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ARTICLE

Characterization of Heat-Induced Changes in Skim Milk Using Asymmetrical Flow Field-Flow Fractionation Coupled with Multiangle Laser Light Scattering

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Separation and size measurement of protein particles are a relevant approach to monitor heat-induced changes in skim milk. Unfortunately, no method is currently available at low cost and without excessive preparation of the samples. Therefore, the present study aimed at evaluating the interest of asymmetrical flow field-flow fractionation (AFlFFF) coupled with multiangle laser light scattering (MALLS) for this purpose. Unheated and heated skim milk samples at pH 6.5 and 7.2 were prepared and comparatively analyzed using AFlFFF−MALLS, size exclusion chromatography (SEC−MALLS) and dynamic light scattering. The results showed that AFlFFF could evidence the conversion of the native whey proteins of unheated milk into heat-induced whey protein/κ-casein complexes in the serum phase of milk and possibly on the surface of the casein micelles. The pH-induced changes in the partition of the complexes between the serum and the micellar phases could also be observed. The results therefore showed the interest of AFlFFF−MALLS to monitor the heat-induced changes in particle sizes in skim milk and to separate the different protein components of unheated and heated skim milk.

KEYWORDS: Asymmetrical flow field-flow fractionation; heat treatment; skim milk

INTRODUCTION

The heat treatment of skim milk at a temperature higher than ∼65 °C for up to several minutes has long been reported to induce denaturation of the whey proteins and the subsequent formation of heat-induced whey protein/κ-casein complexes based on hydrophobic interactions and thiol/disulfide exchanges (*1*−*5*). In skim milk heated at 80−90 °C for 10−15 min, the complexes are found both in the serum phase and bound to the surface of the casein micelles (*6*, *7*). At pH 6.7, the serum complexes are particles of 30−100 nm hydrodynamic diameter (*6*, *8*, *9*), while the micelle-bound complexes seem somewhat smaller (*10*, *11*). Partition of the heat-induced complexes between the serum and colloidal phases of milk is highly dependent on the pH of heat treatment. More complexes are found in the serum phase, and less in the micelle phase, as the pH increases from ∼6.5 to 7.2 (*12*−*15*). The size of complexes also depends on pH, since the hydrodynamic diameter of the serum complexes decreases from 60 to 80 nm at pH 6.5 to 30−50 nm at pH 7.2 (*1*, *15*). These changes with pH are essentially dependent on the activity of the free thiol groups (pK ∼8.5) and on the pH-dependent dissociation behavior of the casein molecules that constitute the casein micelles (*12*−*17*).

Heat-induced changes of the milk protein system have dramatic consequences on its technological ability to be processed into cheese or yogurt (*16*). For that reason, it is essential to provide convenient methods that allow direct, rapid and cost-effective evaluation of the colloidal status of the treated milk. Size exclusion chromatography (SEC) (*3*, *7*, *18*) and agarose gel electrophoresis (*6*, *12*) have been successfully applied on serum fractions of milk but have too low molecular weight cutoffs (MWCO) for milk (*19*). Alternatively, transmission (TEM) or scanning (SEM) electron microscopy have been used to image heated milk or its fractions (*8*, *20*−*22*); however, preparation artifacts clearly prevent quantitative measurements and numbering. In that respect, cryo-TEM or atomic force microscopy represent interesting alternatives, but they also need very heavy investments. Finally, direct light scattering methods yield a fast measurement of particle size in hydrated, nonmodified conditions but can hardly give a proper distribution of heated milk systems because the casein micelles largely outrange the scattering signal of smaller entities like the complexes. For this reason, the coupling of a separative method with multiangle laser light scattering (MALLS) was considered. The reference separation was performed with Sephacryl S-500 SEC column (*1*, *7*).

Asymmetrical flow field-flow fractionation (AFlFFF) belongs to a family of recently developed techniques that allow the fractionation of ultralarge material from 1 kDa to a few micrometers (*23*, *24*) by combining the separating effect of a laminar flow and the concentrating effect of a unidirectional cross-flow (*25*). It was described for the first time in 1960 by J. C. Giddings. This technique is based on a "soft" separation mechanism, which is ideally suitable to maintain the native structure of intact proteins (*26*). Briefly, the cross-flow opposes back-diffusion of the deposited analytes, thus

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preventing excessive dispersion of their residence time. Because of the limited shear applied, the absence of a stationary phase and its very large separation range, flow field-flow fractionation is well adapted to separate macromolecules, supramolecular assemblies and particles in single runs. Compared to other FFF methods like sedimentation FFF (*27*) and thermal FFF (*28*), AFlFFF is more universal and efficient with a broader application range. It has been used for separation and characterization of many types of molecules of ultrahigh molecular weight like biopolymers, colloids and particles (*29*−*32*).

For that reason, the aim of the present study was to evaluate the capacity of the method to separate the full range of protein elements in skim milk, and hence to directly monitor the heat-induced formation of soluble and micelle-bound complexes, in situ.

To date, the technique has yet received little attention in dairy science. Jussila et al. (*33*) successfully used symmetrical flow FFF to study the size distribution of a versatile range of dairy components like fat globules, casein micelles, native or aggregated whey proteins, and bacteria. However, they did not develop a method where an ensemble of these components could be separated from each other, e.g. to analyze milk as a whole, complex system. Only recently, AFlFFF has been applied to isolated casein micelles in order to characterize their structure in the native state (*34*).

Sedimentation field-flow fractionation (SdFFF), which uses centrifugal force rather than cross-flow, has been applied to milk to observe changes in the casein micelle with changes in the ionic environment (*35*, *36*) or with heating (*37*, *38*), but none of the reported methods seemed able to detect the formation of heat-induced complexes in milk. Nevertheless, other works on, e.g., the heat-aggregation of egg white protein (*39*) or the salt-aggregation of β-lactoglobulin (*40*) showed that flow fractionation techniques could be used to separate a wide range of protein particles from monomers to submicrometer particles in single runs, and hence to monitor the aggregation of protein into complexes.

Also, since Raikos et al. (*41*) could evidence the increase in size of milk fat globules as a result of heat-induced aggregation of proteins at their interface, it is hoped that the binding of denatured whey proteins onto the surface of the casein micelles on heating could be monitored by AFlFFF.

Therefore, the purpose of the present study was to evaluate the interest of AFlFFF−MALLS as a means to obtain in situ, direct measurement of the heat-induced changes in the particle distribution in skim milk. For that purpose, a set of well-characterized unheated and heated skim milk samples at pH 6.5 and 7.2 was chosen. Unheated milk samples essentially contain native whey proteins and casein micelles, while in heated milk samples the native whey proteins are converted into heat-induced whey protein/κ-casein complexes. After heating at pH 6.5, the complexes are essentially found on the surface of casein micelles, i.e., there is a small amount of soluble complexes and the size of the casein micelles increases. Conversely, heat treatment at pH 7.2 essentially yields soluble complexes. To date, no method is capable of separating all these fractions at once, and with little sample preparation. The results obtained using AFlFFF−MALLS on these selected milk samples are reported.

MATERIALS AND METHODS

Reconstituted Skim Milk. Milk was reconstituted as 100 g kg^{-1} ultralow heat skim milk powder (*42*) and 0.2 g kg^{-1} sodium azide in stirred deionized water at ∼40 °C. Briefly, ultralow heat skim milk powder is prepared by the skimming of raw milk, then by a Bactocatch microfiltration on 0.8 μm ceramic membrane and by spray-drying. As the milk does not experience temperatures above 50 °C, denaturation of the whey protein is negligible (whey protein nitrogen index = 9.5). The milk was stirred for ∼1 h after reconstitution and then left overnight at 5 °C to complete dissolution.

pH Adjustment. After equilibration at room temperature, the pH of the reconstituted skim milk was adjusted to either 6.5 or 7.2 using 1 and 0.1 M HCl or NaOH, respectively.

Heat Treatment. Heat treatment of 25 g fractions of skim milk was performed in sealed Pyrex tubes immersed in an agitated, thermostated water bath at 95 °C. Come-up time to 90 °C was ∼2 min, then temperature was set at 90 °C and holding time was 10 min. The samples were eventually cooled in ice−water.

Ultracentrifugation. Ultracentrifugation of the unheated or heat-treated milks at pH 6.5 or 7.2 was performed on 15 mL aliquots using a Sorvall Discovery 90 SE centrifuge (Kendro Laboratory Product, Courtaboeuf, France) equipped with a 50.2 Ti rotor (Beckman Coulter, Fullerton, CA, USA). The samples were spun at 19400 rpm (34207 average *g*) for 65 min at 20 °C. The supernatant and pellet were defined as the serum and micellar phases of milk, respectively.

Concentration of the Supernatants. In order to obtain sufficient light scattering signal by SEC−MALLS, proteins in the supernatants were concentrated 4-fold using 10 kDa MWCO Vivaspin 20 tubes (Sartorius Stedim, Aubagne, France). Four grams of each sample were spun at 1800 *g* for 15 min at 20 °C on a Firlabo SV 11 TH centrifuge (Meyzieu, France). The ultrafiltration permeates were used for dilution in the particle size analysis (see below).

Control Samples. Micellar casein or "native phosphocaseinate" (NPPC) was prepared as described by Schuck et al. (*43*). Briefly, raw milk was skimmed at ∼50 °C, then microfiltered through 0.1 μm cutoff ceramic membrane, diafiltered, evapoconcentrated and spray-dried. Reconstitution was at 10 g kg^{-1} in milk ultrafiltrate with 0.2 g kg^{-1} sodium azide. The microfiltration permeate, containing the native whey proteins, was concentrated by ultrafiltration onto 8 kDa MWCO ceramic membrane, diafiltered and freeze-dried to yield native whey protein isolate (WPI). Reconstitution was at 90 g kg^{-1} in deionized water with 0.2 g kg^{-1} sodium azide. The resulting milk ultrafiltration permeate (MUF) was collected and stored at 5 °C after addition of 0.2 g kg^{-1} sodium azide. Aggregated whey protein (WPIA) was prepared according to Vasbinder et al. (*44*). Briefly, a 90 g kg^{-1} solution of WPI in deionized water with 0.2 g kg^{-1} sodium azide was adjusted at pH 7.5 and then heated at 68.5 °C for 2 h to yield ∼100 nm diameter heat-induced complexes. As the diameter of heat-induced complexes obtained in heated skim milk is between 65 and 100 nm (*6*, *8*) and as the WPIA were measured at 62−100 nm (in the current study and in Morand et al. (*45*) and Vasbinder et al. (*12*, *44*)), WPIA is a good model for heat-induced complexes of milk and could be used in SEC or AFlFFF as the particle size is the only significant criterion for the fractionation of particles by these techniques.

Size Exclusion Chromatography coupled with Multiangle Laser Light Scattering (SEC−MALLS). Size exclusion chromatography analysis of the concentrated serum phase of the milk was performed on a Sephacryl S-500 Hi-Prep 16/90 column (Amersham Biosciences, Orsay, France) at room temperature in isocratic conditions using 0.1 M Tris, 0.5 M NaCl, 10 mM $CaCl_2$ and 10 mM NaN_3, pH 7, as the mobile phase, filtered through 0.1 μm and degassed. The concentration in calcium was adjusted to 10 mM, as this is the calcium contain in the serum phase of milk. Loop size was 100 μL, flow rate was 0.5 mL min^{-1} and absorbance was monitored at 280 nm. The system was connected to a 18 angles DAWN-Heleos II multiangle laser light scattering detector equipped with a K5 cell and a 658 nm laser diode and to an Optilab rEX refractometer operating with a 685 nm laser diode and at 25 °C (Wyatt Technology, Santa Barbara, CA, USA). Data were collected at scattering angles θ ranging 20−130°, corresponding to scattering wave vectors q between 4.4×10^{-3} and 2.3×10^{-2} nm^{-1}, where $q = (4\pi n/\lambda) \times \sin(\theta/2)$ and n = 1.333 the refractive index of the solvent. Data was then analyzed using an Astra software version 5.3.4.11 using the Zimm formalism of a Debye plot, as follows. The light scattering intensity I as a function of θ is expressed by

$$I(\theta) \propto M_w c(\delta n_{protein}/\delta c)^2 P(\theta)$$

where M_w is the apparent weight average molecular weight (g mol^{-1}), c is the solute concentration (g mL^{-1} or g cm^{-3}), $\delta n_{protein}/\delta c$ is the refractive index increment of the solute (taken as 0.183 mL g^{-1} for proteins) and $P(\theta)$ is the θ-dependent form factor (dimensionless). $P(\theta)$ expresses the relative

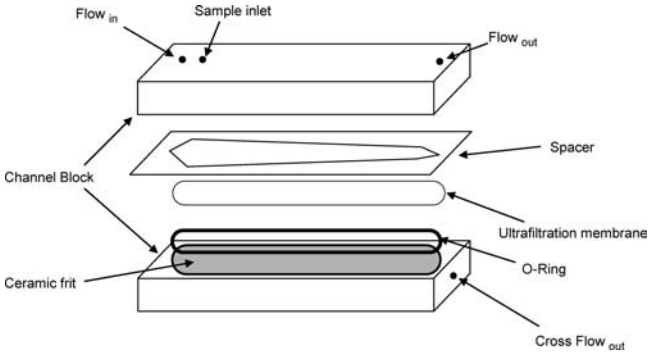

Figure 1. Schematic representation of the AFlFFF channel representing the inlet and outlet of the flow channel, the sample inlet and the cross-flow outlet.

positions of the scattering elements in the system and accounts for the dependence of I on θ as

$$P(\theta) \approx 1\text{-}(16\pi^2 n_0^2/3\lambda^2) * \sin^2(\theta/2) * \langle Rg^2 \rangle + ...$$

and can be truncated into the structure factor $S(q) = [1 + (q.R_g)^2/3]^{-1}$. The Debye plot performed by Astra results from the transformation of $I(\theta)$ into the so-called Rayleigh ratio $R(\theta)$:

$$R(\theta) = KM_w cP(\theta)[1 - 2A_2 M_w cP(\theta)]$$

where A_2 is the second virial coefficient and K the contrast factor as

$$K = (4\pi^2 n_0^2)(\delta n_{protein}/\delta c)^2/(N_A \lambda^4)$$

expressed in mol cm^3 g^{-2}, with N_A, the Avogadro number. In the current conditions, dilution of the protein particles in the elution peak was larger than in Guyomarc'h et al. (*46*) so that interactions were taken as negligible and $A_2 = 0$. When extrapolating $\theta \rightarrow 0$, then $\sin \theta \rightarrow 0$, $P(\theta) \rightarrow 1$ and therefore $R(\theta)/Kc \rightarrow M_w$. The radius of gyration, R_g, is calculated from the expression of the form factor as the slope of $R(\theta)/Kc$ as a function of $\sin^2(\theta/2)$. As a simplification of the Mie theory, Rayleigh scattering is applicable when the radius of the scatterers is much smaller (typically 1 order of magnitude smaller) than the wavelength of the incident light. For red light at $\lambda = 658$ nm, calculation is probably accurate for whey protein complexes and small micelles. However, it might diverge for larger species where multiple scattering is susceptible to occur within the structure of one particle and/or where $P(\theta)$ cannot extrapolate to 1 due to relative (i.e., not independent) positions of multiple scattering items within the particle. Since this error has often been accepted in previous literature, the results of the above calculations are presented for the whole range of particles separated from skim milk or its supernatants using SEC or AFlFFF, essentially for comparison purposes.

Asymmetrical Flow Field-Flow Fractionation Coupled with Multi-angle Laser Light Scattering (AFlFFF−MALLS). The main component in an AFlFFF system is the separation channel (**Figure 1**), in which the sample is carried with an aqueous or organic eluent in a laminar parabolic flow profile. In the case of the AFlFFF, another flow perpendicular to the carrier flow called "cross-flow" is used to generate the force field, to separate the macromolecules as a function of their diffusion coefficient, i.e., their hydrodynamic diameter (Dh) during elution.

The AFlFFF instrument was an Eclipse 2 System (Wyatt Technology Europe, Dernbach, Germany). The AFlFFF channel had a trapezoidal geometry with length 19.5 cm, initial breadth 1.65 cm and final breadth 0.27 cm. A Mylar spacer with a thickness of 250 μm was placed between the ultrafiltration membrane and the upper glass plate. The accumulation wall was an ultrafiltration membrane of regenerated cellulose with 5 kDa MWCO (Wyatt Technology Europe). The same mobile phase as in SEC−MALLS was used. An Agilent 1100 Series isocratic pump (Agilent Technologies, Waldbronn, Germany) with an in-line vacuum degasser and an Agilent 1100 Autosampler delivered the carrier flow and handled sample injection into the AFlFFF channel. The AFlFFF separation consisted of several steps. The first 4 min of elution at an outlet flow rate of 1 mL min^{-1} with a cross-flow rate at 0.15 mL min^{-1} for 2 min and 1 mL min^{-1} for 2 min allowed the obtaining of a correct background. In the first focusing step lasting 1 min, the eluent went into the channel by the inlet and the outlet at 0.5 mL min^{-1} and 2 mL min^{-1}, respectively, and was completely wasted by the cross-flow outlet at 2.5 mL min^{-1}. Then, during the focusing-injection step (8 min), the sample (30 μL) was injected at 0.2 mL min^{-1} while the eluent went by the inlet and outlet at 0.3 and 2 mL min^{-1}, respectively, and the cross-flow rate was fixed at 2.5 mL min^{-1}. This was followed by a new focusing step for 1 min at the same conditions as the first one. During this focusing step, called the relaxation step, the analytes were allowed to diffuse away from the membrane according to their hydrodynamic diameter. The first elution step then began and lasted 5 min, with the eluent going out of the channel at 1 mL min^{-1} and the cross-flow rate fixed at 1 mL min^{-1} (the inlet flow rate was automatically fixed at 2 mL min^{-1}). In the next step, the cross-flow rate was decreased linearly to 0.15 mL min^{-1} in 5 min (and consequently, the eluent went by the inlet at a decreasing rate too, from 2 to 1.15 mL min^{-1}). Next step was at a cross-flow rate fixed at 0.15 mL min^{-1} for 35 min, followed by a step of 10 min with the cross-flow stopped, allowing elimination of the largest particles prior to the next injection.

The AFlFFF was connected to an 18 angle DAWN-DSP multiangle laser light scattering detector (Wyatt Technology, Santa Barbara, CA, USA; $\lambda = 633$ nm), an Optilab rEX refractometer ($\lambda = 685$ nm) and a UV detector Agilent 1100 ($\lambda = 280$ nm). The skim milk samples and micellar casein were diluted 10 times with eluent prior to injection, while the supernatants and other control samples were injected undiluted. Because of the noise generated on the dRI signal by the cross-flow gradient, the UV signal was used as the source data for protein concentration measurement, using an extinction coefficient of 0.729 L g^{-1} cm^{-1} (BSA at 280 nm in the mobile phase).

Particle Size Analysis. Particle size in the different milk samples was measured using dynamic light scattering (DLS) at a set angle θ of 173° ($q = 2.7.10^{-2}$ nm^{-1}) on a Zetasizer nanoZS Malvern (Malvern Instruments, Orsay, France; $\lambda = 633$ nm). The samples equilibrated at 20 °C were diluted in the appropriate milk ultrafiltration permeate (see Concentration of the Supernatants) and left at 20 °C for 15 min to ensure proper equilibrium of the diluted system. The dilutions were then transferred to 4 mL disposable cuvettes and allowed to stand for 5 min prior to measurements. The refractive index and dynamic viscosity of both permeates were 1.348 and 1.187 mPa s at 20 °C, respectively. The data were converted into a particle size distribution using the non-negative least-squares (NNLS) modeling routine. Only one peak was found, of which the mean (Dh) and the width were calculated using the Stokes−Einstein relationship. The calculation assumes the population to be that of hard spheres and is most accurate for monodisperse, dilute (non-interacting) particles with radii in the range of the observation window q^{-1} (~75−100 nm). Due to these limitations, the calculated Dh resulting from the analysis of milk samples is better called the "apparent Dh" of the particles.

Significance. The presented results were obtained from 4 separate sample preparations.

RESULTS

Separation of the Protein Elements of the Unheated Skim Milk. Figure 2A shows typical SEC−MALLS profiles of the unheated supernatants. In variation of the reference method (*7*, *11*, *15*), the separation was performed in the presence of 10 mM calcium for comparison purposes with separation of milk samples using AFlFFF−MALLS. Large amounts of native whey proteins were found at large retention times (290−300 min) essentially corresponding to mono- and oligomers (**Figure 2A**). The collection and analysis of the peak by reverse phase high performance liquid chromatography (RP-HPLC) following a routinely used method (*7*, *13*, *47*, *48*) confirmed that it was > 70% whey proteins with small amounts of α_{s1}- and β-caseins (not shown). The detected MW_w of the whey proteins was ~10^5 g mol^{-1}, which is slightly higher than the expected range of 15−80 × 10^4 g mol^{-1}. Co-elution of various species could shift the MW_w toward that of the largest, most scattering bovine serum albumin, lactoferrin or immunoglobulins. It is also possible that traces of fat (exclusion peak) trailed throughout the profile, thus producing background light scattering. In the case of protein monomers and oligomers that barely scatter light, such a noise may significantly increase

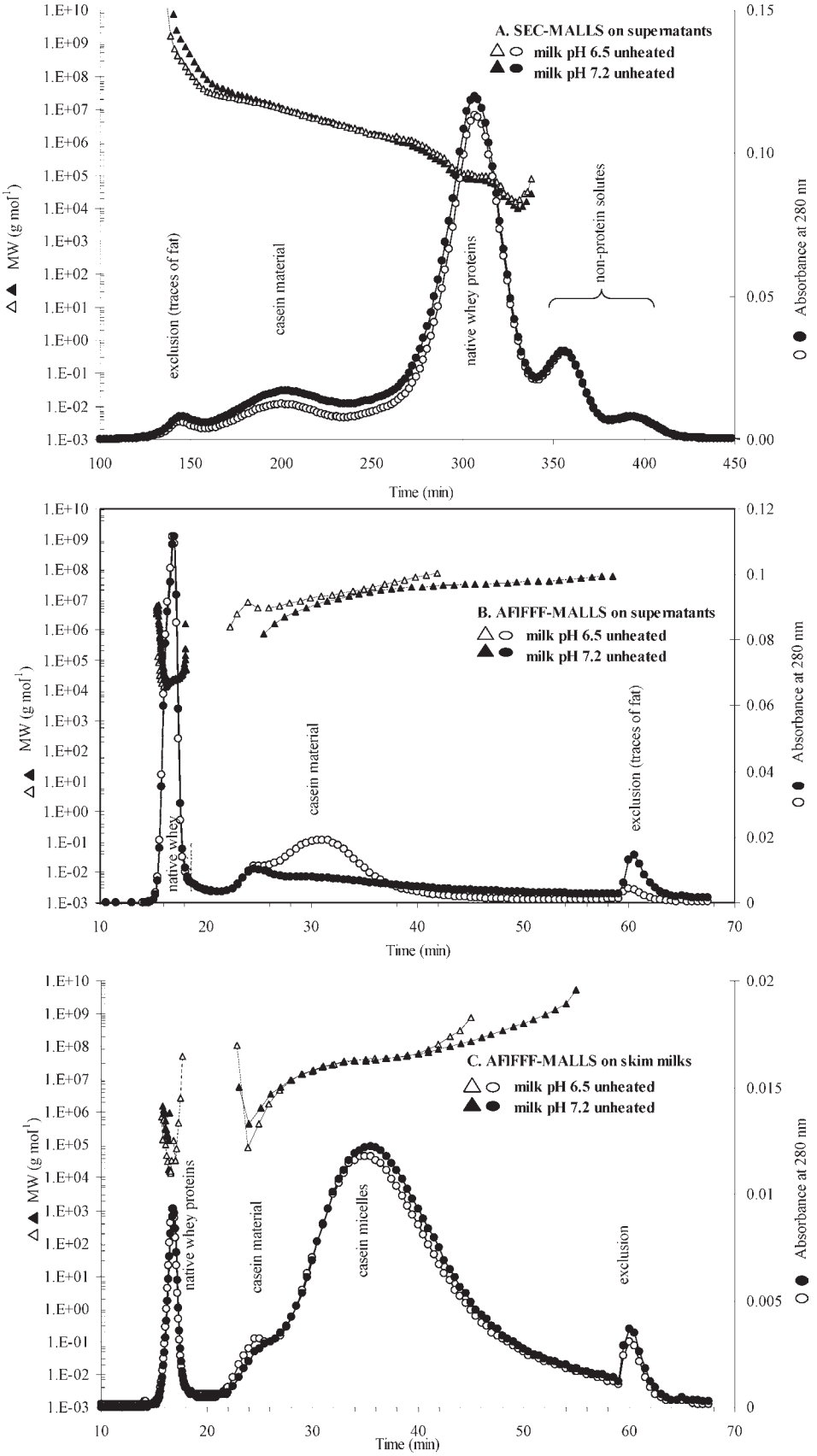

Figure 2. Typical separation profiles at 280 nm (circles) and online calculated molecular weight (triangles) of the unheated samples: centrifugal supernatants of unheated skim milks at pH 6.5 (open symbols) and 7.2 (closed symbols), using (**A**) size exclusion chromatography coupled with multiangle laser light scattering (SEC−MALLS) or (**B**) asymmetrical flow field-flow fractionation coupled with multiangle laser light scattering (AFlFFF−MALLS), and of (**C**) the corresponding unheated skim milks at pH 6.5 (open symbols) and 7.2 (closed symbols) using AFlFFF−MALLS. Separation using SEC takes 450 min, and larger analytes elute first (panel **A**); separation using AFlFFF takes 70 min, and smaller analytes elute first (panels **B** and **C**).

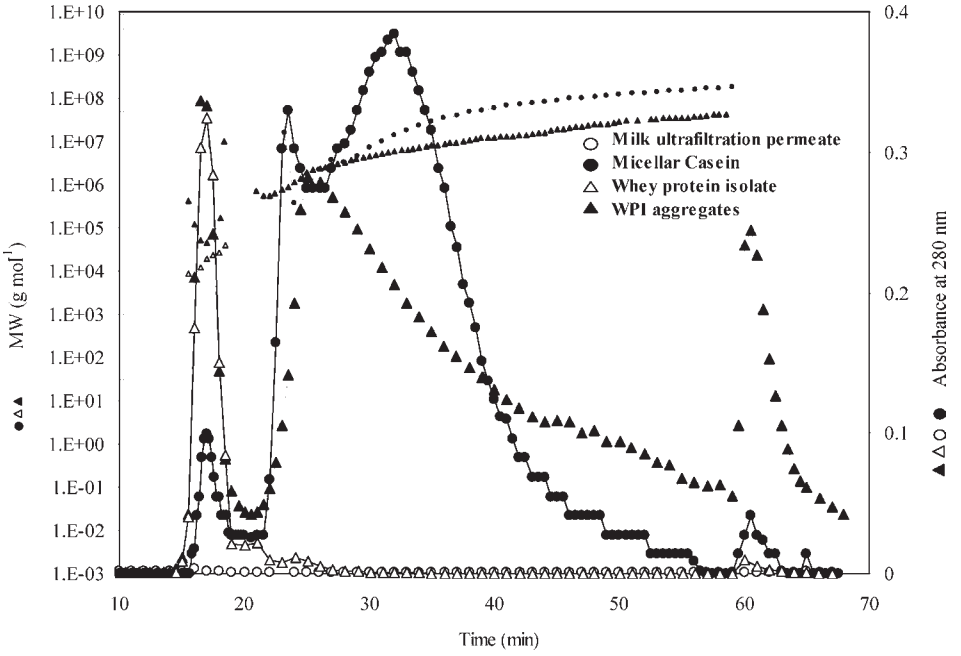

Figure 3. Typical asymmetrical flow field-flow fractograms at 280 nm (large symbols) and calculated molecular weight (small symbols) of milk ultrafiltration permeate (open circles), native micellar casein (closed circles), native whey protein isolate (open triangles) and heat-aggregated whey protein (closed triangles). For practical reasons, absorbance data for native micellar casein and milk ultrafiltration permeate were increased by a factor 90 and 10, respectively. Note that, the concentration of native micellar casein was 10 g kg^{-1}, while that of whey proteins was 90 g kg^{-1}, and that the former sample was diluted 10-fold, as compared to the other control samples that were injected undiluted.

the overall scattering intensity I and, thus, account for overestimated MW_w values ($\propto Ic^{-1}$) of these analytes.

Small amounts of aggregated material were also found at 160−230 min, corresponding to MW_w of $\sim 10^7$ g mol^{-1}. RP-HPLC evidenced that this peak contained small amounts of proteins, namely, 15−35% κ-casein + α_{s2}-casein, ~60% α_{s1}-casein and ~15% whey proteins (in total HPLC area − not shown). Proportions slightly varied with pH. Most likely, the presence of $CaCl_2$ in the mobile phase maintained (or generated) aggregated casein structures that otherwise eluted at ~250 min or in the monomer peak in the absence of calcium (*7*, *11*, *15*). Exclusion contains small fat globules and negligible protein (*7*, *49*) that can be visualized by confocal scanning laser microscopy (not shown). Peaks eluting at 350 and 390 min are non-protein, diffusible materials like orotic acid or vitamins, and were visible on the elution profile of MUF (not shown). As expected, pH of the unheated skim milk samples did not induce any significant change in the area either of the whey proteins or of the non-protein materials ($P > 0.1$). There was, however, slightly more casein material in the supernatant of milk at pH 7.2 than at pH 6.5 (**Figure 2A**, $P < 0.05$). This is in agreement with the increased dissociation of the micellar casein in milk as pH is increased (*13*, *15*).

Figure 2 also shows typical AFlFFF−MALLS fractograms of the same supernatants (**Figure 2B**) and of the corresponding unheated skim milk at pH 6.5 and 7.2 (**Figure 2C**). The separated peaks were identified using the elution profiles of control samples (**Figure 3**). Conversely to SEC, where retention time is inversely related to particle size, AFlFFF in normal mode retains larger particles for longer retention times, providing that all particles have similar densities (e.g., the response of milkfat may differ from that of protein particles, in aqueous flow). According to **Figure 3**, no significant light scattering signal could be measured with the milk ultrafiltration permeate sample. Native whey proteins appeared to elute in a narrow peak at 16−18 min with MW_w at the peak maximum at 2×10^4 g mol^{-1}, in accordance with that of the major whey protein, β-lactoglobulin (18.6×10^3 g mol^{-1}). Micellar casein eluted in a small and narrow peak at 17 min, then in a broad band, with a first peak at 24 min, a second one at 32 min and a small peak at 60 min at the exclusion of very large particles. Corresponding MW_w were from 1×10^5 to 2×10^8 g mol^{-1}, but the first 4 measurements appeared rather noisy between 1×10^5 and 1×10^7 g mol^{-1}. MW_w was $\sim 1 \times 10^7$ g mol^{-1} at the peak maximum at 32 min. Most likely, the material eluting between 24 and 60 min contained the casein micelles. However, the measured MW_w was more than 1 decade smaller than the values from the literature (MW_w 4−10 $\times$ 10^8 g mol^{-1}, McMahon and Brown (*50*), Pitkowski et al. (*51*), Glantz et al. (*34*)). The first reason for this variation was the use of the absorbance signal at 280 nm as the data source for calculation of the protein concentration c. For particles as large as the casein micelles, light scattering significantly adds up to absorption to decrease transmission, and consequently, the measured UV absorbance is increased. For a given light scattering intensity I, the calculated MW_w ($\propto Ic^{-1}$) will therefore be underestimated. To circumvent this problem, one suggestion would be to implement a photodiode array to the MALLS detector in order to determine the dependence of the scattering intensity on wavelength ($I \propto \lambda^{-4}$), e.g., between 300 and 900 nm, so that the sole contribution of absorption can be calculated at 280 nm. Second, as discussed by Udabage et al. (*35*) or Mc Mahon and Brown (*50*), separation of the casein micelles by flow fractionation renders comparison difficult with a majority of the reported values, where light scattering techniques are applied to unseparated samples. In the latter case, contribution of the large particles to scattering intensity shifts the weight average values upward. Eventually, despite the presence of calcium in the eluent, casein micelles may have partially dissociated on dilution and separation and appeared as small casein assemblies. This would account for the peaks observed at 17 and 23 min for the native micellar casein sample in **Figure 3**. The determined MW_w of the 23 min small casein assemblies (1×10^5 to 1×10^6 g mol^{-1}) was furthermore in agreement with a number of reports that indicated the presence of 5−15 nm, 3−5 $\times$ 10^5 g mol^{-1} particles of dissociated casein (*51*−*53*) or minimicelles (*54*) in

various milk systems. WPI aggregates eluted in a broad peak from 22 min up to the flush with a maximum at 24 min and a peak excluded at 60 min. MW_w at the peak maximum was $\sim 2 \times 10^6$ g mol^{-1} (**Figure 3**).

Figure 2B and **Figure 2C** show that the native whey proteins exit the channel first, after ~17 min of elution. Owing to the sufficient protein concentration and light scattering signal in the supernatants, the MW_w of the whey proteins was determined to be $\sim 2 \times 10^4$ g mol^{-1}. As expected, there was no difference in the whey protein peak of the supernatants of milks at pH 6.5 or 7.2. After 22 min of elution, the supernatants exhibit a broad peak (22−34 min), corresponding to sizes ranging from $\sim 7 \times 10^5$ to $\sim 2 \times 10^7$ g mol^{-1}. In accordance with the SEC results and the elution of control samples in AFlFFF, this peak probably contained small assemblies of κ- and α_s-caseins. However, while little difference was visible between the SEC elution profiles obtained at pH 6.5 or 7.2, these small casein assemblies trailed off for longer elution times using AFlFFF on supernatants obtained at pH 7.2, as compared to pH 6.5. The reason for this difference is unknown. At 60 min, a flush liberated the largest, slowest particles into a single peak.

Figure 2C shows the typical AFlFFF profiles of the unheated skim milk at pH 6.5 and 7.2. Again, the whey proteins were found to elute at ~17 min; however, molecular weight determination failed to provide reliable values. Probably, dilution of the milk samples that was necessary to accommodate the high scattering signal of the casein micelles yielded a too noisy scattering signal for the whey proteins. A large broad peak then eluted at ~22 min up to the flush. The peak exhibited one maximum at ~23 min elution, then a large distribution that culminated at ~35 min. The corresponding molecular weights ranged from 1 to 3×10^5 g mol^{-1} for the 23 min peak to $\sim 2 \times 10^9$ g mol^{-1} at 54 min, the highest distribution (i.e., mode) being at 3×10^7 g mol^{-1}. This pattern is in agreement with the elution profile of the NPPC, although its distribution was shifted toward somewhat smaller sizes (**Figure 3**). According to their MW_w, the first peak at 23 min was attributed to small assemblies of caseins, while the one at 35 min contained casein micelles.

Heat-Induced Changes in the Colloidal Organization of Skim Milk. In agreement with previous reports, **Figure 4A** showed that the native whey proteins in supernatants markedly decreased with heat treatment at 90 °C (*7*, *11*, *18*). In the present experiment, the total area decreased by 70 ± 5% across repetitions at either pH value. In the meantime, a new peak appeared at shorter elution times (160−270 min), which has previously been identified as the heat-induced whey protein/κ-casein complexes of the serum phase (*3*, *7*, *15*, *18*). According to Donato and Dalgleish (*15*) or Guyomarc'h et al. (*7*), the casein small assemblies visible in the unheated samples were still present as coeluted analytes. The corresponding MW_w ranged between $\sim 5 \times 10^5$ and $\sim 10^8$ g mol^{-1} and culminated at $\sim 3 \times 10^6$ or $\sim 2 \times 10^7$ g mol^{-1} at pH 7.2 (235 min) or 6.5 (175 min), respectively (**Figure 4A**). These figures were in agreement with previous results (*7*, *8*).

In agreement with SEC analysis, the native whey protein peak eluting at ~17 min using AFlFFF was found to decrease dramatically on heat treatment (**Figure 4B**), while a new peak appeared between 22 and 34 min of elution. Comparisons with control samples of heat-aggregated whey proteins (WPIA in **Figure 3**) and with the SEC profiles (**Figure 4A**) strongly suggested that the heat-induced peak contained the serum whey protein/κ-casein complexes. When the unfractionated heated milk samples were analyzed using AFlFFF (**Figure 4C**), the heat-induced serum complexes could still be visible at elution times of 22−26 min while the casein micelles tended to elute at longer elution times than in the unheated skim milk samples. The MW_w of the heat-induced serum complexes was found to be $\sim 10^6$ g mol^{-1} in these separation conditions, while those of the casein micelles in heated milks were found to range between $\sim 8 \times 10^6$ g mol^{-1} at 28 min and $\sim 10^9$ g mol^{-1} at 58 min with a maximum at $\sim 6 \times 10^7$ g mol^{-1}. Therefore, it appears that the AFlFFF separation method was able to discriminate the heat-induced serum complexes from the residual native whey proteins and from the casein micelles in situ. It also seemed to indicate a heat-induced shift of the population of casein micelles toward larger molecular weights, possibly as a result of the binding of denatured whey proteins on heating. Anema and Li (*10*) previously reported an increase of ~25 nm in the diameter of the casein micelles of skim milk at pH 6.55 heated at 90 °C for 10 min, while Renan et al. (*11*) or Anema and Li (*10*) reported smaller increases at the natural pH of skim milk. In that respect, AFlFFF separation proved to be an interesting method to monitor the heat-induced changes in the protein organization of skim milk.

Effect of the pH of Heat Treatment. As expected from previous studies, more and smaller heat-induced complexes were found in the serum phase of milk as the pH of heat treatment increases (*12*−*14*, *22*, *55*). In agreement with Renan et al. (*1*) and Rodriguez del Angel and Dalgleish (*9*), SEC separation of the milk supernatants clearly showed that the peak of serum complexes showed larger areas ($P < 0.05$) and longer retention times ($P < 0.01$), hence lower MW_w values (see above), after heating skim milk at pH 7.2 rather than 6.5. **Figure 4B** shows that the heat-induced peak of serum complexes as eluted by the AFlFFF method, had a ~1.5 min longer retention time and about twice a smaller area as the pH of heating had been shifted from 7.2 to 6.5 ($P < 0.1$). The MW_w were respectively found at $\sim 10^6$ and 3×10^6 g mol^{-1}. However, when analyzing the milk samples, the difference in elution time between the serum complexes' peaks observed at the two pH values was reduced to ~1 min ($P < 0.1$) and the area of peak between 22 and 29 min was only ~20% higher at pH 7.2 than at pH 6.5 (**Figure 4C**). The difference in corresponding MW_w became negligible. No significant difference was found in the retention time (i.e., hydrodynamic radius) of the maximum of the casein micelles' peak after skim milks were heated at pH 6.5 or 7.2. However, the distribution seemed to lag toward larger sizes (including in the flush) at pH 6.5. Larger amounts of heat-induced complexes are expected to bind to the casein micelles after heat treatment at pH 6.5 (*12*), and Anema et al. (*56*) clearly showed that it proportionally impacted the diameter of the casein micelle. To confirm this, the average hydrodynamic diameter, Dh, of the unheated and heated milk samples was measured at 20 °C using dynamic light scattering (not shown). Because the light scattering properties of the casein micelles largely outrange those of smaller particles (globular proteins or serum complexes), the method is essentially a measurement of the Dh of the casein micelles in skim milk. The casein micelles in unheated milk at pH 6.5 and 7.2 had different apparent Dh of 189 ± 4 nm and 199 ± 8 nm, respectively ($P < 0.01$). After heating at pH 7.2, the apparent Dh of micelles decreased significantly to 194 ± 12 nm ($P < 0.01$), while that of the micelles in milk heated at pH 6.5 increased slightly to 202 ± 6 nm ($P < 0.03$). This was coherent with an increased binding of heat-denatured whey proteins onto the casein micelles as the pH of heating decreases and an increasing content in soluble heat induced complexes at higher pH values. The presented AFlFFF method therefore needs improvement to increase its sensibility to small heat-induced changes on casein micelles (e.g., with pH), albeit those regarding the serum complexes were well evidenced.

DISCUSSION

Despite their potential interest for the study of complex populations of colloid particles, field-flow fractionation techniques have yet been scarcely applied to milk samples. The present results, however, illustrated the interests of asymmetrical flow

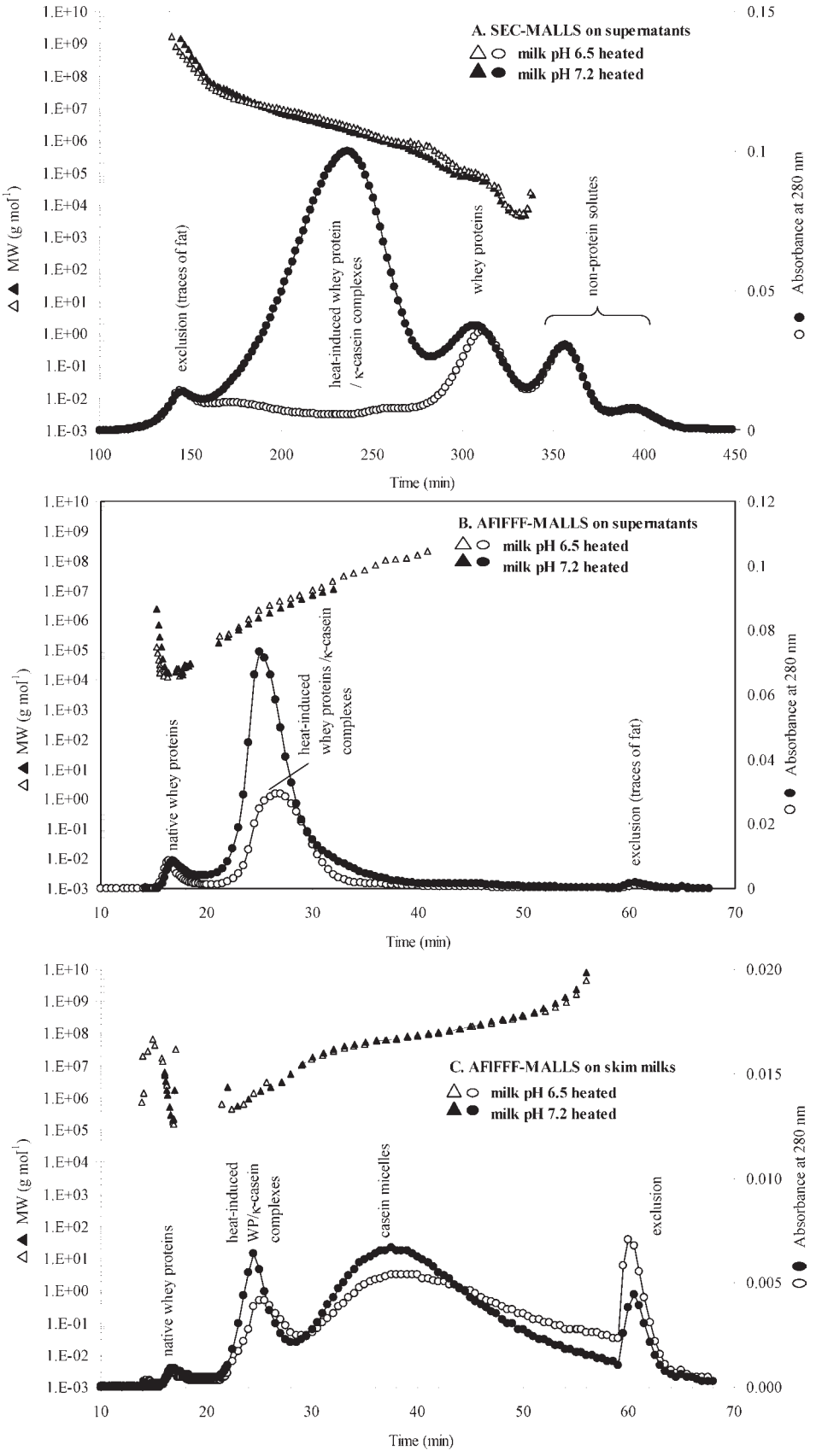

Figure 4. Typical separation profiles at 280 nm (circles) and online calculated molecular weight (triangles) of the heated samples: centrifugal supernatants of heated skim milks at pH 6.5 (open symbols) and 7.2 (closed symbols), using (**A**) size exclusion chromatography coupled with multiangle laser light scattering (SEC−MALLS) or (**B**) asymmetrical flow field-flow fractionation coupled with multiangle laser light scattering (AFlFFF−MALLS), and of (**C**) the corresponding heated skim milks at pH 6.5 (open symbols) and 7.2 (closed symbols) using AFlFFF−MALLS. Separation using SEC takes 450 min, and larger analytes elute first (panel **A**); separation using AFlFFF takes 70 min, and smaller analytes elute first (panels **B** and **C**).

field-flow fractionation to separate the various protein particles in skim milk, from globular proteins to casein micelles, in about 7-fold less time than with SEC. Udabage et al. (*35*), McKinnon et al. (*36*), Glantz et al. (*34*) and de Kruif (*37*) used sedimentation and asymmetrical flow-FFF to separate casein micelles in skim milk samples, but only McKinnon et al. (*36*) mentioned that particles of ~2 and ~15 nm diameter could be separated from the casein micelles. Although not thoroughly identified, these peaks probably corresponded to the individual (whey) proteins and to dissociated casein material, respectively, in agreement with **Figure 2**. To the author's knowledge, the only report of an application of flow fractionation on heated milk was by Mozersky et al. (*38*) and Parris et al. (*57*), who used SdFFF on reconstituted nonfat dry milk preheated at 64−85 °C for up to 30 min. Unfortunately, the method involved extensive dissociation of the casein micelles by dialysis and then artificial reconstitution using 20 mM $CaCl_2$ prior to fractionation. The authors observed the increased propensity of the reconstituted "micelles" to aggregate into larger particles as heat treatment increased. They reported MW_w values of up to over 10^{11} g mol^{-1} after heating at 85 °C for 30 min, probably indicating partial precipitation. In the present study, the reported AFlFFF−MALLS method allowed observation for the first time of the formation of heat-induced whey protein/κ-casein soluble complexes in situ, in unprepared milk samples. It also clearly showed the depletion in native whey protein with heating, as a result of their denaturation and the formation of complexes. The method was accurate enough to evidence changes in the amount of soluble complexes produced at different pH values of heat treatment. However, the AFlFFF technique may prove less capable to evidence small changes that occur on a broad population of particles, like casein micelles. In their use of SdFFF, Udabage et al. (*35*) could only evidence unsignificant changes in the casein micelles' size distribution as a result of addition of calcium, phosphate + calcium, or EDTA to milk. In the present study, it was expected that the size of the casein micelles would increase with heating, to a larger extent at pH 6.5 than 7.2. However the results show that the heat-induced changes in the size of the casein micelles with pH were hardly evidenced using AFlFFF, albeit the casein micelles tended to shift toward larger elution times (larger size) after heating. This could probably be improved if longer elution times or slower gradients were tested. Jussila et al. (*33*) could for instance record the decrease of the size of casein micelles with increasing pH from 6.8 to 9.0.

In the present work, reliable quantification of the heat-induced changes also proved difficult, essentially due to limitations of the method. While it clearly protected the casein micelles from dissociation in AFlFFF (not shown), the presence of $CaCl_2$ in the mobile phase of SEC and AFlFFF also stabilized smaller casein particles that happened to coelute with the heat-induced serum complexes (**Figures 2** and **4**). Also, as mentioned in the Results section, the use of UV absorbance as data source for the online calculation of protein concentration in AFlFFF suffers from the effect of light scattering on absorbance. On the other hand, differential refractive index measurement also has important pitfalls, since the signal-to-noise ratio is very small when analyzing large scatterers. The data is indeed difficult to handle in AFlFFF where flow and cross-flow gradients generate high noise, and rather poor in SEC where long runs made slow baseline shifts eventually significant.

In conclusion, the application of AFlFFF separation to heated milk samples opened the interesting opportunity to fractionate a large range of protein particles, from single proteins to submicrometer casein assemblies, within 1 h and without the need for excessive sample preparation. This is an important improvement over SEC, which requires removal of casein micelles from the sample, longer run times, and more materials (samples and fluids). However, important limitations of the online MALLS detection and calculation were found, especially with respect to the characterization of the large particles, i.e., casein micelles. Thanks to the release of integrated, straightforward equipment, MALLS is growing rapidly in food science. However, further developments are needed to accommodate the characterization of large particle sizes, as often found in food suspensions and emulsions. Large particles may saturate the light scattering detectors, for too little dRI signal-to-noise ratio, and need the Mie theory to be implemented in the calculations. UV absorbance data may also need transformation to discriminate true absorption and light scattering contributions. Fortunately, the characterization of the heat-induced soluble complexes in situ, at two pH values of heat treatment, could yet be obtained despite these limitations. These results therefore opened interesting opportunities for the study of these complexes in conditions that avoid experimental artifacts.

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