Time Domain Measurements and High Resolution Spectroscopy are Powerful Nuclear Magnetic Resonance Approaches Suitable to Evaluate the In Vitro Digestion of Protein-rich Food Products

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TIME DOMAIN MEASUREMENTS AND HIGH RESOLUTION SPECTROSCOPY ARE POWERFUL NUCLEAR MAGNETIC RESONANCE APPROACHES SUITABLE TO EVALUATE THE *IN VITRO* DIGESTION OF PROTEIN-RICH FOOD PRODUCTS

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1 INTRODUCTION

Our knowledge concerning the digestion of dietary proteins, the first step necessary for their proper utilization, is limited and many aspects of this physiological process still remain unexplored.
Digestion is a system of cooperating enzyme reactions, and enzymes are the prime movers in protein digestion. Many factors influence how fast the enzymes act on the protein: among others, the concentration of the enzyme, the amount of protein food needing action, the acidity of the food and of the stomach, the temperature of the food, time, the presence of any digestion inhibitors, such as antacids, and all technological modifications occurred to food before its consumption.
Although chewing helps allowing the reduction of foods in smaller pieces, protein digestion does not begin in the mouth but in the stomach, chiefly with the action of the hydrochloric acid and by the enzyme called pepsin. Protein digestion continues in the upper portion of the small intestine under the action of the pancreatic protein enzymes; the protein-containing foods are broken apart, separating out the protein, then the proteins are broken into their constituent parts, the amino acids. Chemically, the hydrolytic enzymes brought along by the digestive juices solubilise peptides from the biopolymers as schematically represented in figure 1, where meat is considered as an example.
The amino acids are absorbed by the blood capillaries of the small intestines, carried through the liver, and then go into the blood of the general circulation. A peptide can be absorbed through the surface of the *villi* if it is soluble at the intestine physiological conditions, and it is formed by no more than three amino acids. The digestibility of a protein-rich food can be thus estimated by characterizing the fraction of proteins that can be hydrolyzed into peptides suitable for absorption[1] .

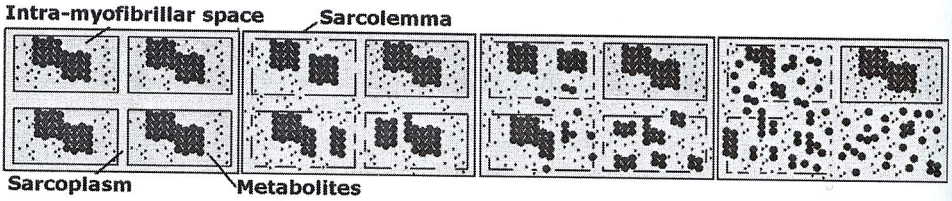


Figure 1. *The digestion attack on the matrix structure and on the proteins (aggregates of dark filled circles) in meat is schematically represented by parallel steps of fragmentation (cut in large polypeptides) and disintegration (small oligopeptides release), with different kinetics.*

It is commonly accepted that the digestibility of a food can be affected by the spatial arrangement of its components[2] , so that two foods with similar macronutrient composition may show different digestibility if produced through different technological processes. Interestingly few studies address this topic, due to the difficulties in setting up a suitable *in vivo* protocol to study the relationship between digestibility and food structure. At the opposite a great variety of *in vitro* digestion tests are described in the literature. The simplest tests simulate human digestion by means of static systems, which are containers where the bolus is mixed by a magnetic stirrer, and the environment is kept at physiological conditions with a heating system, and where the aqueous solutions of enzymes are manually added at the proper pH.

Protein digestion is quantified according to the simplest protocols[3] by collecting digestate samples at fixed intervals and quantifying the total amino acidic content by the Kjeldahl method (AOAC 11984) or colorimetric assays.[4] When information is desired about the molecular weight distribution of peptides, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and HPLC analysis are performed[5]. The spatial arrangement of the food at the microscopic level is typically investigated only prior to digestion, by means of microscopy.[3, 6] Information about the food structure during digestion is collected mainly at the macroscopic level, by means of magnetic resonance imaging performed *in vivo*.[7] In addition the particles detached from the bolus are characterized by means of sieves and a scale.[2] In a recent paper we reported a protocol for investigating the relationship between structure and digestibility of a protein-rich food based on a single analytical technique, namely nuclear magnetic resonance.[8]

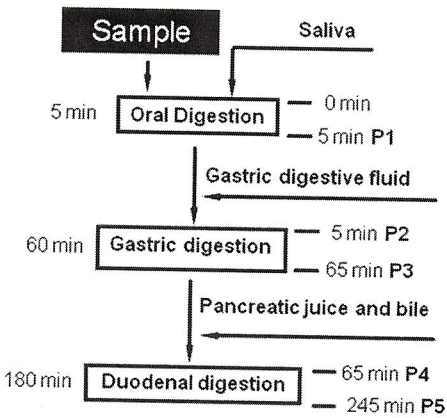
Nowadays time domain (TD) NMR measurements performed at 0.23-0.46 Tesla are commonly applied to get information about the spatial arrangements of several kinds of food through the interactions between biopolymers and water.[9] Amino acids, peptides and proteins solubilised by digestion can be simultaneously observed by means of high resolution (HR) NMR spectroscopy, which offers qualitative information about the size distribution of macromolecules by means of the measure of signal linewidth.[10-11] The quantification of amidic protons, and the observation of molecular auto-diffusion weighted spectra are other information so far acquirable.The application of such techniques for this purpose is far from straightforward, due to the great spatial and compositional heterogeneity of the studied matrices. During the entire course of digestion at least two spatially resolved phases can be distinguished, one represented by the bolus mass, the second constituted by the digestive juices where food particles are suspended and solutes accumulate. In addition soluble molecules of very different molecular weight are simultaneously present, ranging from single amino acids, giving rise to sharp signals in the

NMR spectra, to pretty large proteins, either folded or denatured, which originate broad bands.

The present paper focuses on practical aspects of the application of TD- and HR-NMR to the setting up of protocols for the study of the relationships between the structure of protein-rich foods and their digestibility. For this purpose, details on the recently published study[8] describing the protocol set for evaluating the *in vitro* digestion of Parmigiano cheese by TD- and HR-NMR are given in the next section. Some comparisons with other matrices, such as fresh and cured meat, are provided as well.

2 METHODS AND RESULTS

2.1 Samples and *in vitro* digestion



Scheme 1. *Pictorial representation of the protocol used to simulate the in vitro digestion of a protein-rich food*

Two samples of Parmigiano Reggiano cheese, aged 15 and 30 months respectively, were bought from a local retailer. Human digestion was simulated in vitro as described by Bordoni et al.[8], by following the protocol in scheme 1. Samples were collected by taking aliquots from the mixture at five check points: P1 at the end of the oral digestion; P2 at the beginning of gastric digestion (after the decrease of pH to 2, before the addition of pepsin); P3 at the end of the gastric digestion; P4 at the beginning of the duodenal phase (after the increase of pH to 5, before the addition of pancreatin and bile); and P5 at the end of duodenal digestion. In samples taken at P2 and P3, the pH was increased to 8 with 35% NaOH to stop pepsin action, while samples taken at P4 and P5 were acidified to pH 2 with 37% HCl to stop pancreatic hydrolysis. All samples, except those undergoing LR-NMR, were centrifuged at 4000 g for 5 min, and the supernatant was filtered with 0.2 mm membranes. An aliquot of the supernatant was sequentially ultrafiltered with Amicon Ultra (Millipore) at 3 kDa of molecular weight cut-off. In this way, two aliquots (one filtered with 0.2 mm membrane, and another containing the molecules with molar mass <3 kDa) were obtained for each sample.

2.2 Analytical techniques

SDS-PAGE analysis and Bradford assay

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed using handcast 16% polyacrylamide gels and a Mini-PROTEAN system (Bio-Rad). The molecular weight marker (Fermentas) contained a mixture of proteins from 10 to 200 kDa. Peptide concentration during the digestion process was measured using the Protein Assay kit from Bio-Rad, based on the Bradford assay. The standard curve (R^2 of 0.9875) was obtained using seven dilutions (from 1 to 20 mg) of bovine serum albumin. All protein solutions (standards and samples) were assayed in duplicate or triplicate.

TD-NMR

Low-field proton transverse relaxation time curves were acquired on cheese and its suspension collected at each digestion check point. The latter was taken directly from the digestion flask and analyzed without filtration or other treatments. Four hundred milligrams of both solid and liquid samples were placed inside 10-mm (outer diameter) NMR tubes, so that they did not exceed the active region of the RF coil, and analyzed at 24 °C with the CPMG pulse sequence using a Bruker Minispec PC/20 spectrometer (Bruker Optics Inc., Billerica, MA, USA) operating at 20MHz. Each measurement comprised 30000 echoes, with a 2τ interpulse spacing of 80 ms and a recycle delay of 3.5 s. The specified t, chosen to avoid sample and radio frequency coil overheat, allowed the observation of the protons with T_2 higher than a few milliseconds. The CPMG decays were normalized to the sample weight and analyzed with the UPEN program,[12] which inverts the CPMG signal using a continuous distribution of exponential curves.

HR-NMR

Samples were prepared for high resolution ^{1}H-NMR analysis by adding to 1 ml of each sample, 160 µl of 1 M phosphate buffer in deuterium oxide (D_2O), containing 10 mM 3-TrimethylSilyl-Propanoic-2,2,3,3-d4 acid sodium salt (TSP) as internal standard. After adjusting the pH to 7, the samples were centrifuged at 14000 rpm for 5 min in order to further remove particles. Finally, the samples were stored at 80 °C until NMR analysis. All ^{1}H-NMR spectra were recorded at 300 K on a Bruker US+ Avance III spectrometer operating at 400 MHz, equipped with a BBI-z probe and a SampleCase™ sampler for automation. The spectra were collected with a 90° pulse of 13 ms with 7.7W of power, a relaxation delay of 4 s and an acquisition time of 2.5 s. For each sample, 32 scans were collected into 32 K data points covering a 20-ppm spectral width. The HOD residual signal was suppressed by employing the first increment of the NOESY pulse sequence and a spoil gradient. The data were Fourier transformed into 64 K data points. Phase and baseline corrections were automatically performed using TopSpin version 3.0 (Bruker BioSpin, Karlsruhe, Germany). DOSY experiments have been performed by applying the water-suppressed LED pulse sequence described by Altieri et al.[13] For the purpose, 32 ^{1}H spectra were registered by attenuating the signal through field gradient pulses 1.5 ms long, with power from 1 to 47 G cm, separated by 80 ms. Each peak height was then fitted to a mono-exponential curve by means of the TopSpin 3.0 routine eddosy.

2.2 Information about the digestive juices permeation by TD-NMR

TD-NMR, generally performed with instruments equipped with a permanent 0.23-0.46 T magnet, is employed to obtain information about the spatial arrangement of a food by observing the changes occurring in some measurable parameters affected by the interaction between water and biopolymers.

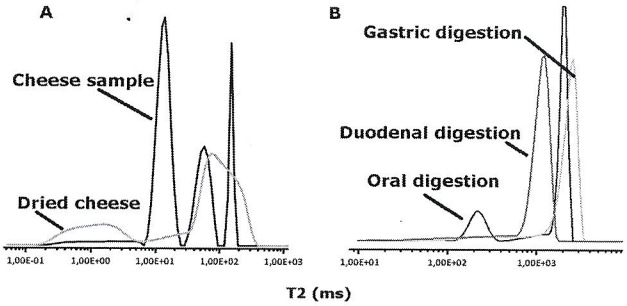


Figure 2. *Relaxograms obtained by inverting T_2-weighted TD-NMR signals to continuous distributions of exponential curves. A. Relaxograms from one Parmigiano cheese sample before and after drying, aiming at assigning the peaks to specific proton populations. B. Typical relaxograms obtained during in vitro digestion of Parmigiano cheese samples.*

When employed on foods undergoing digestion, such interaction allows studying the extent of water flowing from the digestive juices towards the inner structures of the bolus. The parameter most often followed to investigate the water-biopolymers interaction is the transverse relaxation time by means of the CPMG sequence. During the acquisition of T_2 weighted signals, the acid protons of protein bound amino acids and of macromolecular solutes (with a T_2 of microseconds) exchange with water (with a T_2 around 1.6 s). In the fast exchange limit, all protons located in the same compartment have a T_2 proportional to a function of the molar fraction of water in the mixture with solutes and biopolymers. In practical situations, the subtle differences in T_2 among the observed proton populations, and the sub-optimal signal–to–noise ratio often associated with the short acquisition time, make difficult to assign the signals to specific food structures[14] or even to identify the correct number of proton populations which give rise to the recorded signal. In such cases it is advisable to check whether T_1 or diffusion experiments offer a higher contrast, by means of 2D relaxograms, which can be calculated by means of fast 2D Laplace inversion methods.[15] Once the experiment capable of emphasizing the differences among proton populations is set up, the subsequent description of the system under investigation must rely on the correct assignment of each discriminating feature to the corresponding structure within such system. The correct assignment is obtainable by modifying the system with specific treatments, and by annotating the features which are consequently modified. With this approach, Marigheto et al.[16] and Panarese et al.[17] assigned specific relaxograms features to extracellular or vacuoles water, by acquiring T_2 weighted signals on fruit slices and by comparing the relaxogram after dipping the same slices in a paramagnetic relaxation agent solution. The same strategy was successful applied not only in vegetable tissues, but also in products of animal origin, since in fresh meat 3 proton populations have been assigned by means of T_2 weighted signals. The protons located inside the myofibrils give rise to 92% of the signal, with a T_2 around 50 ms. The protons from the extra-myofibrillar spaces account for some 5% of the signal, with a T_2 around 180 ms, while the last population, with a T_2 of a few microseconds, is often loosely described as due to matrix bound water. To perform the so described proton assignment Bertram et al.[18] registered T_2-weighted curves of centrifuged meat, to selectively remove water located in the extra-myofibrillar spaces.

As a practical example of system modification easing signal assignment, figure 2A shows a relaxogram obtained for the present paper on Parmigiano cheese aged 30 months before and after oven drying. Water dehydration marginally modifies the signals with T_2 above 30 ms, thus ascribable to fat, while removes most of the signals around 10 ms, due to water exchanging with biopolymers. Figure 2B shows that most of the signal registered on the digestate of Parmigiano cheese is due to bulk water, and the marked difference of T_2 between bulk and bolus water suggests that a T_1 filter can help in focusing on bolus water.

2.3 Oral digestion

One of the critical steps of *in vitro* digestion is the simulation of mastication. Although proteins are not enzymatically hydrolyzed in the mouth, this first stage of digestion modulates the contact surface between food and digestive juices. A scarce reproducibility of mastication has exponential consequences on each following step, leading to noisy observations hindering the capturing of interesting features. A way to check the reproducibility of the simulated mastication can be based on the calculation of the matrix degradation index (MDI), recently set up by Lamothe et al.[2] The MDI is the ratio between the weight of the particles with a diameter below a certain threshold, due to the mechanical action of mastication, and the digestate total weight. As the separation of the particles according to the diameter is performed by a tedious method, it could be worth checking for possibilities of predictions of MDI from TD-NMR. Preliminary experiments carried out in our laboratory showed that TD-NMR is suitable to quantify the water absorbed on the surface of the bolus particles, since it is characterized by T2 between that of water within the bolus particles and that of bulk water. This measure has been utilized to evaluate the reproducibility of *in vitro* mastication among different repetitions of digestion on the same food.

2.4 Information about the water soluble molecules

Handling the normalization issue to obtain useful information from high resolution spectra

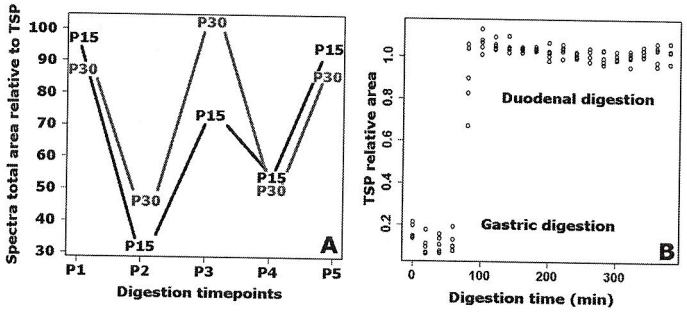


Figure 3. *A - Total area of ^{1}H-NMR spectra registered during digestion and scaled to the internal standard (TSP) signal. P15 represent samples of cheese aged 15 and 30 months, respectively. B - In vitro digestion of a cured beef sample. Intensity of the signal of the internal standard (TSP) obtained from spectra which vertical scale was adjusted to the peaks at 1.33 ppm from lactate.*

Human digestion times are sufficiently long to allow the acquisition of spectra in almost real time, in particular if the instrument used is equipped with an auto sampler.

Unfortunately the obtained spectra, even if acquired with the same parameters, cannot be directly compared, since during digestion the concentration of solutes increases dramatically, modifying the magnetic permeability of the solution and the subsequent proton responses to the excitation pulses.[19] Between oral and gastric digestion and between gastric and duodenal digestion, the addition of digestive juices leads to a dilution of the samples, giving rise to problems similar to those encountered in the analysis of urines. To circumvent these two problems it is common practice to employ the total area of the spectrum as a referring standard, assuming that all the substances interconvert to each other along digestion, leading to an almost constant total area.[20] This assumption does not hold for samples undergoing digestion, as the biopolymers hydrolysis leads to a progressive release of solutes from the undetectable matrix, thus increasing the spectral total area. Another possibility with biological samples consists in adjusting the spectral vertical scale to the area of an internal standard, usually the molecule used as the chemical shift reference for NMR spectra. Obviously, this choice is prone to problems caused by dilution when the internal standard is added after the sampling. For a visual impression, figure 3A shows the total area of the spectra obtained during the *in vitro* digestion of Parmigiano cheese, scaled to the area of the internal standard (TSP). Indeed the passages from oral to gastric digestion and from gastric to duodenal digestion lead to dramatic decreases of the total area, only partially compensated by the area increase due to the solubilisation of peptides. The second problem introduced by scaling to the internal standard area is represented by possible interactions between the internal standard and the matrix in different chemical environment. TSP, for example, possesses an aliphatic short chain, causing its binding to hydrophobic proteins or lipid micelles. As an extreme case, the figure 3B shows the area of TSP peak of spectra scaled with respect to the lactate signal during the digestion of cured beef samples. Lactate was chosen as internal standard because it is expected not to vary during the digestion of cured beef. While the samples from duodenal digestion show a very constant lactate/TSP ratio, samples from gastric digestion show a reduction to one fifth of such value.

The best option to adjust the vertical scale of spectra obtained during digestion is finding molecules which are naturally present in the sample, whose concentration is not influenced by the digestion process. For cheese based foods the peak originating by the methylic protons of lactate and the doublets due to the anomeric protons of glucose could be considered as good options. For meat based foods, carnosine and creatine peaks give the best performances.

Quantification of the total soluble peptides

Quantification of total soluble peptides on aliquots of the digestate is typically obtained by removing all the particulate by means of centrifugation, followed by quantification of nitrogen through Kjeldahl method. The colorimetric assays may represent a less time consuming alternative, with the limit that the binding ability of certain dyes is peptide weight dependant. The Bradford assay, based on Coomassie Brilliant Blue dye, reveals peptides with a molecular weight above 5-6 KDa. Despite such limitation, the observation of Parmigiano cheese digestion by the Bradford assay appears as quite informative (Fig 4A). According to the absence of proteolytic enzymes in the saliva, the oral digestion does not have noticeable consequences on total soluble peptides, while dramatic effects can be noticed during gastric digestion (P3). At P5, a significant decrease in peptide concentration was detected by the Bradford assay. When HR-NMR is simplistically used for total quantization purposes (figure 4B) a marked signal increase was evidenced after duodenal digestion (P5), allowing concluding that the decrease observed with the Bradford assay is due to peptide hydrolysis to a size below the detection limit. In this very context, Bradford

colorimetric assay with HR-NMR spectroscopy constitute a classical stereotype of a couple of approaches in which a costless technique, unfortunately missing a key information, is compared to a costly technique, giving otherwise missing information.

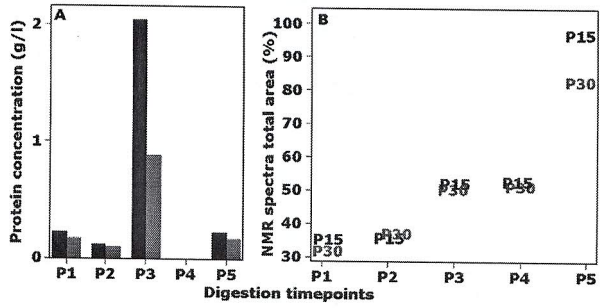


Figure 4. *A - Protein concentration (g/l) in the digestate of Parmigiano cheese aged 15 (black) and 30 months (gray) revealed by Bradford assay. Data from the beginning of duodenal digestion (P4) could not be obtained. B - Total area of ^{1}H-NMR spectra registered during digestion and scaled to the signal at 1.33 ppm from lactate. The areas have been scaled to the area of the spectrum giving rise to the highest signal*

Characterization of the peptides

Since extension to no more than 3 amino acids is a prerequisite for peptide intestinal absorption, as well as its solubility at duodenal physiological conditions, a molecular weight limit of 3KDa was set for peptides, considering their possible further hydrolysation by the mucosal enzymes. To specifically obtain information about the molecular weight profile of these peptides both SDS-PAGE and HR-NMR can be used, since they offer complementary information, the latter showing greater detail.

SDS-PAGE can be used to quantify peptides fractions even if no dedicated instruments and software are at hand. As an example figure 5 shows densitograms obtained by scanning electrophoresis gels obtained on digestate of Parmigiano cheese with a bench top scanner and extrapolating the color intensity by means of the "read.jpeg" function written in R computational language.[21]

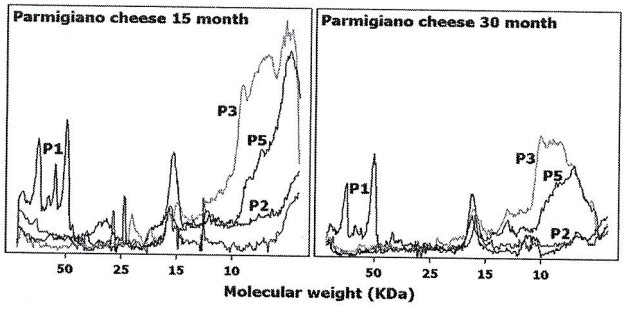


Figure 5. *Densitograms from sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Parmigiano cheese aged 15 and 30 months. P1-P5 are the check points for the in vitro digestion sampling*

It is evident that the soluble peptides from both kinds of cheese undergo a shift towards low molecular weight, 15 months old cheese showing higher concentrations.

In the SDS-PAGE analysis (figure 5) the amount of small peptides appears higher at the beginning than at the end of the duodenal digestion, due to the hydrolysis of peptides to size not detectable by the SDS-PAGE method. It is worth noting that NMR (figure 4) is able to detect all soluble molecules, thus providing the correct information about the continuous increase of small peptides concentration during the duodenal digestion.
^{1}H-NMR spectra can be analyzed from several perspectives in order to focus on peptides characterized by different molecular weight. The most straightforward application consists in observing the digestate where molecules are selectively separated by ultrafiltration according to the molecular weight. However, the ^{1}H-NMR spectrum directly recorded on centrifuged digestate is able to catch sharp signals over broad bands, being the former belonging to very small peptides, whilst the latter are associated to larger denatured peptides. In this way a processing directed to selectively integrate the area associated to sharp signals is capable of giving a measure of the peptides already suitable to be absorbed. Pieces of information about the fate of the peptides can be obtained from the ratio between specific regions of the spectra. The ratio aromatic/CH_α protons represents the relative amount of aromatic amino acids with respect to the total amino acids content, while amide/CH_α protons ratio indicates the relative fraction of amino acids bound to large fragments with respect to their total amount in the digestate.

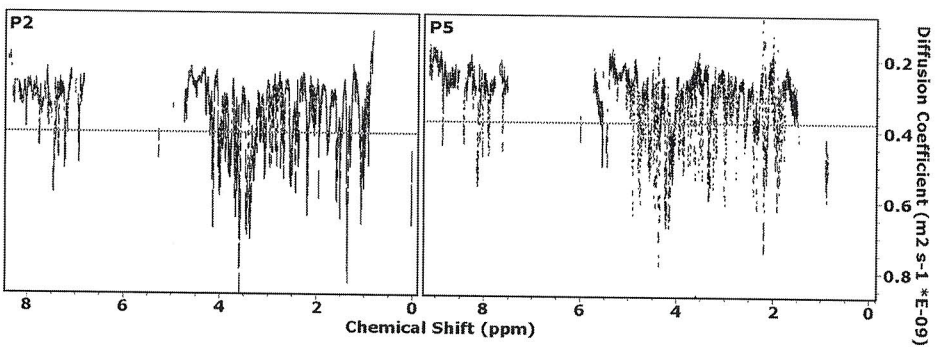


Figure 6. *DOSY plot obtained on samples P2 and P5 digestion check points for the 30-months-aged cheese. The peaks intensity has been conveniently scaled for optimal comparison. To ease the visual comparison of the two plots, dashed lines have been added indicating the average diffusion coefficient of the molecules*

Diffusion spectroscopy, applied to matrices where peptides are distributed over a wide continuum of molecular weights, leads to 2D spectra with several overlapped regions. Nevertheless, figure 6 shows that the overall information, even for a so complex matrix, can be quite easily grabbed. Peptides from samples obtained at the beginning of gastric digestion show an average diffusion coefficient lower than the corresponding from the end of digestion.

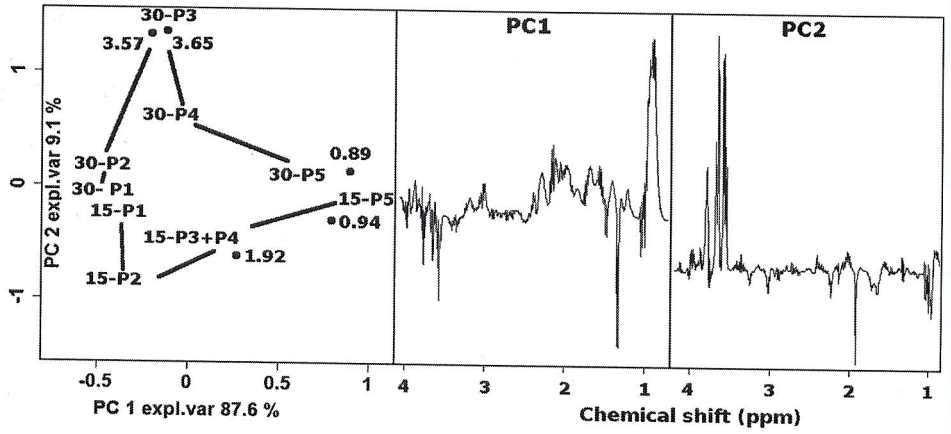


Figure 7. *Principal component analysis of the centered and unscaled ^{1}H-NMR spectra registered on Parmigiano cheese during digestion. In the left part a superimposition of the scoreplot with the loading plot is given. The peaks which mostly contribute to the obtained rotation are presented by means of the corresponding chemical shift. The complete loading plots are then given for PC1 and PC2.*

Multivariate analytical techniques have great potentialities in both offering an overview of the phenomenon under investigation and details about the characteristics of the solubilised molecules. For an overview of the phenomenon a scoreplot, for example from a principal component analysis, seems the right instrument. In the case of Parmigiano cheese *in vitro* digestion, the scoreplot in figure 7 allows to follow along PC1 the digestion process itself, while PC2 seems to focus on the differences between the analyzed samples. The richness of information given by the loading plots makes them the right instrument to focus on details about the molecules undergoing major changes during digestion. This is particularly true when, as in the present case, the spectra are used without any variable reduction prior to multivariate analysis. The only necessary pre-treatment of the spectra consists in a alignment of the peaks, obtained through the "icoshift" algorithm recently described by Savorani et al.[22] This choice leads to loadings appearing as a subsample of the original spectrum, thus easing the grabbing of useful information.

3 CONCLUSIONS

Simply defined, digestion is cutting big nutritional molecules down to a size in which they can be absorbed into the body. Whatever we eat flows through the gastro-intestinal system, but until it is absorbed through the intestinal mucosa, the nutrients in food are physically outside of the body, so that cannot exert either their structural or energetic function.
Food is a very complex mixture of different types of very large (proteins and some carbohydrates); mid-range sized (fats); and smaller molecules (vitamins, minerals, small carbohydrates like sugars, phytonutrients), all having in common the need to become "bio-accessible" in order to be absorbed. Digestion is the process regulating bio-accessibility, and therefore absorbability of nutrients.
In the body, proteins are extremely important because they constitute the majority of the structural tissue. Enzymatic proteins are involved in just about every function in the body,

and proteins are also message carriers. Proteins in the human body are made up of amino acids which are in part derived from foods; food amino acids are made available for absorption and further body protein synthesis by the digestion. In this light, not only the concentration and pattern of food proteins, but also their digestibility represents an important characteristic of the food itself.

The evaluation of protein digestibility in a food represents a step ahead in both food and nutrition sciences; NMR, in its TD or high resolution version, can be a valuable instrument to assess the digestibility of a protein rich food, as long as some problems hindering the application of such technique are properly faced.

Acknowledgements

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