ELSEVIER

Trends in Food Science & Technology 10 (1999) 313–320

TRENDS IN FOOD SCIENCE & TECHNOLOGY

Review

Application of NMR spectroscopy to milk and dairy products

Josefina Belloque* and Mercedes Ramos

Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain (fax: +91 5644853; e-mail: ifibm44@ifi.csic.es)

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, it is structure-sensitive, i.e. 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 ^{1}H, ^{13}C and ^{31}P 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 ^{1}H or ^{13}C 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 ^{1}H-NMR spectrum, since many ^{1}H comprise its structure. Different structural species of the same compound can also show resonances with different chemical shifts. One-dimensional (1D) ^{1}H-NMR spectra are commonly used but, in complex mixtures, such as milk, they are difficult to interpret, since every single ^{1}H 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-^{1}H-COSY,-HOHAHA or-DQ spectra provide information about which nuclei are bound to each other while 2D-^{1}H-NOESY give information on the spatial distance between these nuclei. Heteronuclear 2D and 3D spectra provide information on the binding

* Corresponding author.

0924-2244/99/$ - see front matter
PII: S0924-2244(00)00012-1

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 T_1 or R_1, and transverse relaxation, characterized by T_2 or R_2. Each nucleus within a single molecule has a characteristic T_1 and T_2, 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 T_2 as $LW = 1/\pi T_2$. As the molecular size increases, the mobility decreases and T_2 decreases, causing a broadening of the spectral lines. Determination of T_2 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. ^{1}H- and ^{13}C-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].

^{31}P-NMR has been applied to the analysis of milk [7] and milk fractions [8], allowing the simultaneous analysis of phospholipids, phosphorylated carbohydrates, inorganic phosphate (P_i), 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]. ^{31}P-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 ^{13}C-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, ^{2}H/^{1}H 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].

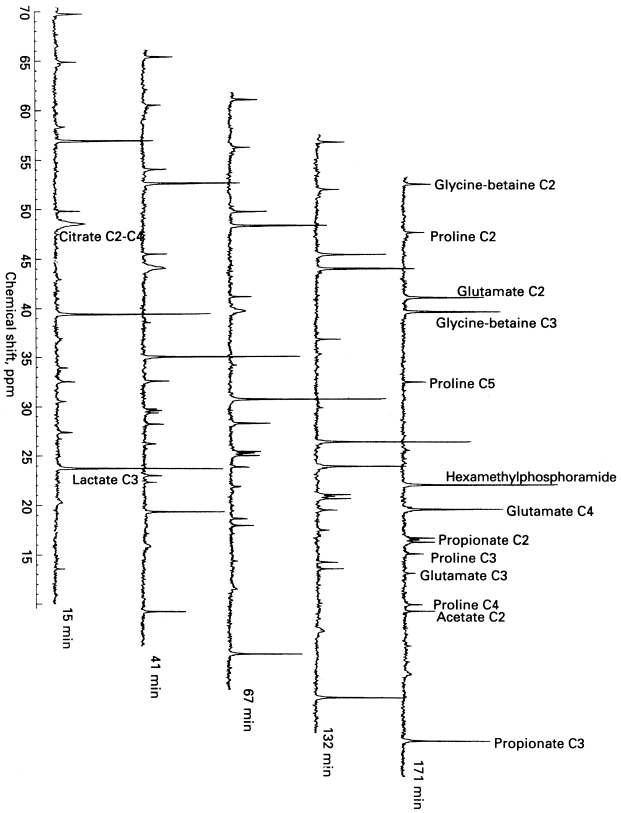

Fig. 1. ^{13}C-NMR spectra of the time course of the consumption of C-sources (citrate and lactate) obtained *in vivo* from a cell suspension of *Propionibacterium freudenreichii* subsp. *shermanii*. The C numbers indicate the carbon positions in the compounds. Observe that the intensity of lactate and 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.

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

Microorganism	Metabolic study	Reference
Lactococcus lactis subsp. *lactis* CNRZ 125	Initial rate of ^{13}C glucose utilization. Monitoring of ^{31}P glycolytic phosphorylated intermediates	[36]
Lactococcus lactis HP, C2, 11007 (lab strains) and S1 and S2 (industrial strains)	Catabolism of ^{13}C-methionine, involved in cheese flavour development	[37]
Propionibacterium freudenchii subsp. *shermanii*	Catabolism of C-sources (^{13}C-citrate and-lactate), possible involvement in the formation of eyes in Swiss-type cheese	[38,39]

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

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

Molecule studied/matrix	Objective of study	Reference
Physical properties of milkfat		
Milk fat/milk fat	Test crystallization of fat subjected to:	
	supercooling	[40]
	high pressures	[41]
	different extraction methods	[42]
Milk fat/milk fat	Comparative thermal properties of milk fat from different species	[43]
State of water		
H_2O/casein systems	T_2/R_2 with casein concentration	[44,45]
H_2O/milk powder	State of water upon rehydration	[46]
H_2O/casein systems	State of water by using different means for rehydration (water vapour and liquid water)	[47]
H_2O/calcium caseinate/casein micelles	Speed for solubilization and time required for complete reconstitution	[48]
H_2O/whey proteins	R_2 in gels and sols	[49]
H_2O/milk rennet curd	State of water during coagulation and syneresis; quantitative estimation in and outside the curd	[50]
H_2O/whey protein	Identify water states by T_1 measurements	[51]
H_2O/casein-NaCl	Preferential interactions	[45]
H_2O, Na^+/β-lactoglobulin-NaCl	Preferential interactions	[52]

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 PP3 glycopeptide κ-casein macropeptide	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]

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 ^{1}H-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 a ^{13}C, ^{15}N enriched form, heteronuclear techniques facilitate the assignment work. Full ^{1}H, ^{13}C and ^{15}N 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 ^{31}P-NMR to study both PSer and P_i present in the micelle, although other nuclei, ^{25}Mg and ^{43}Ca, 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 Ca^{2+} more strongly than P_i does and there are no Ca bridges between PSer and P_i [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. ^{31}P-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, ^{31}P-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 ^{31}Pser, ^{25}Mg and ^{43}Ca [24–26].

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 β-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_i, causing line narrowing of the $^{31}P_i$ 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].

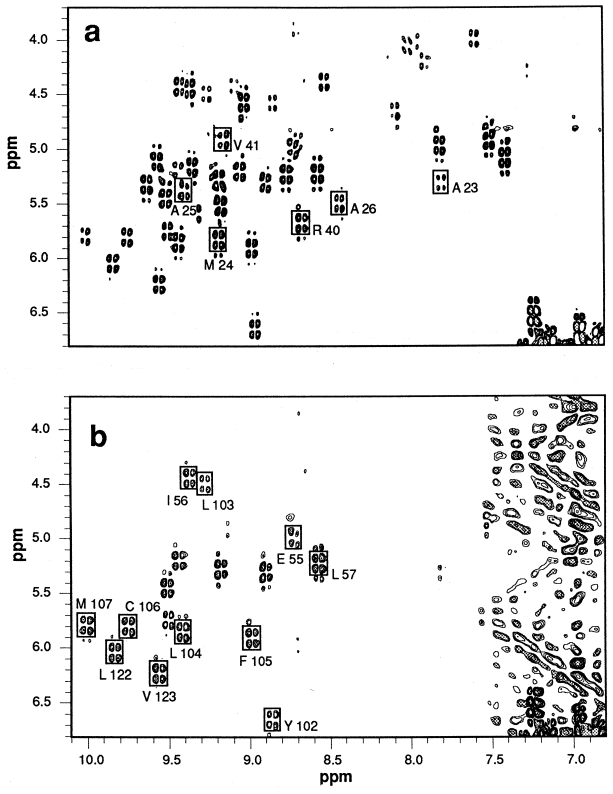

Fig. 2. Partial view of a 2D-^{1}H-COSY spectrum of β-lactoglobulin, dissolved in $^{2}H_2O$ at pH 2, before (a) and after (b) heating for 8 hours at 55°C. The first dimension (horizontal axis) comprise chemical shifts belonging to NH groups while the second dimension (vertical axis) comprise those of the $(CH)_\alpha$ groups. Each cross-peak consists of four small peaks, and arise because the ^{1}H of the NH and $(CH)_\alpha$ groups "comunicate" 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.

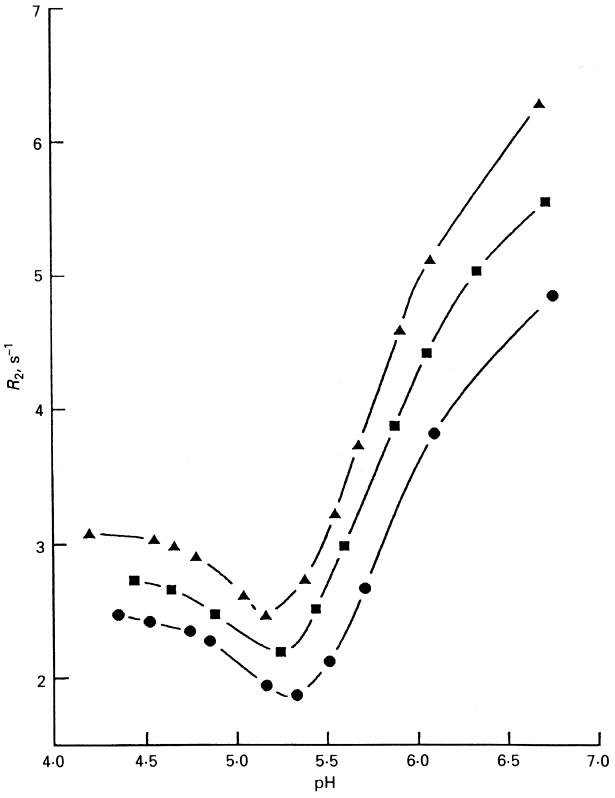

Fig. 3. Variation of R_2 of reconstituted skim milk with pH at different dry matter contents: ●, 8%, ■, 9%, ▲ 10% (from Ref. [33]). Reprinted with permission from Journal of Dairy Research, 60, 175–183, 1993, Cambridge University Press.

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 δ, with another nucleus (B), with chemical shift δ', giving rise to a cross-peak at (δ, δ'). 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 ^{2}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 macroscopic 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.

Acknowledgements

The authors would like to acknowledge project ALI97-0630 (CICYT, Spain) and a fellowship to J. Belloque (C.A.M., Spain).

References

1 Belton, P.S., Hills, B.P. and Webb, G.A. (eds). (1999) '*Advances in Magnetic Resonance in Food Science*'. The Royal Society of Chemistry, Cambridge, UK
2 Official Journal of the European Communities (1990). Vol. 33, L272
3 Wahlgren, N.M. and Drakenberg, T. (1995) 'Milk' in *Ann. Rep. NMR Spectr.* 31, 275–312
4 Smolnyi, A.V., Chesnov, V.O., Shepeleva, E.V., Geraimovich, O.A. and Denisovich, E.Y. (1996) 'Spectroscopic NMR Determination of Chemical Composition of Milk Fat' in *Moloch. Promysh* 4, 20–22
5 Kalo, P., Kemppinen, A. and Kilpelainen, I. (1996) 'Determination of Positional Distribution of Butyryl Groups in Milkfat Triacylglycerols, Triacylglycerol Mixtures, and Isolated Positional Isomers of Triacylglycerols by Gas Chromatography and ^{1}H Nuclear Magnetic Resonance' in *Lipids* 31, 331–336
6 Dewettinck, K., de Greyt, W. and Huyghebaert, A. (1992) 'Lipase Catalysed Interesterification of Milkfat: Influence on the Free Fatty Acid Profile' in *Medelin. Fac. Landbowwet. Rijsunivt. Gent.* 57, 1905–1907
7 Belton, P.S., Lyster, R.L.J. and Richards, C.P. (1985) 'The ^{31}P Nuclear Magnetic Resonance Spectrum of Cows' Milk' in *J. Dairy Res.* 52, 47–54
8 Wahlgren, M., Drakenberg, T., Vogel, H.J. and Dejmek, P. (1986) '^{31}P-nuclear Magnetic Resonance Study of Milk Fractions' in *J. Dairy Res.* 53, 539–545
9 Van Hekken, D.L. and Dudley, R.L. (1997) 'Analysis of Modified Whole Casein with Different Phosphorous Contents Using Phosphorous-31 Nuclear Magnetic Resonance and Fourier Transform Infrared Spectroscopy' in *J. Dairy Sci.* 80, 2751–2759

10 Ward, L.S. and Bastian, E.D. (1998) 'Isolation and Identification of Beta-casein A-1-4P and Beta-casein A-2-4P in Commercial Caseinates' in *J. Agric. Food Chem.* 46, 77–83

11 Day, M.P., Ben-Li, Z. and Martin, G.J. (1995) 'Determination of the Geographical Origin of Wine Using Joint Analysis of Elemental and Isotopic Composition. II. Differentiation of the Principal Production Zones in France for the 1990 Vintage' in *J. Sci. Food Agr.* 67, 113–123

12 Martin, G.G., Wood, R. and Martin, G.J. (1996) 'Detection of Added Beet Sugar in Concentrated and Single Strength Fruit Juices by Deuterium Nuclear Magnetic Resonance (SNIF-NMR Method): Collaborative Study' in *JAOAC Int.* 79, 917–928

13 Remaud, G.S., Martin, Y.L., Martin, G.G. and Martin, G.J. (1997) 'Detection of Sophisticated Adulterations of Natural Vanilla Flavors and Extracts: Application of the SNIF-NMR Method to Vanillin and p-hydroxybenzaldehyde' in *J. Agr. Food Chem.* 45, 859–866

14 Hills, B.P., Manning, C.E. and Godward, J. (1999) 'A Multistate Theory of Water Relations in Biopolymer Systems' in *Advances in Magnetic Resonance in Food Science*, (Belton, P.S., Hills, B.P., Webb, G.A., eds), pp. 45–62, RSC, Cambridge, UK

15 Uhrinova, S., Uhrin, D., Denton, H., Smith, M., Sawyer, L. and Barlow, P.N. (1998) 'Complete Assignment of ^{1}H, ^{13}C and ^{15}N Chemical Shifts for Bovine β-lactoglobulin: Secondary Structure and Topology of the Native State is Retained in a Partially Unfolded Form' in *J. Biol. NMR* 12, 89–107

16 Kim, T.R., Goto, Y., Hirota, N., Kuwata, K., Denton, H., Wu, S.Y., Sawyer, L. and Batt, C.A. (1998) 'High Level Expression of Bovine b-lactoglobulin in Pichia Pastoris and Characterization of its Physical Properties' in *Prot. Eng.* 10, 1339–1345

17 Creamer, L.K., Plowman, J.E., Lidell, M.J., Smith, M.H. and Hill, J.P. (1998) 'Micelle Stability: κ-casein Structure and Function' in *J. Dairy Sci.* 81, 3004–3012

18 Kumosinski, T.F, Brown, E.M. and Farrell, H.M. Jr. (1993) 'Three-dimensional Molecular Modelling of Bovine Caseins: a Refined, Energy-minimized κ-casein Structure' in *J. Dairy Sci.* 76, 2507–2520

19 Holt, C, Wahlgren, N.M. and Drakenberg, T. (1996) 'Ability of a β-casein Phosphopeptide to Modulate the Precipitation of Calcium Phosphate by Forming Amorphous Dicalcium Phosphate Nanoclusters' in *Biochem. J.* 314, 1035–1039

20 Gaucheron, F., Le Graet, Y., Sinbandhit, S., Guenot, P. and Brule, G. (1995) 'Binding of Calcium to β-casein in the Presence of Inorganic Phosphate' in *Sci. Alim.* 15, 481–489

21 Thomsen, J.K., Jakobsen, H.J., Nielsen, N.C., Petersen, T.E. and Rasmussen, K. (1995) 'Solid State Magic Angle Spinning ^{31}P-NMR Studies of Native Casein Micelles' in *Eur. J Biochem.* 230, 454–459

22 Mora-Gutierrez, A., Farrell, H.M. Jr. and Kumosinski, T.F. (1996) 'Comparison of Hydration Behaviour of Bovine and Caprine Caseins as Determined by Oxygen-17 Nuclear Magnetic Resonance: Temperature Dependence of Colloidal Stability' in *J. Agric. Food Chem.* 44, 48–53

23 Rasmussen, L.K., Sorensen, E.S., Petersen, T.E., Nielsen, N.C. and Thomsen, J.K. (1997) 'Characterization of Phosphate Sites in Native Ovine, Caprine and Bovine Casein Micelles and their Caseinomacropeptides: a Solid State Phosphorous-31 Nuclear Magnetic Resonance and Sequence and Mass Spectrometric Study' in *J. Dairy Sci.* 80, 607–614

24 Sleigh, R.W., MacKinlay, A.G. and Pope, J.M. (1983) 'NMR Studies of the Phosphoserine Regions of Bovine as1- and β-casein. Assignment of ^{31}P Resonances to Specific Phosphoserines and Cation Binding Studied by Measurement of Enhancement of ^{1}H Relaxation Rate' in *Biochim. Biophys. Acta* 742, 175–183

25 Baumy, J.J., Guenot, P., Sinbandhit, S. and Brulé, G. (1989) 'Study of Calcium Binding to Phosphoserine Residues of β-casein and its Phosphopeptide (1-25) by ^{31}P NMR' in *J. Dairy Res.* 56, 403–409

26 Wahlgren, M., Dejmek, P. and Drakenberg, T. (1993) 'Binding of Mg2+ and Ca2+ to β-casein A1: A Multinuclear Magnetic Resonance Study' in *J. Dairy Res.* 60, 65–78

27 Tanaka, N. and Kunugi, S. (1996) 'Effect of Pressure on the Deuteration Exchange Reaction of α-lactalbumin and β-lactoglobulin' in *Int. J Biol Macromol.* 18, 3339

28 Belloque, J. and Smith, G.M. (1998) '^{1}H-FT-NMR Studies on the Conformational Changes Related to Foaming Properties of β-lactoglobulin' in *J. Dairy Sci.* 81, 2580–2589

29 Belloque, J. and Smith, G.M. (1998) 'Thermal Denaturation of β-lactoglobulin A ^{1}H-FT-NMR study' in *J. Food Agric. Chem.* 46, 1805–1813

30 Lambelet, P., Berrocal, R. and Renevey, F. (1992) 'Low-field Nuclear Magnetic Resonance Relaxation Study of Thermal Effects on Milk Proteins' in *J. Dairy Res.* 59, 517–526

31 Rollema, H.S. and Brinkhuis, J.A. (1989) 'A ^{1}H-NMR Study of Bovine Casein Micelles; Influence of pH, Temperature and Calcium Ions on Micellar Structure' in *J. Dairy Res.* 56, 417–425

32 Roefs, S.P.F.M., Van As, H. and Van Vliet, T. (1989) 'Pulse NMR of Casein Dispersions' in *J. Food Sci.* 54, 704–708

33 Mariette, R., Tellier, C., Brule, G. and Marchal, P. (1993) 'Multinuclear NMR Study of the pH Dependent Water State in Skim Milk and Caseinate Solutions' in *J. Dairy Res.* 60, 175–188

34 Famelart, M.H., Gaucheron, F., Mariette, F., Graet, Y., Raulot, K. and Boyaval, E. (1997) 'Acidification of Pressure-treated Milk' in *Int. Dairy J.* 7, 325–330

35 Mariette, F., Maignan, P. and Marchal, P. (1997) 'Relaxation NMR: A Sensor for Following Acidification of Milk' in *Analusis* 25, M24–M27

36 Foucaud, C., Herve, M., Neumann, J.M. and Hemme, D. (1995) 'Glucose Metabolism and Internal pH of Lactococcus lactis subsp. Lactis Cells Utilizing NMR Spectroscopy' in *Lett. Appl. Micr.* 21, 10–13

37 Song, G., Mooberry, E.S. and Steele, J.L. (1998) 'Use of -1-3C Nuclear Magnetic Resonance and Gas Chromatography to Examine Methionine Catabolism by Lactococci' in *Appl. Environm. Micr.* 64, 4670–4675

38 Deborde, C., Rolin, D.B., Bondon, A., Certaines, J.D. and Boyaval, P. (1998) 'In Vivo Nuclear Magnetic Resonance Study of Citrate Metabolism in Propionibacterium Freudenreichii subsp. Shermanii' in *J. Dairy Res.* 65, 503–514

39 Rolin, D.B., Girard, F., Certaines, J.D. and Boyaval, P. (1995) '^{13}C-NMR Study of Lactate Metabolism in Propionibacterium Freudenreichii subsp. Shermanii' in *Appl. Micr. Biotech.* 44, 210–217

40 Breitschuh, B. and Windhab, E.J. (1998) 'Parameters Influencing Cocrystallization and Polymorphism in Milk Fat' in *JAOCS* 75, 897–904

41 Buchheim, W. and Frede, E. (1996) 'Use of High Pressure Treatment to Influence the Crystallization of Emulsified Fats' in *DMZ-Lebensmittelind. Milchwirtsch.* 117, 228–237

42 Bashkar, A.R., Rizvi, S.S.H., Bertoli, C, Fay, L.B. and Hug, B. (1998) 'A Comparison of Physical and Chemical Properties of Milk Fat Fractions Obtained by Two Processing Technologies' in *JAOCS* 75, 1249–1264

43 Patel, A.A. and Frede, E. (1991) 'Studies on Thermal Properties of Cow and Buffalo Milk Fats' in *Lebensmitt. Wissensch. Techn.* 24, 323–327

44 Leung, H.K., Steinberg, M.P., Wei, L.S. and Nelson, A.I. (1976) 'Water Binding of Macromolecules Determined by Pulsed NMR' in *J. Food Sci.* 41, 297–300

45 Curme, A.G., Schmidt, S.J. and Steinberg, M.P. (1990) 'Mobility and Activity of Water in Casein Model Systems as Determined by ^{1}H NMR and Sorption Isotherms' in *Food Sci.* 55, 430–433

46 Brosio, E., Altobelli, G., Yu, S.Y. and Di Nola, A. (1983) 'A Pulsed Low Resolution NMR Study of Water Binding to Powdered Milk' in *J. Food Tech.* 18, 219–226

47 Mistry, A., Steinberg, M.P. and Richardson-Schmidt, S.J. (1990) 'Comparison of Vapour and Liquid Isotherms for Casein and Casein Sucrose Mixture' in *J. Food Sci.* 55, 434–436
48 Davenel, A., Schuck, P. and Marchal, P. (1997) 'Rehydration Kinetics and Water Holding Capacity of Powders Measured by Relaxation NMR' in *Analusis* 25, M21–M23
49 Lambelet, P., Berrocal, R., Desarzens, C., Froehlicher, I. and Ducret, F. (1988) 'Pulsed Low-resolution NMR Investigations of Protein Sols and Gels' in *J. Food Sci.* 53, 943–946
50 Tellier, C., Mariette, R, Guillement, J.P. and Marchal, P. (1993) 'Evolution of Water Proton Nuclear Magnetic Relaxation During Milk Coagulation and Syneresis: Structural Implications' in *J. Agr. Food Chem.* 41, 2259–2266
51 Padua, G.W., Richardson, S.J. and Steinberg, M.P. (1991) 'Water Associated with Whey Protein Investigated by Pulsed NMR' in *J. Food Sci.* 56, 1557–1561
52 Fanni, J., Canet, D., Elbayed, K. and Hardy, J. (1989) '^{1}H and ^{23}Na Relaxation Studies of the NaCl/β-lactoglobulin System Equilibrated at Various Water Activities' in *J. Food Sci.* 54, 1909–1989
53 Henle, T, Walter, A.W. and Klostermeyer, H. (1993) 'Detection and Identification of the Cross-linking Amino Acids N-tau- and N-pi-(2′-amino-2′-carboxy-ethyl)-L-histidine ('histidinoalanine', HAL) in Heated Milk Products' in *Zeitsch. Lebensm. Unters. Forsch.* 197, 114–117
54 Moio, L., Semon, E. and Quere, J.L.le (1994) '3-Hydroxy-5-methyl-2-hexanone, a New Compound Characterized by a Melted Cheese Flavour in Dairy Products' in *It. J. Food Sci.* 6, 441–447
55 Yamamoto, Y, Nunome, T., Yamauchi, R., Kato, K. and Sone, Y. (1995) 'Structure of an Exocellular Polysaccharide of Lactobacillus helveticus TN-4, a Spontaneous Mutant Strain of Lactobacillus helveticus TY1-2' in *Carbohydr. Res.* 275, 319–332
56 Stingele, F., Lemoine, J. and Neeser, J.R. (1997) 'Lactobacillus Helveticus Lh59 Secretes an Exopolysaccharide that is Identical to the One Produced by Lactobacillus Helveticus TN-4, a Presumed Spontaneous Mutant of Lactobacillus Helveticus TY 1-2' in *Carbohydr. Res.* 302, 197–202
57 Gruter, M., Leeflang, B.R., Kuiper, J., Kamerling, J.P. and Vliegenthart, J.F.G. (1993) 'Structural Characterisation of the Exopolysaccharide Produced by Lactobacillus delbrueckii subspecies bulgaricus rr Grown in Skimmed Milk' in *Carbohydr. Res.* 239, 209–226
58 Robijn, G.W., Thomas, J.R., Haas, H., Berg, D.J.C.vanden, Kamerling, J.P. and Vliegenthart, J.F.G. (1995) 'The Structure of the Exopolysaccharide Produced by Lactobacillus Helveticus 766' in *Carbohydr. Res.* 276, 137–154
59 Staaf, M., Widmalm, G., Zhennai, Y. and Huttimen, E. (1996) 'Structural Elucidation of an Extracellular Polysaccharide Produced by Lactobacillus Helveticus' in *Carbohydr. Res.* 291, 155–164
60 Viverge, D., Grimmonprez, L. and Solere, M. (1997) 'Chemical Characterization of Sialyl Oligosaccharides Isolated from Goat (Capra hircus) Milk' in *Biochim. Biophys. Acta* 1336, 157–164
61 Groenberg, G., Lipniunas, P., Lundgren, T., Lindh, F. and Nilsson, B. (1992) 'Structural Analysis of Five New Monosialylated Oligosaccharides from Human Milk' in *Arch. Biochem. Biophys.* 296, 597–610
62 Hermansson, K., Jansson, P.E, Kenne, L., Widmalm, G. and Lindh, F. (1992) 'A 1H and 13C NMR Study of Oligosaccharides from Human Milk. Application of the Computer Program CASPER' in *Carbohydr. Res.* 235, 69–81
63 Kitagawa, H., Nakada, H., Kurosaka, A., Hiraiwa, N., Numata, Y., Fukui, S., Funakoshi, L., Kawasaki, T., Yamashina, L., Shimada, I. and Inagaki, F. (1989) 'Three Novel Oligosaccharides with the sialyl-Le-a Structure in Human Milk: Isolation by Immunoaffinity Chromatography' in *Biochemistry* 28, 8891–8897
64 Strecker, G., Fievre, S., Wieruszeski, J.M., Michalski, J.C. and Montreuil, J. (1992) 'Primary Structure for Four Human Milk Octa-, Nona-, and Undeca-saccharides established by 1H- and 13C-nuclear Magnetic Resonance Spectroscopy' in *Carbohydr. Res.* 226, 1–14
65 Saito, T. and Itoh, T. (1992) 'Variations and Distributions of O-glycosidically Linked Sugar Chains in Bovine Kappa-casein' in *J. Dairy Sci.* 75, 1768–1774
66 Urashima, T., Saito, T., Ohmisya, K. and Shimazaki, K. (1991) 'Structural Determination of Three Neutral Oligosaccharides in Bovine (Holstein-Friesian) Colostrum, Including the Novel Trisaccharide GalNAcαl-3Galβl-4Glc' in *Biochim. Biophys. Acta* 1073, 225–229
67 Girardet, J.M., Coddeville, B., Plancke, Y., Strecker, G., Campagna, S., Spik, G. and Linden, G. (1995) 'Structure of Glycopeptides Isolated from Bovine Milk Component PP3' in *Eur. J. Biochem.* 234, 939–946
68 Alexandrescu, A.T., Evans, P.A., Pitkeathly, M., Baum, J. and Dobson, C.M. (1993) 'Structure and Dynamics of the Acid Denatured Molten Globule State of α-lactalbumin: A Two-dimensional NMR study' in *Biochemistry* 32, 1707–1718
69 Molinari, H., Ragona, L., Varani, L., Musco, G., Consonni, R., Zetta, L. and Monaco, H.L. (1996) 'Partially Folded Structure of Monomeric Bovine β-lactoglobulin' in *FEBS Lett.* 381, 237–246
70 Wahlgren, N.M., Dejmek, P. and Drakenberg, T. (1994) 'Secondary Structures in β-casein Peptide 1-42: a Two Dimensional Nuclear Magnetic Resonance Study' in *J. Dairy Res.* 61, 495–506
71 Plowman, J.E., Smith, M.H., Creamer, L.K., Lidell, M.J., Coddington, J.M., Gibson, J.J. and Engelbretsen, D.R. (1994) 'Proton Assignment and Structural Features of a Peptide from the Chymosin Sensitive Region of Bovine κ-casein Determined by 2D-NMR Spectroscopy' in *Magn. Res. Chem.* 32, 458–464
72 Plowman, J.E., Creamer, L.K., Lidell, M.J. and Cross, J.J. (1997) 'Solution Conformation of a Peptide Corresponding to Bovine κ-casein Residues 130-153 by Circular Dichroism and ^{1}H-nuclear Magnetic Resonance Spectroscopy' in *J. Dairy Res.* 64, 377–397

Letters to the Editor

TIFS welcomes letters to the Editor concerned with issues raised by published articles or by recent developments in the food sciences. Letters should usually be supported by reference to published work.

Please address letters to:

Paul Finglas, Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7VA, UK
e-mail: paul.finglas@bbsrc.ac.uk

and indicate clearly whether they are intended for publication.