ARTICLES

Partition of Main and Trace Minerals in Milk: Effect of Ultracentrifugation, Rennet Coagulation, and Dialysis on Soluble Phase Separation

Miguel Angel de la Fuente, Javier Fontecha, and Manuela Juárez*

Department of Dairy Products, Instituto del Frío (CSIC), Ciudad Universitaria s/n, 28040 Madrid, Spain

A study was carried out on the effect of soluble-phase separation on mineral distribution in milk. Fractioning was carried out by rennet coagulation (RC), ultracentrifugation (UC), and dialysis (D) against a large volume of water (1:10). Reconstituted skim milk powder was used and calcium, magnesium, sodium, potassium, phosphorus, manganese, zinc, citrate, lactose, and nitrogen were determined in the soluble fraction. The results of the first two procedures were comparable, except for Zn, which was lower in RC; greater precision was achieved with UC, especially for manganese. On fractioning by dialysis, Ca and Zn levels in the diffusate were higher than in the soluble fractions obtained by UC and RC as a result of long dialysis times (24 h) and low operating temperature (5 °C). With dialysis, Na and K levels in the diffusate were lower than with the other procedures. Of the three procedures assayed, UC appeared to be the best suited for work in series.

Keywords: *Dialysis; rennet coagulation; ultracentrifugation; mineral balance; milk salts*

INTRODUCTION

The partitioning of mineral salts between colloidal and soluble forms and the balance between cationic and anionic minerals influence the physical state and stability of milk proteins. The equilibrium of salts between the aqueous and the dispersed phases of milk therefore affects its heat stability, its rennet coagulability, and the physical properties of the curd (Polychroniadou and Vafopoulou, 1986).

The proportions of the various minerals in the soluble and colloidal phases depend on the procedure followed in phase separation. The aqueous phase obtained on fractioning ought ideally to have the same composition as the aqueous phase of the milk.

Leaving aside techniques of ultrafiltration (Davies and White, 1960; Brulé and Fauquant, 1981; Papajová, 1982) which present a number of drawbacks for routine work, several methods of fractionation of the soluble and colloidal constituents have been applied. The procedures most widely used for the preparation of a suitable aqueous phase for determination of soluble components are analysis of rennet whey (Sindhu and Roy 1976b; O'Connor and Fox, 1977), high speed centrifuging (Sindhu and Roy, 1976a; Rajput *et al*., 1983; Polychroniadou and Vafopoulou, 1986), and different types of dialysis (Davies and White, 1960; Sindhu and Roy, 1973; Singh *et al*., 1989; Reykdal and Lee, 1991).

Comparisons of different separation mechanisms with the same substrate do not abound in the literature and are confined to the study of main elements, particularly calcium (Davies and White, 1960; Papajová, 1982). Data about such trace elements as Mn and Zn, over 90% of which are found in skim milk, are scarce (Singh *et al*., 1989).

The present study deals with the partitioning of minerals, namely, calcium, magnesium, potassium, sodium, phosphorus, zinc, manganese, citrate, nitrogen, and lactose, by means of rennet coagulation (RC), ultracentrifugation (UC), and dialysis (D) against a large volume of water. This paper seeks to ascertain what separation procedure is most suitable and offers most advantages for systematic and routine analysis of a milk-soluble fraction.

MATERIALS AND METHODS

Samples. In order to circumvent the defatted step previous to the sample analysis and simplify the study and to arrange during all the work a more stable sample, the material studied was commercial skim milk powder with medium high treatment. This was reconstituted by dilution (1:10) in high purity water with a metered resistivity of 18 MΩ for subsequent use in the various analyses.

Separation of Soluble and Colloidal Phases. Three procedures were assayed for partition into two phases. First was high speed centrifuging: 30 mL of milk was centrifuged at 100000*g* for 1 h using a 50-RT-1250 rotor at 20 °C in an ultracentrifuge (Sorvall Combi Plus, Wilmington, DE). The supernatant fluid was carefully removed and filtered through Whatman 40 paper. It was then stored for analysis at 5 °C.

Dialysis was performed according to the method of Sindhu and Roy (1973), encasing 10 mL of milk in a tubular porous membrane of 3500 MW cutoff (Cellu-Sep T1, Membrane Filtration Products Inc., San Antonio, TX), dipping the resultant in 100 mL of distilled water, and stirring for 24 h at 5 °C. The diffusate was collected for analysis of the different compounds. In expressing the analytical results, the dilution factor (1:10) was taken into account.

Finally, 40 mL of the same milk sample was warmed to 37 °C in a water bath and a rennet solution (Chr. Hansen Laboratorium, Copenhagen, Denmark) was added at a rate of 0.1 g/L. After allowing a firm curd to form, the curd was sliced into 1−2 mm cubes and centrifuged (Beckman J2-MC, Palo Alto, CA) at 6000 rpm for 10 min. The rennet whey was drained off and collected after filtration, as described above. The filtrate from rennet whey was stored like ultracentrifugal supernatant.

Total and soluble fractions (supernatant, dialyzable, and rennet whey) of milk sample were analyzed for Ca, Mg, Na, K, P, Mn, Zn, citrate, total, and non-casein nitrogen content and lactose.

S0021-8561(95)00694-7 CCC: $12.00

Table 1. Mean Values and Standard Deviation of Calcium (Soluble and Ionic), Magnesium, Phosphorus, and Citrate in Skim Milk and Soluble Fractions Separated by Clotting (Rennet Whey), Ultracentrifuging (Supernatant), and Dialysis (Diffusate)[e]

name of constituent	total in milk mg/kg	rennet whey		supernatant		diffusate	
		mg/kg	%	mg/kg	%	mg/kg	%
calcium	1257 ± 19	248[a] ± 14	19.7	259[a] ± 9	20.6	384[b] ± 31	30.6
ionic Ca	99.7 ± 0.4	72.3[a] ± 0.3	29.2[d]	86.0[b] ± 0.4	33.2[d]	224[c] ± 1	58.3[d]
magnesium	119 ± 1	57.9[a] ± 1.6	48.7	59.0[a] ± 0.8	49.6	63.0[b] ± 4.0	52.9
phosphorus	996 ± 21	406[a] ± 14	40.8	415[a] ± 10	41.7	435[b] ± 19	43.7
citrate	1760 ± 24	1509[a] ± 31	85.7	1510[a] ± 49	85.8	1271[b] ± 59	72.2

[a–c] Different letters in the same row indicate significant differences ($p \leq 0.05$). [d] Percentage calculated in soluble Ca. [e] Concentrations are shown in mg/kg and in percentages. The number of samples analyzed by each fractionation procedure was 12.

Four different partition assays were made for each one of the three procedures (rennet coagulation, ultracentrigugation, and dialysis) and three alicuots were taken in each separation. In total, 12 determinations were made for analyte, for each one of the three different soluble fractions. The mean values of concentration in the soluble fractions were converted to the concentration in the milk as explained below.

Methods of Analysis. Ca, Mg, Na, and K were measured by flame atomic spectrometry in filtrates following precipitation of the samples with trichloroacetic acid (Brooks *et al*., 1970). Technical specifications of the atomic absorption spectrophotometer, composition of the flame, and instrumental conditions for measurement of Ca, Mg, Na, and K are explained in a previous paper (De la Fuente and Juárez, 1995a). The limit of detection, precision, and accuracy of these determinations are also included in the article above mentioned. Mn and Zn were measured by graphic furnace atomic absorption spectrometry after sample digestion in a microwave oven using the method proposed by De la Fuente *et al*. (1995). Phosphorus was estimated colorimetrically by a molybdenum blue method after sample digestion described by De la Fuente and Juárez (1995b).

Citrate was determined enzymatically (Boehringer, 1992) and using ionic calcium with a selective electrode according to Geerts *et al*. (1983). Lactose content was determined by HPLC (Richmond *et al*., 1987). Nitrogen fractions (total and non-casein nitrogen (NCN)) were determined by Kjeldahl procedures (FIL-IDF, 1993). Caseins were separated to determine NCN by precipitation with a buffer acetic/acetate to pH = 4.6, and subsequently the nitrogen content is determined in the filtered soluble fraction.

Statistical Analysis. Results were analyzed by means of a multiple range analysis, using the LSD test with a 95% confidence interval for the comparison of the test means.

RESULTS AND DISCUSSION

Calculation of the Correction Factors Used for the Partition of Salt Data. To convert mineral concentration in the aqueous phases to mineral concentration in the skim milk, some corrections were necessary. Corrections, to offset the excluded-volume effect of cosolutes, were determined for the UC and RC procedures. The concentrations of the constituents in the soluble fractions, determined as mg/100 g of soluble fraction, were converted to mg/100 mg of milk by multiplying by the factor (weight of water in 100 g milk/weight of water in 100 g soluble fraction) proposed by Davies and White (1960). The corrected result were used for comparison with the total concentrations in the milk. The correction factors were 0.979 and 0.982 for RC and UC, respectively.

Moreover, in rennet whey and supernatant fluid, the concentration of soluble salts (Na and K) was corrected for casein-bound water on the basis of the lactose content in the milk and soluble fractions, assuming that the trapped fluid in the pellet had the same composition as the aqueous phase. Lactose contents were 5.14, 5.21, and 5.30%, respectively, for milk and for the aqueous phases obtained by clotting and ultracentrifugation. Correction factors were 0.987 (rennet coagulation) and 0.970 (ultracentrifugation). This correction was only used for Na and K because the factor becomes important when the concentrations of colloidal salts approach zero (Holt, 1982).

In dialysis, the lactose content of the dialysate was 4.08%. Since the milk was dialyzed against a large volume of distilled water Na and K values were only corrected for dilution occurring during dialysis.

Distribution of Calcium, Magnesium, Phosphorus, and Citrate. Table 1 shows calcium (total and ionic), magnesium, phosphorus, and citrate levels in the milk and in the soluble fractions obtained by each procedure. The results showed the separation process to be decisive, giving rise to considerable differences in soluble calcium content depending on the fractioning procedure employed.

Dialysis gave the highest soluble calcium levels and rennet whey and supernatant contained 10% less Ca. These differences may be due to the nature of binding of these minerals to the casein micelles (Sindhu and Roy, 1976a). Dialysis may induce diffusion of ionic minerals from the colloidal minerals and protein to the soluble fraction. This would result in a gradual shift of Ca, in ionic form, from colloidal fraction to soluble state. The methods which apply centrifugal force, on the other hand, will favor passage of minerals in molecular form from supernatant to colloidal structures, thus causing a proportionately greater drop in the concentration of these structures in the soluble fraction. In the present study, Ca^{2+} concentrations of about 224 mg/kg (58% of soluble Ca) were found in the diffusate, which could explain the higher levels of total soluble Ca there. In the UC and RC supernatants, ionic Ca levels were considerably lower (86 and 72 mg/kg, respectively). These last values represent approximately 30% of soluble Ca. The differences in the Ca^{2+} levels between soluble fractions in UC and RC (Table 1) could be attributed to that a part of this ionic specie is participating during the milk coagulation process linking casein micelles.

The dialysis procedure assayed, one volume of milk versus 10 of water, may give rise to changes in the mineral fraction comparable to those caused by dilution of milk with water. Although Brulé and Fauquant (1981) found solubilization slow, depending on temperature during the first 5 h after diluting ultrafiltered milk retentates with water, dilution of milk with water for longer periods (24 h at 4 °C) allowed a greater degree of solubilization (Singh *et al*., 1989). Sindhu and Roy (1973), fractioning by dialysis over 12 h, reported soluble Ca levels to be much the same as when phases were separated by ultracentrifugation (Sindhu and Roy, 1976a); however, where dialysis was prolonged to 24 h,

Table 2. Mean Values and Standard Deviations of Microelements (Zinc and Manganese) in Skim Milk and Soluble Fractions Separated by Clotting (Rennet Whey), Ultracentrifuging (Supernatant), and Dialysis (Diffusate)

name of constituent	total in milk μg/kg	rennet whey		supernatant		diffusate	
		μg/kg	%	μg/kg	%	μg/kg	%
zinc	4040 ± 60	118[a] ± 8	2.9	228[b] ± 9	5.6	693[c] ± 59	17.3
manganese	33.3 ± 1.9	1.78[a] ± 0.23	5.4	1.74[a] ± 0.05	5.1		

[a–c] Different letters in the same row indicate significant differences ($p \leq 0.05$). [d] Concentrations are shown in μg/kg and in percentages of total content in milk. The number of samples analyzed by each fractionation procedure was 12.

soluble Ca levels were 50% higher. Again, the temperature (5 °C) at which dialysis occurs favors solubilization of Ca from the colloidal phase.

The same tendency toward increased soluble mineral content on fractioning by dialysis applies, though to a lesser degree, to concentrations of magnesium and phosphorus. Singh *et al.* (1989) found that dilution of milk with water in excess allowed an increase in the concentrations of nonsedimentable Ca, but there was no significant change in nonsedimentable phosphorus.

Regarding citrate content, dialysis gave lower values than were found with the other two procedures. The explanation for this result could be that dialysis took place at 5 °C whereas the operating temperature for the other two procedures are 20 and 37 °C (Sindhu and Roy, 1976a). This increase in temperature might shift the citrate from colloidal to the soluble form.

Irrespective of the simplicity of the separation procedures used, for the purpose of method selection, their analytical validity is what is important to consider. In general, ultracentrifugation and rennet coagulation offer better precision than dialysis for the four chosen analytes. Between the first two, ultracentrifugation provided lower variation coefficients (3.4, 1.7, and 2.4%) than rennet coagulation (5.6, 3.4, and 3.6%) for Ca, Mg, and P, respectively. Statistical analysis (Table 1) showed that dialysis was significantly different from the others two separation procedures. Between rennet whey and supernatant fluid only ionic calcium levels were significantly different as discussed above.

Partition of Microelements. Within the trace elements occurring in cow's milk, those analyzed here (Zn and Mn) are found largely in the skim milk fraction, with only a small proportion in the lipid fraction (Lönnerdal *et al.*, 1981; Brulé and Fauquant, 1982; Blakeborough *et al.*, 1983; Singh *et al.*, 1989).

Analyses of Zn content differed, according to the separation procedure followed, although Zn was generally primarily retained in the colloidal fractions. This is not surprising considering that cow's milk zinc is bound almost exclusively to high molecular weight compounds, and colloidal calcium phosphate is the major zinc-binding ligand in all milk except human milk (Brulé and Fauquant, 1982; Singh *et al.*, 1989; Kiely *et al.*, 1992).

The percentages of Zn found in the soluble fraction were 2.9, 5.6, and 17.3% in RC, UC, and D, respectively. The differences among the three procedures may be explained by the conditions in which the various assays were performed. The values for ultracentrifugation and rennet coagulation may be attributed to the differences in in-process temperature, 20 °C as opposed to 37 °C. The strong affinity of Zn for the precipitating calcium phosphate was demonstrated by the finding that over 90% of Zn milk aqueous phase coprecipitated, even when only a small amount (5%) of phosphorus was removed from solution by raising the temperature of the milk soluble fraction from 20 to 40 °C, which causes some phase separation of calcium phosphate (McGann *et al.*, 1983). Significant differences were found (Table 2) for Zn levels between RC and UC. The explanation for the different levels of Zn found in separation by RC as opposed by UC (Table 1) may lie in the lower concentrations of Ca and P (2 and 4%, respectively) found in the RC compared to UC process.

The high Zn content in the diffusate (17.3%) may be a consequence of the effect referred to in the preceding section to explain Ca levels. Singh *et al.* (1989) reported concentrations in the region of 20% in the diffusate after dialyzing milk against a large volume of water for 24 h.

There is little information on the localization of Mn in cow's milk. In the present study (Table 2), Mn levels in ultracentrifugation supernatant and rennet whey were similar (5.46 and 5.22%), although it should be noted that analytical precision was considerably greater with ultracentrifugation. These percentages are consistent with the literature. In separation by ultracentrifugation, Lönnerdal *et al.* (1983) reported that around 5% of Mn was found in the soluble fraction, linked to low molecular weight ligands. Again using ultracentrifugation, Brulé and Fauquant (1982) found the bulk of Mn (95%) in the pellet. In dialysis, Mn in the diffusate was not measured because the dilution factor was too high and the Mn concentration was too low for reliable measurement.

Analysis of Other Compounds. Figure 1 shows Na and K present in milk and in the soluble fraction of skim milk obtained by the experimental procedures. The percentages with respect to total content in the fractions obtained by RC and UC were 94.9 and 95.5% for Na and 93.4 and 91.0% for K, respectively. These data are close to those reported previously by Davies and White (1960) in skimmed cow's milk (approximately 5% for Na and 6% for K in the dispersed phase) using ultrafiltration and dialysis of 10 volumes of milk to 1 of water.

A number of more recent studies on distribution of salts between milk soluble and colloidal phases give no data for these minerals (Sindhu and Roy, 1973; O'Connor and Fox, 1977; Polychroniadou and Vafopoulou, 1986). Other authors consider that Na and K occur almost entirely as free ions (Renner *et al.*, 1989). The present findings indicate that the loss of Na and K in the supernatant confirms the presence of protein-bound sodium and potassium in milk.

Where separation was performed by dialysis, levels of soluble Na and K were lower (87 and 82%, respectively) than when ultracentrifugation and clotting were used. The explanation of these findings may be connected with differences in the separation procedures, given that in the present case the water to milk ratio was 10:1. Singh *et al.* (1989) found that in this type of dialysis the quantity of Na remaining in the milk after 24 h was even higher, reaching more than one third of the total content.

With regard to the nitrogenated fractions, leaving aside the diffusate, which exhibited very low levels of nitrogen (0.013%), nitrogen levels in the rennet whey

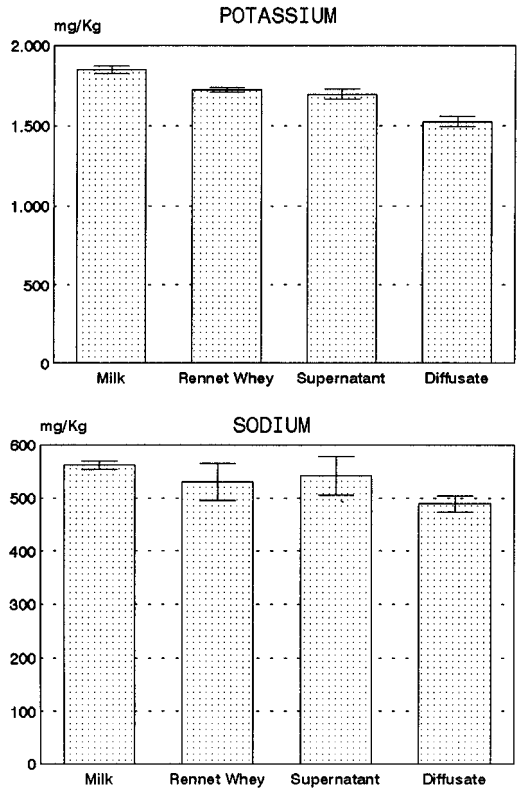

Figure 1. Mean values of Na and K contents (mg/kg) in skim milk and the soluble fractions obtained by rennet coagulation (rennet whey), ultracentrifugation (supernatant), and dialysis (diffusate) against a large volume of water (1:10). Values are means of 12 determinations with standard deviation indicated by vertical bars.

and the UC supernatant were comparable to those in milk (0.124 and 0.125%, respectively). On the other hand, in the fraction obtained by ultracentrifugation, the proportion of non casein nitrogen was slightly lower, 82.4 against 97.6% of total nitrogen, probably due to a higher proportion of soluble β-casein in this fraction.

CONCLUSIONS

The differences in mineral concentrations in soluble fractions obtained, by means of the procedures assayed, reflect the complexity of the mechanisms governing salt balance in milk. It is therefore not easy to recommend any one of these procedures in preference to the others as a routine method. However, given its rapidity and greater precision in dealing with most of the elements assayed here, ultracentrifugation would seem to be the most suitable procedure of the three. Rennet clotting compares less favorably in that higher temperatures are required for milk coagulation, and it is less precise.

Although the simplest of the three procedures, the results of dialysis of small volumes of milk against large volumes of water are more difficult to interpret. At all events, however, any of these procedures is acceptable for studies in series provided that fractioning conditions remain strictly the same from one assay to the next so as to allow interpretation of the influence of the various physicochemical factors on the saline balance.

ACKNOWLEDGMENT

We thank G. Guerrero for technical assistance in the spectrophotometric measurements, Dr. C. Romero for the carbohydrate analysis, and Dr. A. Olano for helpful discussion of this work.

LITERATURE CITED

Blakeborough, P.; Salter, D. N.; Gurr, M. I. Zinc binding in cow's milk and human milk. *Biochem. J.* **1983**, *209*, 505−512.

Boehringer. UV-Method for the determination of citric acid in foodstuffs and other materials. Cat. No. 139076, 1992, Boehringer, Mannheim, Germany.

Brooks, I. B.; Luster, G. A.; Easterly, D. G. A procedure for the rapid determination of the major cations in milk by atomic absorption spectrophotometry. *At. Absorpt. Newsl.* **1970**, *9*, 93−94.

Brulé, G.; Fauquant, J. Mineral balance in skim-milk and milk retentate: effect of physicochemical characteristics of the aqueous phase. *J. Dairy Res.* **1981**, *48*, 91−97.

Brulé, G.; Fauquant, J. Interactions des protéines du lait et des oligoéléments. *Lait* **1982**, *62*, 323−331.

Davies, D. T.; White, J. C. D. The use of ultrafiltration and dialysis in isolating the aqueous phase of milk and in determining the partition of milk constituents between the aqueous and disperse phases. *J. Dairy Res.* **1960**, *27*, 171−190.

De la Fuente, M. A.; Juárez, M. Rapid determination of calcium, magnesium, sodium and potassium in milk by flame atomic spectrometry after microwave oven digestion. *Analyst* **1995a**, *120*, 107−111.

De la Fuente, M. A.; Juárez, M. Determination of phosphorus in dairy products by sample wet digestion in a microwave oven. *Anal. Chim. Acta* **1995b**, *309*, 355−359.

De la Fuente, M. A.; Guerrero, G.; Juárez, M. Manganese and zinc analysis in milk digested in microwave oven by platform graphite furnace atomic absorption spectrometry. *J. Agric. Food Chem.* **1995**, *43*, 2406−2410.

FIL-IDF, International Dairy Federation. 1993, Standard 20B.

Geerts, J. P.; Bekhof, J. J.; Scherjon, J. W. Determination of calcium ion activities in milk with an ion-selective electrode. A linear relationship between the logarithm of time and the recovery of the calcium ion activity after heat treatment. *Neth. Milk Dairy J.* **1983**, *37*, 197−211.

Holt, C. Inorganic constituents of milk. III The colloidal calcium phosphate of cow's milk. *J. Dairy Res.* **1982**, *49*, 29−38.

Kiely, L. J.; Flynn, A.; Fox, P. F. Association of zinc with colloidal calcium phosphate in bovine and non-bovine milks. *J. Dairy Sci.* **1992**, *75 (Suppl. 1)*, 105.

Lönnerdal, B.; Keen, C. L.; Hurley, L. S. Iron, copper, zinc and manganese in milk. *Annu. Rev. Nutr.* **1981**, *1*, 149−174.

Lönnerdal, B.; Keen, C. L.; Hurley, L. S. Manganese binding in human milk and cow's milk-an effect on bioavailability. *Fed. Proc.* **1983**, *42*, 926.

McGann, T. C. A.; Kearney, R. D.; Buchheim, W. Zinc phosphocitrate−casein complexes in bovine milk systems. *Kiel. Milchwirtsch. Forschungber.* **1983**, *35*, 409−411.

O'Connor, P.; Fox, P. F. The proteins and salts of some non-bovine milks. *J. Dairy Res.* **1977**, *44*, 607−609.

Papajová, H. Contribution to the analytics of calcium forms soluble in milk. *Zborník Prác Vyskumného Ústavu Mliekárskeho v Žiline* **1982**, *6*, 231−243.

Polychroniadou, A.; Vafopoulou, A. Salt distribution between the colloidal and soluble phases ewe's milk. *J. Dairy Res.* **1986**, *53*, 353−358.

Rajput, Y. S.; Bhavadasan, M. K.; Ganguli, N. C. Changes in the chemical status of calcium in casein micelles with the pH of milk. *Milchwissenschaft* **1983**, *38*, 211−213.

Renner, E.; Schaafsma, G.; Scott, K. J. Micronutrients in milk. In *Micronutrients in milk and milk-based food products*; Renner, E., Ed.; Elsevier Applied Science Publishers Ltd.: London, UK, 1989.

Reykdal, O.; Lee, K. Soluble, dialyzable and ionic calcium in raw and processed skim milk, whole milk and spinach. *J. Food Sci.* **1991**, *56*, 864−866.

Richmond, M. L.; Harte, B. R.; Gray, J. I.; Stine, C. M. Determination of sugars in yogurt and microbiological media by High Performance Liquid Chromatography during processing and subsequent storage. *J. Dairy Sci.* **1987**, *70*, 1140−1147.

Sindhu, J. S.; Roy, N. K. Partitioning of buffalo milk minerals. Part I: Study through dialysis. *Milchwissenschaft* **1973**, *28*, 573−575.

Sindhu, J. S.; Roy, N. K. Partitioning of buffalo milk minerals. Part II: Study through ultracentrifugation. *Milchwissenschaft* **1976a**, *31*, 479−483.

Sindhu, J. S.; Roy, N. K. Partitioning of buffalo milk minerals. Part III: Study through rennet coagulation. *Milchwissenschaft* **1976b**, *31*, 671−673.

Singh, H.; Flynn, A.; Fox, P. F. Zinc binding in bovine milk. *J. Dairy Res.* **1989**, *56*, 249−263.

Received for review October 20, 1995. Revised manuscript received April 29, 1996. Accepted May 8, 1996.[⊗] We acknowledge the financial support for this research project (ALI 93-0005-CP) furnished by the Comisión Interministerial de Ciencia y Tecnología.

JF9506949

[⊗] Abstract published in *Advance ACS Abstracts,* July 15, 1996.