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The use of sedimentation field flow fractionation and photon correlation spectroscopy in the characterization of casein micelles

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Sedimentation Field Flow Fractionation (SdFFF) was combined with Photon Correlation Spectroscopy (PCS), to characterize changes in the structure of the colloidal particles of reconstituted skim milk of diameter >50 nm (aggregates of casein and calcium phosphate known as casein micelles) with the changes in partitioning (with the addition of salt) of calcium (Ca), inorganic phosphate (P_i) and casein between the serum and colloidal phases of the milk. The number weighted particle size distributions are determined. These are well represented by a log-normal distribution. Methods are presented for estimating the relative contributions of scattering and absorbance to the SdFFF detector signal and for taking both into account when analysing SdFFF data. The values found for the effective density of the casein micelles were in good agreement with the literature and ranged from (1·06–1·08 g cm^{-3}) according to the composition of micelles. The changes in the scattering intensity as determined by PCS correlated with the changes in the particle composition. Although the concentrations of colloidal calcium phosphate (CCP) (1·1–3·5 g/kg milk) and micellar casein (18·1–27·2 g/kg milk) varied considerably only small changes in the size distribution of particles >50 nm diameter were observed except for milk to which 30 mmol P_i+10 mmol Ca/kg milk had been added where the particle size distribution shows a swelling of the particles consistent with a lower than expected value for the particle density. These observations suggest that the micelles have the ability to both lose (depleted micelles) and accommodate (enriched micelles) more casein, calcium and inorganic phosphate in their interior, thus confirming the model of the micelles which postulates an open structure allowing freedom of movement of casein and small ions.

Keywords: Field flow fractionation, photon correlation spectroscopy, casein micelles, mineral composition, particle size distribution, particle density.

The aggregates of casein and calcium phosphate in milk, known as casein micelles contain more than 95% of the milk caseins together with Ca and P_i. These particles are roughly spherical in shape with a diameter ranging from about 10–300 nm (Lin et al. 1971; Schmidt et al. 1973; Holt et al. 1978; Walstra & Jenness, 1984; Wade, 1996). The particles possess an open structure with the space within the structure filled by milk serum, permitting easy access by solute molecules (Walstra & Jenness, 1984). The reported hydrated density of the particles ranges from 1·06–1·11 g cm^{-3} (Kirchmeier, 1973; Walstra & Jenness, 1984). The particles are highly voluminous with a voluminosity of approximately 4 ml g^{-1} of casein and contain approximately 3·3 g water per gram of casein (Walstra & Jenness, 1984). This water is the major component of the particles and is a solution in Donnan equilibrium with the external serum. The composition of the serum phase affects the composition of the colloidal phase. Addition of salts, calcium chelating agents, heat treatment and changing the pH all result in the redistribution of Ca, P_i and casein between the particles and the serum. These changes are reversible and have limited effect on the particle diameter as determined by PCS. There are limits to the reversibility of the changes induced in the micelles. It has been shown that consistency of particle size is a measure of particle integrity and a reliable indicator of the reversibility of changes in the distribution of components between the particles and the serum (Udabage et al. 2000).

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SdFFF, a sub-technique of Field Flow Fractionation (FFF) is an established elution based separation technique, the theory of which is well documented (Giddings, 1973; Caldwell, 1988; Mozersky et al. 1988; Hansen et al. 1989; Kesner & Giddings, 1989; Beckett & Hart, 1993; Giddings, 1993, 1995). Past applications of SdFFF to milk related systems include the study of the adsorption of milk proteins on polystyrene latexes (Caldwell et al. 1992), the examination of the aggregation of proteins isolated from non-fat dry milk powders (Mozersky et al. 1991a), the study of the effects of sucrose and lactose on the sizes of synthetic micelles reconstituted from bovine caseins (Mozersky et al. 1991b) a preliminary examination of the particle size distribution in reconstituted skim milk (Udabage et al. 1997; McKinnon et al. 1999) and an examination of the particle size distribution in skim milk and skim milk that has been heated to 85 °C for 15 min (de Kruif, 1998). In SdFFF all of the parameters relating the buoyant mass of the eluting particles to the experimentally determined retention volume are defined by the geometry of the apparatus or the experimental procedure and are independently measurable. The major factor limiting the application of SdFFF to the determination of the particle size distribution is the requirement that the relative densities of the particles and the medium in which they are dispersed be known.

For porous entities such as the casein micelles of milk the values of properties of the particle such as size, composition and density depend on the way in which the particle is defined. The solution to this problem used in this work is to define the ''particle'' as the hydrodynamic unit, the equivalent diameter (a) of which is related to the particle diffusion coefficient (D), the temperature (T) and the viscosity (η) of the medium by the Boltzmann equation.

$$a = \frac{kT}{3\pi\eta D} \quad (1)$$

The particle size distribution as determined by SdFFF is then directly related to the intensity weighted diffusion coefficient ($\langle D \rangle$) and the corresponding effective diameter as determined by photon correlation spectroscopy. This provides the extra information necessary for the absolute determination of both the particle size distribution and particle density.

The object of this work is to use SdFFF and photon correlation spectroscopy to determine the effect of composition on the size distribution and density of the particles of skim milk greater than 50 nm in diameter.

Appropriate interpretation of the quantity determined by the SdFFF detector is important. In earlier work (McKinnon et al. 1999) it was assumed that variation in the detector signal was due to variation in particle absorbance and that the SdFFF data represented the mass weighted particle size distribution. In this work we present methods for estimating the relative contributions of scattering and absorbance to the detector signal and for taking these into account when determining the particle size distribution from SdFFF data.

Materials and Methods

Materials

Skim milk powder (SMP) was the same batch as used in previous work (Udabage et al. 2000, 2001).

Six different milk solutions, containing various levels of added calcium, phosphate or EDTA, were prepared as previously reported (Udabage et al. 2000, 2001).

Simulated Milk Ultrafiltrate (SMUF) was prepared according to Jenness & Koops (1962) with lactose (50 g/l), and the solution filtered through a plain white millipore 0·22 μm (GS) filter (Selby-Biolab, Clayton, Victoria 3168, Australia) to remove any particulate matter.

SdFFF channel dimensions and run conditions

A stainless steel ribbon like channel of thickness 0·0283 cm, breadth 2·00 cm, length 89·1 cm, void volume 4·91 ml and centrifuge radius 15·1 cm with a channel flow rate of 2·03 ± 0·01 ml/min (Bluebird consultancy, Computing flow meter model 100) was used throughout the runs. A Milton Roy Constametric (ii) metering pump operated at a pressure of 1000–2000 psi was used to deliver the carrier fluid. The pre channel dead volume was 0·29 ml and the post channel dead volume was 0·34 ml.

SdFFF measurements were carried out using SMUF with 50 g lactose/l as the carrier fluid. Milk ultrafiltrate (UF) could not be used as the carrier fluid due to its high absorbance at the detector wavelength of 254 nm. Samples were diluted with the carrier fluid immediately prior to injection (30 μl) into the SdFFF channel to avoid overloading effects and consequent adhesion to the walls of the channel. All runs were performed at least in duplicate.

Samples were injected into the stationary channel with the carrier flow on. The flow was turned off (by pass channel) at a fixed time of 15 s. The centrifugal field was turned on and the particles were allowed to equilibrate across the channel. At the end of the relaxation period the carrier flow was turned on and a centrifugal field applied using the protocol of Williams & Giddings (1987). The optimum conditions were found to be a relaxation period of 10 min, an initial centrifugal field of 1600 $\boldsymbol{g}$ and decay parameters ($t_1 = 8$ min and $t_a = -64$ min). There was no ability to control the temperature of runs, which were therefore carried out at room temperature (22 ± 2 °C). Once separated, the eluted sample was detected using a UV–Visible detector (LDC/Milton Roy spectro Monitor D) at 254 nm and sensitivity of 0·02. The signal was recorded and processed by computer using in-house software to generate fractograms. The fractograms (A_m, the measured UV detector response versus V the elution volume) generated were analysed using FFF analysis software (FFFractionation Inc., Salt Lake City, UT 84112, USA). This software uses the

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known difference in density between the particles and the medium ($\rho_p - \rho_m$) to determine from the fractogram the differential detector response distribution function $A(a)$ such that the detector response due to particles with diameters in the range (a to $a+\delta a$) can be represented as $A(a)\delta a$.

$$A(a) \propto \frac{dA_{measured}}{dV} \frac{\partial V}{\partial a(V)} \quad (2)$$

Calculation of the particle size distribution from SdFFF

The SdFFF UV detector response (optical density $A(a)$ at 254 nm) at a given elution volume (thereby at a given particle diameter a) is proportional to the number of particles eluting per unit volume ($N(a)$) and the scattering (C_s) and absorption (C_a) cross-sections of the particles.

$$A(a) = \frac{N(a)}{\ln 10} [C_s + C_a] \ell \quad (3)$$

The cross-sections are functions of both the wavelength of the light source and of the particle size. The absorption cross-section is assumed to be proportional to the mass of absorbing species in the particle so that we can write

$$C_a = \varepsilon_a a^3 \quad (4)$$

where ε_a is a proportionality constant assumed to be independent of particle size.

Consideration of the total light scattered by a particle gives, for the scattering cross section the following (Hunter, 1993).

$$C_s = a^6 \frac{\pi^5 n_0^4}{4\lambda^4} \left(\frac{(n^2-1)}{(n^2+2)}\right)^2 \int_0^\pi (1+\cos^2\theta)\sin\theta\, P(\theta) d\theta \quad (5)$$

For other than very small particles ($P(\theta)=1$), $P(\theta)$ is a function of particle size.

Writing equation (5) in the form

$$C_s = \varepsilon_s a^6 \int_0^\pi (1+\cos^2\theta)\sin\theta\, P(\theta) d\theta \quad (6)$$

equation (3) becomes

$$A(a) = \frac{N(a)}{\ln 10} \left[\varepsilon_a a^3 + \varepsilon_s a^6 \int_0^\pi (1+\cos^2\theta)\sin\theta\, P(\theta) d\theta\right] \ell \quad (7)$$

The scattering factor $P(\theta)$ is evaluated using the Raleigh, Gans Debye approximation:

$$P(\theta) = \left(3 \frac{\sin(qr) - (qr)\cos(qr)}{(qr)^3}\right)^2$$

where the product of the scattering vector and particle radius

$$qr = \frac{2\pi n_0}{\lambda} \sin(\theta/2) a$$

In order to obtain the contribution of absorbance and the scattering by casein micelles to the detector response, the following analyses were performed.

a) The apparent absorbance of the milk ($A_{app.\ milk}$) was measured at 254 nm using a UV–Visible spectrophotometer (Shimadzu UV-265). This is the sum of the contributions to scattering and absorbance from all sources.
b) Next the apparent absorbance of milk with 50 mmol EDTA/kg milk was measured. The scattering from this solution is negligible (~2 %).
c) The apparent extinction coefficient for the proteins present in solution was calculated assuming negligible absorbance by EDTA and the other non protein components of the serum and similar extinction coefficients for the caseins and whey proteins in solution.
d) The absorbance due to the casein micelles ($A_{micelles}$) was then calculated assuming that the extinction coefficient of the caseins in the micelles is approximately that of the caseins in solution and that absorption by the other micellar components is negligible.
e) The ratio

$$\frac{\text{micellar absorption}}{\text{micellar scattering} + \text{micellar absorption}}$$

is then calculated as

$$\frac{A_{micelles}}{A_{app.\ milk} - A_{micelles}}$$

Equations (4) and (6) together with an approximation to the particle size distribution $N(a)$ give

$$\frac{\text{micellar absorption}}{\text{micellar scatterting}} = \frac{\varepsilon_a}{\varepsilon_s} \frac{\int_a N(a) a^3 da}{\int_a N(a) a^6 \left[\int_0^\pi (1+\cos^2\theta)\sin\theta\, P(\theta) d\theta\right] da}$$

enabling the determination of $\frac{\varepsilon_a}{\varepsilon_s}$.

Equation (7) is then used to recalculate the particle size distribution N_a and the process repeated until convergence is obtained.

Photon correlation spectroscopy

The intensity weighted diffusion coefficient ($\langle D \rangle$) and the effective diameter of the particles at 25·0±0·1 °C were determined by photon correlation spectroscopy carried out

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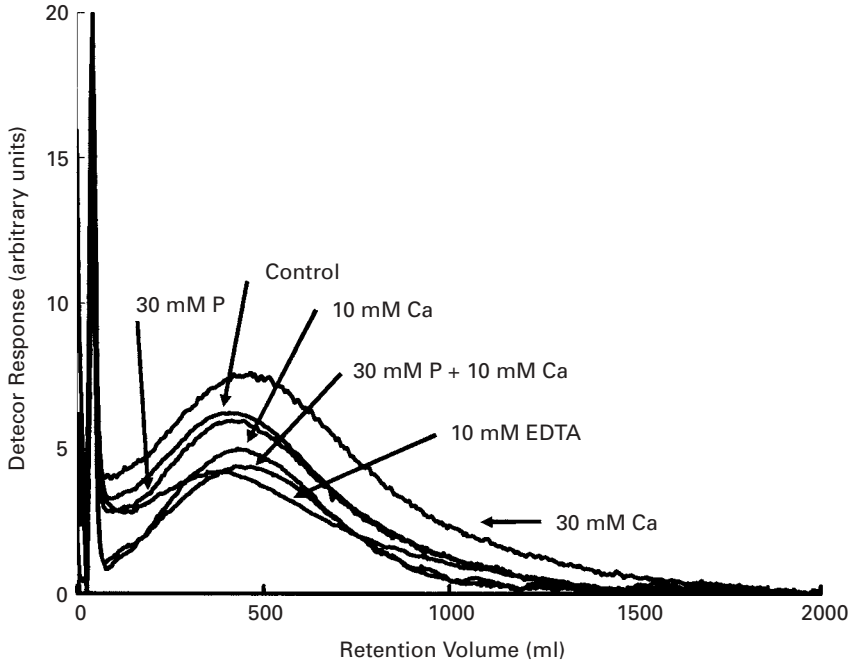

Fig. 1. Non-normalised fractograms of milk suspensions with and without additives as determined by Sedimentation Field Flow Fractionation (mM = mmole additive/kg milk, *y*-axis arbitrary units).

on suitably diluted samples in a Brookhaven Zeta Plus fitted with a BI 90 correlator board (Brookhaven Instrument Corporation, Holtsville, New York 11742, USA), using a laser of 675 nm and a scattering angle of 90°. The data was analysed using the method of cumulants (Brown & Pusey, 1975). SMUF was used as the diluent. The density (ρ) of the SMUF was determined pyknometrically, the viscosity (η) using an Ubbelohde viscometer and the refractive index (n_0) using an Abbe refractometer (Nippon Optical works Co. Ltd., Tokyo, Japan). The values at 20 °C and 25 °C respectively were:

$\rho = 1{\cdot}023, 1{\cdot}022 \pm 0{\cdot}001\ \mathrm{g\,cm^{-3}}$,
$\eta = 1{\cdot}165, 1{\cdot}029 \pm 0{\cdot}003\ \mathrm{mPa\,s}$,
$n_0 = 1{\cdot}341 \pm 0{\cdot}001$.

Absolute determination of particle size distribution and particle density

Equations (1) and (6) together with the estimate of the particle size distribution $N(a)$ calculated from SdFFF are used to calculate the intensity weighted average diffusion coefficient and the effective diameter of the particles. The latter is given by

$$a_{\mathrm{eff}} = \frac{\int_0^\infty a^6 N(a)\, P(90°)\mathrm{d}a}{\int_0^\infty a^5 N(a)\, P(90°)\mathrm{d}a} \qquad (8)$$

The result of this calculation is compared with the value obtained from the PCS measurements. A new estimate of the particle density is made and the whole analysis repeated until the calculated and experimental effective diameters agree.

Results

SdFFF

The fractograms showing the apparent absorbance (the detector response) of the eluent as it leaves the channel as a function of eluent volume for the six milks studied are shown in Fig. 1. Although the fractograms are not markedly different from each other, there was a small increase in the retention volume at peak maximum in samples with added $CaCl_2$ and with addition of 30 mmol P_i+10 mmol Ca/kg milk, a small decrease on addition of 10 mmol EDTA/kg milk and no change on addition of 30 mmol P_i/kg milk relative to the control milk suspension.

The densities of casein micelles as determined by PCS and SdFFF

The values of particle density required to be used in the determination of the particle size distribution $N(a)$ from the SdFFF fractograms in order that the calculated effective diameter (equation 8) match that previously determined by PCS (Udabage et al. 2000) are shown in Table 1 together with the values of the number average particle diameter. The experimental values for the differential number distribution of the particles as a function of particle diameter were able to be represented almost within experimental error by a log-normal distribution. The values of the

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Table 1. The calculated micellar densities and number average diameters using the combined data from photon correlation spectroscopy and particle size distribution of sedimentation field flow fractionation and the parameters obtained from fitting of the experimental. *Number distribution* of particle size to a *ln-normal* distribution function

Additions to milk (mmol/kg milk)	micellar densities (g cm^{-3})	mean diameter (nm)	ln-normal distribution	
			a_0 (nm)	β
Control (none)	1·066	121	112·0	0·340
10 Ca	1·079	121	113·4	0·364
30 Ca	1·072	119	109·5	0·356
30 P_i	1·066	127	113·7	0·353
30 P_i+10 Ca	1·059	140	132·5	0·359
10 EDTA	1·059	124	116·5	0·332

Pooled SD of micellar densities = 0·004
Equation for the normalised ln-normal distribution function

$$N(a)/(\mathrm{nm}^{-1}) = 1/(a/(\mathrm{nm})\beta\sqrt{(2\pi)})\ \exp\{-0{\cdot}5(\ln(a/a_0)/\beta)^2\}$$

the parameter a_0 = the median particle diameter
Pooled SD (a_0) = 0·5 nm
Pooled SD (β) = 0·01

parameters (a_0, β) for each of the systems obtained by fitting the distribution function

$$N(a) = \frac{1}{\sqrt{2\pi}\,a\beta}\ \mathrm{e}^{-0{\cdot}5\left(\frac{\ln(a/a_0)}{\beta}\right)^2}$$

(where $N(a)\delta$ = fraction of particles with diameters between a and $(a+\delta)$) are listed in Table 1. (The parameter a_0 corresponds to the median particle diameter and β is the standard deviation of ln(a) about ln(a_0) when $N(a)$ plotted as a function of ln(a).) The full particle size distributions are shown in Fig. 2.

In the calculation of the particle size distribution function from the experimental data it was found that for the control milk 8% of the apparent absorbance of the particles was due to absorption and 92% scattering and that for milk containing 10 mmol EDTA/kg milk the contributions of absorption and scattering to the apparent particle absorption were 7% and 93% respectively. Also it should be noted that in calculating the effective diameter from the particle size distribution the smallest 90% of the particles do not contribute significantly to the integrals in equation (8). Thus the density determined is that of the largest particles.

Discussion

The particle size distributions are very broad with, for the control milk, β = 0·34. A consequence is that the value for the median diameter based on the number distribution (111 nm) is approximately half that of the effective diameter (198 nm) determined by PCS and the median diameter based on the distribution of scattering intensity with particle size (225 nm). As previously noted the Number distribution could be represented almost to within experimental error by a ln-normal distribution function, however, the experimental intensity weighted distribution (not shown) had a larger tail than that of a ln-normal distribution.

For convenience in discussing the effect of milk composition on the properties of the micelles, earlier data concerning the effect of milk composition on the amounts of micellar casein and colloidal calcium phosphate (Ca^{2+}+ PO_4^{3-}) within the micellar structure (Udabage et al. 2001) and the effective diameters and scattering intensities as determined by PCS (Udabage et al. 2000) are given in Table 2.

The differences between the retention volume at peak maximum in the fractograms (Fig. 1) could be due to changes in the particle size distribution, changes in particle density or both. However the values of the median diameters (Table 1) and particle size distributions (Fig. 2) demonstrate that, except for the milk with 30 mmol P_i+10 mmol Ca/kg milk there was little variation in the particle size distribution over a wide range of CCP and casein content. Thus it is clear that whilst there is some relaxation of the micellar structure with change in micellar composition, with the exception of the milk with 30 mmol P_i+10 mmol Ca/kg milk the change is small. The ratio of the scattering intensity with the addition of 10 mmol EDTA/kg milk compared to the control milk (∼0·53) approximates to the square of the ratio of the scattering mass (micellar casein+CCP) (∼0·48) (Table 2). This indicates a negligible decrease in the number of the larger casein micelles in line with the observed constancy of the particle size distribution.

The changes in the effective diameter (as determined by PCS) are, with the exception of the milk with 30 mmol P_i+10 mmol Ca/kg milk, small, and related qualitatively to the concentration of CCP and micellar casein in the micelle structure (Table 2). In PCS, the effective diameter is very strongly weighted towards the larger sizes with the degree of the weighting depending on the details of the particle size distribution. Thus, in order to show a change in the effective particle diameter, an actual change in the size of the larger particles and/or a very large change in the fraction of smaller particles must occur.

The small effect on the particle size distribution of quite large changes in micellar casein Ca^{2+} and P_i (Table 2) is consistent with the micelles being highly hydrated sponge like particles having an open, porous structure. On the removal or incorporation of a considerable fraction of CCP and casein the framework of a particle is preserved. It is the porosity and densities of the particles that change rather than their number or their size. The concept of an increase in the porosity of casein micelles with added EDTA has previously been suggested by Wade (1996) on the basis of electroacoustics. The milk with 30 mmol P_i+10 mmol Ca/kg milk did not conform to the above patterns. Here a quite substantial increase in CCP and micellar casein was accompanied by a uniform swelling of the particles as indicated by the particle size distribution (Fig. 2), the increase in effective diameter (Table 2) and the small decrease in micellar density (Table 1).

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Table 2. Effect of Additives on the Composition, Effective Diameter and Scattering Intensity of Casein Micelles (Udabage et al. 2000, 2001)

Additions to Milk (mmol/kg milk)	Casein in Micelle† (g/kg milk)	CCP† (g/kg milk)	Effective Diameter‡ (nm)	Scattering Intensity (kC/s)
Control (none)	25·72	2·00	198	290 ± 11
10 Ca	27·21	2·63	193	317 ± 8
30 Ca	26·99	3·44	202	292 ± 5
30 P_i	26·11	2·53	198	277 ± 8
30 P_i+10 Ca	27·04	3·46	209	306 ± 9
10 EDTA	18·07	1·10	199	153 ± 5

† Data from Udabage et al. 2001

‡ Data from Udabage et al. 2000. Note that the effective diameter of milk+10 mmol $CaCl_2$/kg milk given in that reference was in error (see erratum below)

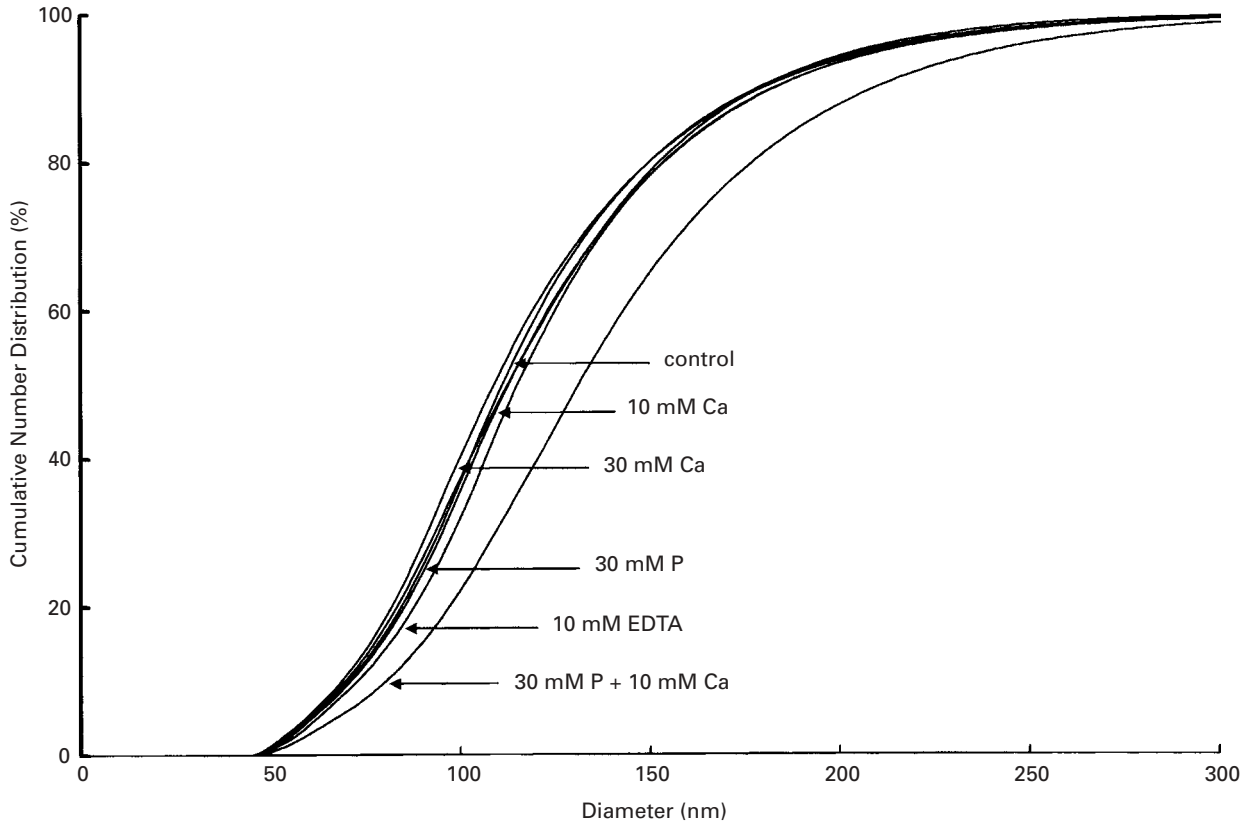

Fig. 2. The cumulant number distribution of particles >50 nm diameter for the milk suspensions depicted in Fig. 1.

Particle diameters were calculated using the values for the particle densities listed in Table 1.

The particle size distributions reported by de Kruif (1998) are intensity weighted distributions and hence are not readily compared with those of Fig. 2 and Table 1. The value of the polydispersity parameter ($\beta = 0{\cdot}38$) is in accord with that in Table 1. When corrected for the difference between the particle density used by de Kruif and that used in this work de Kruif's two milks have median diameters of 207 and 198 nm – approximately 10% less than that reported here. He also found that experimental intensity weighted distribution had a larger tail that that of a ln-normal distribution.

The lack of change in the particle size distribution and effective diameter on the addition of 10 mmol EDTA/kg milk is in accord with the results of Griffin et al. (1988) who observed essentially no change in the value of the diffusion coefficients and, using Controlled Pore Glass (CPG) chromatography, no change in the number frequency size distribution (up to 13·6 mM added EDTA) except at high EDTA concentrations. The lack of change in the particle size distribution is also in accord with reported observations that no sensible change in micellar diameter is observed in the pH range 5·5–6·5 although considerable

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amounts of casein and CCP were solubilized (Visser et al. 1986; Banon & Hardy, 1992).

The observed effective diameters as determined by PCS for casein micelles for the control milk and for milk with added calcium and calcium chelating agents were well in line with that reported in literature (Lin et al. 1972; Schmidt et al. 1973; Holt et al. 1978; Walstra & Jenness, 1984; de Kruif, 1998). The mean micellar density calculated for control milk (using the SdFFF and PCS results) was in good agreement with the literature value of 1·063 g cm^{-3} (Kirchmeier, 1973).

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Erratum

Correction to paper by Udabage P, McKinnon IR & Augustin MA 2000 Mineral and casein equilibria in milk: effects of added salts and calcium chelating agents. *Journal of Dairy Research* **67** 361–370. The authors confirm that the value of effective diameter of casein micelles measured in milk with added 10 mmol $CaCl_2$/kg was 193 nm and not as shown in the above paper (Table 2, p. 366).