The new frontier in foodomics: the perspective of nuclear magnetic resonance spectroscopy

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Nuclear magnetic resonance (NMR) spectroscopy is an investigation technique to study matter. It is based on the properties of magnetically active nuclei, which respond to a perturbation induced in a sample by applying a radio wave pulse. The nuclei, if immersed in an intense magnetic field, respond to the pulse by oscillating at a particular frequency, thus generating a signal which is recorded and transformed by the instrument as a graph, the so-called spectrum, reporting the intensity of the response as a function of the oscillation frequency. The frequency of each nucleus is characteristic of its position in the molecule and depends on the physico-chemical state of the substances. The response depends, in fact, not only on the structure of the molecule to which the atom belongs to, but also on the chemical environment in which the molecule is immersed.

Not all nuclei present in natural substances are magnetically active; therefore, not all of them are detectable by NMR. However, the atoms mainly present in foods, such as H, O, C, N, P, etc., have at least one detectable isotope. For some elements, the most abundant isotope, such as ^{1}H and ^{31}P, is visible, while the technique sensitivity for ^{13}C, ^{17}O and ^{15}N results is very low, since they are low-abundance isotopes in nature for their corresponding element. Sensitivity could be improved by synthetically enriching the substances with the most abundant stable isotopes. The presence of the magnetically active ^{1}H isotope in all natural substances gives NMR spectroscopy, practically, the title of universal detector.

From this preliminary description, it follows that every substance responds to the radio wave pulse with a set of signals, one for each atom constituting it. Moreover, the frequency of oscillation, expressed as the position of the

corresponding signal on the spectrum, changes if the molecule interacts with others present in the surrounding chemical environment, or if the molecule is ionised as a consequence of a different pH, or in the presence of high salt concentrations in solution.

Thus, a set of information can be derived by inspecting the NMR spectrum: the frequency of the signal, normally expressed as the chemical shift (in ppm) with respect to a reference frequency, is diagnostic for the presence of a specific substance in the mixture, and its area is directly proportional to the concentration of the substance; the signal width depends on the rate at which the atom, corresponding to that signal, relaxes back to the equilibrium state in which it was before being perturbed by the radio wave pulse. The relaxation rate depends on the size and rigidity of the compound, and on the energy of the interactions with the surrounding molecules. Other information may be obtained by inspecting further signal features, such as the shape / multiplicity, and by looking at other physical properties, such as the magnetic coupling, which are the basis of multi-dimensional spectroscopic techniques. This additional information is considered unique, allowing the scientist to elucidate the molecular structure of an unknown substance, and was the reason for the success of NMR spectroscopy in past decades.

A more complete description of the theoretical aspects of the technique, and possible information obtainable on foods on the basis of the analytical instruments, is available[1].

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What NMR spectroscopy offers

Nuclear magnetic resonance spectroscopy offers the possibility to obtain a molecular fingerprint of a mixture without having to go through a tedious preparatory phase for the separation of the various components. Many signals originate from the same molecule, one for each atom, each one with its own characteristic frequency. Whatever the instrument used, the resonance frequency, expressed in ppm, corresponds to a specific atom of a molecule present in a particular mixture, and it is constant across all laboratories. Therefore, the same mixture analysed in different laboratories always produces the same spectrum, superimposable to those obtained elsewhere. In contrast to other techniques, the response is not dependent on the column usury or on the manufacture characteristics, which can change from batch to batch. Instead, it can evolve over time if the sample is unstable. Thus, two different spectra necessarily imply a different mixture and not an irreproducible experiment.

Another interesting feature of NMR spectroscopy is a direct proportionality between the concentration of equivalent atoms in the solution and the area of the corresponding signal. In practice, this feature can determine the absolute quantity of a molecule by measuring the area of just one of its signals, assigned to a specific atom within the molecule, and by using, as a reference standard, the area of a signal from another different compound, present at a known concentration in the same solution. Contrary to any other technique, it is not necessary, in fact, to possess a standard solution with known concentration of the molecule to

be titrated. Finally, another feature of NMR spectroscopy is 'dynamic range', i.e. the ability to measure, with a single analysis, substances even billions of times more diluted than others, in the same solution. For other analytical techniques, such as chromatography or spectrophotometry, it is often necessary to proceed with an opportune dilution of the solutions, if the concentration of an analyte exceeds the saturation limit of the detector. In this case, the analyst has to deal with the problem of the choice of solvent for dilution, so that the 'chemical environment' of the mixture is not altered.

MR shows an ease of sample preparation, often limited to the simple extraction phase, to obtain the solution to be placed directly in the instrument probe while for many techniques, derivatisation is a necessary step to add functional detectable groups to the molecule or to convert it in volatile derivatives for the analysis in the gas phase. Through the use of a special probe, it is possible to record the NMR spectrum directly on samples of soft matter, that is, foods existing in an intermediate state between solid and liquid, such as the semi-rigid matrix rich in water (e.g., fruit, meat or cheese). In this case, we refer to High Resolution Magic Angle Spinning (HRMAS) NMR spectroscopy, and the analysis of samples is even easier, because the spectrum of intact food can be directly recorded on a few mm^3 of sample.

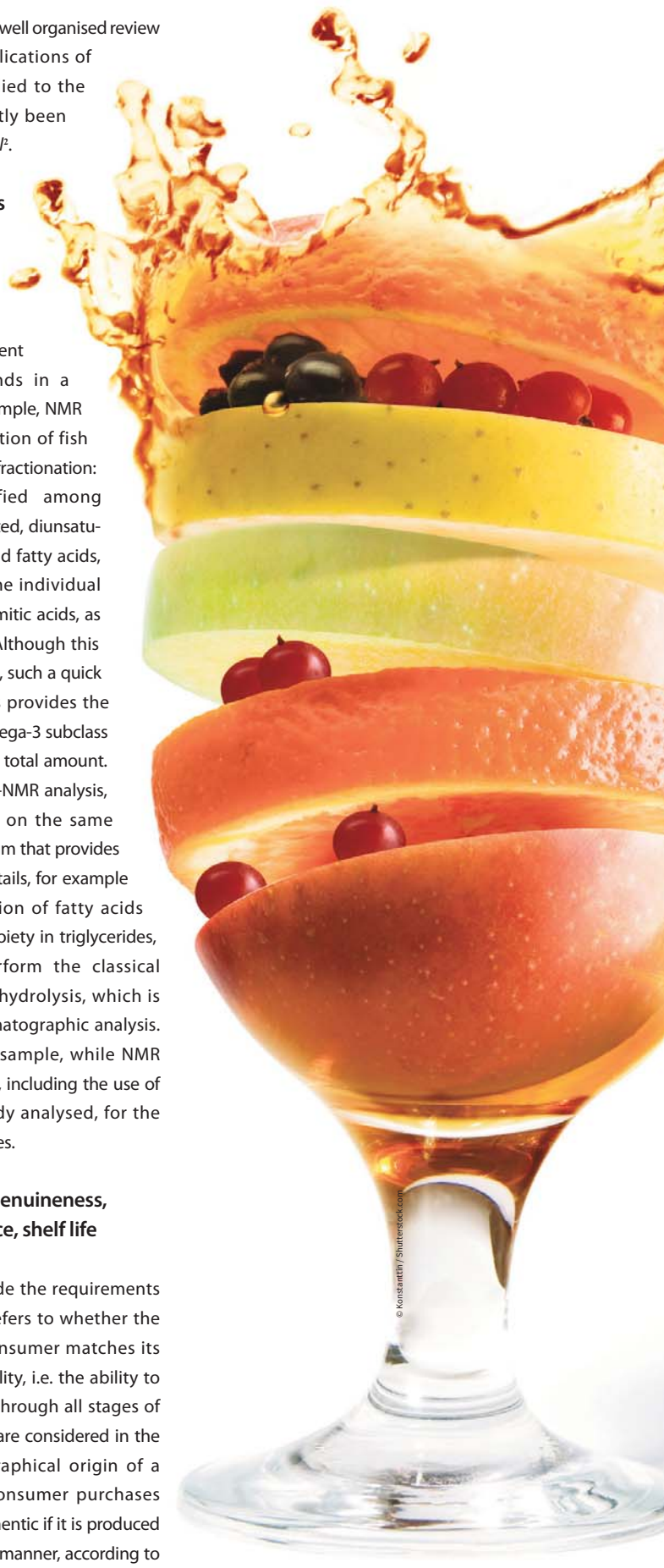
"Food quality must include the requirements of authenticity, which refers to whether the food purchased by a consumer matches its description, and traceability"

Obviously, there are not only laurels for this technique but, among the few disadvantages associated with it, the most important is the poor sensitivity. The comparison with other widely used, sophisticated techniques, such as Mass Spectrometry (MS), leaves no doubt: MS requires as low as sub-femtomole and a few microliters volumes, whilst NMR relies on tens of picomoles and hundreds of microlitres. This makes NMR spectroscopy not so useful when the trace amounts of some compounds have to be determined. So, if the quantitative analysis of individual molecules (target analysis) is sought, then the choice must fall to other techniques. Instead, if molecular details are investigated, such as structural features or interactions with other compounds, then NMR spectroscopy is the principle technique.

A comprehensive and well organised review about the different applications of NMR spectroscopy applied to the food analysis has recently been published by Mannina *et al*[2].

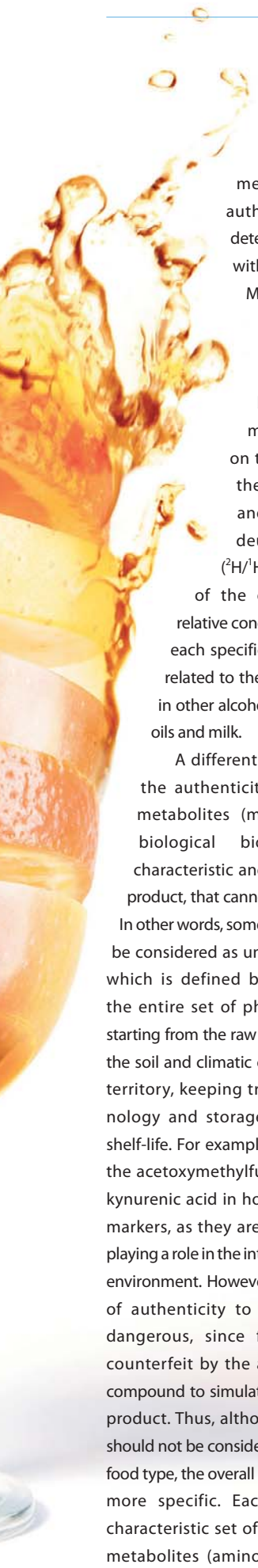
NMR and food analysis

In some cases, the ^{1}H-NMR spectrum has the advantage of allowing a rapid assessment of the relative content of classes of compounds in a complex mixture. For example, NMR analysis of the lipid fraction of fish extracts results in a class fractionation: the mixture is classified among saturated, monounsaturated, diunsaturated and polyunsaturated fatty acids, rather than obtaining the individual signals of stearic and palmitic acids, as they are superimposed. Although this may seem like a limitation, such a quick non-destructive analysis provides the very handy amount of omega-3 subclass as a percentage of the fat total amount. After such preliminary ^{1}H-NMR analysis, it is possible to record, on the same sample, a ^{13}C-NMR spectrum that provides many more molecular details, for example the positional distribution of fatty acids classes on the glycerol moiety in triglycerides, without having to perform the classical and tedious enzymatic hydrolysis, which is preliminary to the chromatographic analysis. The latter destroys the sample, while NMR preserves it for future use, including the use of the same sample, already analysed, for the following nutritional studies.



Geographical origin, genuineness, substantial equivalence, shelf life and freshness

Food quality must include the requirements of authenticity, which refers to whether the food purchased by a consumer matches its description, and traceability, i.e. the ability to trace and follow a food through all stages of production. Both criteria are considered in the definition of the geographical origin of a food product, as the consumer purchases a food believed to be authentic if it is produced in a precise and traceable manner, according to



a protocol or to a disciplinary ensuring a certifiable quality.

The case study, often mentioned to represent authenticity, concerns the determination of wine origin, with a method proposed by Martin, according to a well-known approach that employs the technique ^{2}H-SNIF NMR approved by the EU in 1990. This methodology is based on the correlation between the geographical origin and the isotopic ratio of deuterium to hydrogen (^{2}H/^{1}H) in two specific positions of the ethanol molecule. The relative concentration of deuterium in each specific molecular position was related to the geographical origin also in other alcoholic beverages, fruit juices, oils and milk.

A different approach to determine the authenticity of a food is to seek metabolites (molecules produced by biological biosynthetic pathways) characteristic and unique for a particular product, that cannot be found in other ones. In other words, some specific compounds may be considered as unique markers for a food, which is defined by its history, including the entire set of phases of its production, starting from the raw material, passing through the soil and climatic conditions of the farming territory, keeping track of changes in technology and storage conditions during its shelf-life. For example, the theanine in tea, or the acetoxymethylfurfural in vinegar, or the kynurenic acid in honey are species-specific markers, as they are secondary metabolites playing a role in the interaction of the cell with its environment. However, entrusting a testimony of authenticity to a single metabolite is dangerous, since foods could be easily counterfeit by the addition of the specific compound to simulate the genuineness of the product. Thus, although a single metabolite should not be considered as a reliable marker of food type, the overall set of metabolites is much more specific. Each food type shows a characteristic set of primary and secondary metabolites (amino acids, sugars, organic acids), and altogether contribute to build the metabolome of the species.

Metabolic fingerprinting is used when classification of individual samples, without any preliminary identification of specific metabolites, is required. In this case, the NMR spectrum can be considered as the representation of the molecular profile of the foodstuff, and all the NMR resonances are quantified as belonging to unknown compounds. Molecular profiles are particularly useful when a holistic view is required to describe the food, to study the transformations occurring in a product due to technological processes, or to follow the natural aging during storage. In this case, the comparison between the profiles recorded at different times, or acquired with samples obtained with different technologies, can select the signals, although not yet identified, that undergo an area integral variation. Afterward, the molecules corresponding to the variant signals are identified, to determine the molecular pathways through which the product evolves or to select patterns capable of distinguishing that food from others.

"The NMR metabonomics approach seems to be without limits in investigable food types"

How many compounds can be detected simultaneously by NMR? The answer depends on the selectivity of the extraction. It is clear that the number of compounds actually present in a food extract can be much smaller than the real number of metabolites present in a crude sample. For example, in a single plant species about 5000 to 10,000 metabolites can be present, but only a small selection can be detected in a single NMR experiment. In fact, the concentrations of the major selections of metabolites in plants are less than 1 μM, that is below the limit of detection for NMR spectrometers (above 10 μM).

Metabolomics is a non-biased identification and quantification of the whole metabolome in a biological system

The term 'metabolome', introduced by Stephen G. Oliver in 1998 (University of Cambridge), refers to the quantitative complement of all of the low molecular weight molecules present in cells in a particular physiological or developmental state. Clearly, no analytical technique alone can record the entire metabolome, least of all NMR because of its low sensitivity.

Metabonomics, defined by Jeremy K. Nicholson in 2006 (Imperial College of London), seeks to identify the metabolites that correlate with changes of physiological conditions. By extension, we can consider the effect of natural, environmental and technological perturbations as sources of variance in the entire food metabolome during its life. For this reason, 'food metabonomics' gives the opportunity to gain deeper insights into and have a better control of the fundamental biochemical basis of the things we eat.

While the NMR spectrum of a food extract can be considered as a graphical representation of the entire pool of detectable metabolites, multivariate data analysis applied to the entire set of spectra, corresponding to different food groups, classes or categories, is able to extract the useful information to select the signals, and then the molecules, which are responsible for possible identification, in accordance with the principles of metabonomics. For example, the same product collected in different geographic regions, or produced according to different standards, or even observed during different storage times, may be studied through the metabonomics approach. Olive oil is one of the foodstuffs investigated by ^{1}H-NMR metabonomics, clearly indicating that NMR spectroscopy is uniquely capable of distinguishing between olive oils on the basis of their geographical origin and no other analyses are appropriate for this type of determination of quality and genuineness.

"Entrusting a testimony of authenticity to a single metabolite is dangerous"

Explorative unsupervised multivariate data analysis (e.g., principal components analysis) applied to ^{1}H-NMR spectra of beers allowed their classification according to type (ale, lager, alcohol-free), brewing site (Portugal, Germany, Belgium, England, Spain, the Netherlands and the USA), production dates and malt type, according to the content of dextrins, maltose and glucose. The pattern of these molecules has been recognised after the evaluation of the spectral data according to the principle of the unsupervised analysis. However, the prediction of their respective identity class,

applied to unknown samples, is possible by application of supervised methods (e.g., partial least square regression, linear discriminant analysis, soft independent modelling of class analogies, genetic algorithms and genetic programming, artificial neural network) on trial sets of well-known samples.

Interesting predictive classifications have been obtained by using the NMR-metabonomics approach to investigate the differences in wines produced by using different grape varieties and by the same grape variety harvested in different areas. Metabolic profiling of white wines from different varieties, determined by ^{1}H-NMR and both gas chromatography-coupled time-of-flight mass spectrometry (GC-TOF-MAS), has been correlated to the wine sensory property body using supervised partial least square regression (PLS). The GC-TOF-MS and NMR-based PLS models have been suggested as predictive models complementary to the traditional panel in wine sensorial analysis.

"NMR metabonomics has been exploited to understand and interpret metabolic changes occurring in vivo as a consequence of the farming system for gilthead sea bream"

The metabolic profiles of transgenic tomatoes over-expressing maize transcription factors have been compared with control fruits by PLS statistical treatment at three ripening stages. Clear separation of samples according to ripening stage and genotype has been achieved, confirming the increased production of flavonols in the transgenic tomato line. A similar approach has been applied to grapes in our laboratories, by comparing two different cultivars of transgenic grapes with their corresponding control fruits. The experiments have shown that the extent of the metabolic changes depends on the genotype of the cultivar hosting the extraneous gene, as well as the number of gene copies, showing no proportionality between such a number and the resulting metabolic perturbations.

NMR metabolic profiles of canned tomato have been correlated to their sensory descriptors (bitterness, sweetness, sourness, saltiness, tomato and metal taste, redness and density), suggesting that NMR might be a very useful tool for the characterisation of sensory features of tomatoes.

NMR metabonomics has been exploited to understand and interpret metabolic changes occurring *in vivo* as a consequence of the farming system for gilthead sea bream (tanks, cages and lagoons), as well as to measure the freshness loss of wild and reared fish after storage in different conditions.

The NMR metabonomics approach seems to be without limits in investigable food types, since many examples have appeared in literature, including distillings, soy sauce, vinegar, coffee, tea, fruit juices, mandarin oranges, kiwifruit, mango, melon, watermelon, black raspberry, lettuce, carrot, maize, brassica rapa, potato, wheat, milk, cheese, butter, margarine, honey, fish, meat, truffles, pine-mushrooms, saffron and many others (see the abovementioned review by Mannina *et al*[2] for the broadest coverage of applications).

The next frontier in metabonomics is represented by the application of this approach to nutrition, the so-called nutri-metabonomics. Food is the source of nutrients which must be bioaccessible and bioavailable. Digestion processes, including the role of the gut microbiota, play a main role in the necessary conversion of food in the actual source of macro- and micro-nutrients. For this reason, the food cannot be considered as a static pool of molecules embedded in the matrix. According to this perspective, holistic nutrition studies must consider the evolution of the food matter along the gut and its interaction with the human host. The challenge we are facing up in our 'Laboratory of Foodomics' is the meta-analysis of the food-human metabolome, evolving during digestion, in the most comprehensive approach provided by nutritional metabonomics. This approach has been included in a project funded by the European Commission (CHANCE – grant agreement no. 266331), aiming at developing low-cost, nutritionally correct food for populations at risk of poverty. Both the food products and the metabolic state of the recruited consumers will be defined by applying the nutri-metabonomics (visit www.chancefood.eu). The science behind this innovative project requires a multi-disciplinary integration, which is not easy to achieve because food technologists, nutritionists, chemists, clinicians and other specialists lack common ground for their interactions.

For this reason, since 2009, we have organised the biannual international conference on Foodomics (www.foodomics.eu), consisting of four sessions, namely: i) foodomics for discovering foods, food components and nutraceuticals; ii) foodomics for discovering digestion, bioaccessibility and the role of microbiota; iii) foodomics for discovering the nutrients mechanisms of action; iv) foodomics for discovering nutrition in clinical sciences. Of course, while aiming for this objective, we do not limit the field of application only to the metabolome realm, but in the effort to complete the -omics picture, we extend the coverage to genomics and proteomics sciences. But that is another story!

References

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Biography

Since 1998, **Francesco Capozzi** has been Associate Professor of Chemistry at the University of Bologna. He is leader of the BIO-NMR group at the Campus of Food Science of the University of Bologna, with research activities aimed at studying biological systems (of bacterial, vegetable or animal origins) and foodstuffs by applying a system chemistry approach.

He is developing spectroscopic methods to be adopted in the assessment of food quality by discovering correlations between nuclear relaxation times of water contained in foodstuffs and the chemical-physical characteristics of the food matrix. He is also deeply involved in research aimed at developing chemical descriptors defining the qualitative characteristics of foodstuffs by extracting simple numerical parameters from spectroscopic data. The case studies include vegetable products protected by collective quality labels, requiring particular attention for their traceability and vegetables GMOs, demanding a careful assessment of the variations introduced in their metabolic profiles by genetic modifications. Currently, he is developing a traceability system for fruit and vegetable products based on univocal molecular fingerprints, derived by spectroscopic data, and he is supporting the Italian Ministry of Agriculture in the definition of 'fish freshness' with molecular descriptors capable of providing a holistic definition (Foodomics) of the food composition.

He is the European coordinator of the project KBBE FP7-266331 'CHANCE – Low cost technologies and traditional ingredients for the production of affordable, nutritionally correct foods Improving Health in Population groups at risk of poverty' (www.chancefood.eu). He is the work group leader on 'Food Structure and Nutrient Bioavailability' of the European COST Action FA1005 INFOGEST 'Improving Health Properties of Food by Sharing our Knowledge on the Digestive Process' (www.cost-infogest.eu).

He is the scientific coordinator of the project funded by the Italian Ministry of Agriculture, Food and Forestry on 'FRESH FISH: Development of the fresh fish system' to support the General Directorate of Maritime Fishing and Aquaculture in the drafting of regulatory guidelines.