Application of NMR spectroscopy to milk and dairy products

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NMR spectroscopy is a technique in increasing use for dairy research. It provides us with unique information that can be applied to research or to quality control of dairy samples. In addition, it is a non-destructive and very versatile technique, providing data on the same sample under different parameters. This review intends to give an overview of the type of information that can be obtained, based on some results obtained in dairy research. It includes the application of NMR for qualitative and quantitative analysis, monitoring reactions in vivo, isotopic analysis, study of the physical state of milk fat and water, structural characterization and studies on the conformational and aggregation state of proteins.

Since the first detection of signals in 1945 by Bloch, Purcell and their colleagues, Nuclear Magnetic Resonance (NMR) spectroscopy has evolved greatly through the development of instruments and methods leading to its application in all fields. Good examples of the growing interest of food scientists in this technique is the regular International Conference on Applications of Magnetic Resonance in Food Science [1], and the approval of NMR methods as official by the European Union [2]. The particular application to the study of dairy products has been previously reviewed [3]. It is not only the unique information that NMR can provide, but also the versatility of the instrument that makes it an important potential tool for food analysis. However, due to its limited availability, it is not as popular as other techniques, being sometimes overlooked by food researchers/technologists.

The main characteristics of NMR spectroscopy are: it is a non-destructive method, that it makes possible to perform different analyses on the same sample; it is able to detect different nuclei, allowing a study of the sample under different perspectives, i.e. it is structure-sensitive, capable of investigating structural features; it is sensitive to dynamics, which allows differentiation between molecules or portions of molecules with different mobility.

Among relevant nuclei 1H, 13C and 31P have been extensively used in the dairy field. For the same sample, the researcher can choose among nuclei to detect different characteristics, or to improve the spectrum. For instance, milk fat lipids can be analysed from either 1H or 13C spectra, the latter giving better resolution through lower signal-to-noise. Within one single type of nucleus, each of the resonances present in a spectrum are characterized by the chemical shift, measured in ppm, which varies depending on the chemical and structural environment. A single compound can give rise to many different resonances on a 1H-NMR spectrum, since many 1H comprise its structure. Different structural species of the same compound can also show resonances with different chemical shifts. One-dimensional (1D) 1H-NMR spectra are commonly used but, in complex mixtures, such as milk, they are difficult to interpret, since every single 1H atom present in the sample gives rise to a resonance, leading to significant overlap. The use of other nuclei, the application of two-dimensional (2D) techniques, or a previous fractionation scheme may help to obtain better spectra. 1D spectra can provide a qualitative assessment of the sample but can also be applied to quantitative determinations, as the area under the resonance can be made proportional to the concentration.

Nuclei are able to “communicate” with each other, which is the basis for 2D and higher dimensional spectra. 2D-1H-COSY, -HOHAHA or -DQ spectra provide information about which nuclei are bound to each other while 2D-1H-NOESY give information on the spatial distance between these nuclei. Heteronuclear 2D and 3D spectra provide information on the binding
between different nuclei. These types of spectra have been used for the structural characterization of a number of molecules in dairy products.

The NMR signal, which is detected while the nuclear spins are relaxing back to their equilibrium state, fades with time, a phenomenon caused by two types of relaxation: longitudinal relaxation, characterized by T1 or R1, and transverse relaxation, characterized by T2 or R2. Each nucleus within a single molecule has a characteristic T1 and T2, which depends on its own mobility, which also depends on the mobility/size of the molecule it belongs to. The line width (LW), depends on T2 as LW = 1/πT2. As the molecular size increases, the mobility decreases and T2 decreases, causing a broadening of the spectral lines. Determination of T2 or LW of resonances, provides a means to study characteristics that depend on size or mobility, such as the determination of the proportion of liquid and solid fat, the aggregation state of proteins, or the binding of water or ions to solute molecules.

All the above NMR features can be applied to reach different goals in dairy samples and their applications are exemplified in the following sections.

**Qualitative and quantitative analysis**

NMR can be applied as a common analytical tool, capable of detecting many different compounds. 1H- and 13C-NMR have been used to analyse the lipid composition of milk fat, providing quantitative data on the relative molar fractions of oleic, palmitic, butyric acids and triglycerides [4]. Positional isomers of triglycerides have great nutritional importance and, because of the structural sensitivity of NMR, distribution of acyl groups between sn-1(3) and sn-2 positions in milk fat triglycerides has been possible [5, 6].

31P-NMR has been applied to the analysis of milk [7] and milk fractions [8], allowing the simultaneous analysis of phospholipids, phosphorylated carbohydrates, inorganic phosphate (Pi), phosphoserine (PSer) and other phosphorylated compounds from a single spectrum [8]. Quantitative analysis on the PSer resonance has been useful to determine the degree of phosphorylation in super- and dephosphorylated casein [9]. 31P-NMR analysis of commercial caseinates has shown some PSer-depleted β-casein fractions compared to natural caseins [10], which might provide a means for distinguishing them.

**Monitoring reactions ‘in vivo’**

Biological transformations by microorganisms are important in developing organoleptic characteristics in dairy products. As NMR is a non-destructive method, it allows the monitoring of biological reactions in vivo. The microorganism can be incubated in the NMR tube, and the metabolite of interest can be followed by obtaining spectra of the sample at different times (Fig. 1). It is very useful to employ 13C-labeled substrates to follow the transformation. Some metabolic pathways have been studied by NMR in a number of microorganisms (Table 1).

**Isotopic analysis**

The natural distribution of isotopes is a powerful tool for authentication of foods, since identical chemical species from different origins can be distinguished. The distribution of isotopes is dependent on a variety of factors or processes that are able to select favourably certain isotopes, enriching the final product with them. Due to its accuracy, 2H/1H is the preferred ratio detected by NMR. Stable-Natural-Isotope-Fractionation (SNIF)-NMR has been applied to products, such as wine [11] or fruit juices [12]. However, its application to the analysis of dairy products may be limited, since the manufacture of these products is often complex and can involve the use of components from different sources, that can alter the isotopic ratio. Even though, it is feasible to use it for quality control of some authorized additives, such as distinguishing between natural and synthetic vanillin in ice cream and yoghurt [13].

![Fig. 1. 13C-NMR spectra of the time course of the consumption of C-sources (citrate and lactate) obtained in vivo from a cell suspension of Propionibacterium freudenreichii subsp. shermanii. The C numbers indicate the carbon positions in the compounds. Observe that the intensity of lactate and citrate resonances decrease as the reaction proceeds, while those of propionate, acetate and glutamate increase, as a consequence of metabolic transformations (from Ref. [38]). Reprinted with permission from Journal of Dairy Research, 65, 503–514, 1998, Cambridge University Press.](http://www.itran24.com/landing1.html)
Studies on the physical state of dairy components

Physical characterization of milk fat

One of the main applications of NMR to dairy products has been the evaluation of the physical properties of milk fat. The use of $^1$H-NMR has proved to be an excellent tool for the determination of the proportion of liquid fat relative to solid fat. The different mobility between the solid and liquid fractions provide the basis for the determination. Solid fat, having less mobility shows rapid relaxation and a wide signal, while that from liquid fat is sharper. Both signals can be separated by mathematical methods and quantified. Some examples of such studies are shown in Table 2.

Physical state of water

When water binds other components in solution, its mobility changes. Because of this, the measurement of water-T$_2$ has been used to evaluate the state of water in dairy samples. Water relaxation measurements can provide similar information to that from sorption isotherms about the rehydration of food systems [14]. It has been applied for the evaluation of rehydration of caseins, caseinates and milk powder (Table 2). Gelation or coagulation processes lead to water trapped into the network, processes that involve changes in water mobility, thus some authors have studied relaxation phenomena in protein gels (Table 2).

Structural characterization of molecules from dairy origin

The spectrum of a compound depends on its chemical and structural nature, providing 1D or 2D spectra unique fingerprints. If the identification of a suspected compound is to be confirmed, the spectral pattern can be compared to that of a standard compound, providing the latter is available. On the other hand, if a new compound needs to be characterized, 2D and 3D spectra can provide bonding and spatial distance information, from which the structure of the compound can be obtained. A number of molecules/macromolecules of different nature found in dairy products have been characterized (Table 3).

Carbohydrates

Extensive work has been done on the structural characterization of carbohydrates naturally found in milk or produced by microbial strains found in dairy products (Table 3). A combination of mass spectrometry and

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Table 1. Examples of NMR studies on metabolic pathways in microorganisms

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Metabolic study</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactococcus lactis</em> subs. lactis CNRZ 125</td>
<td>Initial rate of $^{13}$C glucose utilization. Monitoring of $^{31}$P glycolytic phosphorylated intermediates</td>
<td>[36]</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> HP, C2, 11007 (lab strains) and S1 and S2 (industrial strains)</td>
<td>Catabolism of $^{13}$C-methionine, involved in cheese flavour development</td>
<td>[37]</td>
</tr>
<tr>
<td><em>Propionibacterium freudenreichii</em> subs. shermanii</td>
<td>Catabolism of C-sources ($^{13}$C-citrate and-lactate), possible involvement in the formation of eyes in Swiss-type cheese</td>
<td>[38,39]</td>
</tr>
</tbody>
</table>

Table 2. NMR relaxation studies on the physical characterization of milk fat and water in dairy systems

<table>
<thead>
<tr>
<th>Molecule studied/matrix</th>
<th>Objective of study</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical properties of milkfat</td>
<td>Test crystallization of fat subjected to:</td>
<td></td>
</tr>
<tr>
<td>Milk fat/milk fat</td>
<td>supercooling</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>high pressures</td>
<td>[41]</td>
</tr>
<tr>
<td></td>
<td>different extraction methods</td>
<td>[42]</td>
</tr>
<tr>
<td>Milk fat/milk fat</td>
<td>Comparative thermal properties of milk fat from different species</td>
<td>[43]</td>
</tr>
<tr>
<td>State of water</td>
<td>T$_2$/R$_2$ with casein concentration</td>
<td>[44,45]</td>
</tr>
<tr>
<td>H$_2$O/casein systems</td>
<td>State of water upon rehydration</td>
<td>[46]</td>
</tr>
<tr>
<td>H$_2$O/milk powder</td>
<td>State of water by using different means for rehydration (water vapour and liquid water)</td>
<td>[47]</td>
</tr>
<tr>
<td>H$_2$O/casein systems</td>
<td>Speed for solubilization and time required for complete reconstitution</td>
<td>[48]</td>
</tr>
<tr>
<td>H$_2$O/whey proteins</td>
<td>R$_2$ in gels and sols</td>
<td>[49]</td>
</tr>
<tr>
<td>H$_2$O/milk rennet curd</td>
<td>State of water during coagulation and syneresis; quantitative estimation in and outside the curd</td>
<td>[50]</td>
</tr>
<tr>
<td>H$_2$O/whey protein</td>
<td>Identify water states by T$_1$ measurements</td>
<td>[51]</td>
</tr>
<tr>
<td>H$_2$O/casein-NaCl</td>
<td>Preferential interactions</td>
<td>[45]</td>
</tr>
<tr>
<td>H$_2$O, Na$^+$/$\beta$-lactoglobulin-NaCl</td>
<td>Preferential interactions</td>
<td>[52]</td>
</tr>
</tbody>
</table>
NMR is usually employed for the characterization of oligosaccharides, the latter giving important structural information, such as the specific linkages between the sugar elements.

Whey proteins

Even though structural characterization by NMR becomes more difficult as the size of the molecule increases, a number of milk proteins and peptides have been studied, in detail (Table 3). The first step that has to be taken for any structural characterization is to assign each resonance to each nucleus in the molecule, which is not an easy task, particularly for macromolecules. 2D 1H-NMR spectra may suffice for the assignment of peptides, but for larger proteins this may only provide a partial assignment. If the protein of interest is available in enriched form, heteronuclear techniques facilitate the assignment work. Full 1H, 13C and 15N assignments have been obtained recently for β-lactoglobulin [15], information that will allow in-depth study of many conformational features, as this is a recombinant protein, cloned in Pichia pastoris, that can be subjected to specifically designed changes in the amino acid sequence [16].

Caseins

Due to the large size of caseins in the micellar state, detailed structural studies are unattainable by NMR, it being necessary to take other approaches. A likely overall structure of κ-casein has been proposed [17] by combining the information from NMR structural characterization of κ-casein peptides (Table 3) and from structure prediction methods [18].

Micelles

The nature of the interactions between the components that form the micelle has been studied by focusing on dynamic features. Most studies have employed 31P-NMR to study both PSer and P, present in the micelle, although other nuclei, 25Mg and 43Ca, have been useful to study the behaviour of the divalent cations.

Due to the difficulties associated with the large micellar size, some authors have approached the problem by using peptides. Results obtained from β-casein phosphopeptide suggested that the peptide binds calcium phosphate through its sequence of phosphorylated residues, while maintaining in the termini the conformational freedom of the unbound peptide [19]. Other studies have suggested that PSer residues in β-casein bind Ca2+ more strongly than P, does and there are no Ca bridges between PSer and P [20]. The use of solid-state techniques, i.e. magic-angle-spinning (MAS)-NMR, has made possible the study of dynamic features on native micelles. 31P-MAS-NMR data indicated that the major fraction of the casein PSer is in an immobilized state within the micelle [21], supporting the idea that the micelle has a compact internal structure. In the same work it was suggested that the micellar calcium phosphate structurally resembled hydroxyapatite. MAS-NMR has also provided comparative data on the micellar structure from different species [22, 23].

Other studies have focused on the characterization of PSer cation-binding sites. Due to their particular location and environment, not all the PSer groups in caseins give rise to a single resonance, on the contrary, 31P-NMR spectra of individual caseins show different PSer resonances, and some of them have been assigned [24]. As the ionization state of PSer causes chemical shift changes, the pK of some specific PSer residues can be determined from titration curves (chemical shift vs pH). Binding of cations modifies the PSer pK but, furthermore, it alters the mobility of both PSer and the bound cation. Because of this, researchers have mainly focused on variations on PSer pKs as well as relaxation measurements of 31Pser, 25Mg and 43Ca [24–26].

| Table 3. Examples of dairy components structurally characterized by NMR |
|-----------------------------|-----------------------------|-----------------------------|
| Molecule                  | Origin                      | Interest                                  | Reference |
| **Miscellaneous molecules** |                             |                                           |           |
| Histidino-alanine (HAL)    | Milk                        | Chemical reactions during heating of milk  | [53]      |
| 3-hydroxy-5-methyl-2-hexane| Cheese                      | Aroma from melted cheese                 | [54]      |
| **Carbohydrates**          |                             |                                           |           |
| Exopolysaccharides         | Lactobacillus spp.          | Potential functional agents               | [55–59]   |
| Free oligosaccharides      | Goat’s and human milk       | Nutritional/biological effects             | [60–64]   |
| Carbohydrate moieties in proteins | κ-casein                  | Deeper knowledge of milk components       | [65–67]   |
|                            |                             |                                            |           |
| **Proteins/peptides**      |                             |                                           |           |
| α-lactalbumin              | Bovine milk                 | Deeper knowledge of milk constituents     | [68]      |
| β-lactoglobulin            | Bovine milk                 | Deeper knowledge of milk constituents     | [29,69]   |
| β-lactoglobulin            | P. pastoris (recombinant)   | Deeper knowledge of milk constituents     | [15]      |
| β-casein phosphopeptide    | Bovine milk                 | Deeper knowledge of milk constituents     | [70]      |
| κ-casein macropeptide      | Bovine milk                 | Deeper knowledge of milk constituents     | [17,71,72]|

Changes in conformational and aggregation state of milk proteins

Some processes, such as pressure, heating or changes in pH can alter the milk protein conformation and/or the aggregation state. Both aspects can be studied by NMR means.

The evolution of the unfolding processes of whey proteins, induced by heat, pressure and foaming, have been followed by the use of $^1$H-NMR in combination with deuterium exchange reactions [27–29]. This method is based on the disappearance of backbone NH resonances due to the exchange of $^1$H for $^2$H atoms as unfolding proceeds. If deuteration is combined with 2D-$^1$H-NMR (Fig. 2) conformational details that take place during unfolding can be obtained [28].

Aggregation, which follows unfolding upon heating of whey proteins, can also be studied from water-T2 measurements, as water bound to the aggregate has a slower mobility than if bound to the non-aggregated protein. In fact, a decrease of water-T2 was observed upon heating of $\beta$-lactoglobulin, this effect being more significant at higher protein concentration or when caseins were added [30].

The aggregation behaviour of caseins, has been studied under different environments and after processing. Depletion of calcium in milk leads to the increased mobility of P, causing line narrowing of the $^{31}$P resonance [7]. The consequent micellar disaggregation also causes line narrowing of casein resonances, thus enhancing the signal intensity in a $^1$H-NMR spectrum of calcium-depleted micellar casein [31].

Upon acidification of skim milk, an ($R_2$ vs pH) plot gives a characteristic curve, that shows how $R_2$ decreases to a minimum at pH 5.2, attributed to the maximum disaggregated state of caseins (Fig. 3) [32, 33]. This type of curve can be used to compare the behaviour of different systems. For instance, a curve similar to the above was obtained upon addition of phosphate and calcium to a caseinate–whey mixture [33], while a completely different behaviour of milk was found after pressure treatment [34]. The kinetics of the acidification process has consequences on protein aggregation mechanisms and it has been suggested that $R_2$ can provide information concerning the type of acidification treatments [35].

![Fig. 2. Partial view of a 2D-$^1$H-COSY spectrum of $\beta$-lactoglobulin, dissolved in $^2$H$_2$O at pH 2, before (a) and after (b) heating for 8 hours at 55°C. The first dimension (horizontal axis) comprise chemical shifts belonging to NH groups while the second dimension (vertical axis) comprise those of the $(\text{CH}_m)$ groups. Each cross-peak consists of four small peaks, and arise because the $^1$H of the NH and $(\text{CH}_m)$ groups “communicate” to each other, when they belong to the same residue. Observe that a portion of the resonances disappear upon heating, as NH groups become deuterated, due to the unfolding of the protein, which allows for exchange with the solvent (from Ref. [29]). Reprinted with permission from J. Agric. Food Chem., 46(5), 1805–1813, 1998, American Chemical Society.](http://www.itrans24.com/landing1.html)

![Fig. 3. Variation of $R_2$ of reconstituted skim milk with pH at different dry matter contents: , 8%, ▲, 9%, △, 10% (from Ref. [33]). Reprinted with permission from Journal of Dairy Research, 60, 175–183, 1993, Cambridge University Press.](http://www.itrans24.com/landing1.html)
Box 1. Glossary of NMR terms

NMR: Nuclear Magnetic Resonance. Technique based on the properties of certain nuclei that are able to absorb and emit electromagnetic radiation in the radio frequency range when they are placed in a strong magnetic field.

Resonance: Signal (peak) that appears on a spectrum. It represents a single type of nucleus in the sample. For instance, in a $^1$H-NMR spectrum of glucose, the $^1$H that belongs to C-4 give rise to one resonance, and the $^1$H from C-2 give rise to another one, with different chemical shift.

Chemical shift: Frequency, measured in ppm, at which a resonance is observed in the spectrum.

$^1$H-, $^{13}$C-, $^{31}$P-NMR: Refers to the type of nucleus detected. Spectral conditions are different for the detection of either $^1$H, $^{13}$C or $^{31}$P nuclei.

1D, 2D, 3D spectra: Refers to the number of dimensions of the spectrum. The resonances that appear on a 2D spectrum correlate one nucleus (A), with chemical shift $\delta$, with another nucleus (B), with chemical shift $\delta'$, giving rise to a cross-peak at $(\delta, \delta')$. Each cross-peak gives different information depending on the experiment. In a 2D 1H-COSY, A is bound to B by three bonds. In a 2D 1H-NOESY, A is close in space to B.

Homo- and heteronuclear spectra: Applies to two- and higher dimensional spectra. If the same nucleus is detected in both dimensions, it is called homonuclear spectroscopy. If different nuclei are detected, it is called heteronuclear spectroscopy. For instance, 2D-$^1$H-COSY, HOHAHA or-NOESY are homonuclear experiments; 2D $^{13}$C-$^1$H-HMQC is heteronuclear.

Relaxation: Process by which the nuclear spins return to equilibrium. Two types of relaxation are important: longitudinal relaxation, characterized by $T_1$ or $R_1$, and transverse relaxation, characterised by $T_2$ or $R_2$. Relaxation time, $T$, is the time required to relax and $R$, relaxation rate, is the rate at which relaxation occurs. They are related as $R = 1/T$. Relaxation times and rates are measured on a specific resonance on the spectrum and depend on the dynamics of the nucleus involved, among other factors.

Assignment of resonances: Before any further study is done, the resonances that appear on the spectrum have to be assigned to the nucleus to which they belong. It can be tedious work, particularly in the case of large molecules, such as proteins.

SNIF-NMR: Stands for Stable Natural Isotope Fractionation NMR. It is a technique by which the same resonance is analysed for its isotopic proportion. For instance, the H attached to C-2 in wine ethanol is mainly $^1$H, but presents some proportion of $^2$H. The resonance that belongs to this nucleus can be quantified for the relative proportion of $^1$H/$^1$H.

MAS-NMR: Stands for Magic Angle Spinning NMR. Technique that is used to obtain spectra from solids.

Perspectives for the use of NMR

The versatility of NMR spectroscopy has been demonstrated in this review. With a few exceptions, NMR spectroscopy has been mostly applied to the basic knowledge of dairy products and components. Dairy research in structural chemistry is an increasing field, as structural features are known to have nutritional, immunological and technological impact. NMR allows us to get a deep knowledge at a molecular/atomic level of the mechanisms underlying the macromolecular processes. This alone has a significant importance. But, furthermore, NMR could also become a unique tool for quality control, since its sensitivity to structural changes can provide alternative insights for analysis. Isotopic differences or slight structural changes between alike components are fine tools useful for quality control and authentication. The evolution of dairy products is fast, they are getting more complex, and more ingredients are used. This picture requires the use of new and powerful technologies. Nowadays, NMR is a high-cost technique, but if applications were to be more widespread, it may be available for many laboratories in the future.

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and indicate clearly whether they are intended for publication.