

## Microparticle-Enhanced Nephelometric Immunoassay. 3. Application to Milk and Dairy Products<sup>1</sup>

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### ABSTRACT

A microparticle-enhanced nephelometric immunoassay (NEPHELIA<sup>®</sup>) has been developed for the measurement of milk, whey, and curd proteins ( $\alpha_s$ -casein,  $\kappa$ -casein,  $\alpha$ -lactalbumin, and  $\beta$ -lactoglobulin). This new method was applied to measure milk protein variations in a year-long study. The protein levels and their chronological evolution agree with other published data. The effects of some technological treatments on these measurements were studied:  $\alpha$ -lactalbumin and  $\kappa$ -casein were not modified during freezing-thawing cycles;  $\beta$ -lactoglobulin and  $\alpha_s$ -casein measurements were strongly influenced by freezing; the determination of heat-processed  $\beta$ -lactoglobulin in the presence of  $\kappa$ -casein was also altered; the technological treatments applied to raw milk for pasteurization and fat standardization of milk had no influence on the proteins' measured values. The  $\beta$ -lactoglobulin: $\alpha$ -lactalbumin ratio was determined as a good indication of heat denaturation. It was unmodified in standardized milk as

well as in whey. The  $\kappa$ -casein level in milk was correlated with some cheese-making parameters, particularly with soft and pressed cheese yield, which could be a good predictive factor in cheese making.

(Key words: nephelometric immunoassay, milk technology, dairy products)

Abbreviation key: CN = casein,  $K_{20}$  = the time required for curd to achieve a specified firmness giving a 20-mm width of the recording line, LA = lactalbumin, LG = lactoglobulin.

### INTRODUCTION

The dairy industry is confronted with many problems associated with milk proteins and milk composition as affected by technological treatments. Milk cooling and cold preservation at the farm or the factory, heat treatment, and milk standardization processes could induce irreversible physicochemical and microbiological changes. Those changes could involve a loss of cheese yield or the appearance of organoleptic defects.

A microparticle-enhanced nephelometric immunoassay (NEPHELIA<sup>®</sup>, Diagnostics Pasteur, Marnes la Coquette, FR) has been previously described (7, 26) for the measurement of the main milk proteins,  $\alpha_s$ -casein (CN),  $\kappa$ -CN,  $\alpha$ -lactalbumin (LA), and  $\beta$ -lactoglobulin (LG). This new analytical method is based on the ability of antigen-coated microparticles to ag-

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glutinate in the presence of corresponding antibodies and to induce turbidity. Free antigen solution inhibits the immunoagglutination of microparticles, and the measurement of scattered light during inhibition allows the determination of antigen concentration.

We used this new method to measure levels of  $\alpha_s$ -CN,  $\kappa$ -CN,  $\alpha$ -LA, and  $\beta$ -LG in milk, whey, and curd during a year-long study of cheese making (Camembert-type and Saint Paulin-type cheeses) and to study the influence of some technological treatments on measurements of these proteins.

## MATERIALS AND METHODS

### Reagents

All chemical reagents (analytical reagent grade) were as previously reported by Collard-Bovy et al. (7). Caseins were purified according to Mercier et al. (28) and  $\alpha$ -LA and  $\beta$ -LG according to Armstrong et al. (2). Polyfunctional hydrophilic microparticles were synthesized as previously described (12). Protein-coated microparticles and specific antisera used as nephelometric reagents were obtained following methods of Collard-Bovy et al. (7) and Marchal et al. (26).

### Milks, Wheys, and Curds

Fresh raw milks were collected from nine herds with an average size of 28 cows (Friesian and Red and White French breeds) over 17 mo (November 1985 to March 1987). Bulk raw milk (5000 L), stored from 24 to 48 h at 4°C, was obtained each week by mixture of four successive milkings.

A part of the milk from each collection was pasteurized (72°C  $\pm$  2°C, 20 s) and fat standardized (25 g/L) by partial skimming and then used in an experimental pilot-plant cheese making (60 cheese makings during the studied period) of soft cheese (Camembert-type cheese) and pressed cheese (Saint Paulin-type cheese). In both classical cheese-making processes, animal rennet (Boll-Hansen, Copenhagen, DK, chymosin concentration: 520 mg/L) was used to coagulate the milk (.104 mg of chymosins for 1 L of milk) with maturation for 45 min at 37°C. The milk contained calcium chloride (.1 g/L) and several starters (*Streptococcus lactis* and *Streptococcus cremoris*

MAO16, Eurozyme, Sassenage, FR, .02 units/L and *Streptococcus diacetylactis* MD88, Eurozyme, .005 units/L; protease from *Bacillus subtilis* B500, Rapidase, Seclin, FR, .2 g/L). The wheys and curds obtained were treated as previously reported (7, 26) before protein determination.

### Heat and Freezing Treatments

A buffered solution (.05 M phosphate, pH 6.6) of either purified  $\beta$ -LG (2.7 g/L) with or without  $\kappa$ -CN (3.2 g/L) in sealed tubes was heated to temperatures from 70 to 100°C in a water bath for 90 s and then cooled rapidly before protein assays.

Fresh milk was frozen at -18°C for 24 h, then quickly thawed in a 40°C water bath, and equilibrated 2 h at room temperature before protein measurement and frozen again. The same protocol was repeated for eight cycles.

### Analytical Methods

Proteins ( $\alpha_s$ -CN,  $\kappa$ -CN,  $\alpha$ -LA, and  $\beta$ -LG) were measured in raw milks, pasteurized and fat-standardized milks, wheys, and curds from Camembert-type and Saint Paulin-type cheeses, and heating and freezing processed mixtures by microparticle-enhanced nephelometric immunoassay as previously reported (7, 26). Measurement of total protein and whole casein was performed by the Kjeldahl method (23). Levels of proteins in raw and fat standardized milks were compared by using this defined statistical coefficient =  $\sum_i | Y_i - X_i | \times 100 : n \times \bar{X}_i$ , where  $Y_i$  = level in standardized milk,  $X_i$  = level in raw milk,  $\bar{X}_i$  = mean level in raw milk, and  $n$  = number of samples.

Casein micelle sizes were estimated with a laser granulometer (Autosizer II, Malvern Instr., Worcs, Engl.) after dilution of skim milk by the salt solution of Jenness and Koops (22). The rheological  $K_{20}$  parameter, representing the time required for the curd to achieve a specified firmness giving a 20-mm width of the recording line (21), was determined using the modified thromboelastograph Formagraph (Foss and Co., Hillerod, DK).

Cheese yield represents the weight of curd after turning out of the molds that is obtained from 100 L of standardized milk. Yields from

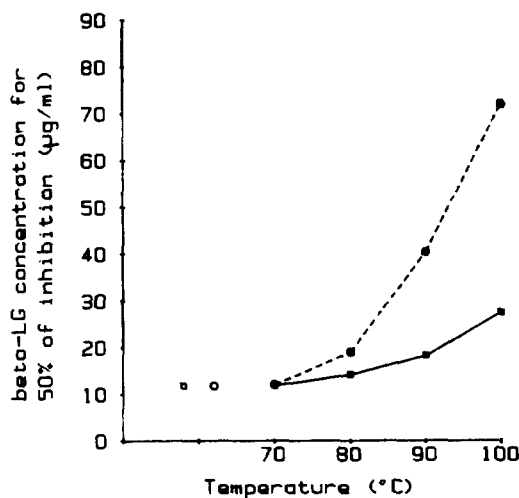


Figure 1. Concentration of heat-processed (90 s, .05 M phosphate, pH 6.6)  $\beta$ -lactoglobulin (LG) inducing 50% of inhibition of the agglutination of the  $\beta$ -LG-coated microspheres (25 mg/L) with anti- $\beta$ -LG antiserum (3800-fold diluted) as a function of the temperature: heat-processed  $\beta$ -LG solution (■, 2.7 g/L), heat-processed  $\beta$ -LG (●, 2.7 g/L) solution in the presence of  $\kappa$ -casein (3.2 g/L), and unheated controls (□, ○).

Saint Paulin-type cheese were multiplied by a preliminary experimentally defined correcting factor (ratio mean yields of soft cheese:mean yield of pressed cheese = 1.43) because of loss of material by washing of curd and to allow their comparison with those from Camembert-type cheese.

## RESULTS AND DISCUSSION

### Influence of Heating and Freezing on the Measurement of Milk Proteins

Heat-processed  $\beta$ -LG solutions were used as the inhibiting agent for the  $\beta$ -LG-coated microparticle immunoagglutination as previously studied (26), and  $\beta$ -LG concentrations inducing 50% inhibition were calculated (Figure 1). The results display a partial loss of the antigenicity (increase of  $\beta$ -LG concentration for 50% of inhibition) for  $\beta$ -LG heated to high temperature (above 90°C), which could indicate some denaturation, resulting in spatial modifications or the possibility of masking antigenic sites. Immunoreactivity of  $\beta$ -LG was lower when  $\beta$ -LG was heated at the same

temperatures in the presence of  $\kappa$ -CN than without  $\kappa$ -CN. The formation of a stable  $\beta$ -LG- $\kappa$ -CN disulfide-bonded complex (16, 20, 25) between the two proteins, involving a decreasing accessibility of antigenic sites on the  $\beta$ -LG, may explain this result, although the immunoreactivity of  $\kappa$ -CN was not modified by the same treatment (no variation of the  $\kappa$ -CN concentration inducing 50% of inhibition of the agglutination of  $\kappa$ -CN-coated microparticle with anti- $\kappa$ -CN antiserum).

In the study of the effect of freezing on the measurement of milk proteins, no modification was observed for  $\alpha$ -LA (reproducibility CV = 2.9%) and  $\kappa$ -CN (reproducibility CV = 5.5%) after eight freezing-thawing cycles of whole milk, but great variability in results was obtained for  $\beta$ -LG (reproducibility CV = 15.9%) and for  $\alpha$ <sub>s</sub>-CN (reproducibility CV = 17.6%). Measurements of  $\beta$ -LG and  $\alpha$ <sub>s</sub>-CN could not be performed on frozen whole milks.

### Chronological Study of Raw Milk Proteins Levels

Two proteins ( $\alpha$ -LA and  $\kappa$ -CN) were measured on 67 fresh or frozen milk samples between November 1985 and March 1987, whereas  $\beta$ -LG and  $\alpha$ <sub>s</sub>-CN were determined only on 21 fresh milks from October 1986 to March 1987. The results for the estimation of these four proteins showed concentration ranges from 1.12 to 1.56 g/L (mean = 1.36 g/L; SD = .10 g/L) for  $\alpha$ -LA; 2.19 to 3.40 g/L (mean = 2.70 g/L; SD = .22 g/L) for  $\kappa$ -CN; 3.04 to 3.64 g/L (mean = 3.30 g/L; SD = .15 g/L) for  $\beta$ -LG; and 9.28 to 11.93 g/L (mean = 10.71 g/L; SD = .56 g/L) for  $\alpha$ <sub>s</sub>-CN. The distribution of the data followed a standard pattern with identical median, modal, and average classes for each protein. The observed results agree with most of the published values (Table 1) but sometimes indicate an influence of the techniques used on the result of the measurements.

Seasonal variations already reported (9, 11, 19, 24) were observed for  $\alpha$ -LA and  $\kappa$ -CN concentrations (Figure 2) with alternation of minima (February and November for  $\alpha$ -LA; January and August for  $\kappa$ -CN) and maxima (December, May, and January for  $\alpha$ -LA; February and October for  $\kappa$ -CN) showing an opposite cyclic evolution for these two proteins.

The briefness of the study for  $\beta$ -LG and  $\alpha_s$ -CN did not permit comparison of the variation of their concentrations with published data.

#### Comparison Between Protein Levels In Raw and Fat-Standardized Milk

The comparison between protein levels in raw milks before and after pasteurization and fat standardization permitted evaluation of the influence of these technological treatments on

the immunological measurement of the main proteins. The statistical coefficient (2.9, 1.4, 4.0, and 3.2%, respectively, for  $\alpha$ -LA,  $\beta$ -LG,  $\alpha_s$ -CN, and  $\kappa$ -CN), compared with the reproducibility of the nephelometric assays (CV from 1 to 4%) previously determined (7, 26), indicated that the technological treatments applied to raw milks had no influence on the protein concentrations as measured by NEPHELIA®.

#### Variation of the $\beta$ -LG: $\alpha$ -LA Ratio In Milk, Whey, and Curd

The  $\beta$ -LG: $\alpha$ -LA ratio appeared to be a good indicator for the denaturation of proteins, because  $\beta$ -LG was a more thermosensitive protein than  $\alpha$ -LA. This ratio has been calculated for 6 mo from October 1986 to March 1987. The results obtained in raw milks, standardized milks, whey, and curd showed a decrease in the ratio from October to January, followed by a period of no change until March, which is explained by the seasonal variation observed for each protein in the raw milks.

The  $\beta$ -LG: $\alpha$ -LA ratios for standardized milks and whey samples were not significantly different from those calculated for raw milks

TABLE 1. Comparative review of  $\alpha_s$ -casein (CN),  $\kappa$ -CN,  $\alpha$ -lactalbumin ( $\alpha$ -LA), and  $\beta$ -lactoglobulin ( $\beta$ -LG) content of bovine milk.

Protein	Range (g/L)	Reference
$\alpha_s$ -CN	13.3–15.6	(18)
$\alpha_{s1}$	9.96	(8)
$\alpha_{s2}$	2.72	(8)
$\alpha_{s1}$	9.68–10.82	(9)
$\alpha_{s2}$	2.53–2.95	(9)
$\alpha_{s1}$	8.3–10.1	(27)
$\alpha_{s2}$	2.9–4.1	(27)
$\alpha_{s1}$	10.0	(23)
$\alpha_{s2}$	2.6	(23)
	9.28–11.93	Present study
$\kappa$ -CN	1.6–1.8	(18)
	3.2	(8)
	2.7	(4)
	3.13–3.77	(9)
	2.7–3.9	(27)
	3.3	(23)
	2.19–3.40	Present study
$\alpha$ -LA	1.1–1.5	(30)
	2.7–2.8	(18)
	1.15–1.29	(17)
	1.14–1.32	(9)
	.53–1.10	(15)
	.98–1.13	(27)
	1.18–1.76	(5)
	1.24–1.30	(1)
	1.2	(23)
	1.12–1.56	Present study
$\beta$ -LG	3.0–3.9	(30)
	2.5–3.7	(18)
	1.03–7.16	(6)
	3.0–4.1	(3)
	2.8–4.0	(3)
	2.9–3.9	(3)
	3.18–3.61	(17)
	2.95–3.33	(9)
	1.75–3.11	(15)
	3.0–3.6	(27)
	3.61–5.73	(5)
	3.2	(23)
	3.04–3.64	Present study

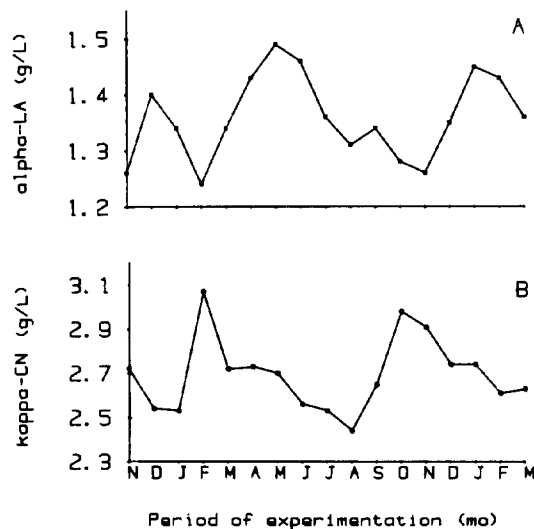


Figure 2. Chronological variation from November 1985 to March 1987 of  $\alpha$ -lactalbumin (LA) (A) and  $\kappa$ -casein (CN) (B) content (monthly mean of weekly measures) of collected raw milks.

(statistical coefficient = 2.9 and 4.3%), contrary to the same ratio obtained in curd samples that was significantly lower (statistical coefficient = 12.6%), indicating a greater denaturation of proteins in curd.

#### Correlation Between $\kappa$ -CN Level and Some Physicochemical and Technological Parameters

The weekly variations from January 1986 to March 1987 of  $\kappa$ -CN concentration, total protein concentration, casein micelle size in raw milks,  $K_{20}$  parameter, and cheese yield are reported in the Figure 3. Total protein concentration and cheese yields followed in parallel to  $\kappa$ -CN concentration, contrary to casein micelle size and  $K_{20}$  parameter. Multiparametric linear regression analysis of these results showed that  $\kappa$ -CN concentration in raw milk was significantly correlated with total protein concentration ( $n = 60$ ,  $r = .26$ ,  $P < .05$ ), cheese yields ( $n = 60$ ,  $r = .36$ ,  $P < .01$ ), casein micelle size ( $n = 60$ ,  $r = -.39$ ,  $P < .01$ ), and  $K_{20}$  parameter ( $n = 60$ ,  $r = -.26$ ,  $P < .05$ ).

The correlation observed between  $\kappa$ -CN concentration of milk and casein micelle size confirms the results of Ekstrand and Larsson-Raznikiewicz (13) and Davies and Law (10) showing a decreasing size of the micelles when the concentration of  $\kappa$ -CN increases. The model of Schmidt (31) for the micelle of casein, in which micellar growth stops when its surface consists essentially of  $\kappa$ -CN, also agrees with the correlation observed in this study because it gives to  $\kappa$ -CN a regulatory function on micelle size. Rennet activity involves the formation of hydrophobic *para*- $\kappa$ -CN on the surface of casein micelle, making association easier and leading to the formation of massive coagulation (29). The negative correlation between  $\kappa$ -CN concentration in raw milk and the  $K_{20}$  parameter, already observed by Ekstrand et al. (14), may be so explained.

The correlation between  $\kappa$ -CN concentration in raw milk and the yield of cheese from this milk suggests that the  $\kappa$ -CN level in raw milk could be used in the dairy industry as a predictive parameter. The  $\kappa$ -CN concentration appears, in our study, to be a better factor to predict cheese yield than the total protein or the whole casein concentration, which were not significantly correlated with cheese yield ( $r = .14$  and  $.18$ , respectively).

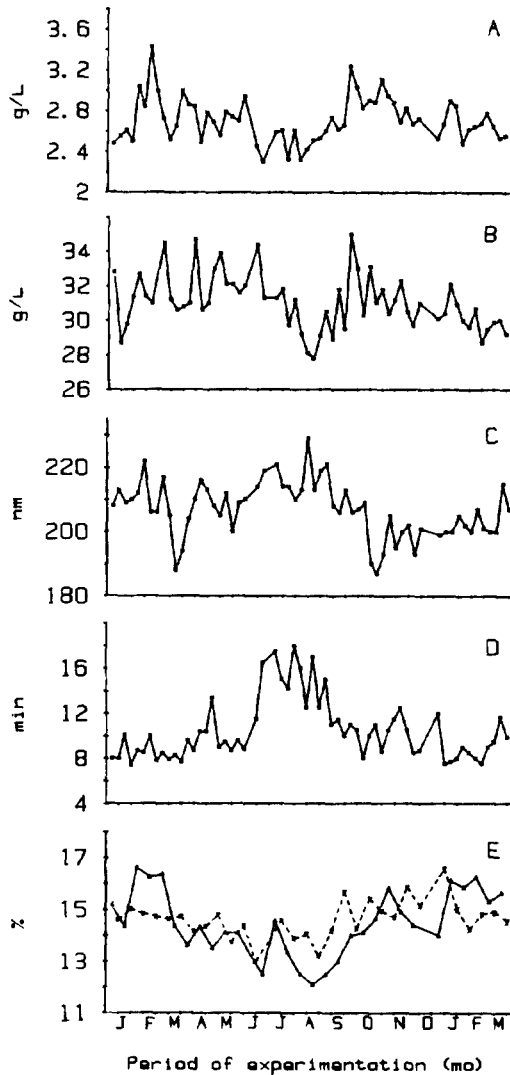


Figure 3. Comparative variation from January 1986 to March 1987 of weekly measured  $\kappa$ -casein (CN) concentration (A), total protein concentration (B), and casein micelle size (C) of collected milks, with  $K_{20}$  parameter (D), and cheese yield (E) for soft ( $\blacktriangle$ ) or pressed ( $\times$ ) cheese.  $K_{20}$  = The time required for curd to achieve a specified firmness giving a 20-mm width of the recording line.

#### CONCLUSIONS

Microparticle-enhanced nephelometric immunoassay (NEPHELIA<sup>®</sup>) appears to be a suitable and easy method for the measurement of the main milk proteins,  $\alpha$ -LA,  $\beta$ -LG,  $\alpha$ -

CN, and  $\kappa$ -CN. Their assay by this new method on milks, wheys, and curds from a 17-mo cheese-making study gives concentrations and a chronological evolution of the proteins that agree with published data obtained by other, more complicated methods.

The technological treatments applied to raw milk for the manufacture of pasteurized and fat-standardized milk have no significant influence on the measured concentrations of these proteins. The  $\beta$ -LG: $\alpha$ -LA ratio can be considered as a good indicator of the heat denaturation of proteins because of the great thermosensitivity of the  $\beta$ -LG. This study confirms that these proteins are not altered in standardized milks and wheys, but the lower ratios, observed in curds from Camembert-type and Saint Paulin-type cheeses, show a decreased immunoreactivity of  $\beta$ -LG that could be due to the formation of the  $< \beta$ -LG- $\kappa$ -CN  $>$  complex.

The measurement of milk  $\kappa$ -CN concentration by NEPHELIA<sup>®</sup> confirms the already known correlations between the  $\kappa$ -CN level, casein micelle size, and the  $K_{20}$  parameter. The  $\kappa$ -CN concentration in raw milk is better correlated with cheese yield than either the total protein or whole casein concentration. This  $\kappa$ -CN concentration, easily determined by NEPHELIA<sup>®</sup>, could be a good predictive factor for the dairy industry.

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