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EFFECT OF HIGH-PRESSURE HOMOGENIZATION ON A STERILIZED INFANT FORMULA: MICROSTRUCTURE AND AGE GELATION

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Abstract

Age thickening and gelation of an infant formula was induced by applying high pressure homogenization prior to in-can sterilization. The initial viscosity of the preparation increased with increasing homogenization pressure. Thickening rate upon storage, as monitored by viscosity changes, was also proportional to the pressure applied during homogenization. Optical and electron micrographs of 6 month-old samples showed evidence of aggregation. The effect of dissociating agents on the viscosity and microstructure of these samples suggested a contribution of hydrogen bonds and calcium bridges to the gel integrity. The alteration of the mineral balance of aged samples also indicated a gel strengthening effect by the colloidal calcium phosphate.

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Introduction

In the U.S., sterilized liquid infant formulas (ready to feed and concentrates) are more popular than the corresponding powdered product (McDermott, 1987). The shelf-life of these products can however be limited by the occurence of fat separation during storage. Therefore, the homogenization treatment must be designed carefully in order to prevent this defect.

Homogenization is generally applied to fat containing dairy products in order to reduce the diameter of fat globules. This physical treatment reduces creaming and coalescence of the dispersed fat phase. The pressures usually applied range from 3.5 to 35 MPa, and are chosen according to several parameters: type of valve, number of stages, temperature and physico-chemical properties of the emulsion and the desired product's characteristics.

The age gelation of UHT (ultra-high temperature) and of HTST (high temperature-short time) sterilized milks have been reviewed by Harwalkar (1982). This storage defect is characterized by an increase of viscosity (age thickening) followed by the formation of a gel, usually free from syneresis, appearing after approximately three months. Age gelation has been observed in UHT, HTST sterilized milk and in-can sterilized concentrated milk. Two distinct hypotheses based on proteolytic activity and physico-chemical destabilisation have been proposed to explain the age gelation mechanism. However, there is still a lack of experimental evidence to support either of those hypotheses. Several factors influence age gelation of sterilized products: the processing conditions, the composition and microbiological quality of the product, the presence of additives and the storage temperature.

Among the processing conditions, homogenization has been identified as having a specific effect (Harwalkar, 1982). In fact, the sequence of processing steps is the determining factor, e.g., homogenization placed before the concentration step yields a product with reduced stability against gelation. When used together with a controlled heat treatment (holding at 94°C), allowing the development of a critical viscosity, homogenization can prevent age gelation. However,

the effect of homogenization pressure or of repeated homogenizations has never been reported.

Age gelation also occurs in sterilized infant formulas (McDermott, 1987) and the phenomenon is similar to age gelation of sterilized milks. In the present study, gelation of infant formula was induced by applying high-pressure homogenization prior to in-can sterilization. The gelation upon storage was followed using viscosity measurements. Dissociating agents were added to gelled samples in order to investigate the nature of interactions leading to the gel formation. Gel microstructure was examined using optical and transmission electron microscopy of resin-embedded samples.

Materials and methods

Preparation of infant formulas

A commercial liquid mix containing 7.2 % fat, 14.0 % carbohydrates, and 3.1 % proteins (60% casein, 40% whey proteins) was prepared and the pH was adjusted to 7.0. A commercial double-stage homogenization (17-3.5 MPa) was applied to the product with a valve homogenizer. The pH was readjusted and then a second homogenization step was performed on some samples at pressures of 48 MPa or 77 MPa with a Microfluid laboratory scale homogenizer (Microfluidic Corp., Boston, MA USA). The mix was finally in-can sterilized (121°C-20 min). The cans were stored at 25°C for 6 months. Samples were taken after processing and after three and six months.

Viscosity measurements

Apparent viscosity of the samples was determined at 25°C using a Brookfield LV viscometer fitted with spindles, no. 3 and 4, (effective shear rate between 8 and 20 s^{-1}). Before samples were taken for analysis, the cans were agitated for 15 minutes with a magnetic stirrer in order to redisperse their contents and to ensure representative sampling.

Gel dissociation

The following dissociation agents were used: EDTA (ethylenediamine tetraacetate) (30 mM), urea (4 M) and ß-mercaptoethanol (40 mM). The agents were added directly to the samples up to the selected concentration, the samples were agitated for 60 min before their viscosity was measured. After EDTA additions the pH was adjusted back to 6.8 using 0.1N NaOH, before viscosity measurement. 0.1N HCl and 0.1N NaOH, or NaH_2HPO_4 and Na_2HPO_4 were added to the samples in order to study the effect of pH changes on the product's viscosity.

The results of viscosity measurements on dissociated gels are reported as dimensionless relative viscosity. This term expresses the ratio between the viscosity of samples treated with dissociating agents and the viscosity of untreated samples. This expression has been chosen to better compare the effect of the various dissociating agents.

Microscopy

Sample preparation The samples were encapsulated in agar according to Salyaev (1968), fixed at 4 °C in buffered-glutaraldehyde (Sorensen buffer, 0.1M) at pH 6.8 for 16 h, washed in phosphate buffer for 4 h and post-fixed in 1% OsO_4 for 2 h. Dehydration was done in increasing concentrations of ethanol (30% to 100%). The capsules were impregnated overnight in a dimethylaminomethyl phenol (DMP 30)-catalysed resin mixture, and were thereafter embedded in Epon 812 for sectioning. Ultrathin preparations were made using a LKB 8800 Ultratome III (LKB-Produkter AB, Bromma, Sweeden) microtome.

Optical microscopy Sections of 2 µm thick were stained in 0.1 % toluidine. Microscopic examination was done using a photomicroscope (Zeiss 63500) with phase contrast illumination at 80 X magnification. Micrographs were taken on Panatomic-X, 32 ASA, Kodak film.

Transmission electron microscopy (TEM) Sections of 80 nm thick were double-stained with 2 % uranyl acetate and lead citrate according to Reynolds (1963). The examination was done using a Jeol 1200 EX transmission electron microscope, operating at 80 kV, at 8000 X magnification.

Fat globule size determination Average diameters of fat particles have been measured from enlarged electron micrographs. We calculated the volume/surface average diameter ($d_{v/s}$) which is obtained from the following equation:

$$d_{v/s} = \frac{\Sigma i\, d_i^3}{\Sigma i\, d_i^2} \frac{4}{\pi}$$

where d_i is the fat particle diameter measured on the micrograph and $(4/\pi)$ a correction factor to take into account the random fracture plane of fat particles during sample preparation (Bird and Stainsby, 1974). The volume/surface diameter is equivalent to the particle diameter of a monodisperse emulsion having the same oil phase volume and the same interfacial area.

Results and discussion

Viscosity of homogenized infant formulas

Homogenization reduces fat particle size and creates new oil/water interface according to the pressure used (Mulder and Walstra, 1974). During the homogenization process, the newly formed interface is rapidly covered with amphiphilic material (mainly proteins) (Oortwijn and Walstra, 1979). We observed that the initial viscosity of infant formula increased with the pressure applied (table 1). As a first approximation, the increased viscosity resulting from homogenization could be attributed to the newly formed membrane which increases the effective dispersed phase fraction of the emulsion (Bird and Stainsby, 1974). High pressures generate larger interface which allow more proteins to be integrated in the dispersed phase of the emulsion.

However, it is unlikely that the increase of dispersed phase fraction upon homogenization is the only factor responsible for viscosity of infant formula. Other variables such as polydispersibility, degree of aggregation, particle size, shape, deformability, and viscoelastic properties of the membranes have been shown to affect the viscosity of suspensions (Dickinson and Stainsby, 1982). One should consider the effect of homogenization on

Table 1 Effect of homogenization pressure on the viscosity of homogenized infant formulas immediately after processing.

Homogenization pressure (MPa)	Viscosity (mPa.s)
17-3.5 (double stage)	10
19	15
48	37
77	46

these parameters to better understand the phenomenon.

Thickening and gelation upon storage

Effect of high-pressure homogenization. The viscosity changes upon storage have been measured after three and six months (figure 1). The formula homogenized at high pressures (48 MPa and 77 MPa) showed evidence of age thickening and gelation. The rate of change of viscosity was related to the pressure used to homogenize the product. It appears that high-pressure homogenization accelerates the phenomenon of age gelation according to the pressure used. This trend has also been observed for UHT and sterilized concentrated milk (Harwalkar, 1982).

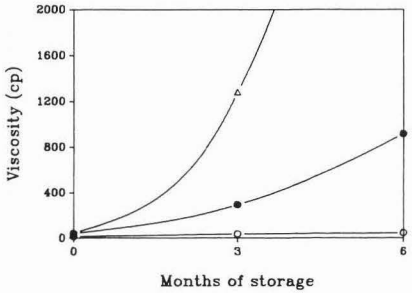

Figure 1. Viscosity changes (over 6 months at 25°C) of sterilized infant formula homogenized at 17-3.5 MPa (o), 48 MPa (●) or 77 MPa (Δ).

Age thickening and gel formation are related to the aggregation of protein material in the product. Therefore, according to its effect on these phenomena, homogenization is believed to alter the interaction potential of proteins. However, it has been shown that even the large casein micelles are too small to be directly affected by homogenization (Walstra, 1980). It is then likely that protein adsorption and spreading onto fat droplets, which occur during homogenization, are responsible for increased sensitivity to aggregation. Unfolding of proteins might expose new regions of the structure that promote interactions. Absolom et. al. (1987) studied the surface properties of protein-coated polymers and reported an important drift of protein surface energy upon adsorption. Assuming that proteins adsorbed onto fat droplets are more sensitive to aggregation than non-adsorbed proteins, one could relate the aggregation potential of an emulsion to the proportion of the total proteins adsorbed onto the fat droplets.

Considering the fat and protein content of infant formula, one could approximate the effect of reducing fat droplets size on the proportion of adsorbed and non-adsorbed protein. For that purpose, we assumed a constant protein load of fat droplets of 10 mg/m^2 (Melsen and Walstra, 1989). There is however limitation to this assumption. Fisrt it has been shown, in emulsions containing casein micelles, that the protein load tends to increase with decreasing particle size (Walstra and Oortwijn, 1982), and second, for very fine particle dispersion, the lack of protein could limit the amount adsorbed which in turn would limit the protein load. Despite these limitations, the calculation has been done and the results are presented on figure 2. Decreasing particle size of the fat droplets induces an approximately hyperbolic increase of the proportion of adsorbed proteins. Inversely, the amount of free proteins is reduced. Average particle size was determined on our samples from the electron micrographs. According to these calculations, the average diameter (volume/surface) of low (17-3.5 MPa) and high (77 MPa) pressure homogenized samples were respectively 0.71 and 0.25 um. In relation with figure 2, the high pressure conditions would increase the proportion of adsorbed protein from 19 to 55%. Therefore, it is believed that the large amount of proteins sensitized to aggregation (via adsorption onto fat droplets) could explain the rapid viscosity increase during the storage of these samples.

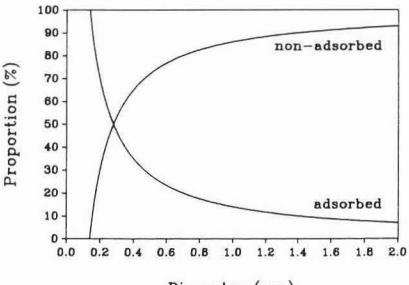

Figure 2. Distribution of proteins in infant formula as a function of the mean particle size of the fat dispersion.

The inclusion of small oil droplets into the protein matrix could also be responsible for accelerated age gelation of high pressure homogenized samples. Tiny oil droplets, presumably covered with proteins, participate in gel formation and increase the volume fraction of the gel matrix.

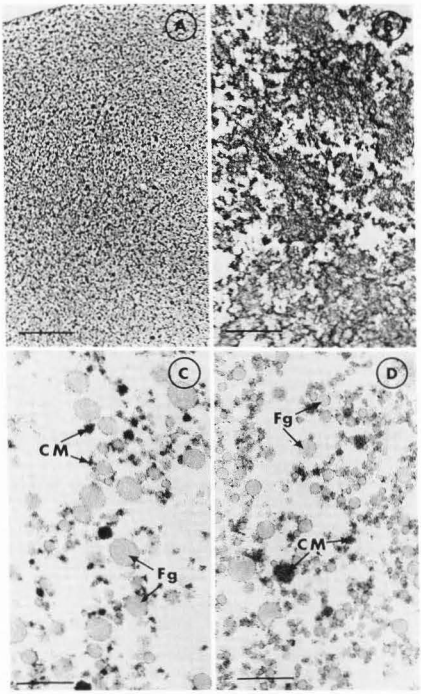

Figure 3. Microstructure of sterilized infant formula after 3 months storage: (A) commercial sample under optical microscope; (B) 77 MPa-homogenized sample under optical microscope; (C) TEM micrograph of commercial sample; (D) TEM micrograph of 77 MPa-homogenized sample. Bar = 100 µm for A and B, 1 µm for C and D.

Integration of tightly attached fat in protein clusters has been shown by Geyer and Kessler (1989).

As seen from figure 3, the coarse structure of products homogenized at low and high pressure can be observed using optical microscopy as the microstructural details may be better seen on the electron micrographs. We observed from optical micrographs that the gelled sample (figure 3B) showed uneven morphology composed of large aggregates. The aggregates in the ungelled sample (figure 3A) are much smaller and uniformly distributed. The electron micrographs revealed the presence of both fat globules (fg) and casein micelles (cm). Large globules (up to 1 µm) have been found in the commercial sample (figure 3C). The high pressure homogenized sample (figure 3D) gelled and showed some "chain-like" arrangements composed of fat globules and casein micelles. The large colloidal surface associated with the size of fat droplets might have promoted associations between colloidal particles. The absence of free casein micelles is also noticeable in that sample. Again, considering the average size of fat particles (0.25 µm), most of the micelles are believed to be adsorbed on fat surface. Such interactions between fat particles and casein micelles could be suspected from the work of Wilson et. al. (1963) on gelled concentrated milks.

Breakdown of gel matrix

Effect of dissociating agents. Different dissociating agents have been tested in order to break down the the gel matrix obtained after storage. The reagents have been chosen in order to determine the types of interactions responsible for gel formation. Selective dissociating agents were: EDTA (for Calcium bridges), urea (for H-bonding) and b-mercaptoethanol (for S-S bonds).

From viscosity data (table 2), it was observed that EDTA increased the viscosity of the sample. Calcium chelation may have caused some swelling of the casein micelles, which would explain the viscosity increment despite the dissociating action of EDTA. The two other dissociating agents (urea and ß-mercaptoethanol) produced a decrease in viscosity. When a binary mixture of dissociating agents was used, a synergetic effect was observed for those containing urea. This effect suggests that not all H-bonds are accessible to urea. Calcium bridges or S-S bonds need to be disrupted to allow an extensive action of urea. The very low viscosity of the EDTA-Urea treated samples emphasizes the importance of H-bonds and calcium bridges in the gel structure.

Table 2 Effect of dissociating agents on the relative viscosity of infant formula gels.

Treatment	Relative viscosity
Untreated (gelled)	1.00
Mercaptoethanol (40 mM)	0.52
Urea (4 M)	0.46
EDTA (30 mM)	1.31
Mercapto. + Urea	0.37
Mercapto. + EDTA	0.82
Urea + EDTA	0.06

The optical micrographs provided some confirmations of the previous findings using viscosity measurements. Samples treated with EDTA exhibited some gel disaggregation (figure 4B). The combination EDTA-Urea (figure 4D) produced the finest dispersion, suggesting an extensive dispersion of the gel. However, when urea was used alone (figure 4A) or with ß-mercaptoethanol (figure 4E), no evidence of disaggregation was found. When ß-mercaptoethanol was used alone (figure 4C) or with EDTA (figure 4F), large

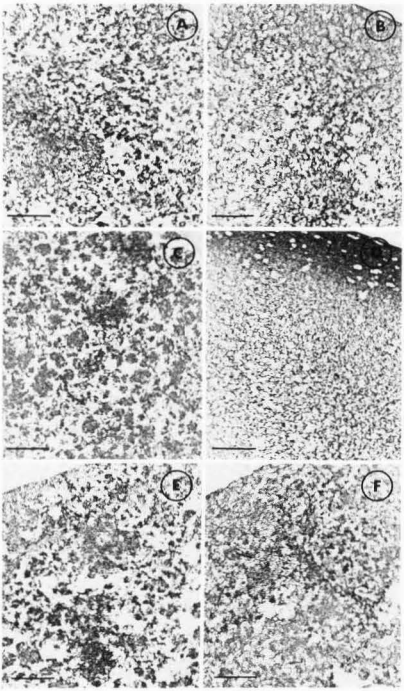

Figure 4. Microstructure (optical micrograph) of infant formula gels upon added dissociating agents: (A) Urea 4 M; (B) EDTA (30 mM); (C) β-Mercaptoethanol (40 mM); (D) Urea (4 M) + EDTA (30 mM); (E) Urea (4 M) + β-Mercaptoethanol (40 mM); (F) β-Mercaptoethanol (40 mM)+EDTA (30 mM). Bar = 100 µm.

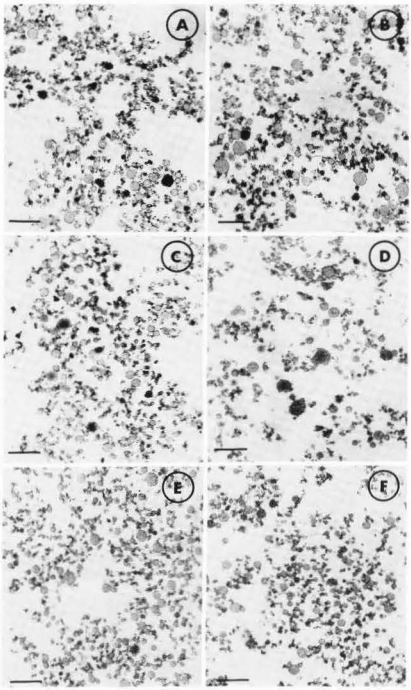

Figure 5. Microstructure (TEM micrograph) of infant formula gels upon added dissociating agents: (A) Urea 4 M; (B) EDTA (30 mM); (C) β-Mercaptoethanol (40 mM); (D) Urea (4 M) + EDTA (30 mM); (E) Urea (4 M) + β-Mercaptoethanol (40 mM); (F) β-Mercaptoethanol (40 mM) + EDTA (30 mM). Bar = 1 µm.

aggregates were found, questionning the implication of S-S bonds in gel matrix. However, Wilson et. al. (1963) observed a quick release of fat globules from the protein-fat aggregates in the presence of reducing agents (Na_2SO_3 and ascorbic acid). According to the authors, this effect was due to the occurence of disulfide bonds in the gel structure, surrounding the fat particles.

The electron micrographs (figure 5) provided limited information on the gel structure since the occurence of "chain-like" structure was found on all micrographs. However, one should remember that TEM is not the best suited method to reveal associations between particles since only the associations in the plane of the observation can be observed (Kalab et. al., 1976). None of the tested dissociating agents affected the size of fat particles.

Effect of pH changes. Since the effectiveness of the dissociating agents used in our study was due to their action on the proteins, it was thoughtthat other variables affecting the proteins would also affect the gels. So the pH was modified using separately NaOH and HCl, or NaH_2PO_4 and Na_2HPO_4, and the viscosity was monitored over the 6.5-7.5 pH range.

In fact, altering the pH of the gelled samples with NaOH or HCl had an important effect on viscosity (Figure 6). This phenomenon is believed to be related to the effect of pH on the casein micelle's colloidal calcium phosphate (Pyne and McGann, 1960) which might contribute to gel formation. Increasing the pH, presumably increased the colloidal forms of calcium phosphate, and thus, increased the viscosity of the sample. Inversely, decreasing pH solubilized some colloidal calcium phosphate and reduced sample viscosity. When the pH was altered using sodium phosphate salts, the excess of phosphate ions possibly exceeded the solubility product of calcium phosphate. Thus, higher proportions of colloidal calcium phosphate were expected than with HCl or NaOH at any pH. As seen from fig. 6, samples having had their pH altered with sodium phosphate salts showed higher viscosity than samples treated with HCl or NaOH. This result suggests a contribution of the equilibrium between colloidal and soluble forms of calcium phosphate to the age gelation of infant formula.

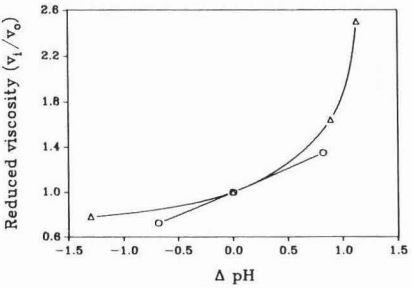

Figure 6. Effect of pH changes on the relative viscosity of infant formula gels, using HCl and NaOH (o), or NaH_2PO_4-Na_2HPO_4 (Δ).

Conclusion

The results of this study suggest that high pressure homogenization removes casein micelles from the serum phase as they form a tightly bonded fat protein complex. Spreading of casein on fat particles is believed to increase their interaction potential and their availability for association, which in turn explains the acceleration of age gelation phenomenon. Breakdown of gel matrix when using dissociating agents suggests that hydrogen bonds and calcium bridges contribute to the gelation phenomenon upon storage. Furthermore, the equilibrium between the colloidal and soluble forms of calcium phosphate presumably has a determining effect on the gel strength in the process of age gelation of sterilized infant formula.

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Discussion with reviewers

P. Walstra: The 15 min agitation using a magnetic stirrer may considerably lower the apparent viscosity, or even redisperse the gel, so that the results of the viscosity test may be rather meaningless. Please comment.
Authors: We agree with the idea that mechanical stress reduced the apparent viscosity of our samples. However, the gravitational separation of the product upon storage forced us to re-establish the homogeneity before sampling. Another aproach would have been to store the products in a device which maintains slow rotation in order to avoid gravitational effects. However, even then, the transfer of the product from the can to the rheometer cell would have induced some gel disruption which is still difficult to control. For this reason, we chose the first aproach which consisted in applying a controled mechanical stress prior to the viscosity measurement. The product's viscosity was then related to the mechanical resistance of the gel which depends on the strenght of interactions within the matrix.

P. Walstra: I thought it was fairly clear that one mechanism (proteolysis) is responsible for age gelation in UHT unconcentrated milk products, and the other one (physico-chemical) normally in evaporated milk ?
Authors: From the evidences presented by Harwalkar (1982), we believe that it is not clear wether the two hypotheses can be associated with one or the other type of sterilized products (see pages 260 to 264). In this review, it is demonstrated that age gelation can occur in UHT unconcentrated milk products showing no proteolysis. The age gelation phenomenon can be seen as the result of more than one mechanism.

B.E. Brooker: When using the agar tube technique with your material, are milk solids uniformly distributed along the tube after embedding or are there local concentrations resulting from the removal of water during dehydration ?
Authors: We have not observed such concentration gradient in our preparations. It seemed that the glutaraldehyde fixation maintained an homogeneous solids distribution within the sample. From the observation of larger embedded sections under optical microscope, we could not notice differences in structure between the center and the edge of the capsule. Jewell (1981) used succesfully the agar tube technique for fruit juices, which are less viscous and contains much less protein than our product. These points suggest that the solid distribution in our samples was not affected by the preparation technique.

D.N. Holcomb: While the analytical composition of the liquid mix is given, details of the ingredients are not given. Without such details, a reader could not duplicate the experiment.
P. Walstra: Was the milk pre-heated ? Is the casein present in micellar form ?

B.E. Brooker: Since the observations made in this paper hinge on the chemical composition of the milk, more details of the preparation of the infant formula should be given. Thus, is all of the fat butterfat ? Is the 14% carbohydrates predominantly lactose ? Was the mixture prepared from reconstituted milk powder ?
Authors: As forementionned, the liquid mix was a commercial product of which detailed formulation and process could not be divulged. However, we know that a pre-heating treatment was applied to the product but we could not have details about the exact conditions. Also, from a partial list of ingredients, we know that this product contains skim milk solids (thus, the caseins were added in micellar form), lactose, soya and coconut oil, mono- and di-glycerides, lecithin from soya and carraghenin.

M. Ruegg: The role of whey proteins have not been discussed enough. The high temperatures applied to the samples suggest a complete denaturation of whey proteins and it is known that the hydrophobic whey proteins are adsorbed on both, casein micelles and fat globules. The authors should comment on this.
Authors: For the purpose of this study, we have considered the protein fraction of infant formula as a whole. However, we agree with the reviewer that this type of protein could affect the viscosity change upon storage. During the sterilization treatment, whey proteins are believed to adsorb onto fat droplets and casein micelles, changing their surface properties. It is also believed that the heat treatments alter whey protein molecules already adsorbed onto fat droplets. Those changes could affect the gelation kinetics of infant formula. However, since the amount of whey proteins and the severity of heat treatment were the same for all samples, it is not possible from our data to further discuss those points.

M. Ruegg: The high temperatures and pressures applied to the infant formulas cause denaturation and precipitation of whey proteins and possibly alteration of caseins. What is the authors opinion about the application of chromatographic methods to investigate the state and role of the various proteins on the fat globule surface ?
Authors: The homogenization pressure and sterilization temperature could affect the composition and surface properties of the protein layer which surrounds fat droplets. To study the fat droplet surface properties, one could use hydrophobic chromatography in the same way as used to characterize the surface properties of bacteria (Dahlback et. al., 1981). However, the polydispersity of fat droplet could interfere with the method. We would also suggest the methods which have been used to characterize proteins adsorbed onto polymers, such as sedimentation volume and contact angle measurements (Absolom et. al., 1987). Those approached could be adapted to study the surface properties of the protein membrane adsorbed onto fat droplets.

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