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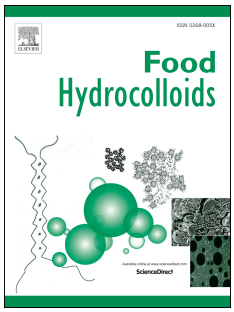

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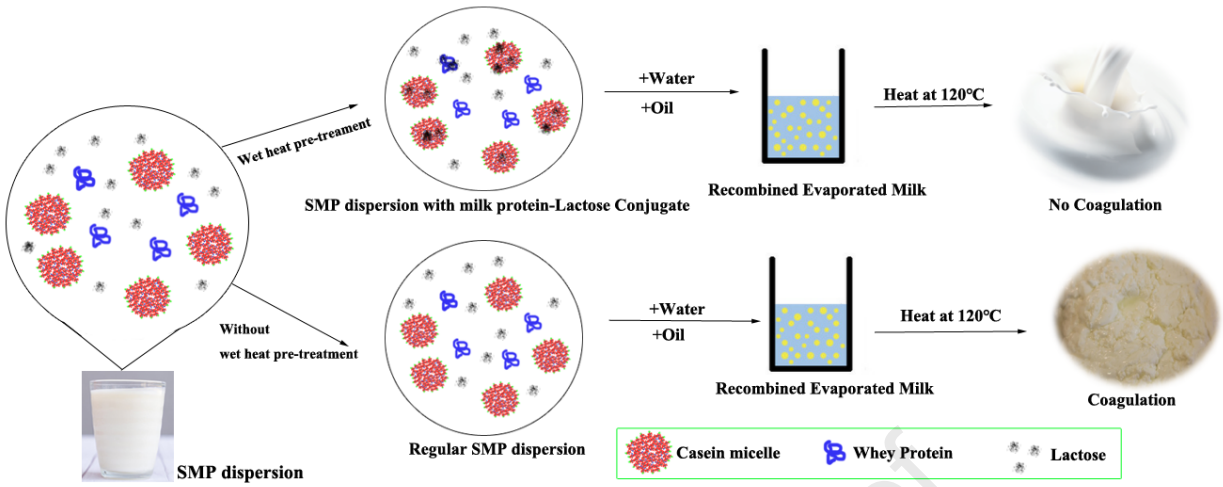

Improved heat stability of recombined filled evaporated milk emulsions by wet heat pretreatment of skim milk powder dispersions

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Abstract

In this study, skim milk powder (SMP) aqueous dispersions were subjected to a preliminary wet heat incubation at various temperatures (70-90 ℃) to improve the heat stability of recombined filled evaporated milk (RFEM) emulsions. The determination of the free amino group and free lactose content and SDS-PAGE revealed that both whey proteins and caseins were involved in the conjugation with lactose upon preliminary wet heating at different temperatures. The results of the non-sedimentable serum protein content, the particle size and relative viscosity of the SMP dispersion indicated that both association of whey proteins with casein micelles and casein dissociation from the micelles occurred during the incubation. The heat stability of the subsequently produced RFEM emulsions was greatly enhanced: RFEM emulsions could withstand a typical in-container sterilization procedure (i.e. 30 min of heating at 120 ℃) without coagulation ($D_{4,3}$ <5 μm and consistency coefficient <50 mPa·s) when selecting an appropriate incubation time. Generally, the higher the incubation temperature, the shorter the incubation time needed to enable a heat-stable RFEM emulsion. However, prolonged incubation had a negative effect on the heat stability of RFEM emulsions, and a narrower incubation time interval that enabled an improved heat stability was observed at a higher incubation temperature.

Key words: skim milk powder; recombined filled evaporated milk emulsion; wet heat pretreatment; conjugation; heat stability

1. Introduction

According to the United States Code of Federal Regulations (April 2006), evaporated milk is the liquid food obtained by partial removal of water only from milk. It contains not less than 6.5 percent by weight of milk fat, not less than 16.5 percent by weight of milk solids not fat, and not less than 23 percent by weight of total milk solids. Recombined evaporated milk (REM) is an O/W emulsion product of similar composition which is typically obtained by homogenization of a highly concentrated aqueous dispersion of skim milk powder (SMP) and anhydrous milk fat (Kasinos, Le, & Van der Meeren, 2014). When the milk fat is replaced by an alternative fat source,

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such as a (cheaper) vegetable oil, these products are referred to as recombined filled evaporated milk (RFEM; (Kjaergaard, 1982). Native and recombined (filled) evaporated milk is widely used for drinking after dilution, as well as for infant food applications, or as a coffee whitener. One of the biggest challenges in the manufacturing of (recombined) evaporated milk is the possible coagulation during the intense sterilization (Crowley, et al., 2015; Dumpler & Kulozik, 2016). According to Liang, et al. (2017), this heat-induced emulsion droplet aggregation is a direct consequence of the heat-induced unfolding and association of non-adsorbed globular whey proteins (WPs), such as β-lactoglobulin, with interfacial and other non-adsorbed proteins to initiate large flocculates and/or aggregates via non-covalent and/or disulfide bonds. Finally, this may lead to the formation of a visible coagulum, which severely damages the product quality and production equipment. Until now, various strategies have been proposed to improve the heat stability of (recombined filled) evaporated milk, such as the addition of a heat stabilizer and pre-heating. Heat stabilizers like lecithin can be used before packing and sterilization of milk, but the addition of heat stabilizers is against the current trend of natural and clean-label products (Asioli, et al., 2017). Intense pre-heat treatment is now a standard practice in manufacturing high-heat SMP or evaporated milk. It refers to a relatively short-time-high-temperature heat treatment prior to evaporation, e.g. 90 ℃ for 5 min or 120 ℃ for 1 min.

As the heat instability of (recombined) evaporated milk largely depends on protein interactions and other protein-related changes, the Maillard conjugation of milk protein and sugar via controlled heating, which has been proven to be an effective strategy to improve the functional properties of milk proteins including their heat stability, might be an effective approach to improve the heat stability of (recombined filled) evaporated milk (Wu, et al., 2021). Liu and Zhong (2015) found that a short-time-high-temperature dry heating (130 °C for less than 30 min) of whey protein isolate (WPI) and lactose could improve the heat stability at various acidity and ionic conditions. A'yun, et al. (2020) reported that dry heating of WPI and lactose enabled a constant particle size and viscosity of WPI-stabilized O/W emulsions against heating at 80 °C for 20 min. Thanks to the indigenous sugars (mostly lactose) present, no additional ingredient and processing is needed to produce glycated proteins starting from SMP, which is favored for industrial application. Hiller and Lorenzen (2010) reported the Maillard conjugation in SMP upon dry heat incubation, but a detrimental effect on the heat stability of SMP dispersions was found. Some studies focused on the lactosylation in dairy products during processing, such as concentration, sterilization, and storage (Balde & Aider, 2019; Chandra Roy, Zhang, Liu, & Zhou, 2020; Liu, et al., 2019). However, an enhanced heat stability of dairy emulsions has not been reported under these conditions. Previously, Wu, Chen, Sedaghat Doost, A'yun, and Van der Meeren (2020) found that the dry heating of SMP greatly improved the heat stability of RFEM emulsions. Compared with dry heating, the wet heat treatment of aqueous SMP dispersions is much easier to be operated and automated in industry. Furthermore, the time required to obtain a desired extent of conjugation is mostly reported to be shorter in wet heating (Doost, Nasrabadi, Wu, A'yun, & Van der Meeren, 2019).

In this study, SMP dispersions were incubated at different temperatures (70-90 ℃) at such a concentration (i.e. 17.6% SMP, w/w) that RFEM with 6.5% fat and 23% of total solids was obtained upon addition of a fat phase and subsequent homogenization. The characterization of the

Maillard conjugation during incubation was carried out by color measurement, the determination of the free amino group and free lactose content, and SDS-PAGE. Moreover, the protein composition of the non-sedimentable serum protein, as well as the particle size and relative viscosity of the SMP dispersion was determined. Finally, the effect of a preliminary wet heat incubation of the SMP dispersion on the heat stability of the resulting RFEM emulsions was investigated via particle size analysis and viscosity measurements.

2. Materials and methods

2.1 Materials

Low-heat SMP, characterized by a whey protein nitrogen index (WPNI) of 6.1, was obtained from Milcobel Dairy Corporation (Kallo, Beveren, Belgium). According to the manufacturer, this SMP contained 37.7% of protein (7.6% whey protein, 30.1% casein), 51.5% of lactose and 0.8% of fat (w/w). Sodium caseinate and whey protein isolate (WPI) used as controls in SDS-PAGE were purchased from Sigma-Aldrich (Overijse, Belgium). The sunflower oil used for the preparation of the recombined filled evaporated milk was purchased from a local supermarket.

2.2 Wet heat pre-treatment of SMP dispersions

A typical composition of recombined evaporated milk without fat was used to prepare the SMP dispersions: 16.5 g SMP was reconstituted in 77 g of 0.02% NaN_3 solution (to prevent microbial contamination). Hence, the concentration of SMP was 17.6% (w/w). These SMP dispersions were subsequently incubated in a water bath at 70, 80 and 90 ℃. As chemical reactions occur faster at higher temperatures, the incubation times were reduced at higher temperatures accordingly, i.e. up to 24 h, 4 h and 2 h at 70, 80 and 90 ℃, respectively. The level of water inside the water bath was sufficient to completely immerse the SMP dispersion in the plastic containers. After that, the incubated dispersions were cooled down in cold water immediately.

2.3 Characterization of Maillard reaction during wet heat incubation of SMP dispersions

2.3.1 Color measurements

Color measurements in terms of the L^*, a^*, and b^* values at the specular component excluded (SCE) mode were performed using a colorimeter (Minolta Model CM-2500D Spectrophotometer, Konica Minolta Sensing, Tokyo, Japan). The values were calculated from the average of 10 measurements. The total color difference (ΔE) was calculated using Eq. (1),

$$\Delta E = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad (1)$$

where L^*, a^* and b^* were the color parameters of the incubated SMP dispersions, whereas L_0^*, a_0^* and b_0^* were the color parameters of the untreated SMP dispersions.

2.3.2 Determination of free amino group content

As described in previous studies (Setiowati, Vermeir, Martins, De Meulenaer, & Van der Meeren, 2016), all samples were diluted 10 times in 1% SDS solution before further analysis. Subsequently, 0.25 ml of each sample was added into a test tube with 2 ml of phosphate buffer (0.2125 M, pH 8.2) and 2 ml of 0.01% 2,4,6-trinitrobenzene sulfonic acid (TNBS) solution. The mixture was shaken and incubated in a water bath in a dark environment at 50 ℃ for 1h. Subsequently, 4 ml of

0.1 M HCl was added to terminate the reaction. The absorbance of the samples was finally read at a wavelength of 340 nm (UV-1600 PC spectrophotometer, VWR, PA, USA).

2.3.3 Determination of free lactose content

The content of free lactose in reconstituted SMP was determined by the 3,5-dinitrosalicylic acid (DNS) method as reported previously (Listiohadi, Hourigan, Sleigh, & Steele, 2005; Svorc, Miertus, & Barlikova, 1990). DNS in alkaline solution can be reduced by the free carbonyl group of reducing sugars to 3-amino 5-nitro salicylic acid with an orange color. A concentration series of lactose (0-1.5 mg/ml) was used to determine the standard curve. The DNS reagent contained 1% (w/w) DNS, 0.4 M NaOH and 30% (w/w) of potassium sodium tartrate. Subsequently, 0.5 ml diluted sample and 0.5 ml DNS reagent were well mixed and heated in a boiling water bath for 5 min. The mixtures were immediately diluted with 5 ml H_2O and cooled down in cold water. The absorbance was measured at 540 nm against the blank, and the free lactose content was calculated from the standard curve.

2.3.4 SDS-PAGE analysis

SDS-PAGE, as described by Laemmli (1970), was performed on 15% polyacrylamide gels under reducing conditions to analyze the compositional changes in the reconstituted SMP during the wet heat incubation. Sodium caseinate and WPI were used as controls. The samples were diluted 30 times with demineralized water to a protein concentration of 2.2 mg/ml, while the concentration of the controls was 0.5 mg/ml. The sample volume was 15 µl. The electrophoresis was performed at 110 V for 1.5 h. The proteins were finally visualized by gel staining with Coomassie Brilliant Blue R-250.

2.4 Characterization of the incubated SMP dispersions

2.4.1 Non-sedimentable protein analysis

SMP dispersions before and after incubation were ultracentrifuged using a Beckman L7-55 ultra-speed centrifuge with SW40 Ti-rotor (Pasadena, CA, USA) at 120,000×g and 25 ℃ for 60 min to separate the serum from the casein micelles. The protein concentration of the supernatant was determined by a simplified Lowry method as described by Schacterle and Pollack (1973).

The supernatant was diluted 30 times and subjected to SDS-PAGE analysis as mentioned in 2.3.4. The protein concentration of the diluted supernatant was 0.5 mg/ml, while the concentration of the controls was 0.5 mg/ml. The gel was scanned with a gel imager (Bio-Rad, CA, USA). For quantitative analysis, the band densities of individual bands in the gel were calculated using the software ImageJ in grayscale mode to obtain integrated grey values.

2.4.2 Particle size determination

The particle size of the SMP dispersions was measured before and after wet heat pre-treatment using photon correlation spectroscopy (PCS) with a spectrometer 100 SM (Malvern Instruments, Malvern, UK) equipped with a 15 mW He–He laser and a K7032 multi 8-bit correlator (Malvern Instruments, Malvern, UK). The dispersions were diluted 1500 times using a Ca-imidazole buffer (20 mM imidazole, 5 mM $CaCl_2$, pH 6.8) to prevent micelle disruption during the measurement (Joyce, Brodkorb, Kelly, & O'Mahony, 2017). All of the measurements were done within 15min after the dilution. The measurements were carried out at a scattering angle of 150° at 25 °C with 100 µm aperture. The Z-average mean diameter was obtained by cumulant analysis.

2.4.3 Relative viscosity of SMP dispersions

The relative viscosity (η_r) of the SMP dispersions was defined as the ratio of the dynamic viscosity of the SMP dispersion (η) to the dynamic viscosity of the serum phase after ultracentrifugation of the SMP dispersion as described in 2.4.1 (η_o), which were measured using an Ubbelohde viscometer (Type 53201/0a, Schott-Geraete, Germany).

The voluminosity of the casein micelles in SMP dispersions can be calculated from their viscosity using the model developed by Krieger and Dougherty (1959). This model enables to calculate the volume fraction of casein micelles (ϕ) from the relative viscosity (η_r) and the maximum packing fraction of the dispersed particles (ϕ_{max}) using Eq. (2)).

$$\phi = \phi_{max} \cdot \left(1 - \eta_r^{-1/[\eta] \cdot \phi_{max}}\right) \quad (2)$$

The voluminosity ν (in ml/g of casein) can be directly calculated by. Eq. (3), based on the volumetric concentration of casein in the SMP dispersion (c), assuming the intrinsic viscosity [η] to be equal to 2.5. The volumetric casein concentration (0.0564 g/ml) follows from the mass fraction of casein in the SMP dispersion (0.0531 g/g), multiplied by the density of the SMP dispersion (1.0615 g/ml).

$$\nu = \frac{\phi}{c} \quad (3)$$

2.5 Heat stability of recombined filled evaporated milk (RFEM) emulsions

2.5.1 Preparation of RFEM emulsions

Sunflower oil (6.5g) was added to the SMP dispersions (93.5 g) to prepare RFEM emulsions. Hence, the final composition of the RFEM emulsions was 16.5% SMP and 6.5% oil (w/w). The mixtures were then pre-homogenized using a high-speed blender (Ultra-Turrax, type S50N–G45F, IKA-Werke, Germany) for 1 min. They were subsequently homogenized using a Microfluidizer 110S (Microfluidics Corporation, Newton, MA, USA) at 55 °C and a compressed air pressure of 0.2 MPa (corresponding to a liquid pressure of 28 MPa) for 2 min. After emulsification, the recombined filled evaporated milk emulsions were cooled down at room temperature. All of the emulsions were adjusted to an identical pH value (pH 6.40 ± 0.02) and stored in a refrigerator for at least 12h before the heat coagulation test for ionic equilibration.

2.5.2 Heat stability test

According to the heat stability test developed by Kasinos, Karbakhsh, and Van der Meeren (2015), the RFEM samples (10ml) were contained in 20-mL headspace vials (75.5×22.5 mm, 1st hydrolytic class; Grace, Deerfield, IL, USA) with an aluminum crimp seal and immersed in a temperature-controlled oil bath (Fritel turbo SF, 5L capacity, Vanden Borre, Gent, Belgium). The level of the oil inside the bath was sufficient to completely immerse the RFEM emulsions in the glass vial. The temperature of the oil bath was set at 120±4 ℃ and kept uniform by stirring the oil by an IKA RW20 stirrer (Janke & Kunkel). After heating for 20 or 30 min, the samples were taken out and cooled in cold water immediately.

2.5.3 Particle size and viscosity of RFEM emulsion

The particle size of the RFEM emulsions was measured before and after heating using a Mastersizer 3000 (Malvern Instruments Ltd, Malvern, UK), assuming a refractive index of 1.47 and an absorption index of 0.01 for sunflower oil. Viscosity measurements of RFEM emulsions were carried out using a programmable LV-DV-II+ viscometer (Brookfield, Stoughton, MA, USA) as described previously (Kasinos, et al., 2015).

2.6 Statistical analysis

The single factor analysis of variance (one-way ANOVA) of SPSS19.0 software was used to

analyze significance of the data. Excel 2010 and Origin Pro 8 was used to calculate the mean and standard deviation and to draw figures.

3. Results and discussion

3.1 Characterization of Maillard reaction during wet heating of SMP dispersions

3.1.1 Color measurements

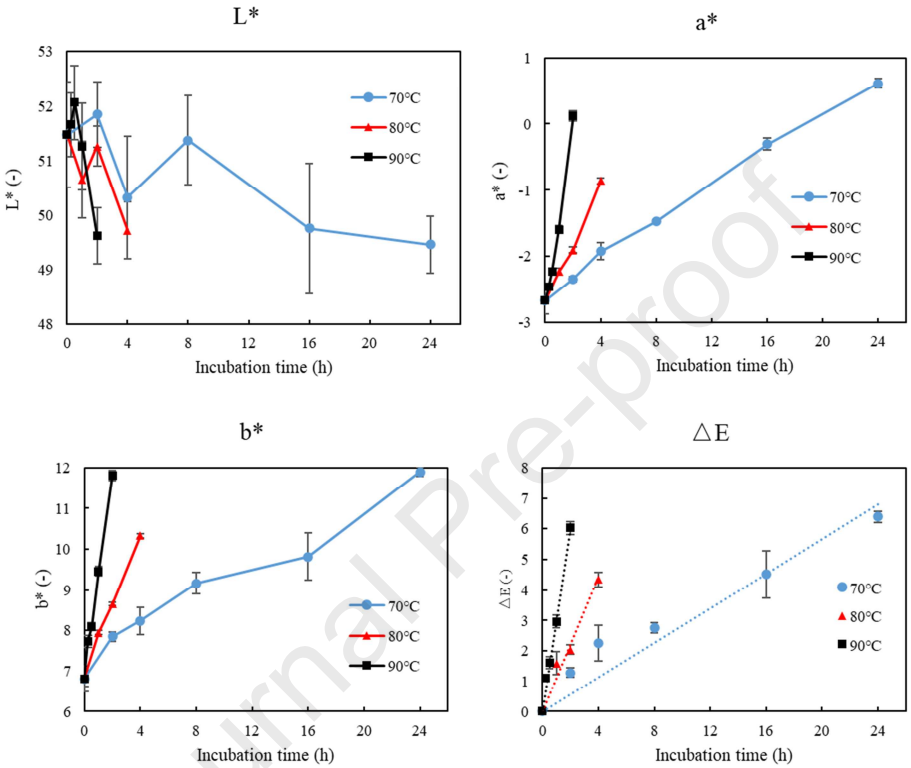

Fig. 1 Changes in color parameters of the SMP dispersions during wet heat pre-treatment at different temperatures; L^* represents the luminance, a^* expresses the redness and greenness, b^* indicates the yellowness and blueness and △E is the total color difference.

Whereas browning is desired for some food systems like bakery products, it is undesirable for dairy products. Hence, it is necessary to monitor the browning development to avoid over-processing. From Fig. 1, a decrease in luminance (L^*) was observed during incubation at the different temperatures, while a more pronounced increase in redness (a^*) and yellowness (b^*) was shown. Additionally, the total color difference data in Fig.1 show that a high temperature provoked a higher reaction rate: the slope of the linear regression is 0.28, 1.10 and 3.03 h^{-1} for 70, 80 and 90 ℃, respectively.

Brown color formation can be due to both Maillard reaction and sugar caramelization. The production of brown color is primarily related to the products formed in the advanced stages of the Maillard reaction, including unsaturated and nitrogenous polymers (such as melanoidins), and nitrogen-free products formed from condensation reactions of furfurals and dehydroreductones

(Drapala, Auty, Mulvihill, & O'Mahony, 2016; Liu, Kong, Han, Sun, & Li, 2014). Amongst these, the nitrogen in nitrogenous polymers is commonly provided by the lysyl residues in milk proteins (Devi, Buckow, Singh, Hemar, & Kasapis, 2015). In a kinetic study of brown color formation during the conjugation of casein and either lactose or glucose, Morales and Van Boekel (1998) also reported an increasing reaction rate of browning at a higher temperature. For completeness, it has to be mentioned in previous research that brown color formation may also be attributed to the caramelization of lactose (Devi, et al., 2015).

3.1.2 Determination of the free amino group content

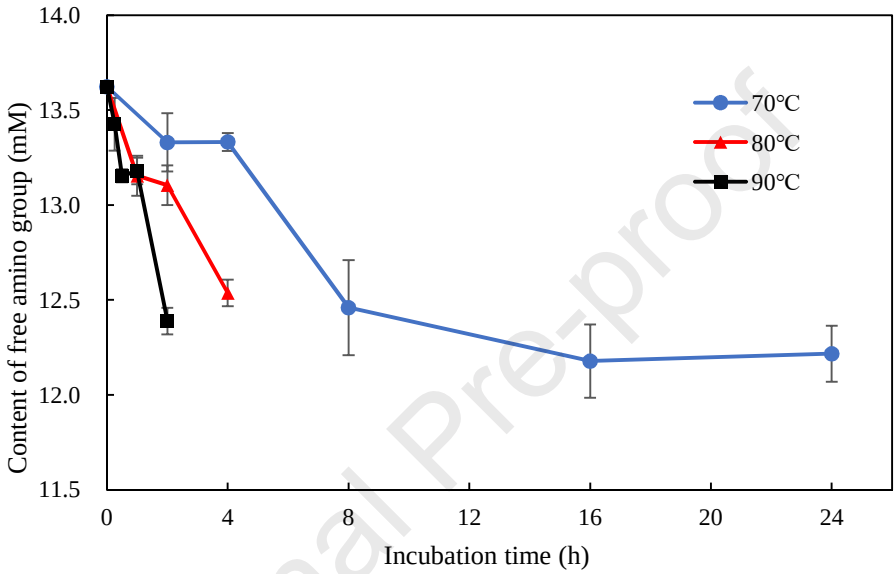

Fig. 2 Changes in free amino group content in reconstituted SMP during wet heat pre-treatment at 70, 80 or 90 ℃

It can be assumed that the free amino groups of milk proteins were consumed by saccharides via the Maillard reaction during wet heat incubation. In Fig. 2, the content of free amino groups at 70 ℃ decreased gradually in the initial 8 h, whereas the speed slowed down afterwards until 24 h. The concentration decreased from 13.6 mM to 12.2 mM after 24 h of incubation at this temperature, deducing (13.6-12.2)/13.6=10.3% of degree of glycation. A similar trend was observed at 80 and 90 ℃: the content of free amino groups decreased gradually until 2h and 4h, respectively. Generally, the glycation between milk proteins and lactose proceeds faster at a higher temperature. It took 4 h and 2 h at 80 and 90 ℃, respectively, to reach a comparable content of free amino groups with wet heating at 70 ℃ for 8 h.

In the following discussion, β-lactoglobulin (β-lg), α-lactalbumin (α-la) and bovine serum albumin (BSA) in a 5:3:1 weight ratio were considered for the whey protein fraction, while α_{s1}-casein (α_{s1}-CN), α_{s2}-casein (α_{s2}-CN), β-casein (β-CN), and κ-casein (κ-CN) in a 4:1:4:1 weight ratio were considered for the casein fraction. Considering the amino acid composition and molar weight of the different proteins (Supplementary material), it is estimated that the amount of free amino groups linked to the casein fraction, the whey protein fraction and the total milk protein in the original SMP dispersion corresponds to 49.1, 15.1 and 64.2 mM, respectively. By comparing the

amount of amino groups in reconstituted SMP before and after ultracentrifugation (13.6 mM versus 5.1 mM), it was deduced that the amount of amino groups of the caseins and whey proteins was 8.5 mM and 5.1 mM, respectively. Hence, the predicted values based on the composition are much higher than the measured values. This is possibly due to the compact structure of proteins. A'yun, et al. (2020) suggested that TNBS had a limited access to whey proteins due to their globular structure, leading to a higher degree of glycation calculated from the results of the TNBS method as compared to that estimated by diffusion NMR. The casein micelle structure might also adversely affect the accessibility of TNBS, although most of the micellar structure was destroyed in the presence of 1% SDS during the measurement (Lefebvre-Cases, Gastaldi, & Tarodo de la Fuente, 1998). Finally, the limited accessibility of TNBS to milk proteins might lead to an overestimation in the degree of glycation.

According to our previous research, a degree of glycation of 51.0% was achieved after dry heat incubation of SMP for 24 h at 60 ℃ and 74% relative humidity based on the free amino group content determined by TNBS method (Wu, et al., 2020). In the present study, the achieved degree of glycation is lower (i.e. 10.3%) after the same incubation time at an even higher temperature (70 ℃). Generally, it is believed that the Maillard reaction proceeds slower in dry state than in wet state due to the limited mobility of the reactants and the less effective contact between reactants (Doost, et al., 2019). However, the high concentration of SMP (i.e. 17.6%, w/w) during the incubation in this study could play an important role in the Maillard reaction. Zhuo, et al. (2013) demonstrated that reactions at a high concentration (e.g. 10%, w/w) in wet state preferably proceeded in the direction of products that have smaller excluded volumes, such as Maillard reaction products, whereas products with larger excluded volume produced by protein denaturation and polymerization were minimized. Hence, the lower degree of glycation in this study (as compared to dry heating) might be explained from a lower degree of protein polymerization which may also reduce the availability of free amino groups (Setiowati, et al., 2016).

3.1.3 Determination of free lactose content

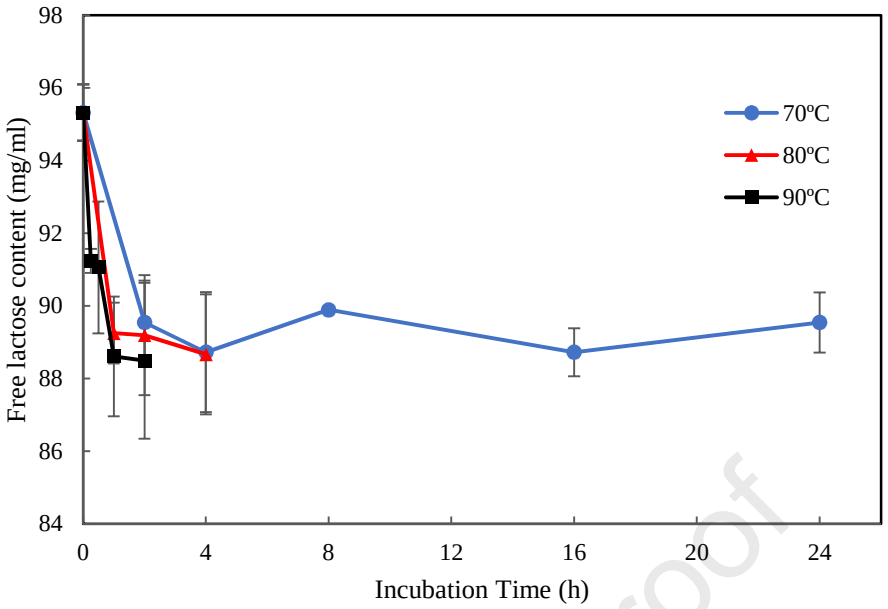

Fig. 3 Changes in free lactose content of reconstituted SMP during wet heat pre-treatment at 70, 80 or 90 ℃

As the Maillard reaction involves both free amino groups and reducing sugars, it follows that the extent of this reaction can be quantified not only by the determination of the free amino group content in milk proteins, but also from the free sugar content. Hereby, lactose was assumed to be the only reducing sugar present in SMP dispersions, despite of traces of other reducing sugars being present in milk. Since the lactose bound with milk proteins no longer exhibits a reducing ability (Lertittikul, Benjakul, & Tanaka, 2007), it is feasible to assess the Maillard reaction by determining the free lactose content, which can be done via the DNS method.

It was observed in Fig. 3 that the free lactose content decreased rapidly from 95.3 mg/ml to 89.5 mg/ml in the first 2 h of incubation at 70 ℃, whereas it fluctuated around 90 mg/ml afterwards with a free lactose content of 89.5 mg/ml after 24 h. A faster decrease in lactose concentration was found at a higher temperature in the initial period: it took 1h at 80℃ and 90℃ to reach a comparable free lactose content compared to 2 h incubation at 70 ℃ without significant difference *(P>0.05)*.

The measured free lactose content in the original sample (i.e. 95.3 mg/ml) is close to the theoretical value: mixing 16.5 g SMP with 77 g of water, a volume of 88 ml was obtained with an expected lactose concentration of 96.5 mg/ml based on a lactose content of 51.5% in the SMP, indicating that this method is reliable to analyze the free lactose content. After 24 h of incubation at 70 ℃, (95.3-89.5)/95.3=6.1% of the lactose was bound with milk proteins. Considering the average molecular weight of milk proteins (21.4 kDa) and lactose (342 Da), the number of reactive groups per molecule (i.e. 19.1 in protein, as compared to 1 in lactose), and the ratio of protein to lactose in SMP (i.e. 37.7% protein, and 51.5% lactose, w/w), the estimated ratio of amino groups in protein to carbonyl groups in lactose is approximately (37.7/21400*19.1)/ (51.5/342*1) =1:4.46. As one carbonyl group in lactose reacts with one amino group in protein

during the Maillard reaction, the estimated degree of free amino group reduction is 6.1%*4.46=27.2%. On the other hand, the degree of glycation estimated by the TNBS method was only 10.3% when considering that all the amino groups are accessible. If part of the amino groups is not accessible for TNBS, the degree of glycation should be even less. The discrepancy between the results from the free lactose and the free amino group is even more pronounced at a higher incubation temperature: 31.2% versus 8.0% at 80 ℃ after 4 h and 32.0% versus 9.0% at 90 ℃ after 2 h. This could be attributed to lactose caramelization which is also an important pathway during heating of milk. Generally, in the presence of proteins, the Maillard reaction is considered as a low-energy pathway for the decomposition of sugars compared with caramelization, and hence the Maillard reaction is preferable (O'Brien, 2009). However, lactose caramelization is also occurring during heating of milk, especially at a temperature above 100 ℃ (Van Boekel, 1996). Pellegrino, De Noni, and Resmini (1995) even reported that a considerable amount of lactulose (23 mg/ml), which is an indicator of lactose caramelization, was generated in milk after heating at 70 ℃ for 1h, and the lactulose level at a higher temperature could be several times higher than the furosine level which is a Maillard reaction indicator. Rozycki, Buera, Piagentini, Costa, and Pauletti (2010) also confirmed the significant role of caramelization in the development of color in concentrated milk during heating. Therefore, lactose caramelization could play a role in the loss of free lactose under the conditions of this study, whereby concentrated skim milk was heated at 70-90 ℃ for several hours.

3.1.4 SDS-PAGE

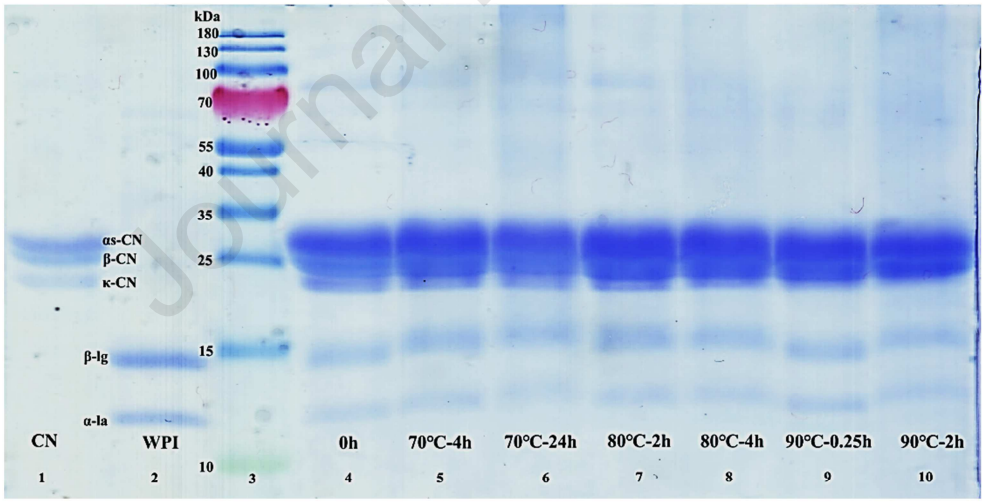

Fig. 4 SDS-PAGE of SMP dispersions incubated at different temperatures for various times. Lane 1–2, Sodium caseinate and WPI samples, respectively; Lane 3, Molecular weight marker; Lane 4–10, samples incubated at different temperatures for different periods. The concentration in Lane 1-2 was 0.5 mg/ml, while the protein concentration in Lane 4-10 was 2.2 mg/ml.

As shown in Fig. 4, several bands representing the major proteins in the original SMP dispersion were well visualized in lane 4, including caseins (α_s-CN, β-CN and κ-CN) and whey proteins (β-lg, α-la). Compared with the original sample (lane 4), the bands corresponding to β-lg and α-la, of samples incubated at 70 ℃ (lane 5 and 6) shifted towards a higher molecular weight with incubation time. Similarly, the bands of β-lg and α-la in samples heated at 80 and 90 ℃ (lane 7-10)

obviously moved to higher molecular weight to various degrees, except from the sample incubated at 90℃ for a very short time (i.e. 0.25 h). This is most probably due to the fact that it takes some time before the temperature of the sample increases from room temperature to the temperature of the heating bath. As expected, the molecular weight of the whey proteins increased during wet heating of SMP at 70-90 ℃, confirming the formation of conjugates of whey proteins and lactose. A similar trend in SDS-PAGE was found in the study of wet heating of a mixture of WPI and lactose performed at 50 ℃ for 7 days (Wang, Bao, & Chen, 2013).

As compared to the whey proteins, the shift of the bands representing the caseins was less obvious. Nevertheless, the casein bands became smeared and less featured after wet heating, which indicated the formation of a mixture of conjugates of caseins and lactose with various molecular weights. It was observed that the shift of the bands of α_s-casein and β-casein was limited in the samples incubated at 90 ℃ (lane 9 and 10), whereas the band of κ-casein almost disappeared because it moved upwards and became mixed with the bands of α_s-casein and β-casein. It is well known that caseins are embedded in micellar structures in milk, in which the casein aggregates are wrapped with a hairy κ-casein layer (Horne, 2020). The effect of the casein micellar structure on the conjugation between caseins and sugar still remains controversial. According to the model of casein micelles, it can be assumed that the κ-casein on the surface of casein micelles is readily accessible in the conjugation with lactose. A limited improvement in the emulsifying properties after the conjugation of casein and pectin was possibly due to the existence of the micellar structure of casein (Einhorn-Stoll, Ulbrich, Sever, & Kunzek, 2005). In the study of Moeckel, Duerasch, Weiz, Ruck, and Henle (2016), a comparable quantity of Amadori products in wet heating of micellar casein or nonmicellar sodium caseinate with glucose was reported, whereas the advanced Maillard reaction products in the glycated casein micelles and sodium caseinate were significantly different. Anema, Pinder, Hunter, and Hemar (2006) observed that incubation at 50 ℃ for 10 days induced a comparable lactosylation level of individual casein proteins in the micellar state in milk protein concentrate, indicating that the micellar structure did not affect the conjugation between caseins and lactose. A similar pattern in the changes of molecular weight for α_s-casein, β-casein and κ-casein in SDS-PAGE was also observed upon dry heating of SMP in the study of Wu, et al. (2020).

When samples were incubated at 70 ℃ for 24 h and 90 ℃ for 2 h (lane 6 and 10), a distinct smeared band in the range of 40 kDa to 180 kDa (and possibly above 180 kDa) was found. This corresponds to protein aggregates formed during the incubation at a high temperature (e.g. 90 ℃) or at a relatively low temperature for a long time (e.g. 70℃ for 24h). Whereas protein aggregates formed by disulfide bonds should be eliminated under the reducing conditions used in SDS-PAGE, aggregates via Maillard reaction products could remain present (Le, Bhandari, Holland, & Deeth, 2011). Overall, it was confirmed from the results in Fig. 4 that both whey proteins and caseins were engaged in the conjugation with lactose.

3.2 Characterization of the incubated SMP dispersions

3.2.1 Non-sedimentable protein fraction

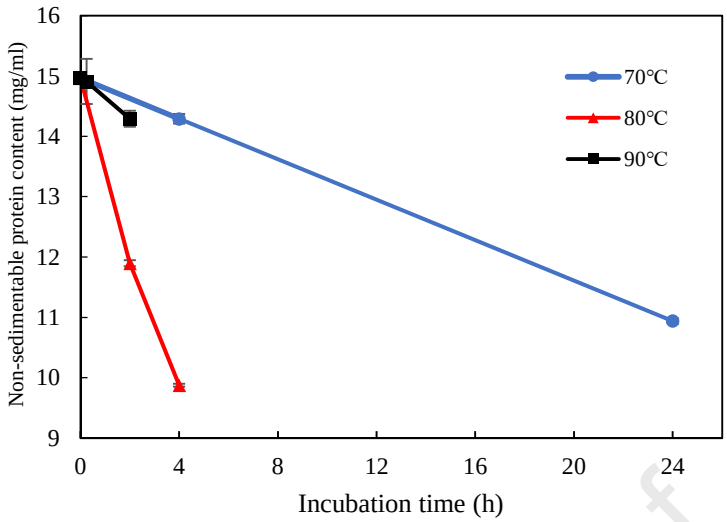

Fig. 5 Changes in non-sedimentable protein content in SMP dispersions with wet heat pre-treatment at different temperatures

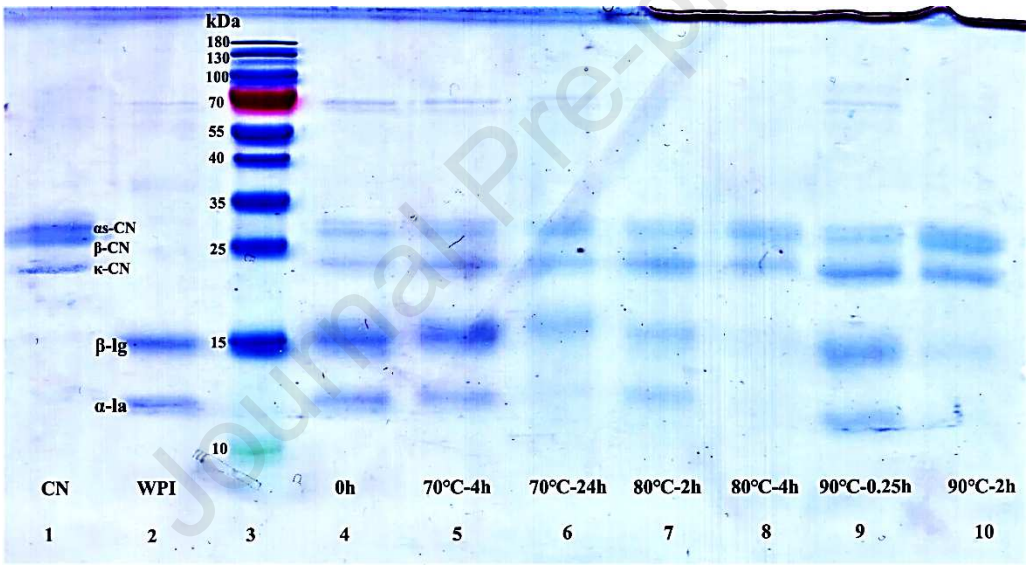

Fig. 6 SDS-PAGE of the non-sedimentable protein fraction in SMP dispersions with wet heat pre-treatment at different temperatures for various times. Lane 1–2, Sodium caseinate and WPI samples, respectively; Lane 3, Molecular weight marker; Lane 4–10, non-sedimentable protein fraction in samples incubated at different temperatures for different periods. The concentration in all lane was 0.5 mg/ml.

Table 1 Integrated grey values of various compositions in non-sedimentable protein in SMP dispersions with various wet heat pre-treatment obtained from bands in SDS-PAGE

T (°C)	t (h)	Absolute grey value (a.u.)			
		β-lg	α-la	α_s/β-CN	κ-CN
-	0	29.6	23.6	19.8	18.9
70	4	29.6	23.0	20.0	22.7
	24	22.6	18.0	21.1	21.7
80	2	19.9	20.5	20.2	23.2

	4	16.9	16.1	22.0	21.1
90	0.25	25.3	23.3	22.1	27.2
	2	18.2	17.9	27.5	27.4

As shown in Fig. 5, the non-sedimentable protein content exhibited a considerable decrease with incubation time at 70 ℃: it decreased from 14.9 mg/ml to 10.9 mg/ml after 24 h. The original level is in line with the whey protein content: as the SMP dispersions contained 16.5 g of SMP in a total volume of 88 ml, the whey protein content is supposed to be 14.3 mg/ml based on 7.6% (w/w) of whey protein. The observed decrease in the non-sedimentable protein content may be attributed to the formation of WP-casein micelle complexes which were removed from the serum after ultracentrifugation. The decrease in the non-sedimentable protein content during wet heating at 80 ℃ was faster: it took only 2 h or 4 h to reach a value of 11.9 mg/ml and 9.9 mg/ml, respectively. However, the change in the non-sedimentable protein content after the incubation at 90 ℃ for 2 h was limited as compared to the incubation at 80 ℃.

During wet heating, there are complicated protein interactions leading to the changes in non-sedimentable protein content, such as the association of whey proteins with casein micelles and the dissociation of caseins from casein micelles. To better understand the protein interactions during the incubation, SDS-PAGE analysis of the non-sedimentable protein fraction was performed. As shown in Fig.6 the bands representing the major whey proteins (β-lg and α-la) of the original sample (lane 4) were well visualized with the less distinct bands corresponding to caseins. To quantitatively analyze the compositional changes in the non-sedimentable protein fraction during the incubation, the band intensities (i.e. integrated grey values) were calculated via ImageJ software in grayscale mode. Due to the low content of casein in the serum after ultracentrifugation, coupled with the unresolved bands after conjugation, α_s casein and β-casein were regarded as a whole in the calculation of the absolute grey values. As shown in Table 1, no apparent changes in the bands of β-lg and α-la were observed after 4h of incubation at 70 ℃, whereas prolonged incubation (24h) induced a considerable decrease in the absolute grey values obtained from the bands of β-lg and α-la. On the other hand, 2 h of incubation at 80 and 90 ℃ was found to be sufficient to induce a considerable decrease for both β-lg and α-la. Denaturation of the whey proteins, especially of β-lg, occurs upon heating at a temperature above 70 ℃ at native pH, and subsequently the denatured whey proteins associate with casein micelles through sulfhydryl/disulfide bond exchange (Wijayanti, Brodkorb, Hogan, & Murphy, 2019). Hence, the micelle-bound complexes were removed from the serum after ultracentrifugation, leading to a decrease in the non-sedimentable whey protein fraction. Donato and Guyomarc'h (2009) suggested that increasing the heating temperature (70-95 ℃) promotes the whey protein denaturation and the formation of micelle-bound complexes for a constant heating duration. Corredig and Dalgleish (1999) demonstrated that both β-lg and α-la were engaged in the interaction between whey proteins and casein micelles. They also found that this interaction was generally faster at a higher temperature (75-90 ℃). The interaction between β-lg and casein micelles may proceed directly through κ-casein binding, whereas β-lg may also be involved in complexes of α-la and κ-casein via a soluble intermediate formed between these two whey proteins.

The changes in α_s/β-casein and κ-casein after wet heat incubation at 70 or 80 ℃ were limited, but a considerable increase in both α_s/β-casein and κ-casein in the serum was found after incubation at

90℃. The heat-induced dissociation of caseins from micelles, which leads to an increase in casein content in the serum, has been widely studied. The dissociation of caseins is generally considered to be pH-dependent: the dissociation of caseins occurs at pH above 6.7 in skim milk regardless of the temperature (Anema & Klostermeyer, 1997). However, the high concentration of SMP in this study could play an important role. As compared with normal milk, the critical pH of dissociation in concentrated milk shifts toward a lower value, and a higher extent of κ-casein release in concentrated milk is found at the same pH (Singh, 2004). Consequently, casein dissociation could occur during heating at pH lower than 6.7. The pH of the SMP dispersions in this study was around 6.60 before wet heat incubation, and dropped to varying degrees after incubation, reaching pH 6.39 upon incubation at 70 ℃ for 24 h, and pH 6.46 and 6.44 upon incubation at 80 ℃ for 4 h or 90℃ for 2 h, respectively. Anema (1998) reported that a considerable quantity of casein, especially κ-casein, was found to be soluble in concentrated milk (with total solid content of 25%) after heating at the normal pH of milk (pH 6.6-6.7). Furthermore, the quantity of soluble caseins, including α_s-casein, β-casein and κ-casein, increased with higher temperature.

Combining the results of the non-sedimentable protein content and the SDS-PAGE results, the combined effect of whey protein deposition onto casein micelles and of casein micelle dissociation becomes clear. With limited dissociation of casein, the non-sedimentable protein content decreased with the incubation time at 70 and 80 ℃ due to the association of whey proteins with casein micelles. On the other hand, the more pronounced casein micelle dissociation during the incubation at 90 ℃ compensated the loss of whey proteins in the serum, leading to a limited overall change in the non-sedimentable protein content.

3.2.2 Particle size of SMP dispersions

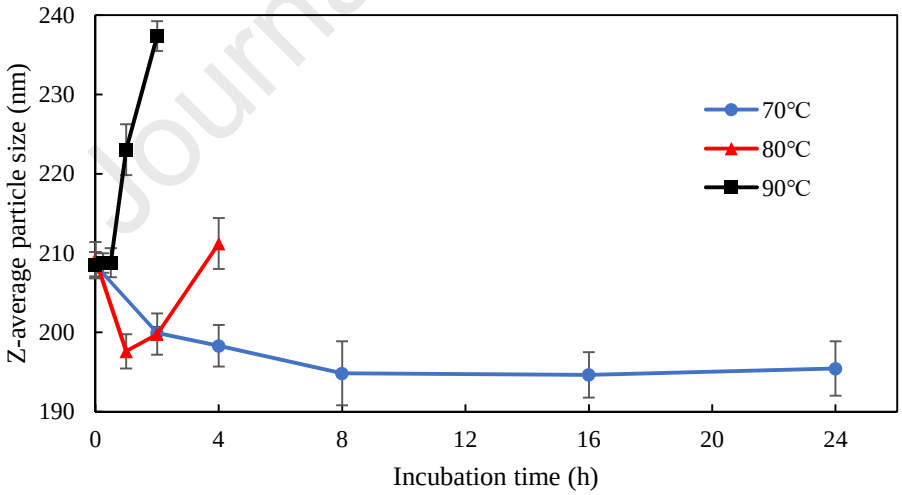

Fig. 7 Z-average hydrodynamic diameter of particles in SMP dispersions upon wet heat pre-treatment at different temperatures

The measured particle size in reconstituted SMP using PCS primarily refers to the hydrodynamic diameter of casein micelles. Horne (2020) mentioned that the size of casein micelles is closely related to their functional properties, including their emulsifying activity and heat stability.

Before wet heat incubation, a z-average casein micelle diameter of about 210 nm was found, which is in line with the value of about 220 nm, reported by Gebhardt, Doster, Friedrich, and

Kulozik (2006). As shown in Fig. 7 the mean particle size decreased slightly after incubation at 70 ℃ or at 80 ℃ for less than 2 h, whereas an increase was observed after wet heat treatment at 80℃ for a prolonged time or at 90 ℃. A similar trend was observed in the study of Anema (2008): the particle size in skim milk decreased after heating at a lower temperature, while the size increased upon heat treatment at a higher temperature.

A large number of changes occur in the SMP dispersions during wet heating, which can all contribute to variations in the size of casein micelles. One of the most important changes is the association of whey proteins on the surface of casein micelle which is largely dependent on the heating temperature (Fairise, Cayot, & Lorient, 1999; Martin, Williams, & Dunstan, 2007). Heating temperatures of 80 or 90 ℃ actually are sufficient for whey proteins to unfold their globular structure. These unfolded whey proteins can easily associate with casein micelles via disulfide bonds, resulting in an increase in the casein micelle size. This interaction was considered to be promoted for a longer incubation time at a higher temperature, which has been verified by the results of the non-sedimentable protein content. Moreover, it was observed that the peak in the particle size distribution shifted towards higher size when the Z-average particle size increased rather than appearing an additional peak in the higher size range (data not shown), indicating that the increase in the particle size was more likely due to the association of whey proteins with micelles rather than whey protein aggregation. Anema and Li (2003) suggested that the level of whey protein association with the micelles was more important than whey protein aggregation under slow heating conditions, e.g. using a water bath as is done in this study, due to the fact that whey proteins cannot efficiently form larger aggregates under slow heating conditions. A comparable magnitude of size increase (approximately 30~35 nm) was found as in this study upon the formation of complexes of casein micelle and β-lg after heating at 100 ℃ for 1 h (Anema, et al., 2003). In addition, the conjugation of micellar casein with lactose could further facilitate the increase in the size of the micelles: after glycation, the amount of bound water may be increased, which may affect the hydrodynamic diameter of particles (Wu, et al., 2020).

On the other hand, some changes occurring during wet heating could be responsible for a decrease in the micelle size. Upon heating, the strength of hydrophobic interactions is increased, leading to a more compact micellar structure (Beliciu & Moraru, 2009). Furthermore, dissociation of α_s/β casein and κ-casein during the heating process could be responsible for a slight decrease in the micelle size upon incubation at a lower temperature (such as 70 ℃) or for a relatively short time at 80 ℃ (Dalgleish & Corredig, 2012).

3.2.3 Relative viscosity of SMP dispersions

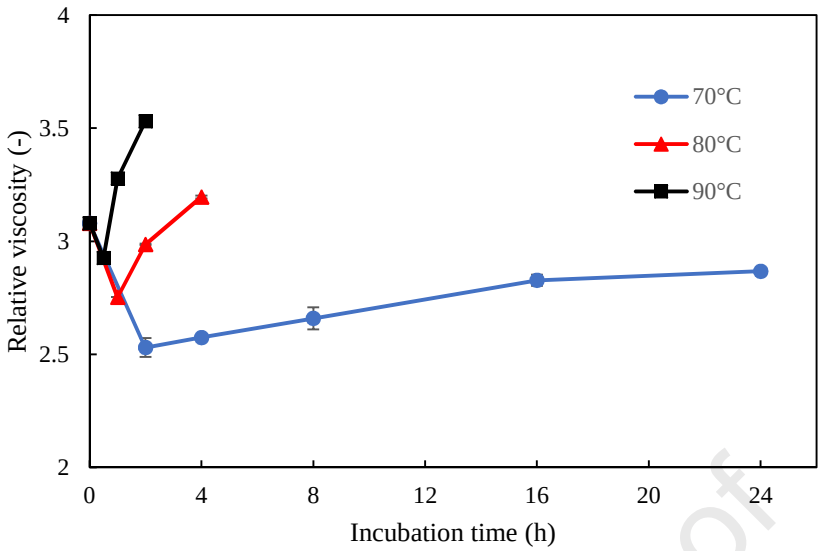

Fig. 8 Changes in relative viscosity of SMP dispersions during wet heat pre-treatment at different temperatures

As the viscosity of SMP dispersions is proportional to the volume fraction of the casein micelles, their relative viscosity was measured to evaluate the voluminosity (which expresses the volume per unit mass of casein) of the casein micelles before and after wet heat pre-treatment. As the relative viscosity was based on the viscosity measured both before and after ultracentrifugation, only the casein micelles were taken into account.

As shown in Fig. 8, the relative viscosity of the original sample was 3.48. Using Eq. (2) and (3), the voluminosity of the casein micelles can be calculated provided that ϕ_{max} in Eq. (2) is fixed. Nöbel, Weidendorfer, and Hinrichs (2012) proposed a maximum volume fraction of 0.64 for casein micelles as ideal monodisperse hard spheres, while Walstra (1999) suggested 0.9 as a reasonable figure as the particles varied widely in size in milk, leading to a difference in the calculated voluminosity (5.73 versus 6.28 ml/g). It was observed that wet heating at 70 ℃ gave rise to a (limited) decrease in relative viscosity, which indicates a smaller voluminosity. The latter is in line with the decreasing trend of the z-average diameter (Fig. 7). In contrast, the relative viscosity initially decreased upon incubation at the different temperatures, whereas it increased after prolonged incubation, particularly at 80 or 90 ℃. The values after 4 h of wet heating at 80 ℃ or 2 h at 90 ℃ were even higher than that of the original SMP dispersion. A similar trend was also observed for the z-average diameter of the casein micelles, as shown in Fig. 7.

Some structural changes of casein micelles during heating might favor a decrease in voluminosity and hence in relative viscosity (Nöbel, Kern, Sonne, Bähler, & Hinrichs, 2016; Nöbel, et al., 2012). For example, wet heat incubation could increase the protein hydrophobicity and retard the migration of highly water binding β-casein from the inside to the surface (Nöbel, et al., 2012). These complicated changes related to the casein micelles may result in a decrease in the voluminosity in the initial period of wet heat incubation. On the other hand, some changes that favor an increased voluminosity of casein micelles become more pronounced upon prolonged

incubation. It is generally considered that the voluminosity of casein micelles increases upon glycation (Colas, Gobin, & Lorient, 1988; Mahran, Haggag, Youssef, & Ali, 2011). Colas, et al. (1988) demonstrated that the viscosity and voluminosity of casein micelle dispersions had a positive correlation with the degree of glycation with galactose, which could be attributed to the increased net charge density of the proteins and steric hindrance provided by the attached sugar molecules. Moreover, more complexes of whey proteins and casein micelles, which greatly favors an increase in the voluminosity, are formed upon a prolonged incubation, especially at higher temperatures, e.g. 80 and 90 ℃. Anema, et al. (2003) suggested that upon heating at 90 ℃, the highly hydrated whey proteins associated with the micelles, or the whey proteins were arranged at the micelle surface in such a way that a substantial quantity of solvent was entrapped at the micelle surface, which led to a measurable increase in the viscosity of the milk and the volume fraction of casein micelles. Furthermore, a higher temperature favors the dissociation of caseins from the micelles (as shown in the SDS-PAGE analysis of the non-sedimentable protein fraction), leading to a looser and more porous micelle structure, and an increase in voluminosity (Beliciu, et al., 2009).

3.3 Heat stability of RFEM emulsions stabilized by wet-heated SMP

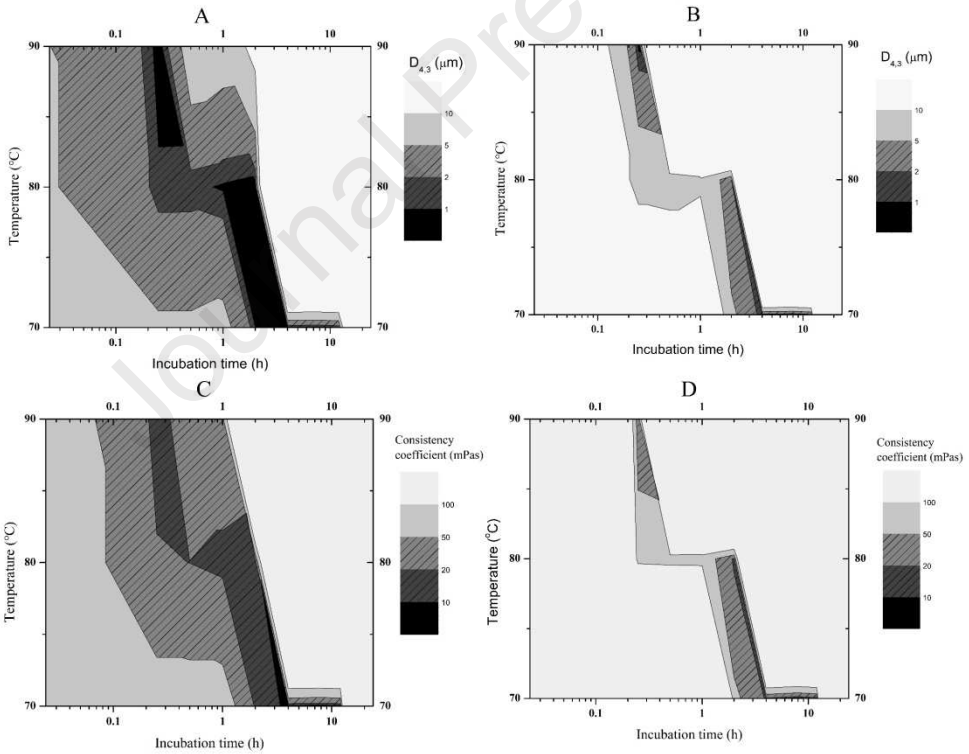

Fig. 9 Contour plot of particle size $D_{4,3}$ (A, B) and consistency coefficient (C, D) of RFEM emulsions stabilized by SMP with wet heat pre-treatment at different temperatures for various incubation times after heating for 20min (A, C) or 30min (B, D) at 120°C

The different wet heated SMP dispersions were subsequently used to prepare recombined filled evaporated milk (RFEM). In order to evaluate their heat stability, the particle size as well as the viscosity of these RFEM samples upon sterilization for 20 or 30 min at 120 °C was measured. The

results are summarized in Fig. 9 as contour plots showing the volume-weighted average diameter ($D_{4,3}$), as well as the consistency coefficient as a function of wet heat incubation temperature and time. In these contour plots, zones with similar values of the volume-weighted average diameter (A, B) or consistency coefficient (C, D) are indicated by the same color. In Fig. 9, darker colors indicate lower values for both particle size and consistency coefficient after heating, which is desirable, whereas lighter colors indicate high values and hence increased heat-induced coagulation. As heat-stable RFEM samples were characterized by a volume-weighted average diameter below 5 μm and a consistency coefficient below 50 mPa·s, it follows that heat stable samples were located within the shaded zones (corresponding to the 3 darkest grey levels), whereas heat-labile samples were located in the non-shaded zones with the 2 weakest grey levels. The average particle size and viscosity results of RFEM emulsions exhibited a similar trend upon heating at 120 ℃ for 20 or 30 min. RFEM emulsions stabilized by the original SMP had a relatively poor heat stability: after 20 min of heating (Fig. 9 A and C), the $D_{4,3}$ and consistency coefficient increased from 0.36 μm and 4.5 mPa·s (for the unheated RFEM) to 5.5 μm and 64.3 mPa·s, respectively, as indicated by the light grey color, and visible coagulation appeared. Wet heating incubation at 70 to 90℃ for appropriate times enabled to improve the heat stability of RFEM emulsions, as reflected by the initially gradually increasing darker shades at increasing incubation times. The optimum heat stability was characterized by the darkest regions in the contour plots. Generally, the higher the adopted incubation temperature, the shorter the incubation time needed to accomplish a desired improvement in heat stability, e.g. withstanding 30 min heating at 120 ℃ without visible coagulation ($D_{4,3}$ < 5 μm and consistency coefficient < 50 mPa·s). At 70 ℃, the incubation time needed to acquire heat stability against 30 min of heating at 120 °C without coagulation was more than 2 h (Fig.9 B and D). Incubation at 80 or 90 ℃ for 2 h and 0.25 h was sufficient to enable RFEM emulsions to withstand 30 min heating at 120 ℃ without coagulation, respectively (Fig. 9 B and D). However, the heat stability of RFEM emulsions decreased when the SMP dispersions were subjected to prolonged incubation, as observed by the lighter colors at long incubation times. In addition, a narrower incubation time interval that enabled an improved heat stability was observed at a higher temperature.

The enhanced heat stability of RFEM emulsions stabilized by incubated SMP can be attributed to the conjugation of milk proteins and lactose, which was clearly observed in the results of SDS-PAGE. Because of conjugation, the denaturation temperature of whey proteins is increased. Hence, the extent of denaturation of the whey proteins during heating will be less, which is thought to be beneficial for the heat stability of RFEM emulsions (Chen, Chen, Guo, & Zhou, 2015). In addition, the steric hindrance provided by glycated sugar could play a role. Liu and Zhong (2013) reported that the glycation of WPI with lactose greatly improved the heat stability and enabled transparent dispersions after heating at 88 °C for 2 min. They attributed the improved heat stability to the extra steric hindrance provided by glycated sugars. Furthermore, the highly hydrated sugar can bring more bound water around the glycated milk proteins against heat-induced aggregation (Wu, et al., 2020). According to Sagis and Scholten (2014), the greater thickness of the interfacial layer in emulsions stabilized by conjugated proteins can also contribute to the stability of oil droplets against flocculation. A higher incubation temperature generally promotes the process of conjugation. Therefore, the incubation time needed to acquire a heat

stable emulsion is shorter. Liu, et al. (2015) reported that WPI became more heat stable by a short-time high-temperature incubation of WPI and lactose (i.e. 130 °C for 30 min). Considering that the degree of glycation was relatively low upon incubation at 90 ℃ for 0.25 h (as shown from the of free amino group results and SDS-PAGE), the enhanced heat stability at 90 ℃ could be associated with the formation of complexes between casein micelles and whey proteins. It is indeed well known that a certain amount of complexes between casein micelles and whey proteins can prevent whey proteins from aggregation (Kelleher, et al., 2020).

The detrimental effects of prolonged incubation on the heat stability of RFEM emulsions could result from complicated protein interactions. As shown by the SDS-PAGE results of the non-sedimentable protein, the dissociation of caseins from micelles was more pronounced after a longer incubation time or at a higher incubation temperature. This also directly explains the narrower incubation time interval that enabled an improved heat stability at a higher incubation temperature. The dissociation of κ-casein from micelles has a greatly negative impact on the colloidal stability of casein micelles as they are prone to aggregate with each other without the protection of the hairy layer of κ-casein (Dumpler, Wohlschläger, & Kulozik, 2017). WP-casein complexes were formed upon prolonged incubation (as shown from the decreasing non-sedimentable protein content), as well as larger casein micelles with a higher voluminosity (relative viscosity). These effects could contribute to the decreased heat stability (Yang, Zhang, Wen, Zhang, & Liang, 2014). Amongst these, the formation of WP-casein complexes has a complicated impact on the heat stability, which depends on the balance between soluble and micelle-bound complexes (Donato, et al., 2009; Donato, Guyomarc'H, Amiot, & Dalgleish, 2007). Considerable complexation between whey proteins and κ-casein is considered to favor the dissociation of κ-casein from the casein micelles upon heating in concentrated milk systems like RFEM emulsions in this study (Huppertz, 2016). It was also reported that the association of whey proteins with caseins plays an important role in the heat-induced coagulation of concentrated milk (Dumpler, et al., 2017).

4. Conclusions

Heat-induced coagulation is one of the biggest challenges in dairy sterilization, particularly in manufacturing products with a high concentration of milk protein, like (recombined) evaporated milk. The coagulation is mostly due to the destabilization of milk proteins during heating. Hence, it is of great importance to improve the heat stability of milk proteins, especially the thermolabile whey proteins. Until now, most studies focused on conjugation in model systems to improve the heat stability of milk proteins, such as WPI and pure sugars. However, in commercial dairy products like SMP, the simultaneous existence of caseins and whey proteins and the more complicated mineral environment could bring more uncertainty when applying the acquired knowledge from model systems to commercial products. Therefore, to fill this knowledge gap, the effects of conjugation of indigenous lactose and milk proteins in SMP via preliminary wet heating on the heat stability of RFEM emulsions was studied. Thereby, no extra ingredient was added, which is in accordance with the current "clean" label trend (i.e. without E-numbers) (Asioli, et al., 2017). Additionally, after wet heating, the SMP dispersions (containing 17.6% SMP, w/w) were directly used to prepare RFEM.

SDS-PAGE indicated that lactosylation of both whey proteins and caseins occurred upon heating of SMP dispersions at 70-90 ℃, which was the most important reason for the enhanced heat stability of the derived RFEM emulsions. RFEM emulsions stabilized by SMP with appropriate incubation times could withstand a typical in-container sterilization procedure (30 min of heating at 120 ℃) without visible coagulation ($D_{4,3}$ <5 μm and consistency coefficient <50 mPa·s). Generally, the higher the incubation temperature, the shorter the incubation time needed to enable a heat-stable emulsion against 30 min of heating at 120℃: 4 h, 2 h and 0.25 h of wet heating was needed upon incubation at 70, 80 and 90 ℃, respectively. However, prolonged incubation had a negative effect on the heat stability of RFEM emulsions, and a narrower incubation time interval that enabled an improved heat stability was observed at a higher temperature. This could be related to the considerable dissociation of κ-casein and the formation of complexes of whey proteins and caseins upon prolonged wet heating, which was indicated from the results of the non-sedimentable protein content and composition, as well as of the particle size and relative viscosity of SMP dispersions.

The current research implies that wet heat pre-treatment of SMP dispersion might be a promising alternative to improve its heat stability, which is significant in the applications where a high heat stability is desired, such as in recombined filled evaporated milk or infant formulas.

Acknowledgements

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Appendix A

Table A1 Basic information[1] of the molecular weight and the number of free amino groups in the various whey proteins and caseins in bovine milk based on UniProt database (Consortium, 2018)

Protein		Molecular weight (kDa)	Number of free amino groups per molecule[2] (-)	Molar ratio[3] (mol/mol)	Average molecular weight (kDa)	Average number of free amino group per molecule (-)
Whey protein	β-lg	18	19	55	17.7	18.8
	α-la	14	14	42		
	BSA	66	83	3		
Casein	α_{s1}-CN	23	21	39	22.2	19.3
	α_{s2}-CN	24	31	10		
	β-CN	23	16	39		
	κ-CN	19	15	12		
Total milk protein		/	/	/	21.4	19.1

[1] At native pH

[2] Based on the number of lysine and arginine residues, as well as the terminal amino group of the protein

[3] Molar ratio of individual proteins within the whey protein or casein fraction, respectively.

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- Wet heating of SMP dispersions gave rise to the formation of conjugates of milk proteins and lactose
- SDS-PAGE revealed glycation of both whey proteins and caseins
- Pre-heated SMP-stabilized REM emulsions could withstand at least 30min of heating at 120℃ without coagulation
- The higher the incubation temperature, the shorter the incubation time needed to enable a heat-stable REM
- Prolonged incubation had a devastating effect on the heat stability of REM
- A narrower incubation time interval that enabled an improved heat stability of REM

Conflict of interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.