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Determination of the casein content in bovine milk by ^{31}P-NMR

By JOSEFINA BELLOQUE* and MERCEDES RAMOS
Instituto de Fermentaciones Industriales (C.S.I.C.), Juan De La Cierva 3, 28006 Madrid, Spain

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Summary. The relative proportion of caseins to total protein is a parameter that can be used to control the protein quality in standardised milk, an increasing tendency in dairy industries. ^{31}P-NMR was used to analyse the casein content of milk, by the quantitation of the area under the resonances belonging to SerP, and using methylenediphosphonic acid as internal standard. This procedure yielded good results, as similar values of caseins were obtained from N Kjeldahl and NMR analysis for slightly heated milk samples. Heating at 95 °C for 15 min did not alter the casein content results. Casein content of raw, pasteurised and UHT milks ($25{\cdot}6 \pm 1{\cdot}4$, $26{\cdot}4 \pm 1{\cdot}8$, $25{\cdot}5 \pm 1{\cdot}6$ g casein/l milk, respectively), obtained by NMR, were not significantly different, giving an average of $25{\cdot}8 \pm 1{\cdot}6$ g casein/l for bulk liquid milk. This work concluded that ^{31}P-NMR could be used as an alternative method to determine casein in raw, pasteurised, dry and UHT milks.

Keywords: Phosphorus, nuclear magnetic resonance, milk proteins.

Milk composition is subject to natural variation. Protein content fluctuates due to factors such as season, breed, nutrition or milking habits. To produce milk with a more consistent quality, standardisation of milk protein has become an important issue for the dairy industry (Marshall, 1995). Protein-standardised milk must keep the natural protein composition of milk, i.e. the serum proteins/casein ratio. Thus, methodology able to monitor this ratio is needed.

The relative proportion of serum proteins and casein can be obtained by the determination of casein/total protein, serum proteins/total protein or serum proteins/casein ratios. While the total protein content of milk is easy to determine by the Kjeldahl analysis of N content, the determination of selected fractions, i.e. casein or serum protein fractions, can be problematic. During heating processes, covalent bonds, disulphide (Jang & Swaisgood, 1990) and non-disulphide (Singh & Latham, 1993), are formed between proteins, particularly in UHT and sterilised milks. Because of this, in severely heated milks, N measurements based on the selective precipitation of caseins lead to erroneous results, as the casein precipitate contains also whey proteins.

Other techniques such as the determination of disulphide/thiol groups by polarography (Lechner & Klostermeyer, 1981), the determination of casein-phosphorus by colorimetry (Douglas *et al.* 1982; Wolfschoon-Pombo & Moreira Furtado, 1989; Feier & Goetsch, 1996), the use of the 4th derivative UV-spectra to

* For correspondence; e-mail: belloque@ifi.csic.es

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obtain a serum proteins/total proteins ratio (Meisel, 1995; Lüthi-Peng & Puhan, 1999; Miralles *et al.* 2000) or the quantitation of caseins and serum proteins by capillary electrophoresis (Recio & Olieman, 1996) seem to be better quantitative methods. However, a good method has not yet been established for the determination of caseins in milk, particularly after severe thermal processes, such as UHT.

High resolution NMR has been applied to several aspects, mainly structural features of milk and dairy products (Wahlgren & Drakenberg, 1995). However, since the detection basis of NMR is very different from other techniques, it can be a powerful tool for chemical analysis. ^{31}P-NMR spectra obtained from milk are very clean since not many phosphorylated compounds are present, and many of the resonances have been assigned (Wahlgren *et al.* 1986; Belton & Lyster, 1991). Phosphorus bound to caseins in the form of SerP is clearly observed and has been used to quantify the phosphorylation degree of super- and dephosphorylated casein (Van Hekken & Dudley, 1997). Recently, in our laboratory ^{31}P-NMR has been employed to analyse the phosphorus content of different phosphorylated compounds in milk and milk fractions (Belloque *et al.* 2000). In this work it has been shown that this procedure is useful to determine the content of SerP in milk, as the analytical parameters such as linearity, inter- and intra-assay variation were good, giving results that were consistent with those obtained by other techniques. To improve and extend the applications of this methodology, the objective of this work has been to apply ^{31}P-NMR to determine the casein content of milk through the quantitative analysis of SerP.

MATERIALS AND METHODS

Standard materials and milk samples

Methylenediphosphonic acid, trisodium salt (Sigma, St. Louis, MO 63178, USA) was used as internal standard (IS).

A commercial dry milk powder (low-heat), containing 273·5 g caseins/kg, as determined in our laboratory by N Kjeldahl analysis, was used for the preliminary studies, as well as for calibration, linearity and reproducibility tests. Five dry milk dispersions (50, 75, 100, 150 and 200 g/l) were prepared. When using internal standardisation, the 200-g/l dispersion could not be used because the IS precipitated from very concentrated samples. Three additional milk types, raw, pasteurised, and Ca-enriched UHT milk were used in selected tests. The Ca-enriched UHT milk was diluted 75:25 (milk:H_2O).

To test whether the results obtained by NMR were altered after heating of milk, the same raw milk, was analysed in duplicate before and after heating. Heating was carried out in Eppendorf tubes, using a Thermomixer 5436 (Eppendorf, Hamburg, D-22339, Germany). Samples were heated first for 3 min to reach 95 °C, maintained for 15 min at this temperature, and cooled in a water bath.

To analyse the casein content in different milk types by NMR, a total of 21 milk samples were analysed: three raw milks, five pasteurised milks, five dry milks and eight UHT milks. Dry milk powders were reconstituted in water to 100 g/l.

Sample preparation for NMR analysis

Samples were prepared as previously reported (Belloque *et al.* 2000). To 800 μl of milk or standard solution, 10 μl of 400 mM-methylenediphosphonic acid (D_2O solution) and 100 μl of 0·5-M EDTA (D_2O solution) were added. The pH was then adjusted to 9·5 with NaOH (D_2O solution), and the volume made up to 1 ml. D_2O solutions were used to obtain a final D_2O concentration of 200 μl/ml. Final

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concentration of the internal standard was 4 mM, i.e., 8 mM in terms of P content (two P atoms *per* methylenediphosphonic molecule). The sample was then centrifuged at 8800 ***g***, 10 °C, 30 min in a Biofuge 22R, 3743 rotor (Heraeus, Hanau, D-63450, Germany). The upper layer was discarded and the sample introduced into a 5-mm NMR tube. For measurements based on external calibration (EC), methylenediphosphonic acid was omitted.

^{31}P-NMR

NMR analysis was done according to previous studies (Belloque *et al.* 2000). ^{1}H-decoupled ^{31}P-NMR spectra were acquired at 30 °C in a 400-MHz Varian UNITYINOVA spectrometer (Varian NMR Instruments, Palo Alto, CA 94304, USA), with a 5-mm probe, at a frequency of 161·892 MHz, using a spectral width of 30000 Hz, a delay time of 2 s (unless otherwise stated), and an acquisition time of 1·6 s. The number of transients accumulated for each sample was 1700. The FID obtained was apodized with a line broadening of 1 Hz, Fourier transformed and phased. Area under the resonances of SerP and methylenediphosphonic acid were integrated using Varian NMR software.

In the preliminary tests, a 2-s delay time was assessed for casein determination. Area(SerP) and area(SerP)/area(IS) ratio were evaluated on the same samples, taking two consecutive spectra with 2 s and 60 s (full relaxation) delay times.

Determination of the T_1 relaxation time of methylenediphosphonic acid was done by an inversion-recovery pulse sequence, using a delay time, between the 180 and the 90° pulses, that ranged from 0·25 to 64 s. T_1 was automatically calculated by the Varian software.

In all cases the reference line of orthophosphate was set at 3·07 ppm.

Determination of milk casein by ^{31}P-NMR

Casein content in milk was determined by internal calibration, using the IS as the reference, and also by an external calibration, using milk powder dispersions.

Determination of caseins using methylenediphosphonic acid as internal standard. The concentration of casein ([CN]) was obtained from the resonance area of SerP, relative to that of the IS, with known concentration, by using the equation:

$$\text{area(SerP)/area(IS)} = k\,[\text{CN}].$$

The constant, k, was obtained from a preliminary linear calibration using four dry milk dispersions (50–150 g/l), with casein contents of 13·7–41·0 g/l. Resonance areas of SerP and IS were measured on the same spectrum, as relative numbers, i.e. area(SerP)/area(IS).

External calibration using milk powder dispersions. Reconstituted skim milk powder, (50–200 g/l), containing concentrations of caseins of 13·7–54·7 g/l (N Kjeldahl analysis) were used to obtain the calibration line (area(SerP) vs. casein content). Concentration of casein in milk samples was then obtained from interpolation of the area(SerP) into the calibration line. For each batch of samples to be analysed, a new calibration was performed. The area from SerP resonance, was obtained in absolute values and, in order to keep the proportionality among standards and samples, NMR parameters such as line broadening, Fourier number, or integration scale were kept constant.

For both IS and EC procedures, the samples were prepared and the spectra were acquired in exactly the same way, but the resonances used to quantify the caseins differed.

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Linearity and reproducibility of the NMR analysis

To test the linearity of the EC procedure, a series of dry milk powder dispersions, made at 50–200 g/l, and covering a casein concentration range of 13·7–54·7 g/l, were employed. To test the linearity of the IS procedure, another series of dry milk powder dispersions, made at 50–150 g/l, which covered a range for casein concentrations of 13·7–41·0 g/l, each containing the same amount of IS, were employed. Samples were prepared and analysed in duplicate.

Intra-assay reproducibility was obtained by using six different preparations, made from the same dry milk dispersion (100 g/l), which were analysed on the same day. Inter-assay reproducibility was done by employing the same dry milk, which was dispersed (100 g/l), prepared and analysed on six different days, with the NMR probe being re-tuned in the meantime.

Determination of milk casein by N analysis

Casein content by Kjeldahl N analysis was obtained by standard methods (International Dairy Federation, 1964, 1993). Comparison of the casein values obtained by Kjeldahl and by the two NMR methods, four pasteurised milks and two powdered milks (dispersed to 100 g/l) samples were used.

results and discussion

^{31}P-NMR spectra of a milk, containing the internal standard methylenediphosphonic acid, showed that the group of resonances belonging to casein SerP, as well as the resonance from IS, were clearly observed and well away from the other resonances (Fig. 1). The areas under these two resonances were used for the determination of the casein content in milk. Internal standardisation of the procedure was very important, since the use of IS offers some important advantages over an external calibration. First, construction of a calibration curve is not required. Second, since only the relative areas of SerP and IS are important, maintaining the same integration parameters is not necessary. Because of its simplicity, determination of caseins in milk was performed by the use of IS, even though an EC approach was also employed to confirm the results.

There were two main considerations to be taken into account for the analytical method presented in this paper. First, the T_1 relaxation time of the different resonances, and second, the SerP composition of caseins.

NMR is usually employed as an absolute measurement, under conditions of full relaxation. However, P nuclei take a long time to relax and, therefore, long acquisition times are required to collect a single spectrum. T_1 relaxation time of casein SerP, under the same conditions used in this work, is ~ 2 s (Belloque *et al.* 2000), and that of the IS was found to be 6 s. Therefore, classic NMR quantitation of SerP and IS, under full relaxation conditions, would take at least 30 s (five times the T_1) to collect each transient, and at least 14 h to collect a spectrum with 1700 transients. The same number of scans can be acquired in less than 2 h by using a short delay time (2 s) between transients. Even though this time does not allow full relaxation of the P nuclei, it has been shown to yield good results for the determination of SerP in milk (Belloque *et al.* 2000). A series of four dry milk dispersions were analysed under both 2 and 60 s delay times. The parameters measured were the area(SerP) (Fig. 2*a*) and the area(SerP)/area(IS) ratio (Fig. 2*b*), as these would be used later for determining the casein contents. Figure 2 shows that

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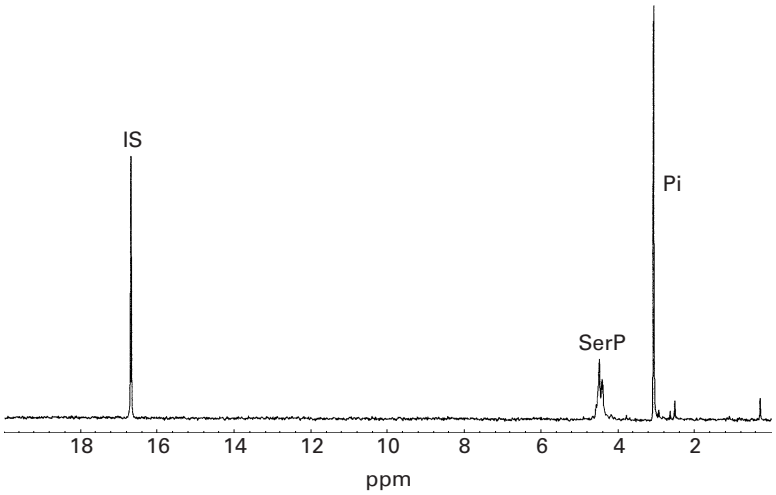

Fig. 1. ^{1}H-decoupled ^{31}P-NMR spectrum of a raw bovine milk sample (30 °C, frequency 161·892 MHz, spectral width 30000 Hz, delay time of 2 s, acquisition time 1·6 s, 1700 transients). Labelled resonances belong to inorganic phosphate (Pi), casein phosphoserine (SerP) and the internal standard, methylenediphosphonic acid (IS).

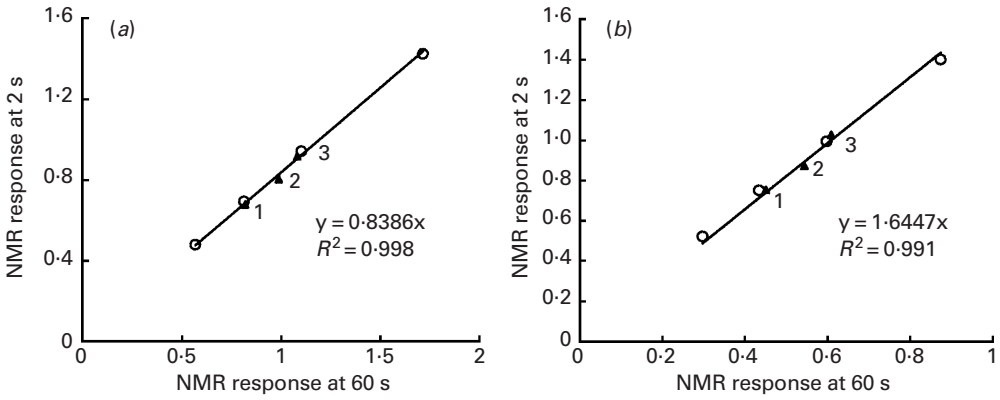

Fig. 2. Linear correlation between the NMR response obtained by the use of 2 s and 60 s (full relaxation) delay times. Parameters evaluated were (*a*) absolute values of SerP resonance, area(SerP) and (*b*) the values relative to the internal standard (IS), methylenediphosphonic acid, area(SerP)/area(IS). ○: Dry milk dispersions (50–150 g/l), used to obtain the linear regression; ▲: 1, Ca-enriched UHT milk (dil. 75:25 (milk:H_2O)); 2, pasteurised milk; 3, raw milk.

the NMR responses at 2 and 60 s delay times were linearly related. To confirm that this relationship was maintained in samples subjected to different thermal processes, three different types of milk were tested. The NMR responses fitted on the straight line (Fig. 2*a*, *b*: labels 1, 2 and 3). Therefore, a 2-s delay time was appropriate for the analysis of the casein content in bovine milk, whether or not IS was employed.

Estimation of casein content by measurement of bound SerP is dependent on casein composition, which varies between species, individuals and among different types of dairy products. In bovine milk, some authors have found that, while the casein/protein ratio changes appreciably throughout the seasons, the casein-phosphorus/casein ratio showed very small variations (Wolfshoon-Pombo & Moreira Furtado, 1989). The SerP/casein ratio was assumed to be constant throughout this work, and the analytical procedure was circumscribed to bulk bovine milk. Analysis

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Table 1. *Linearity of the ^{31}P-NMR method for the determination of caseins, using external calibration (EC) and internal calibration with methylenediphosphonic acid as internal standard (IS)*

Method	Standard material	[CN] range	Equation	R^2	RSD
EC	Dry milk	13·7–54·7	area(SerP) = 0·0341 [CN]	0·996	3·1
IS	Dry milk + IS	13·7–41·0	$\frac{\text{area(SerP)}}{\text{area(IS)}} = 0{\cdot}0359[\text{CN}]$	0·996	2·4

[CN]: casein concentration, in g casein/l milk. R^2: correlation coefficient. RSD: relative standard deviation. Methods as described in Materials and methods.

Table 2. *Comparison between the values of caseins obtained by N Kjeldahl and by ^{31}P-NMR in six different milk samples with low thermal treatment*

	Casein content (g/l milk)		
Milk sample type	N-Kjeldahl	NMR-IS	NMR-EC
Pasteurised 1	26·9	27·2	26·5
Pasteurised 2	27·7	29·3	29·0
Pasteurised 3	26·7	27·1	26·7
Pasteurised 4	24·2	23·8	24·0
Dry† 1	27·3	27·5	27·5
Dry† 2	20·9	19·7	19·3

† Dry: skim milk powder reconstituted at 100 g/l.
NMR-IS using calibration with internal standard.
NMR-EC: using external calibration. Methods as described in Materials and methods.

Table 3. *Casein content of selected milk types, obtained by ^{31}P-NMR analysis, using methylenediphosphonic acid as internal standard*

(Values are means ± SD for n = various (see table))

Milk type	n	Casein content†
Raw	3	25·6 ± 1·4
Pasteurised	5	26·4 ± 1·8
UHT	8	25·5 ± 1·6
Mean, liquid milk‡	16	25·8 ± 1·6
Dry milk powder	5	269·0 ± 45

† Casein content in: g/l milk, for liquid milk, and g/kg powder, for dry milk powder.
‡ Mean, liquid milk is the average of all raw, pasteurised and UHT milk samples.

of milk from a different species, or products other than milk, would require a preliminary study, particularly the SerP/casein ratio, and the relaxation time of SerP signals.

Evaluation of the ^{31}P-NMR method for the quantitation of caseins

Determination of caseins by ^{31}P-NMR, based on the relative response of SerP to that of IS, was evaluated, and the results were compared to those obtained by the Kjeldahl method for lightly processed milk, and confirmed by an NMR external calibration procedure.

The area(SerP)/area(IS) ratio was plotted against the casein concentration, giving a linear relationship within the tested range (Table 1). Intra- and inter-assay reproducibility showed relative standard deviations (RSD) of 1·2 and 2·4%, respectively. There was good agreement between the NMR and Kjeldahl results (Table 2).

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Linearity was also achieved within the tested range (Table 1) with the EC procedure, plotting area(SerP) against casein content. Intra- and inter-assay reproducibility were also good, showing RSD 1·1 and 3·0 %, respectively. Good agreement with the Kjeldahl method was also observed (Table 2).

From these results, it was concluded that the determination of caseins by ^{31}P-NMR with internal standardization was appropriate and, therefore, this procedure was further applied to the determination of caseins in different types of milk.

Effect of milk heating on the determination of caseins

Lower values for casein content of UHT milk were obtained by NMR than by N Kjeldahl (24·8 and 27·8 g/l, respectively). This was consistent with the fact that whey proteins, which become cross-linked to the caseins as a consequence of heating, are included in the N Kjeldahl measurement.

The effect of whey protein denaturation on the NMR method was assessed by heating raw milk at 95 °C for 15 min. Under these conditions, significant interaction between whey proteins and caseins was expected. Values were 24·3 and 24·4 g/l for the unheated and the heated milk samples, respectively, demonstrating that bound whey protein did not interfere with ^{31}P-NMR casein determination.

During heating of milk, lysinoalinine (LAL) is formed by β-elimination from SerP and cysteine residues. This could lead to the underestimation of casein content, as the consequence of SerP depletion. However, some authors have reported a maximum value of 186 mg LAL/kg protein in UHT milk (Faist *et al.* 2000), corresponding to $\sim 0{\cdot}5$ % depletion of the total SerP, which would not significantly alter the results. For milks with greater heat damage, such as sterile milk, this percentage could increase 3–4 times, which should be taken into account.

Determination of caseins in milk by NMR

Casein content of a number of milks was determined by ^{31}P-NMR (Table 3). No significant differences were found between raw, pasteurised and UHT skim milks, further supporting the proposition that the heat processes involved did not alter the results. Since the casein content was not dependent on the thermal process, results obtained from all liquid milk samples were averaged. An average of $25{\cdot}8 \pm 1{\cdot}6$ g casein/l ($n = 16$) in bulk bovine milk was obtained, consistent with values obtained in the literature for bulk bovine raw milk (Lüthi-Peng & Puhan, 1999).

^{31}P-NMR seems to be an alternative method to determine caseins in raw and pasteurised, dry and UHT milks. However, the method could be further optimised, particularly regarding the analysis time, $\sim 1{\cdot}7$ h. There are several strategies that could be investigated to improve signal-to-noise and to shorten analysis time: shortening delay time below 2 s; reducing a number of scans (while maintaining a reasonable signal-to-noise ratio); using 10 mm diameter NMR tubes to increase sample volume. A reduction in analysis time would make this method more useful for potential applications, such as monitoring the casein/protein ratio in commercial milks, especially UHT milk, since the difficulties related to classical methods can be overcome.

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