# Probing the improved heat stabilizing capacity of dry heat conjugated whey protein in oil-in-water emulsions: a microrheological study

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## ABSTRACT

Dry heat conjugation of whey protein has been shown before to improve its heat stabilizing properties when applied as an emulsifier. However, the range of feasible heating conditions of these conjugates has not yet been evaluated. Microrheology, a non-destructive method, was utilized in this study to determine the acceptable heating range for whey protein stabilized-emulsions. Practically, oil-in-water emulsions stabilized by whey protein-lactose conjugates were subjected to either an in-situ thermal treatment, including a heating-cooling cycle, or to an isothermal period. The observed microstructural rearrangements were also confirmed by examining the bulk emulsion behavior through oscillatory rheology.

The obtained results indicated that microrheology could unravel the range of heating temperatures and durations for emulsions stabilized by whey protein concentrate (WPC) that was dry heated for 8 and 48 hours as compared to the native WPC. The improved heat stability was a result of the conjugates' ability to prevent the formation of an oil droplet network. Consequently, the conjugated WPC-stabilized emulsions remained mainly viscous with a low elasticity index (EI) and macroscopic viscosity index (MVI) during heating. Furthermore, the microrheology findings were found to be largely in line with bulk rheological properties: both methods indicated a comparable temperature for the onset of network formation upon applying a temperature sweep. The insights from this study may help to stimulate the industrial application of whey protein-sugar conjugates as heat stable natural emulsifiers.

***Keywords:*** *conjugation, whey protein, lactose, multi-speckle diffusive wave spectroscopy, heat stability, emulsifier*

## 1. INTRODUCTION

In our previous research, the heat stability of whey protein concentrate (WPC) has been improved via dry heat conjugation with the innate lactose (A'yun et al., 2020). As a result, the conjugates-stabilized oil-in-water (o/w) emulsions could maintain their particle size and viscosity upon heating for 20 min at 80 °C, whereas the original WPC-stabilized emulsion became heavily aggregated. However, the heat stability evaluation was only performed at the end of the predefined heat treatment and only the bulk response, such as the viscosity and particle size were evaluated. It would, however, be fascinating to further unravel the range of feasible heating conditions, including the maximum acceptable heating temperature and duration under which the conjugates may retain the emulsions' stability. This information is highly relevant for the further industrial application of these protein conjugates.

Microrheology has been shown to enable the detection of alterations in food products in an earlier stage, i.e. before the bulk characteristics become affected (Tisserand, Fleury, Brunel, Bru, & Meunier, 2012). It is a technique that probes the motion of small tracers (e.g. colloidal particles) to investigate the microstructure of a material (Xia, Xiao, Pan, & Wang, 2018). The particle motion may be detected by methods such as dynamic light scattering (DLS), diffusing wave spectroscopy (DWS), and video-particle tracking (Cicuta & Donald, 2007; Moschakis, 2013). In passive microrheology, the particle movement relies only on the intrinsic Brownian motion due to thermal agitation. In the case of purely viscous (i.e. liquid) samples, particles continuously move do to Brownian motion, which results in a linear correlation between the decorrelation time and the explored area. Viscoelastic products, on the other hand, exhibit a non-linear correlation due to restricted particle movement as they interact with the sample's 3-D network. As the tracer movement is determined by its interaction with the medium, the medium characteristics can be examined, such as the local elasticity and viscosity (Moschakis, 2013; Xia et al., 2018). Importantly, microrheology may overcome some major drawbacks of bulk rheology, as it is non-destructive, and enables to evaluate the local dynamics at a high frequency with high spatial accuracy (Cicuta & Donald, 2007). As passive microrheology does not impose a macroscopic deformation, but uses the intrinsic (limitations in) Brownian motion of small particles in the network, the obtained results are always related to the behavior within the linear visco-elastic region. (Cicuta & Donald, 2007; Moschakis, 2013).

Microrheology has a wide range of applications, such as the evaluation of gelation processes (Houghton, Hasnain, & Donald, 2008; Larsen & Furst, 2008), emulsion stability (Degrand, Michon, & Bosc, 2016; Medronho et al., 2018), viscosity and transition points

(Papagiannopoulos, Sotiropoulos, & Pispas, 2016), rheology at interfaces (Lee, Cardinali, Reich, Stebe, & Leheny, 2011), and movement of intracellular particles (Reverey et al., 2015). However, as far as we know, this method has not yet been applied to investigate the heat stability improvement of conjugated whey protein-stabilized emulsions. Meanwhile, a microstructural understanding of the latter could provide a better insight into the functionality of whey protein-sugar conjugates, which might assist the industrial application of conjugated whey proteins as heat-stable natural emulsifiers.

The current work aims to identify the starting point of emulsion destabilization, which in its turn provides information regarding acceptable heating temperature and duration conditions for WPC conjugate-stabilized emulsions. To achieve that goal, microrheology based on a multi-speckle diffusing wave spectroscopy (MS-DWS) technique was applied to probe the microstructural alterations of o/w emulsions stabilized by whey protein-lactose conjugates during an in-situ thermal treatment, including a heating-cooling cycle, as well as an isothermal period in between. Hereby, the emulsion droplets were used as the tracer particles and their movement profile (i.e. mean square displacement versus time) was determined, from which the elasticity and macroscopic viscosity were derived. Moreover, the microstructural rearrangements were also confirmed by investigating the bulk emulsion behavior through oscillatory rheology.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Both sunflower oil and whey protein concentrate (WPC; Royal Green Organic Whey Protein®, Frenchtop Natural Care Product BV, Al Hoorn, The Netherlands) were purchased from a local shop. According to the manufacturer, the WPC contains 80% protein, 8.6% lactose, 4.4% fat, and 2.8% ash.

An imidazole buffer was used, consisting of 20 mM imidazole ($C_3H_4N_2$; Fisher scientific, ≥99% purity), 50 mM NaCl (VWR, ≥99% purity), 1.5 mM sodium azide ($NaN_3$; Sigma Aldrich, ≥99% purity) and 5 mM $CaCl_2$ (Sigma Aldrich, ≥99% purity). The buffer pH was adjusted to 6.5 ± 0.1 by using 0.5 M HCl.

### 2.2 Methods

#### 2.2.1 Conjugates preparation

Whey protein conjugates were prepared through dry heat conjugation of whey proteins with the innate lactose, as described by A'yun et al. (2020). To that end, a 125 ml plastic tube filled

with 10 gram of WPC was incubated in a desiccator at 80 °C for up to 48 hours. The relative humidity inside the desiccator was conditioned to reach 76% by using a saturated NaCl solution (Greenspan, 1977). During incubation, samples were collected after 0, 8, and 48 hours, which are referred to as 0h, 8h, and 48h.

### 2.2.2 Emulsion preparation

Aqueous solutions were prepared by dissolving 0.5% (w/v) of whey protein concentrate from samples with different incubation times in imidazole buffer (pH 6.5 ± 0.1). Subsequently, 5 g of sunflower oil was added to 45 g of the aqueous solution to produce 10% (w/w) oil in water emulsions. This mixture was prehomogenized using an IKA Ultra Turrax TV45 (Janke-Kunkel, Germany) at 24,000 rpm for 2.5 min, followed by homogenization using a Microfluidizer M110S (Microfluidics, USA) at 560 bar working pressure for 2 min. The emulsions were stored at 4 °C for maximum one day after preparation before (micro)rheological characterization, and were readjusted to 25 °C prior to measurement.

### 2.2.3 Microrheology measurements

A Rheolaser Master™ (Formulaction SAS, France) with multi-speckle diffusing wave spectroscopy (MS-DWS) was utilized to study the microrheological properties of the emulsions. This instrument enables to measure 6 samples in one single run. To that end, 20 ml of the emulsion was filled into a cylindrical flat bottom measuring cell and placed in the instrument's sample chamber. The experimentally acquired speckle images were translated into mean square displacement (MSD) as a function of decorrelation time through a patented algorithm. The emulsions' microrheology was measured both during a heating-cooling cycle and at an isothermal temperature. In the heating-cooling cycle experiment, the temperature was set to increase from 25 °C to 80 °C by using a temperature ramp of 1 °C/min, followed by holding at 80 °C for 30 min and cooling to room temperature. As the device is not equipped with a cooling system, the total measurement duration was 8 hours. In isothermal evaluation, the emulsions were subjected to in-situ isothermal heating at 60 or 80 °C for 1 hour.

The measurements were controlled through the RheoSoft Master_1.4.0.10 software. The expert program (manual setting) was chosen, whereby the $d_2$ cutoff was set at 80%, and no limitation on the maximum decorrelation time was applied. The resulting MSD (mean square displacement), EI (elasticity index), and MVI (macroscopic viscosity index) were collected from the software.

Whereas the elasticity index (EI) is calculated from the inverse MSD value at the plateau (Equation 1), the macroscopic viscosity index (MVI) is deduced from the inverse of the MSD slope at long decorrelation times (Equation 2). More detailed information regarding the MS-DWS technique can be found in the Rheolaser Master™ user guide (Formulaction SAS, 2014) and was also described by Tisserand et al. (2012). Data were collected from two repetitions. The MSD graph was taken from one of the two experiments, while the EI and MVI graphs were constructed based on the means ± standard deviation of the two repetitions.

$$EI = 1 / MSD_{plateau} \quad \text{(Equation 1)}$$

$$MVI = 1 / MSD_{slope} \quad \text{(Equation 2)}$$

### 2.2.4 Bulk rheology measurement

The bulk rheology of the emulsion samples was evaluated through an oscillatory test on an advanced Rheometer AR 2000ex (TA instruments, USA) equipped with a concentric cylinder geometry (cup radius 15 mm, bob radius 14 mm), coupled to a Peltier temperature control system.

Twenty grams of sample were placed inside the bob. The measurements were conducted during a sequence consisting of a heating ramp (25 to 80 °C at 1 °C/min), a time sweep (holding at 80 °C for 30 min) and cooling back to 25 °C (at -1 °C/min). The strain and frequency were controlled at 0.02 and 1.0 Hz, respectively. The rheological profile figures depict one of the parallel experiments, and the gelling point results were presented as mean ± standard deviation, based on 2 individual repetitions.

### 2.2.5 Heat treatment of emulsions

The heat stability of the emulsions stabilized by WPC conjugates was tested by placing 10 ml of sample in 20 ml glass tubes with a plastic cap. Subsequently, the tubes were heated in a water bath at 60 °C or 80 °C for 30 min and cooled using running water. The particle size distribution and apparent viscosity of the emulsions were measured both before and after heat treatment.

### 2.2.6 Particle size measurement

The particle size distribution was examined through the static light scattering method using a Mastersizer 3000 (Malvern Instrument Ltd, Malvern, UK). The device was equipped with a Hydro MV dispersion chamber (Malvern) filled with distilled water (refractive index = 1.33) as the dispersant. The refractive index and absorption index of the droplets (sunflower oil) were set

at 1.47 and 0.01, resp. The emulsion was then added dropwise into the chamber to achieve 10-20% of obscuration along with continuous stirring at 1,500 rpm. The data were analyzed by using the polydisperse model.

### 2.2.7 Apparent viscosity measurement

Using an LV-DVII+pro viscometer, the consistency coefficient was measured at 25°C (Brookfield, USA). Seven ml of emulsion was placed in the small sample holder and examined using a SC4-18 spindle with a 10% minimum torque setting. Consequently, a shear rate of 30 to 100 $s^{-1}$ was used for viscous materials, and 200 to 250 $s^{-1}$ was used for less viscous samples. The viscosity of purely viscous (Newtonian) emulsions was estimated from the average value at various shear rates, whereas the consistency coefficient (K) of shear-thinning samples was calculated from a power law (Equation 3) fit to the data.

$$\tau = K \cdot \gamma^{n} \quad \text{(Equation 3)}$$

In equation 3, $\tau$ represents the shear stress (in Pa), $\gamma$ the shear rate ($s^{-1}$), and $n$ the flow behavior index.

### 2.2.8 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 25 software. Statistical differences between EI and MVI values obtained at different temperatures and measurement times were determined by univariate analysis of variance with Tukey's-b post hoc test. A difference was regarded significant when $p<0.05$.

## 3. RESULTS AND DISCUSSION

The heat stabilizing capacity of WPC conjugates was evaluated from both the microrheology characteristics and the bulk properties of the WPC-stabilized emulsions. Emulsions were considered to become destabilized upon heating when their viscous characteristics changed to viscoelastic or elastic as indicated by the microrheological (MSD, EI, and MVI) and bulk rheological parameters (G′ and G″). The acceptable heating temperature and duration conditions for each WPC-stabilized emulsion were determined. Thereafter, the heat-stability of the emulsions was evaluated both during a heating-cooling cycle and at an isothermal temperature.

### 3.1 Detection of heat-induced destabilization

#### 3.1.1 Microrheology during a heating-cooling cycle

The microrheology of protein-stabilized emulsions is, in fact, the result of the behavior of the proteins acting as the emulsifier. Proteins may form a viscoelastic film covering the oil droplets,

providing inter-droplet repulsive forces (Kaltsa, Michon, Yanniotis, & Mandala, 2013). This interfacial film may contribute to the elasticity of the emulsion (Mackie, Ridout, Moates, Husband, & Wilde, 2007). Furthermore, since proteins also interact with the continuous phase, they also determine the emulsion viscosity. Hence, changes in the protein properties, in this case due to conjugation with lactose, might affect the emulsion rheology.

Microrheological measurements were applied to detect the temperature at which the WPC-stabilized emulsions destabilized. The gathered information served as a recommendation of the acceptable heating temperature for each WPC emulsion. As microrheology enables the direct tracking of the droplet movement at a short length scale, it enables the detection of interdroplet interaction prior to changes in bulk characteristics. In practice, the microrheology of the native and conjugated WPC-stabilized emulsions was examined during a temperature ramp from 25 to 80 °C, followed by holding at 80 °C for 30 minutes and cooling as the final step. Hereby, the emulsion droplets were used as the tracer particles. The mean square displacement (MSD) data were collected and further processed to obtain the elasticity index (EI) and macroscopic viscosity index (MVI) parameters.

**a. Mean Square Displacement (MSD)**

The mean square displacement (MSD) curves provide the initial hint to indicate the emulsion stability in terms of its viscoelasticity. The latter could be identified by the MSD curve shape (i.e. linear or sigmoidal), the MSD value, and the required decorrelation time. In a purely viscous sample, particles diffuse freely, whereby, according to Einstein's diffusion equation, the MSD increases linearly with the decorrelation time (Cicuta & Donald, 2007; Medronho et al., 2018). As WPC-stabilized emulsions are expected to be fully viscous, a linear shape, high MSD values and short decorrelation times are expected provided that heat coagulation does not interfere. For completeness, it is worth noting that the MSD is inversely proportional to the particle size of the droplets (Cicuta & Donald, 2007). Hence, the initial emulsion droplet size will have an effect on the absolute value of the MSD. However, it will hardly have an effect on the observed effect of heat coagulation, i.e. a transition from free diffusion to hindered diffusion/entrapment in a 3-D network, which was the main focus of this study.

The MSD profiles of native and conjugated WPC-stabilized emulsions revealed distinct stability characteristics. A linear MSD versus decorrelation profile, and hence a purely viscous behavior, was observed during the first 35 min of heating, i.e. from 25 to 60 °C (dark blue line, Figure 1a). In this case, all emulsions were only observed during short decorrelation times (<0.1 s), which

was due to the high MSD values. Meanwhile, heating above 60 °C (>35 min) induced destabilization, as could be deduced from the viscoelastic behavior of the native (0h) WPC-stabilized emulsion. A reduced displacement (i.e. drop in MSD values) and the shift to a sigmoidal pattern was observed, whereby a plateau region was present over a longer decorrelation time (Figure 1a).

The lower MSD values at a fixed decorrelation time demonstrated a more limited particle movement due to being entrapped in a network structure. To reach a sufficiently large MSD value, the decorrelation time was largely prolonged. In a viscoelastic sample, an inter-particle network is formed. Whereas the particles freely move inside the network at short decorrelation times, their movement becomes slowed down at longer decorrelation times due to their interaction with the network structure. Finally, at long decorrelation times, the particles move throughout the network and experience the macroscopic viscosity of the bulk medium, which is again reflected by a linear increase of the MSD curve. Hence, the alteration from a linear to a sigmoidal behavior in the MSD trend could be used as an indication of emulsion aggregation. The viscoelastic behavior occurred within a time span from about 39 to 106 min (corresponding to heating from 64 °C onwards, holding at 80 °C for 30 min, and subsequent cooling to 74 °C). After this period, the WPC curve shifted upwards and toward shorter decorrelation times. This upward shifting seemed strange at first sight as network reinforcement is typically observed for heat-induced protein gels upon cooling. Instead, the increased MSD at lower temperature was thought to indicate that entrapped water was expelled from the network, leading to a lower viscosity near the walls of the container, where the light scattering took place. In conditions where the protein interaction is too strong and not in balance with the protein-water interaction, the network is indeed known to collapse, whereby water will be expelled, which is indicated as syneresis (Singh & Havea, 2003).

Interestingly, in the emulsion that was stabilized by WPC which was dry heated for 8 h, the drop in MSD values and hence the viscoelastic behavior was initiated at a higher temperature (i.e. at 80 °C, which corresponds to 57 min; starting from the green line Figure 1b). Moreover, no syneresis was observed in the microrheology experiments as the viscoelasticity continued to grow steadily throughout the holding and cooling step, as showwn by the much slower increase in MSD at increasing decorrelation times ($t_{dec}$), as well as the strong deviation from linearity (Figure 1b).

The ability to prevent syneresis may imply that the conjugated whey protein had an extended water holding capacity. Guo et al., (2022) described that syneresis is more severe in gels containing more free water and decreases as more bound water is present. The latter results from the strong interaction between water and hydrophilic groups of the polymer. Hence, an increased amount of bound hydrophilic groups may enhance the amount of bound water (Liu et al., 2017), thus improving the dehydration stability of a gel towards thermal treatment (Guo et al., 2022; Liu et al., 2017). In the current study, whey protein was conjugated with lactose which has been recognized for its capacity to bind water via its hydroxyl groups (Imberti, McLain, Rhys, Bruni, & Ricci, 2019). Thus, attachment of lactose appeared to increase the whey protein affinity to the aqueous phase, and thereby enhanced its water binding activity.

The emulsions stabilized by WPC that was incubated for 48h generated high MSD values at short decorrelation times, as well as linear curves throughout the complete heating and cooling cycle (Figure 1c). This result implies that these emulsions retained their liquid-like (viscous) behavior during the thermal treatment, albeit with a somewhat increased viscosity as reflected by the slightly decreased MSD when comparing the results obtained before (blue curves) and after the heating-cooling cycle (red curves). Hence, it could be concluded that the emulsions containing WPC conjugates obtained upon 48h of dry heating were the most heat stable. For completeness, it should be mentioned that the experimental window of Figure 1c is much smaller as compared to Figure 1a and 1b.

The above-mentioned MSD results indicate that the conjugated WPC preserved the liquid-like behavior of the emulsions during the applied temperature program, particularly for the 48h conjugates. In the native WPC-stabilised emulsion, on the other hand, destabilization leading to an elastic (rather than viscous) behavior occurred. The latter was a result of particle network formation and its interaction with the aqueous system. The determination of the starting temperature inducing particle network formation in each emulsion will further be detailed by looking at the elasticity index (EI) and macroscopic viscosity index (MVI) derived from the MSD curves.

**b. Elasticity Index (EI)**

The elasticity index (EI) is derived from the MSD value at the plateau. It denotes the formation of a three dimensional network by strongly aggregating particles (Xu et al., 2017). Hence, the increase in EI value is an indication of emulsion destabilization.

The elasticity index (EI) of the emulsion stabilized by native WPC increased prominently during the heating cycle (Figure 2): whereas the EI started to deviate at 51 °C, a significant and relatively steady increment was detected at 69 °C. Moreover, high EI values were observed during the holding period at 80 °C. On the other hand, the EI value decreased upon cooling, which was initiated at 73 °C, as also shown from the MSD results (Figure 1a), indicating a loss in the detected elasticity due to syneresis.

Meanwhile, the EI increment of the emulsions containing WPC that was incubated for 8h happened at a higher temperature (i.e. 79 °C) and was less pronounced than that of the emulsion containing native WPC: the highest EI values were $1.43 \times 10^{-2}$ and $0.75 \times 10^{-2}$ $nm^{-2}$ for the emulsions that were stabilized by WPC that was dry heat incubated for 0h and 8h, respectively. Further on, the EI value tended to increase steadily during the holding and cooling step. This in line with the typical heat-induced protein gelation mechanism, whereby 3-D network formation is initiated upon heating, and becomes reinforced upon cooling. Interestingly, the 48h emulsion exhibited a fairly constant and low EI (of about $0.06 \times 10^{-2}$ $nm^{-2}$) throughout the whole heating and cooling treatment.

The increase in EI during heating as exhibited by the emulsion stabilized by native and conjugated WPC for 8 h is initiated by the increased viscoelasticity of the protein film covering the oil droplet. Further on, the proteins aggregate and form a continuous gel network (Dickinson, 2009; Tan, Wang, Chen, Niu, & Yu, 2016). Hence, the embedded oil droplets in this network become more difficult to be displaced, which is reflected by an increment of the EI.

The delay of the EI increment, as well as the lower EI value in emulsions that were stabilized by dry heated WPC implied that conjugation of WPC with lactose could prevent extensive protein network formation, hence promoting more thermally stable emulsions. Glycation has indeed been found to inhibit the unfolding of the whey protein's tertiary structure upon heating at 80 °C for 30 minutes by increasing the denaturation temperature (Wang & Ismail, 2012). This more limited unfolding consequently reduces the exposure of hydrophobic side chains, and hence the binding to proteins on adjacent droplets to form a network. Moreover, the increased water binding activity as mentioned in the MSD section, may enhance the interdroplet repulsion force as following the Flory-Huggins parameter (McClements, 2016) and hence prevent the network formation by emulsion droplet aggregation.

In conclusion, based on the initiation of network formation, the emulsions stabilized by WPC conjugates may withstand a higher heating temperature than those stabilized by native WPC.

The heat treatment of the emulsions containing conjugates obtained upon 8h of dry heating should be kept below 79 °C, whereas 80 °C is acceptable for emulsions stabilized by conjugates obtained after 48 h of dry heating. Meanwhile, heating of the native WPC-stabilized emulsion should be limited to below 69 °C to prevent extensive heat-induced gelation. However, it has to be noted that this emulsion already began to destabilize from 51 °C onwards.

**c. Macroscopic Viscosity Index (MVI)**

The Macroscopic Viscosity Index (MVI) is derived from the inverse of the MSD slope at long decorrelation times (i.e. after the plateau). It describes the interaction between droplets and the continuous phase at zero shear (Xu et al., 2017). As the droplets are covered by an interfacial protein film, the MVI is affected by the affinity between the interfacial protein and the continuous phase.

Figure 3 displays different MVI profiles of WPC-stabilized emulsions. A series of inclining and declining MVI values was observed in native (0h) WPC-stabilized emulsions. A rapid MVI increase began at nearly 70 °C and an additional significant MVI increase was observed after 5 min holding at 80 °C. Furthermore, the MVI value was steady during holding but then decreased upon cooling from 73 °C downwards along with the syneresis phenomenon as described in the MSD section.

Different MVI patterns were observed in the emulsions that contained conjugated as compared to native WPC. The MVI increment occurred at a later temperature and duration in the emulsions stabilized by WPC that was dry heated for 8h. In this specific case, the MVI was relatively stable during the temperature ramp and holding period but increased continuously during subsequent cooling (from 73 °C downwards). In fact, the final MVI value of this emulsion was higher (0.70 $nm^{-2}.s$) than the maximum MVI value of the native WPC emulsion (0.019 $nm^{-2}.s$). Moreover, no syneresis was visually observed in the 8h conjugates-stabilized emulsion. These results confirm the higher water binding ability of the conjugated whey protein. Furthermore, the higher MVI but lower EI than the native WPC emulsion may confirm the correlation between the higher protein-water affinity with the reduced network compactness (i.e. increased steric repulsion).

The 48h emulsion showed the lowest (~$0.57 \times 10^{-5}$ $nm^{-2}.s$) and most stable MVI value throughout the whole thermal treatment, indicating no alteration in the sample viscosity. This observation is fully in line with the EI evaluation that also did not indicate any network formation.

The formed network in emulsions stabilized by native and 8 h conjugated WPC (as shown by the EI data) entrapped water from the continuous phase, increasing their macroscopic viscosity. Nevertheless, the MVI increase occurred at a greater temperature as compared to the EI increase. This indicates that substantial aggregation is necessary to produce a significant effect on the macroscopic viscosity. Therefore, it appears that the elasticity index was more sensitive for detecting the onset of emulsion destabilization as compared to the MVI.

Considering the MSD, EI, and MVI profiles, it can be concluded that the emulsions containing conjugated WPC, especially the one obtained by dry heating for 48 h, exhibited a greater heating temperature tolerance than those stabilized by native WPC. In addition, the elasticity index (EI) parameter was more sensitive to detect the onset of destabilization than the macroscopic viscosity index (MVI). Overall, MS DWS could clearly display the different heat stability of emulsions stabilized by native and conjugated WPC.

### 3.1.2 Bulk rheology

Oscillatory bulk rheological measurements were conducted as an alternative tool to determine the emulsion destabilization. In this case, the gelling point of the emulsions could be determined. Moreover, these measurements also enabled to investigate the relationship between the emulsions' local microrheology and their overall bulk rheological behavior.

Figure 4 shows a different rheological profile for the emulsions containing native and conjugated WPC. In the emulsions stabilized by native WPC (0h) and by conjugates obtained upon 8h of dry heating, heating initially increased the elastic modulus (G'), whereas the viscous modulus (G'') remained constant. Further heating increased the G". A cross-over between G' and G'', which is often defined as the estimated gelling point (Tung & Dynes, 1982) was observed at 55 ± 4 °C for the emulsion containing native WPC. However, Kim, Choi, and Yang (2002) (Kim et al., 2002) discovered that the cross-over point is dependent upon the frequency and occurs prior to the real gel point. Indeed, Almdal et al. (1993) suggested that a gel should have an elastic modulus (G') with a pronounced plateau region for a timescale length of seconds with a considerably higher G' than G'' value (preferably 10 times higher). Meanwhile, the current results showed that the elastic and viscous modulus were comparable and kept increasing after the cross-over point.

Gelation was also defined as the point when the elastic modulus (G') is greater than 1 Pa in the case of yogurt (Lee & Lucey, 2006) which is a closely related soft material to the heat-induced gelled emulsions in this study. When the starting point of network formation was determined

by considering the studies by Almdal et al. (1993) and Lee & Lucey (2006), it was deduced that the gelation was initiated at 64 ± 4 °C and 79 ± 2 °C for the emulsions containing native and 8 hours conjugated WPC, respectively. Upon cooling, stronger structures were formed, as reflected by higher G' values. Hereby, the final elastic modulus of the 8h emulsion (28 Pa) was lower than that of 0h emulsion (105 Pa), indicating that the 8h emulsion sample was less structured.

The elastic modulus of the emulsions containing WPC that was dry heated for 48 h had the lowest value: the maximum value it reached was only 0.4 ± 0.2 Pa, suggesting that no gelation occurred. Moreover, the viscous modulus was hardly affected by the thermal treatment for this sample. Hence, these data provide additional evidence for the previously formulated hypothesis that no thermal destabilization occurred and thus no structure was formed in the 48h emulsion.

These results confirm our previous finding that gelling occurred at a higher temperature for emulsions stabilized by WPC conjugates. Taking a deeper view, pronounced network formation was detected at a comparable temperature of 69 °C and 80 °C in emulsions containing WPC that was incubated for 0 h and 8 h by both micro- and bulk rheology. However, the release of serum from the gelled matrix by syneresis, as reflected by the MVI and EI data of the 0h emulsion, was not discovered during bulk rheological experiments. This could be due to the different cooling rates (and hence different observation periods) in both techniques; as cooling only occurred due to spontaneous heat transfer to the environment, it happened much slower (during several hours) in the microrheology setup. Sun & Arntfield (2011) indeed described that both the heating and cooling rate could impact gel network formation. On the other hand, the different sensitivity towards syneresis could also result from the fact that microrheology measurements basically probe the surface of the heated sample (due to the limited penetration depth of light in the opaque emulsion), while bulk rheology rather probes the bulk properties of the material between the two surfaces in the sample holder. Anyway, this water release is a local scale phenomenon, as it could not be seen by visual observation of the samples after finishing the experiments (Figure 5). In fact, this observation indicates a clear advantage of microrheology as it enabled to detect the sensitivity towards syneresis before this became apparent in the bulk properties. As also discovered by Tisserand et al. (2012), microrheology could detect sample alterations before they appeared in its bulk properties.

Besides, both techniques corresponded well to each other when characterizing the improved heat stabilizing capacity by conjugation of lactose to whey proteins. A comparable micro- and macro-rheological behavior was also discovered by Cristiano et al. (2020) and Moschakis, Murray, & Dickinson (2006). As shown in the current study, the destabilization in terms of network or structure formation was delayed in the emulsions stabilized by conjugated WPC as compared to the native one. Hereby, the best heat stabilization was shown by the 48h conjugated WPC.

The visual appearance of the emulsion samples after both rheological measurements (Figure 5) supported the experimental data of the improved heat stability of the conjugated WPC emulsion. The structured emulsion produced by the native WPC could be clearly distinguished by the solid-like gel in the inverted glass vial to the left. However, this gel was easily broken when being shaken. The 8h emulsion sample displayed a highly aggregated structure, while the 48h emulsion was a homogenous liquid with hardly any noticeable flocs left on the glass wall. The samples recovered from the rheometer had an aggregated appearance with the degree of aggregation diminishing in the order of emulsions containing native, 8h, and 48h conjugated WPC.

From a practical point of view, microrheology enables the measurement of a sample at rest (without any mechanical force) in a completely closed recipient. This enables product characterization during ageing or storage, without disturbing any previously formed weak network. As the characterization is done in-situ in tightly closed sample tubes, evaporation during long heating tests is also prevented. Besides, samples can be held under a modified atmosphere. A last important advantage of the microrheology setup is that several samples (up to 6 in the setup used) can be measured simultaneously, which is especially important for long measurement sequences.

## 3.2 Detection of isothermal heat-induced destabilization

### 3.2.1 Microrheology

In this second part, microrheologial measurements were performed to detect the time window during which the emulsions remained stable upon heating at constant temperature. Hereby, the EI was used as it was shown above to be the most sensitive parameter to determine the destabilization point. As the above experiments indicated that the destabilization temperature of the different emulsions ranged from nearly 64 to 80 °C, the different emulsions were

continuously monitored at a constant temperature of either 60 or 80 °C throughout 1 hour of measurement.

Figure 6 indicates significant network formation during heating at both conditions of the native WPC emulsion: its EI profile increased significantly at both 60 °C and 80 °C, with a significant increase being initiated at 10 min and 3 min, respectively. At 80 °C, the EI rise was more extensive (more than 1 decade), but largely dropped after 27 min. The fall in EI was due to syneresis as has been discussed in Section 3.1.

The emulsion stabilized by conjugated WPC that was dry heated for 8 h exhibited no significant rise in elasticity index during one hour heating at 60 °C (Figure 6a). Hence, the protein conjugates appeared to possess stronger intermolecular repulsive forces than the native WPC, to impede protein-protein interactions, hence preventing network formation. Nonetheless, a significant increase in EI was detected after 5 min of heating at 80 °C. However, the maximum EI value of the 8 h emulsion ($2.6 \times 10^{-3}$ $nm^{-2}$) was clearly lower than that of 0 h emulsion ($1.4 \times 10^{-2}$ $nm^{-2}$), which implies that the 8-hour emulsion structure was less dense.

The 48 h conjugated WPC-emulsion owed the most extended acceptable heating duration (i.e. one hour) as there was not any indication of network creation within the time frame used: throughout one hour of heating at 60 and 80 °C, the EI values remained rather consistent at low values, ranging from $5.1 \times 10^{-4}$ to $5.6 \times 10^{-4}$ and from $5.5 \times 10^{-4}$ to $7.2 \times 10^{-4}$ $nm^{-2}$, respectively.

In summary, it is demonstrated that by using conjugated WPC, protein-stabilised emulsions could withstand heat coagulation for a longer time. Taking into account the onset of detectable network formation, it is suggested that the acceptable heating duration of native WPC-stabilized emulsions was below 10 min at 60 °C, and less than 3 min at 80 °C. Meanwhile, the 8 h conjugated WPC could keep WPC-stabilized emulsions stable for up to one hour at 60°C, but less than 5 min at 80°C. The 48 h conjugates provided the longest possible heating duration with hardly any heat coagulation during heating up to one hour at both 60 and 80 °C.

### 3.2.2 Particle size and viscosity evaluation

In a last series of experiments, the emulsions were evaluated for their particle size and viscosity, the two most commonly used parameters to evaluate emulsion stability: heat stability is reflected by a limited change in the particle size and viscosity. In these experiments, the emulsions were previously heated at 60 or 80 °C for 30 min.

The results displayed in Figure 7 and Table 1 as well as Figure A2, again denote the prominent improvement of the emulsion heat stability when whey protein conjugates were used as the emulsifier. The native (0h) WPC-stabilised emulsion was unstable at all heating temperatures applied (i.e. both 60 and 80 °C), as shown by the tremendous increase in particle size and viscosity. On the contrary, the emulsion stabilized by the 8 hours-conjugated WPC showed a constant particle size and viscosity upon heating at 60 °C, but was highly aggregated at 80 °C. Whereas the emulsion containing WPC conjugates obtained by 48 h of dry heating showed a higher initial particle size compared to the other samples (which was thought to be caused by emulsion droplet aggregation due to advanced Maillard reaction products, such as melanoidins and polymerised proteins), it was the most stable at both temperatures. Especially at 80 °C, where emulsions containing native (0h) and 8h conjugated WPC were highly destabilized, the 48h conjugated sample's particle size and viscosity only showed a limited increment. Hereby, the slight increase in viscosity indicated that the droplet aggregation (as shown by the increased particle size) did not form a strong packing to affect the bulk viscosity of the emulsion.

The particle size analysis (Figure 7, Figure A2) and viscosity results (Table 1) correlated well with the EI data, whereby higher elasticity index values in emulsions containing (native) WPC that was not dry heated versus WPC that was dry heated for 8h and 48h indicated enhanced droplet aggregation and, consequently, a larger particle size and higher viscosity. Interestingly, although the unheated emulsions with WPC conjugates prepared by 48h dry heat incubation showed a bigger average particle size than that containing WPC after 8h incubation (Figure 7), it performed better towards thermal treatment, as evidenced by the lower EI, MVI and G'. Therefore, a higher heating temperature and longer heating duration were possible in emulsions stabilised by WPC that was dry heated for a longer time.

## 4. CONCLUSIONS

Microrheology could well determine the initial heat-induced destabilization of WPC stabilized-emulsions. Hereby, the heat stabilizing capacity of whey protein-lactose conjugates was proven by their ability to maintain the microrheological properties of the WPC-stabilized emulsions during thermal treatment. The improved heat stability was a further result of the ability of the conjugates to prevent the formation of an oil droplet network. As such, the conjugated WPC emulsions remained mainly viscous (rather than elastic) with a low elasticity (EI) and viscosity (MVI) during heating, consequently providing an extended acceptable heating temperature

and duration. Furthermore, the microrheology findings were found to be largely in line with the bulk rheological properties: both methods indicated a comparable temperature for the initial network formation. Nevertheless, the syneresis phenomenon which was observed in microrheology was not detected in bulk rheology. As such, microrheology seems more sensitive for the early detection of syneresis prior to its appearance in bulk properties.

The conjugates-stabilized emulsion had an extended range of feasible heating temperature and duration. From the obtained results, it is concluded that the recommended heating temperature for native (0h) and 8h conjugated WPC-stabilized emulsions was below 64 and 79 $^{o}$C, respectively. Meanwhile, under isothermal heating, the acceptable heating duration of native WPC-stabilized emulsions was below 10 min at 60 $^{o}$C, and less than 3 min at 80 $^{o}$C, whereas the 8h conjugated WPC-stabilised emulsions could resist up to one hour heating at 60°C, and up to 5 min at 80°C. The WPC conjugates obtained upon 48 h of dry heating provided the highest temperature-tolerance and longest possible heating duration with up to one hour at both 60 and 80 $^{o}$C.

The obtained microstructural understanding may provide a better insight in the functionality of whey protein-sugar conjugates, which in turn might further favor the industrial application of conjugated whey proteins as heat stable natural emulsifiers.

## APPENDIX A. Supplementary material


## ACKNOWLEGEMENT

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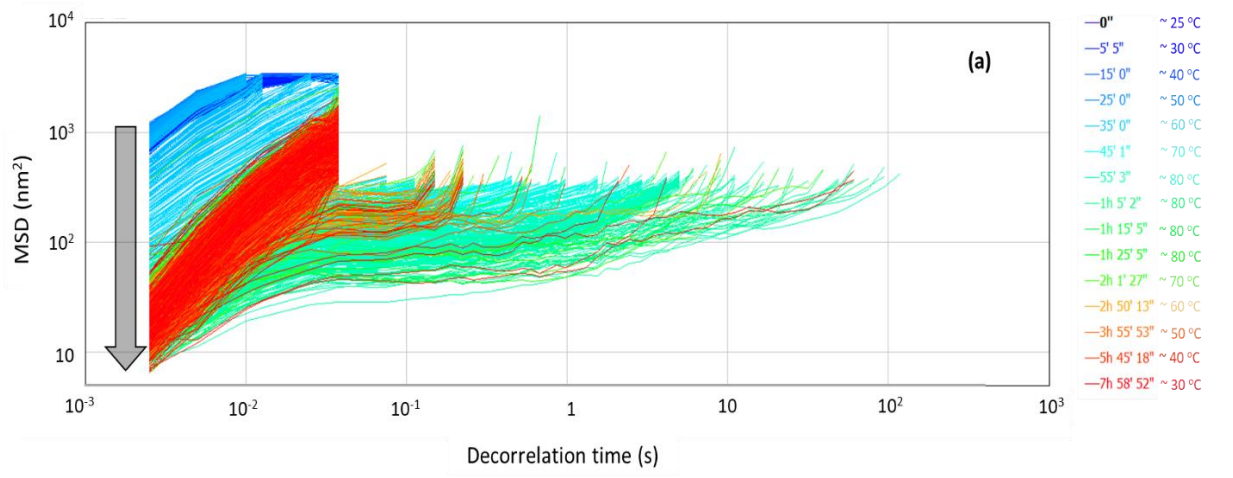


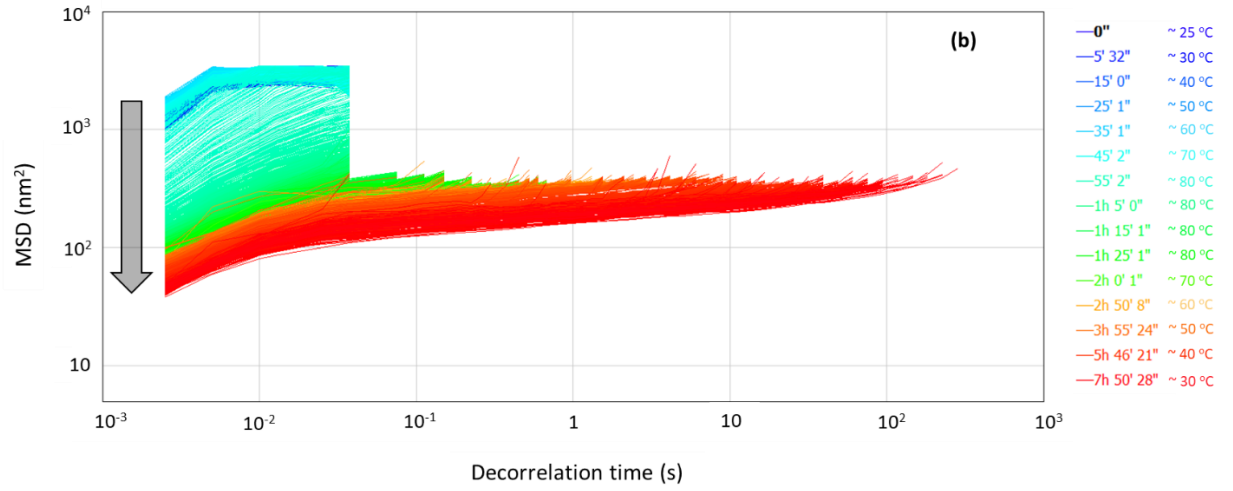


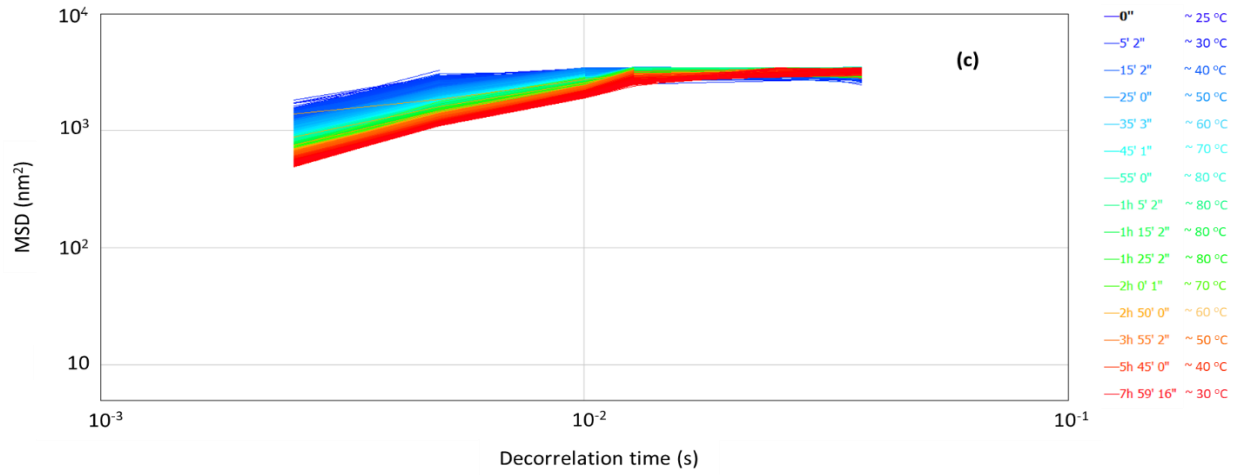


**Figure 1.** Mean square displacement (MSD) versus decorrelation time curves of emulsions stabilized by WPC which was dry heated for **(a)** 0 h, **(b)** 8 h, and **(c)** 48 h. Hereby, the MSD was measured continuously during a cycle consisting of heating from 25 to 80 °C at 1 °C/min (from 0 to 55 min), holding at 80 °C for 30 min (from 55 to 85 min), and subsequent cooling (from

640 85 min onwards). The legend on the right side indicates the measurement time with its
641 corresponding temperature, ranging from 0 s (0"; in blue) up to about 8 hours (in red).
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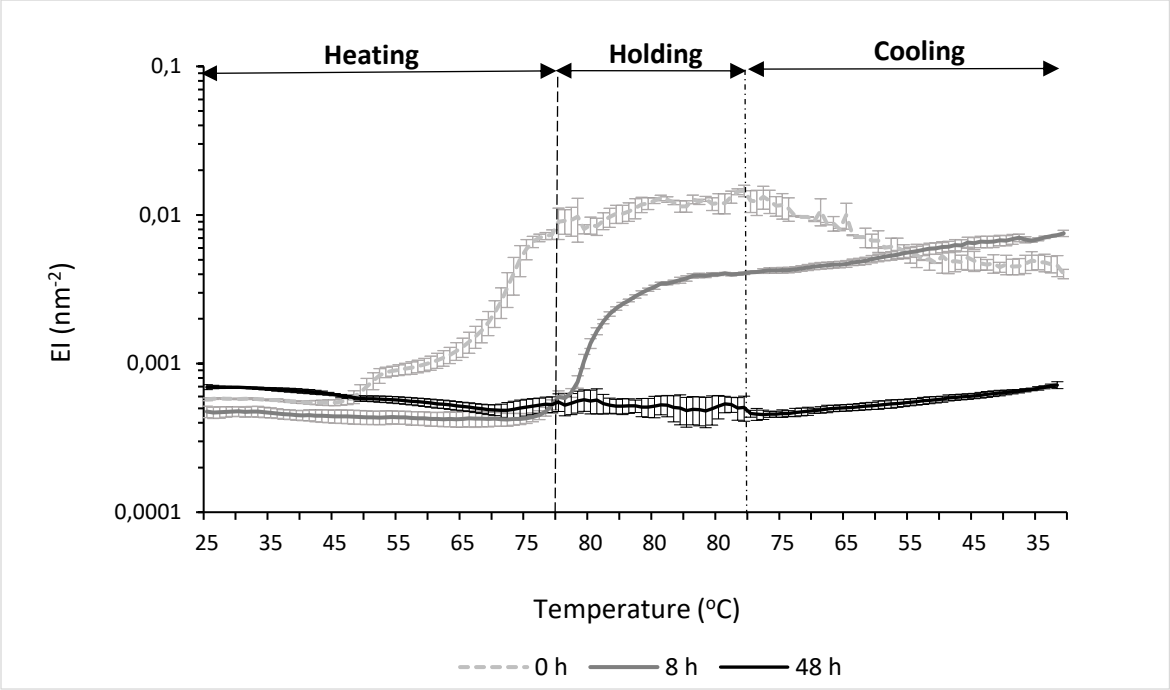



643

644 **Figure 2.** Elasticity Index (EI) of emulsions stabilized by WPC conjugated for different
645 incubation periods (0, 8, and 48 h), during a cycle consisting of heating from 25 to 80 °C with
646 1 °C/min ramp (0 to 55 min), holding at 80 °C for 30 min (55 to 85 min) and subsequent manual
647 cooling (from 85 to 445 min). Holding at 80 °C was presented as one point per minute (30
648 points in total). The detailed data of the temperature profile as a function of the measurement
649 time can be found in Appendix 1. The data represent the average and standard deviation
650 obtained from two repetitions.

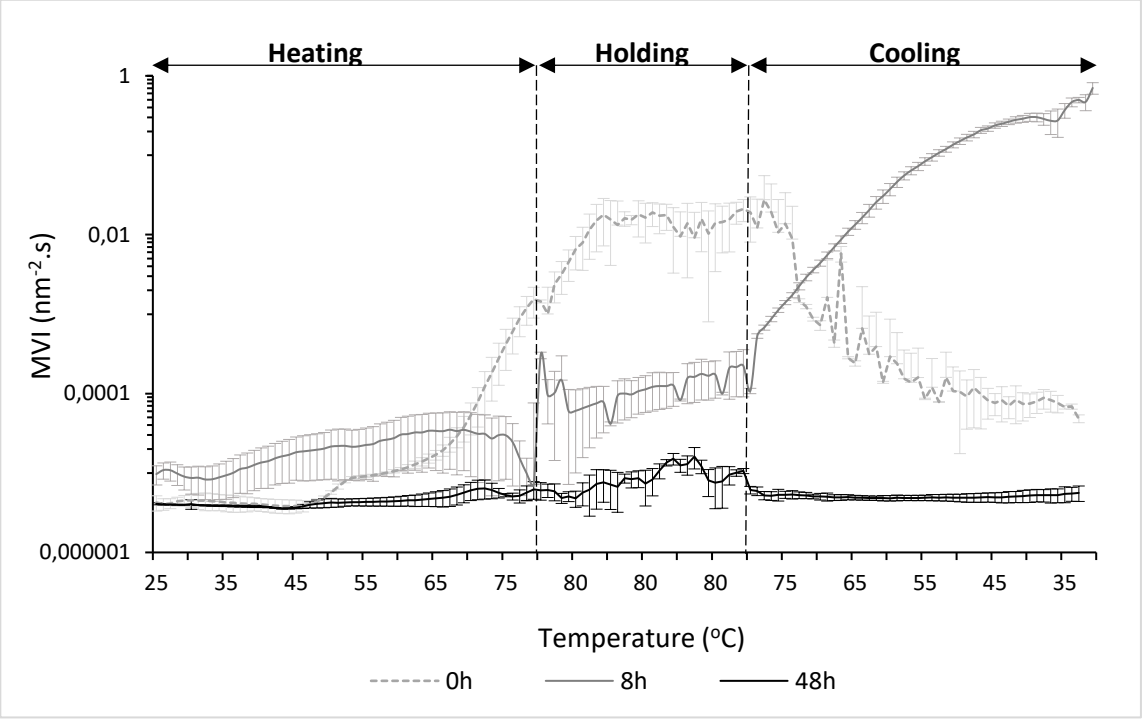
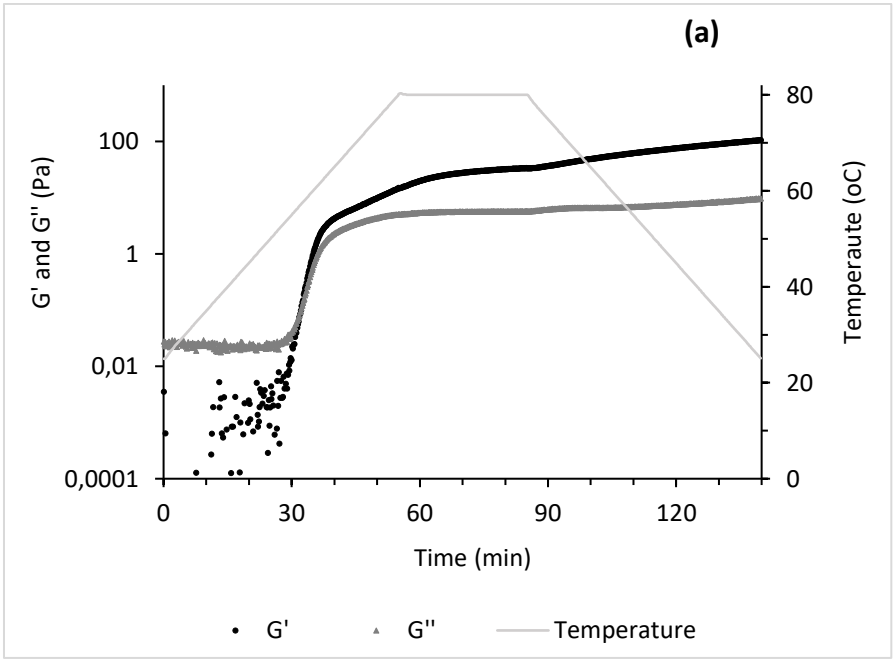
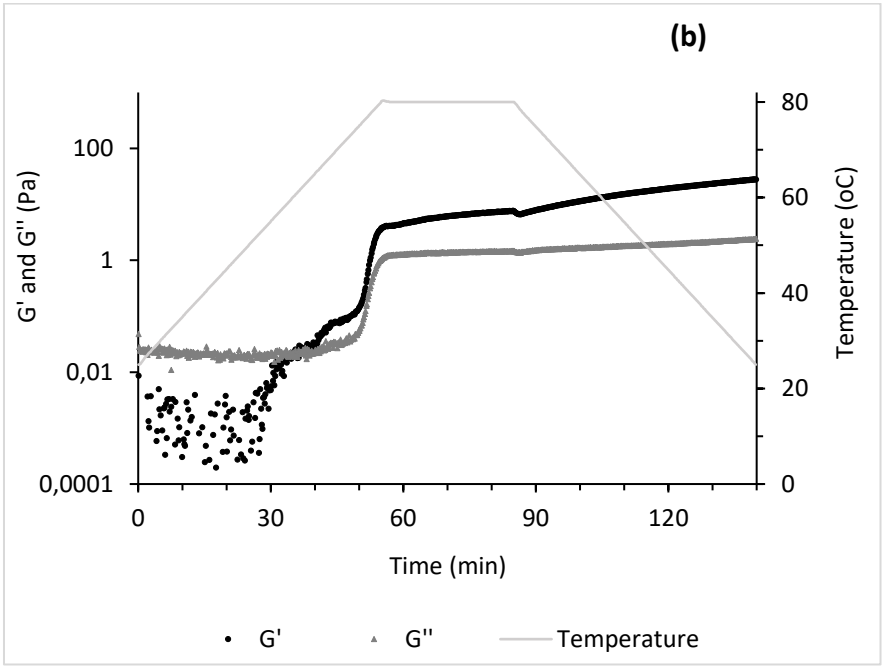



**Figure 3.** Macroscopic viscosity index (MVI) of emulsions stabilized by WPC conjugated for **(a)** 0, **(b)** 8, and **(c)** 48 hours, during a cycle consisting of heating from 25 to 80 ºC with a 1 ºC/min ramp (0 to 55 min), holding at 80 ºC for 30 min (55 to 85 min) and subsequent manual cooling (85 to 445 min). Holding at 80 ºC was presented as one point per minute (30 points in total). The detailed data of the temperature profile as a function of the measurement time can be found in Appendix 1. The presented data were the average of two repetitions.





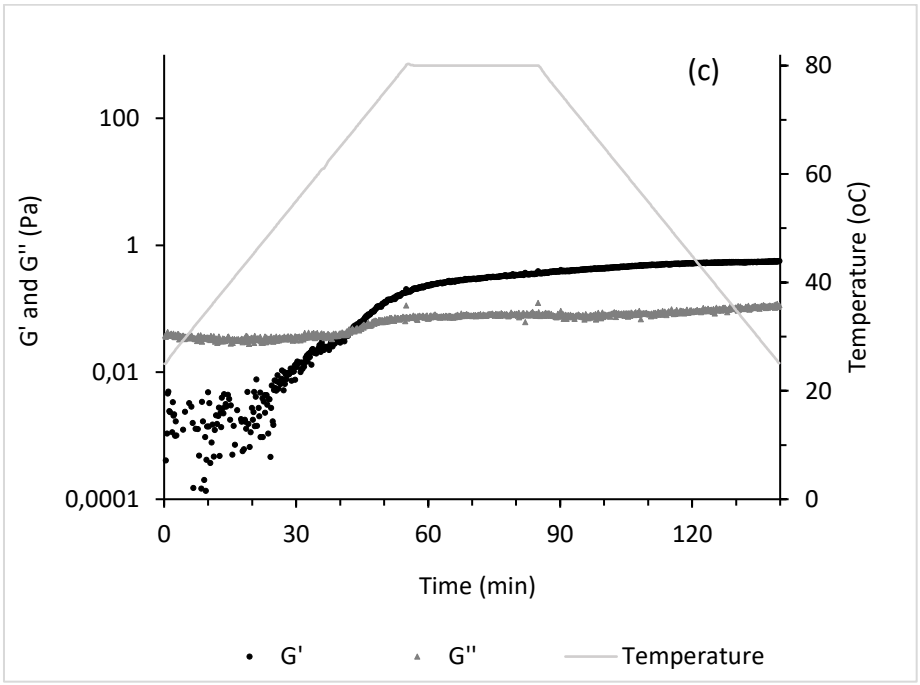




**Figure 4**. Rheological profile of emulsions stabilized by **(a)** native (0h) WPC; **(b)** conjugated WPC that was dry heated for 8 hours (8h); and **(c)** conjugated WPC that was dry heated for 48 hours (48h), during a temperature sweep from 25 to 80 °C at 1°C/min, holding at 80 °C for 30 min, and cooling back to 25 °C at 1°C/min.

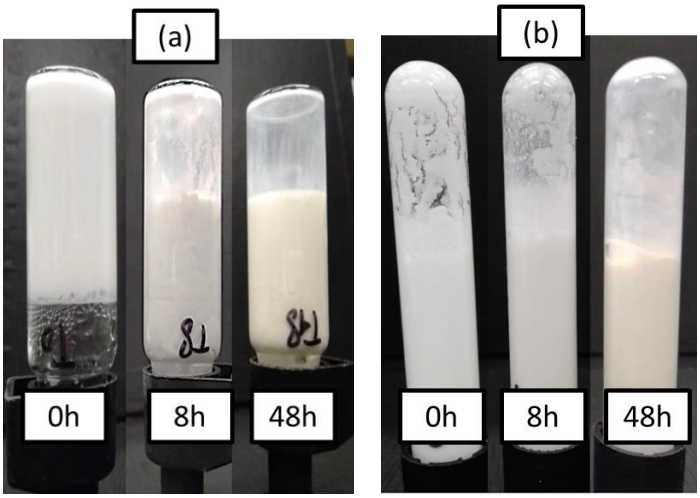



**Figure 5.** Visual appearance of the emulsions stabilized by native (0 h) and conjugated WPC (8 h and 48 h, resp.) after (a) microrheology and (b) bulk rheology measurements.

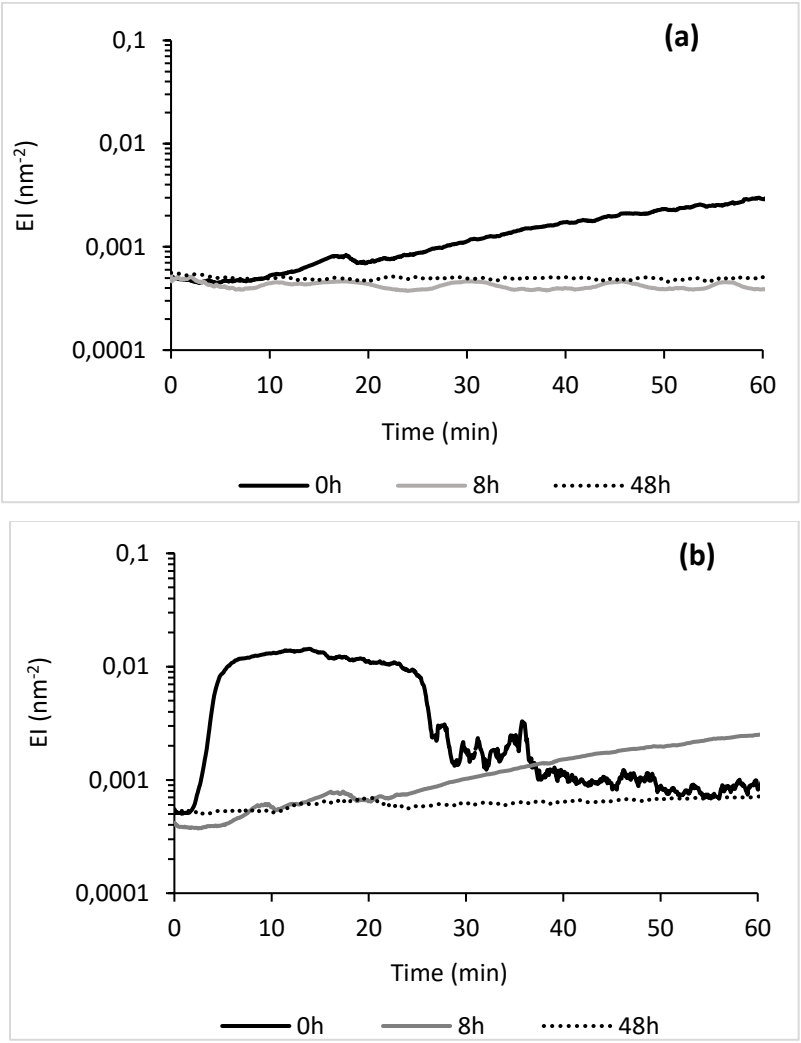



**Figure 6.** Elasticity Index (EI) of emulsions stabilized by WPC conjugated for different incubation periods (0, 8, and 48 h), during heating for 1 hour at (a) 60 °C or (b) 80 °C.

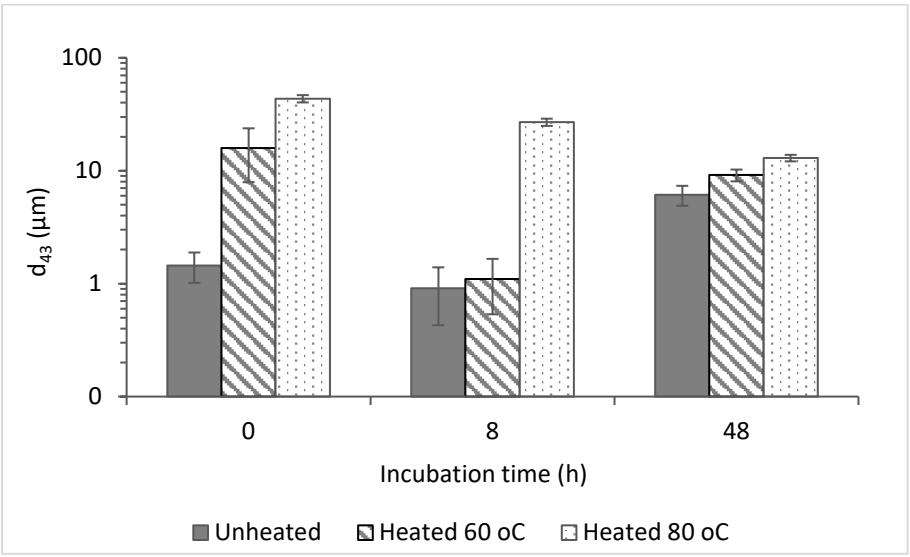



**Figure 7.** Volume weighted average particle diameter ($d_{43}$) of emulsions stabilized by whey protein concentrate that was previously dry heat-treated for 0, 8, and 48 hours, before and after heating at 60 or 80 °C for 30 min.

**Table 1.** Consistency coefficient of emulsions stabilized by native and conjugated WPC that was dry heated for 8 and 48 hours, before and after heating at 60 and 80 °C for 30 min.

| Incubation time (h) | Consistency coefficient (mPa.s) | | |
|---|---|---|---|
| | Unheated | Heated at 60 °C | Heated at 80 °C |
| 0 | 1.8 ± 0.0 | 6.2 ± 1.0 | * |
| 8 | 1.6 ± 0.0 | 1.5 ± 0.0 | 605 ± 109 |
| 48 | 2.5 ± 0.1 | 2.7 ± 0.1 | 7.6 ± 1.0 |

**this sample was highly aggregated and could not be measured accurately using viscometry.*

**Appendix 1.** Temperature profile as a function of measurement time during MS DWS measurements in a heating-cooling cycle

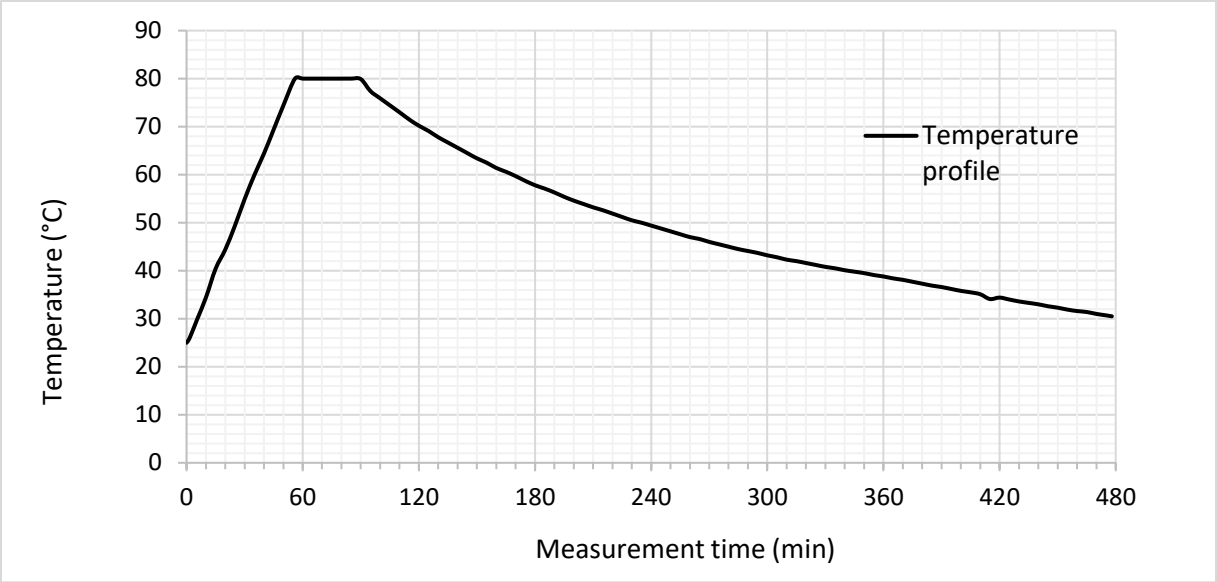
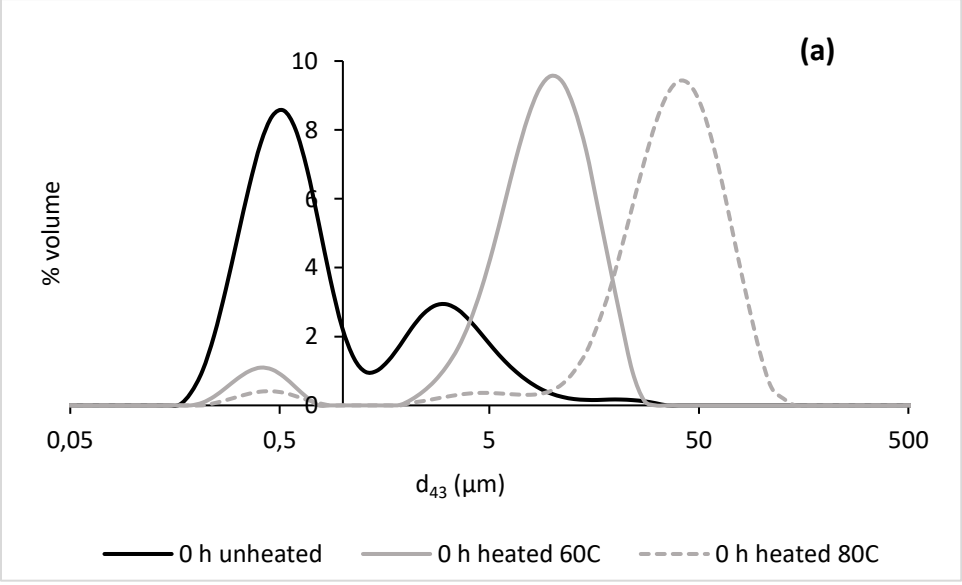
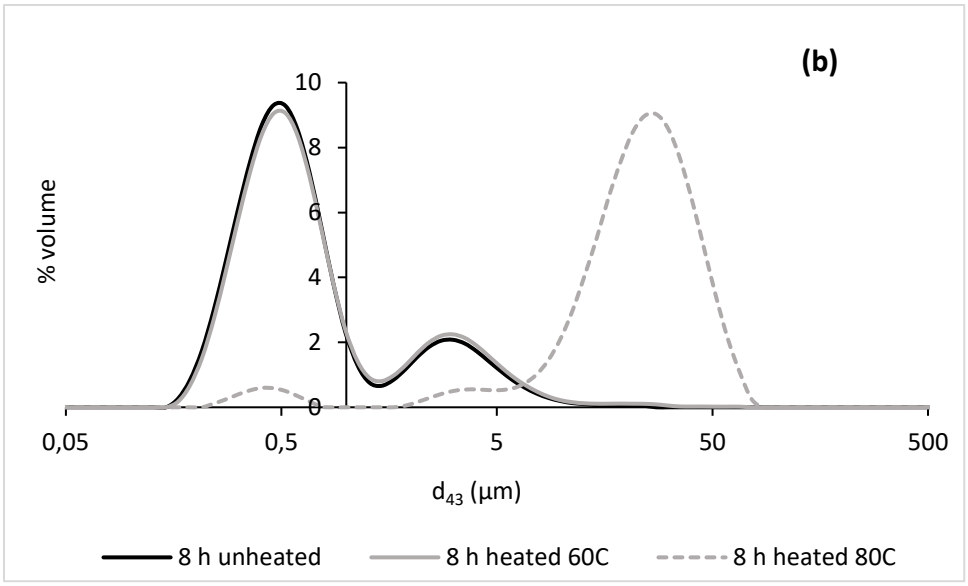



**Figure A1**. Temperature profile as a function of the measurement time during a heating-cooling cycle of microrheological evaluation of the heat stability on native and conjugated WPC-stabilized emulsions.

**APPENDIX 2.** Particle size distribution of emulsions stabilized by whey protein concentrate that was previously dry heat-treated for 0, 8, and 48 hours, before and after heating at 60 or 80 °C for 30 min.





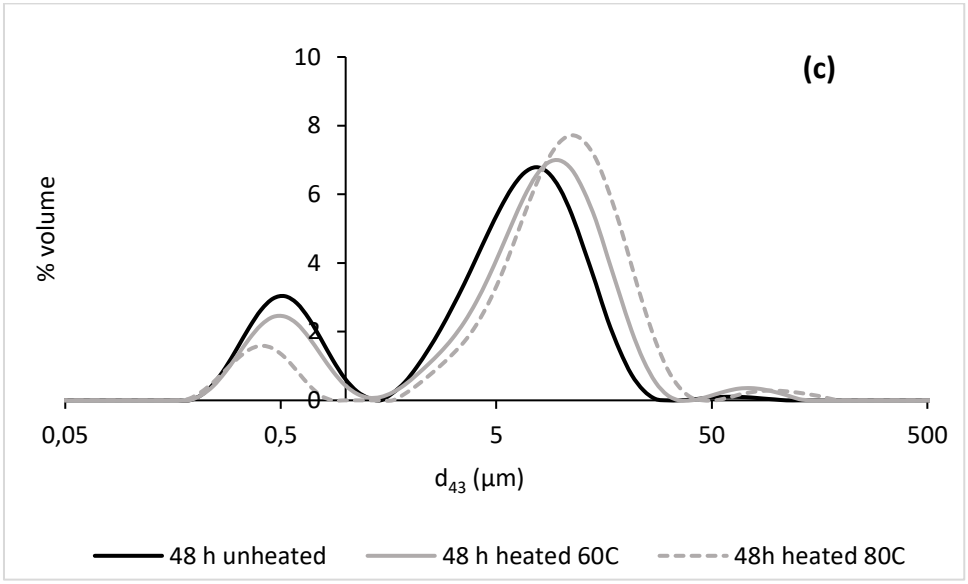




**Figure A2.** Particle size distribution of emulsions stabilized by whey protein concentrate that was previously dry heat-treated for **(a)** 0; **(b)** 8; and **(c)** 48 hours, before and after heating at 60 or 80 °C for 30 min.