illnesses and other physiological states. However, to date, the most widely used biological specimens are blood and urine as they can be readily obtained by non-invasive or minimally invasive methods. In addition, metabolites in blood and urine can report on the pathology of virtually any kind of cell in the human body. Other biological specimens that are useful for identifying biomarkers for diseases, including tissue, bile, and cerebrospinal fluid, are more difficult to collect. Yet others such as sweat, saliva, tear fluid, lung aspirate, etc., have not been as well studied. Recent works have attempted to probe fluids like milk and sweat.

Human breast milk (HBM) is the only food capable of satisfying all the nutrient needs of a newborn infant.[31] There is extensive variation in the composition of HBM among individuals and over the course of lactation period. A detailed metabolic profiling of human breast milk using NMR was carried out by Alfredo Micheli's lab at the University of Rome.[32] Human milk has a very complex chemical composition and contains a plethora of bioactive compounds, the activity of which has not yet been clearly elucidated. At the outset, the detailed characterization of low-molecular-weight metabolites in the hydro/alcoholic phase from milk is very challenging because of the presence of a large number of highly overlapping peaks in the region between 3.5 and 5.0 ppm. The most intense signals at 5.22, 4.69 and 4.47 ppm were assignable to the free anomeric protons of lactose. Fucosylated oligosaccharides could be distinguished by their fucose H-1, H-5 and H-6 rings, using 2D NMR, as were sialic acids. A total of 43 metabolites were identified. The metabolite composition differs significantly from individual to individual but the individuals fall into three broad groups based on the differences in signals from fucosyl oligosaccharides. However, among the three groups only two showed a clear separation during PCA analysis.

In another study, the effect of Mediterranean diet (MD) has been probed using the urine of test subjects. The term 'Mediterranean diet' was coined in 1960 by the American physiologist Ancel Keys and his wife Margaret Keys in their book, *How to Eat Well and Stay Well the Mediterranean Way*.[33] The MD is generally recognized as healthy, characterized by large amounts of beneficial monounsaturated fatty acids (MUFA), net intake of relatively small amounts of carbohydrates, combined with an elevated intake of dietary fiber and antioxidants.[34–36] The effect of MD on urinary metabolome was studied using ^{1}H NMR, by comparing the urine NMR profiles of subjects at 1 and 3 years of follow-up, after an MD supplemented with either extra-virgin olive oil (MD + EVOO) or nuts (MD + nuts), to those on medical advice to follow a control low-fat diet (LFD). Ninety-eight non-diabetic subjects were evaluated who were divided into MD + EVOO ($n = 41$), MD + nuts ($n = 27$), or LFD ($n = 30$) groups. The application of NMR-based metabolomics enabled the classification of individuals regarding their dietary pattern and highlights the potential of this approach for evaluating changes in the urinary metabolome at different time points of follow-up in response to specific dietary interventions.[37,38]

The secretions of the sweat gland are rarely used as sample in metabolomics analysis. The systematic studies of human sweat are relatively few, mainly because of the difficulty in collecting amounts sufficient for any meaningful analysis. As early as 1910, there was an attempt in the microscopical analysis of sweat to identify the presence of amino acids.[39] In a novel work, liquid-state NMR has been used to study the metabolome of human sweat.[40] In the present work, specially designed tools were used for this purpose to collect sweat samples from the forehead and back of the neck from adult and young subjects. The collection tools were washed with 99% pure D_2O before accumulating the metabolites. Taken collectively, the results show that sweat from different parts of the body show a biological similarity allowing one to make an educated assumption that sweat metabolites from different parts of the body are highly conserved. Hot rooms with a median temperature of 43.3 °C and a relative humidity of 65% were used, and so conditions of sweat collection may not be optimal. Mena-Bravo and Decastro (2014) review the advantages of sweat over other biofluids such as blood or urine for routine clinical analyses and the potential when related to metabolomics.[41] With this aim, they critically discuss sweat samplers and equipment

Table 1. Additional recent applications of NMR in food and dietary studies

Type of sample	Observation	Reference
Grape juices	Complete metabolite profiling of 63 commercially harvested Sauvignon Blanc (SB) juices was performed by combining GC-MS and NMR. Juices fermented under controlled laboratory conditions using a commercial yeast strain (EC1118) at 15 °C were used for the study. Correlation of varietal thiol concentration in the wines with initial metabolite profiles identified 24 metabolites that showed a positive correlation ($R > 0.3$) with both 3-mercaptohexanol and 3-mercaptohexyl acetate, whereas only glutamine had a positive correlation with 4-mercapto-4-methylpentan-2-one. Subsets of the 24 metabolites in a 2011 SB grape juice were subjected to juice manipulation experiments in order to validate the hypotheses generated by metabolomics. The results confirmed the metabolomics hypotheses and revealed that grape juice metabolites have a significant impact on the development of the three major varietal thiols and other aroma compounds of SB wines	51
Tomato	Reviewed work of the last 10 years related to metabolic profiling of tomato plant including fruit by using techniques of NMR spectroscopy and GC-MS	52
Lemon oil	Evaluated the potential of volatile and non-volatile fractions for classification purposes. Volatile compounds of cold-pressed lemon oils were analyzed, using GC-FID/MS and FT-MIR, while non-volatile residues were studied using FT-MIR, ^{1}H NMR and UHPLC-TOF-MS. 64 lemon oil samples from Argentina, Spain and Italy were considered. Studying the loadings allowed highlighting of important classes of discriminant variables that corresponded to putative or identified chemical functions and compounds	53
Bilberry	Leaves of bilberry (*Vaccinium myrtillus* L.) and lingonberry (*Vaccinium vitis-idaea* L.), which are potential raw materials for food and healthcare products, were subjected to ^{1}H NMR analysis. Changes in metabolomics profile during the season were apparent in bilberry but not lingonberry leaves. Negative correlation was found between the contents of lipids and phenolics. Consistency between key results obtained by targeted and non-targeted analyses suggests non-targeted metabolomic analysis is an efficient tool for fast screening of various leaf materials	54
Peach	Two different varieties of peach, namely Percoca Romagnola 7 and Flaminia, have different susceptibility patterns to *Ceratitis capitata* pest attack. PR7, the more resistant species, shows greater amounts of the amino acids alanine, QA, CGA and nCGA – molecules involved in the phenylpropanoid pathway – suggesting that this pathway is involved in repulsion of the pest. Two principal components during PCA analysis, namely PC1 and PC2, contain the variations, one emphasizing metabolites involved in defense and other involving scarcity of compounds that have an appealing flavor to attract insects	55
Citrus	Metabolite differences between healthy and diseased orange fruit grown in different locations were investigated. Based on ^{1}H NMR data from Hamlin and Valencia fruits, it was found that the metabolite profiles were different. The location of growth of these cultivars did not influence the ability to differentiate them on the basis of infection status. In both varieties, concentrations of phenyl alanine, histidine, asparagine and limonin were lower in control samples as compared to the infected samples	56
Bean	HR-MAS was considered here because of the simplicity in sample preparation, removing the need for complex extraction procedures. Transgenics was employed at the RNA level; therefore the researchers used the embryo. Here they demonstrate that it is possible to apply metabolite differentiation studies on two genotypically equivalent cultivars, one with conventional gene expression and the other where specific genes are silenced by means of siRNA technology. The study also showed that growth conditions did not influence the amount of flavonoids in both conventional and transgenic varieties. This work successfully established HR-MAS as a viable technique to study samples with little pre-treatment and so conferring a significant advantage	57
Soybean leaves and leaf extracts	Leaf extracts were used for solution NMR and intact leaves were used for HR-MAS NMR. Resonance assignments were made using typical two-dimensional experiments like ^{1}H-^{1}H TOCSY and ^{13}C-HSQC, on both whole leaves and leaf extracts. PCA and PLS-DA were carried out on these data. The authors make an important note that, since HR-MAS signals are indicative not only of their relative concentrations but also their mobility, caution should be exercised while quantifying peak intensities. Results showed that iron-deficient soybean extracts had relatively high levels of expression of amino acids such as valine, asparagine, hypoxanthine and trigoneline, whereas they had relatively low levels of chlorogenate, fatty acids, ethanol and methanol. Polyphenols were present in significantly higher amounts as compared to normal soybean. A strong tendency was seen in the grouping during PCA where Fe-sufficient metabolites tend to group on the positive side of PC1 and Fe-deficient metabolites group on the negative side of PC1, and this principal component accounted for 85% of the variation. The authors posit by citing previous work that the increase in amino acid content in senescent leaves is due to leaves using amino acids as carbon source in anaplerotic reactions	58
Citron and lemon	HR-MAS benefits from the ability to simultaneously study both polar and non-polar metabolites at the same time, without the need for complex extraction procedures. The authors conclude that solid-state NMR has established itself as a tool to study intact plant material. For example, it can be used as a nano-probe to study oil glands in plants without the need for complicated extraction procedures that can lead to isomerization during injection of samples into a gas chromatograph. The authors could almost instantaneously follow changes in sugar, CA, MA and ethanol content, enabling one to follow the ripening process in shelved fruits. They could also observe metabolites from a citrus pathogen, the green mold *Pencillium digitatum*	59
Potatoes	There is strong public opinion that organic food products are healthier than conventional ones, but there is little scientific evidence to corroborate this idea. How agricultural practices like organic farming can influence the nutritional and toxicological patterns in foods is not clearly understood. This study uses organic early potato tubers to probe the ^{1}H NMR profile using solid-state NMR. Q^2 values obtained after subsequent statistical analyses show that metabolomic profiles indeed depend on farming practices. The study indicates that application of fertilizers and pesticides, soil type and season can all affect the chemical composition of the tuber	60

Table 1. Continued

Type of sample	Observation	Reference
Tomato	A domestic cultivar of tomato – M82 – and another highly introgressed tomato cultivar – *Solanum pennelli* (IL) – have their metabolomic profiles analyzed by an array of sophisticated techniques including liquid-state NMR. Results show that introgressed tomato lines have very different metabolomic profiles compared to M82	61
Ginseng	Ontario ginseng landraces were distinguished from each other by ^{1}H NMR, which otherwise could not be accomplished by HPLC. In addition, ^{1}H NMR data from Ontario landrace ginseng and Canadian ginseng were used to characterize key metabolite differences	62
Gastrodia	A detailed comparison of four popular techniques (FT-NIR, ^{1}H NMR, LC-MS, GC-MS) used in metabolomics has been carried out in parallel on two medicinal plants – *Gastrodia elata* and *Rehmannia glutinosa* –cultivated in Korea and China	63
St John's wort	NMR fingerprinting and MS metabolic profiling techniques were applied to species of the genus *Hypericum* (St John's wort), namely *H. perforatum*, *H. polyphyllum*, *H. tetrapterum*, *H. androsaemum*, *H. inodorum*, *H. undulatum* and *H. kouytchense*, and the data were subjected to statistical multivariate analyses. *In vitro* anticancer assays to evaluate the ability of extracts from *Hypericum* spp. in inhibiting prostate and colon cancer growth suggest that bioactivity might be predicted by metabolic profiling	64
Zangyinchen	Detailed characterization and comparison of the complete set of metabolites of *Swertia mussotii* Franch and *Swertia chirayita* Buch.-Ham., which have been commonly used under the same name – 'Zangyinchen' – for the treatment of liver and gallbladder diseases in traditional Tibetan medicine. The results showed that *S. mussotii* had significantly higher contents of gentiopicrin, isoorientin, glucose, loganic acid and choline, whereas *S. chirayita* exhibited higher levels of swertiamarin, oleanolic acid, valine and fatty acids. These findings indicate that ^{1}H NMR-based metabolomics is a reliable and effective method for the metabolic profiling and discrimination of the two *Swertia* species, and can be used to verify the genuine origin of Zangyinchen	65
Spirits	The study employs a specialized NMR pulse sequence called WET1D, which successfully revealed the presence of important minor compounds against a high ethanol background matrix. Two Greek marc spirits, namely Tsipouro and Tsikoudia, along with 86 samples from Greece and around the world, were used for the study. Suppression of signal from the huge ethanol matrix resulted in the identification and assignment of 35 metabolites. Subsequent multivariate analysis of ^{1}H NMR data resulted in PCA and PLSDA models with a goodness-of-fit value R^2 (cum.) > 0.8 and predictive ability Q^2 (cum.) > 0.8, indicating that NMR-based metabolomics profiling can be used to fingerprint such spirits.	66
Wine	Metabolic profiles of 32 Negroamaro red wines were analyzed using ^{1}H NMR spectroscopy. The effects of conventional industrial processing steps on global phytochemical composition of broccoli, tomato and carrot purees were investigated by using a range of complementary targeted and untargeted metabolomics approaches including LC-PDA for vitamins, ^{1}H NMR for polar metabolites, accurate mass LC-QTOF MS for semi-polar metabolites, LC-MRM for oxylipins and headspace GC-MS for volatile compounds. During processing the activity of a series of endogenous plant enzymes, such as lipoxygenases, peroxidases and glycosidases, including myrosinase in broccoli, is key to the final metabolite composition and related quality of the purees (Lopez-Sanchez *et al.*, 2015)	67
Wine	This study used ^{1}H NMR spectroscopy to evaluate the metabolic profiles of two 'Fiano di Avellino' wines produced from the same grape variety and oenological techniques but fermented with either commercial or autochthonous yeast. PCA analysis of the ^{1}H NMR data showed that the PC1 accounted for 96.4% of the variation between the wine varieties produced by fermentation by different yeasts. The autochthonous yeast-fermented wine had higher glucose and sucrose content, suggesting a slower glycolytic activity as compared to commercial yeast strains. The commercial yeast also produced higher ethanol and glycerol for the same grape variety. The results provide insights into the fermentation, as wineries attempt to produce wine with lower alcohol content	68
Wine bottle	A probe and spectrometer was developed to investigate ^{1}H and ^{13}C NMR of a series of Cabernet Sauvignons full intact wine bottles. The authors developed methods to measure acetic acid content, complex sugars, phenols and trace metals	69
Wines	The authors investigated the application of ^{1}H NMR spectroscopy (under an eightfold suppression of water and ethanol) in combination with targeted and non-targeted multivariate statistical analysis to differentiate between German wines based on grape variety, geographical origin and year of vintage	70
Black raspberry urine	The urine of smokers typically contains 8-hydroxy-2′-deoxyguanosine (8-OHdG) is a marker for DNA damage. In this study, smoking males in the age group 20–30 were chosen and classified as black raspberry (BR) responders and BR non-responders, based on the placebo 8-OHdG mean decrement level of 12% from their urine. Proton NMR was performed on the urine samples of these subjects and there was a clear difference between metabolite profiles of the responders and non-responders. The study found that glycine and TMAO levels were much higher in the BR responder group, and amino acids such as histamine, formate, lysine, tyrosine, phenylalanine, acetate and ornithine were higher in the non-responder group. Since BR may be considered as a functional food (FF), and there are responders and non-responders to this particular FF, the authors conclude that functional foods could be personalized based on the prior metabolic profile of people	71
Urine	The lingonberry plant (*Vaccinium vitis* L.) is high in polyphenol content and its intake is supposed to influence pancreatic lipase activity and therefore reduce energy intake. Postprandial urine ^{1}H profiles of 14 non-smoking male subjects after consumption of lingonberries as an addition to an oil-rich meal were subjected to non-targeted analysis. It was found that the sugar:acid ratio was 3.6. The so-called zero sample spectra was measured on lingonberry powder and its purity was verified. PCA analysis revealed a clear difference in postprandial samples as compared to control samples without lingonberry consumption and zero sample of lingonberry extract. They found elevated levels of hippuric acid in postprandial meal, which is not otherwise found in pure lingonberry extracts, suggesting that it was the benzoic acid in the berries that was metabolized to hippuric acid in the liver. A significant decrease of dimethylamine (DMA) was also noted, whereas levels of creatinine were significantly lower. Citric acid in urine was increased. The level of 3-hydroxybutonic acid (HBA) was decreased. The results indicate that postprandial NMR of urine can be now established as a method to study the influence of dietary polyphenols, and also the products excreted after lingonberry consumption were direct results of its metabolism	72

Table 1. Continued

Type of sample	Observation	Reference
Milk	Dairy cows are affected by mastitis, when neutrophils, epithelial cells, macrophages and lymphocytes are secreted into milk in response to invading microorganisms and are collectively called somatic cell count (SCC), which can be an indicator of the usefulness of milk. ^{1}H NMR spectra were acquired and the data were subjected to multivariate analysis. This is the first study where the relation between milk metabolites and SCC was assessed. Distinct differences were noted in the metabolic profile among the milk samples with either lower or higher SCC than normal levels. PLS-DA showed a significant association between NMR data and SCC	73
Cheese	Protected Designation of Origin (PDO) Fiore Sardo cheese was classified using NMR methods on the basis of the bacterial cultures used to ferment the cheese. ^{1}H-^{1}H TOCSY and COSY experiments were done on methanol–chloroform–water extractions from pulverized cheese. These spectra were used for the assignment of metabolites	74
Sea cucumber	Sea cucumbers pre-acclimatized to two temperatures (20 and 25 °C) were used for the study. Extraction was done using methanol and water using powdered muscles from sea cucumbers. ^{1}H NMR spectra were measured and a total of 31 metabolites were identified. PCA analysis of zero thermal stress data showed no metabolite separation, but after 3 days of exposure to the thermal stress the metabolites clearly separated along the lines of temperature	75
Pork	Meat extracts from five different pig crossbreeds were analyzed by NMR-based metabolomics. The results were compared with technological traits and sensory analyses in order to elucidate the potential of NMR-based metabolomics to highlight meat metabolites of importance for technological and sensory attributes of meat. A high content of carnosine in the meat was associated with a low value of many sensory attributes related to meat flavor/taste, while IMP and inosine were in general not correlated with sensory attributes related to meat flavor/taste	76
Polydextrose	This study elucidated the impact of polydextrose (PDX), a soluble fiber, on human fecal metabolome by high-resolution nuclear magnetic resonance (NMR) spectroscopy-based metabolomics in a dietary intervention study ($n = 12$). The establishment of a correlation between the fecal metabolome and levels of *Bifidobacterium* ($R^2 = 0.66$) and *Bacteroides* ($R^2 = 0.46$) demonstrates the potential of NMR-based metabolomics to elucidate metabolic activity of bacteria in the gut	77

for analysis of target compounds. Well-established routine analyses in sweat are those that diagnose cystic fibrosis. The advantages and disadvantages of sweat *versus* urine or blood for doping control have also been discussed. Methods for analytes such as essential metals and xenometals, ethanol and electrolytes in sweat in fact constitute target metabolomics approaches or belong to any metabolomics sub-discipline such as metallomics, ionomics or xenometabolomics. The higher development of biomarkers based on genomics or proteomics as omics older than metabolomics is discussed and also the potential role of metabolomics in systems biology taking into account its emergent implementation.

SENSORY

Using precise quantitative information from different foodstuffs that are preferred for their flavor and specific aroma, it is possible to develop electronic sensors for taste. Because of the high commercial value of foods with preferred flavors, various technologies are being explored around the world for this purpose. Three of the five human senses – sight, sound and touch – have been measured and reproduced successfully, but the remaining two are much more challenging because of the direct involvement of chemistry in those senses. A rapid, non-destructive and highly reproducible way of acquiring accurate knowledge of properties and quantities of the chemicals involved is necessary. NMR is perhaps the only technology with the listed characteristics that can be considered as a kind of 'magnetic tongue' for the characterization and prediction of the tastes of foods, since it provides a wealth of information in a non-destructive and non-targeted manner.[42] NMR metabolic fingerprints have been shown to match with taste parameters in the case of sour cherry juice[43] and tomatoes.[44] In a recent study, Wei and colleagues sought to identify the chemical substances in roasted coffee bean extracts that could distinguish and predict the different sensations of coffee taste by using a combination of NMR-based metabolomics, a human sensory test and application of the multivariate projection method of orthogonal projection to latent structures (OPLS). Four different types of commercial coffee beans were obtained locally. Samples were extracted in 100% D_2O after incubation of the ground powder at 95 °C. ^{1}H NMR spectra were obtained and resonance assignments were carried out. The results showed that NMR-based metabolomics accompanied by multiple statistical models is convenient, fast and accurate for the sensory evaluation of coffee.[45]

PROBIOTICS

Probiotics can be broadly defined as 'Microorganisms that are either alive or in spore form, that when ingested in sufficient quantities are known to confer certain health benefits that are beyond basic nutrition'.[46] The growth and activity of these beneficial microbial symbionts are enhanced by prebiotic foods, such as fructo-oligosaccharide (FOS) and galacto-oligosaccharides, in the human gastrointestinal tract. Therefore, evaluation of the effects of prebiotic foods on the dietary interactive modulations of the host and beneficial microbial symbionts are important for human health. The consumption of probiotic foods has dramatically increased in recent years, since the potential health benefits of these foods have been validated by research. Date *et al.* [47] have developed a simple and rapid *in vitro* evaluation method using an NMR-based metabolomics approach coupled with multivariate statistical analysis for screening and discovery of uncharacterized and untapped prebiotic foods. Metabolic profiles generated by intestinal microbiota after *in vitro* incubation with feces were examined and viscous substances of Japanese bunching onion (JBOVS) were identified as one of the candidate prebiotic foods by this method. The ingestion of JBOVS led to lactate and acetate production by the intestinal microbiota, and were accompanied by

an increase in the populations of *Lactobacillus murinus* and *Bacteroidetes* sp. in the intestine.

Settachaimongkon *et al.* analyzed both natural and commercial yogurts using an array of chromatographic and ^{1}H NMR for interesting metabolic signatures.[48,49] The role of *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB12 in co-fermentation with traditional starters on metabolite formation in set yoghurt has been evaluated using ^{1}H NMR. Thirty-seven volatile and 43 non-volatile metabolites were identified. Results showed that the two probiotic strains did not influence acidity and the key aroma volatile metabolites of set yoghurt, whereas the effect of *L. rhamnosus* on the non-volatile metabolite profile of yogurt was clearly noticed, providing new insights into the impact of probiotics on the metabolome of yogurt. The role of probiotic strains found in human milk in treatment of infectious mastitis, a common condition among lactating women, has been evaluated by NMR. The ^{1}H NMR-based metabolomics approach was applied to detect metabolomics differences in 24 h urine samples from women with lactational mastitis that were collected at baseline and after 21 days of probiotic (PB) consumption. The discriminant metabolites detected by ^{1}H NMR at baseline were lactose, ibuprofen and acetaminophen (drugs commonly used for mastitis pain), while, after PB intake, creatine and the gut microbial co-metabolites hippurate and TMAO were detected. A voluntary desertion of the pharmacological drugs ibuprofen and acetaminophen was observed after probiotic administration. Additional applications of NMR used for food analysis is briefly summarized in Table 1. The use of sensory NMR techniques in evaluating the quality of functional foods with respect to prebiotic carbohydrates has been reviewed by Corradini *et al.*[50]

CONCLUSIONS

NMR has traditionally been used as a tool of choice to elucidate chemical structures of small synthetic and natural molecules and, of late, large biomolecules with increasing precision. With increasing computational power, reduced costs, and development of stronger magnetic fields, cryoprobes, solvent suppression techniques and a large number of versatile 1D and 2D NMR pulse sequences have extended their application in the field of NMR in metabolomics and nutrigenomics, because of the distinct advantage of reproducibility. Application of NMR in the agro-food sector is one of the fastest-growing fields. This review provides recent updates on applications of NMR in food and agricultural research in specific areas of food science, namely nutrition, fingerprinting for food classification, sensory property evaluations and metabolomics. There have been increasing applications in quantitative proton/fluorine NMR (qHNMR and qFNMR) for purity assessment of compounds, quantitation of multiple components without the need for complex chromatographic separations. This advantage has significant potential in the field of biomarkers and other metabolomics studies. However, the common challenge associated with qNMR is lack of selectivity due to inherent overlap of resonances. In addition, developments in solid-state NMR and magnetic resonance imaging provide a non-invasive continuum from liquid-state NMR. Solid-state NMR can, in principle, be used to monitor the chemical composition of food materials, without any kind of additional sample preparation. Modern developments in electronics and magnet construction have enabled table-top portable spectrometers, which are already being used in metabolomics and food composition analysis. Spectrometer sizes will shrink and a concomitant development in computational tools will bring magnetic resonance into the hands of a lay user, say a farmer on the field or a nutritionist. With these unique advantages, magnetic resonance is well on its way to becoming a technique of choice in the study of food.

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