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Protein fortification of model cheese matrices using whey protein-enriched double emulsions

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Abstract

Whey proteins represent 20% of the protein content of milk and are an underutilised by-product of cheese manufacturing. This study was aimed at encapsulating whey proteins in the fat content of cheese using double emulsions. A two-stage power ultrasound (20 kHz) emulsification was used to produce double emulsions with an internal aqueous phase enriched with high concentrations of whey proteins contained within droplets of sunflower oil. Primary water-in-oil (W1/O) nanoemulsions were successfully formed at an applied ultrasonic power of 1.35 W/mL, using 20% w/w and 30% w/w whey protein concentrate (WPC) solutions in the internal phase. The inner water droplets were stabilised by a combination of food grade lipophilic emulsifiers included in the sunflower oil at minimum concentrations of 1% w/w lecithin and 3% w/w PGPR. The secondary oil emulsions were formed by emulsifying the primary W1/O emulsions in 5% w/w WPC solutions, with the whey proteins serving as the emulsifying agent. The encapsulation loading rate of whey proteins within the double emulsion droplets was investigated in relation to ultrasound parameters and formulation loading rates. A very high encapsulation loading rate of ~45 $g_{\text{whey protein}}/L_{\text{double emulsion}}$ was achieved using 0.81 W/mL of ultrasound, with oil droplets of comparable diameter to native milk fat globules (~10 μm). These double emulsions were successfully incorporated into renneted and cooked curd

systems to enable the retention of whey protein in cheese matrices. This study demonstrates the potential of ultrasound emulsification to form whey protein-enriched double emulsions with minimum food-grade emulsifiers to fortify the protein content of cheese and other food products.

Key words

Double emulsions, encapsulation, functional food, cheese, ultrasonication, protein fortification

1. Introduction

The nutritional value of bovine milk and dairy products is important to consumers. Milk includes casein and whey protein, both of which are a good source of nutritional protein (Barłowska, Szwajkowska, Litwińczuk, & Król, 2011). However, during cheese production, water soluble whey proteins are drained out from the coagulum during syneresis (Heino, Uusi-Rauva, Rantamaki, & Tossavainen, 2007), representing a loss of approximately 20% of the protein from cheese milk. The protein in cheese whey retains much of its native nutritional and functional properties (Heino, et al., 2007) and can be used to produce protein supplements (e.g. whey protein concentrate powder) or animal feed (Siso, 1996). While these are useful products, the former requires further processing and a certain minimum scale of production to be commercially viable, and the latter is of much lower value than the protein in cheese.

Attempts have been made to incorporate whey protein back into cheese, to make better use of the whey protein and to increase the protein content of the cheese. Different technologies have been applied to cheese whey to facilitate incorporation of whey protein into cheese, including the use of membrane filtration to pre-concentrate the protein, thermal denaturation to aggregate the protein, and the addition of spray-dried whey protein powders (Hinrichs, 2001). However, these technologies each have their limitations. The addition of membrane concentrated whey protein has been associated with the development of bitter flavours due to the retention of undesirable water-soluble enzymes (Hinrichs, 2001). Incorporating thermally denatured whey proteins can alter the rate of coagulation (Gamlath, Leong, Ashokkumar, & Martin, 2020; Kethireddipalli, Hill, & Dalgleish, 2010; Singh & Waungana, 2001; Vasbinder, Rollema, & De Kruif, 2003) and lead to curds with excessive moisture. The addition of whey protein powders can result in poor binding of the curd (Brown & Ernstrom, 1982; Hinrichs, 2001; Punidadas, Feirtag, & Tung, 1999). The aim of the current study was to investigate a novel approach to enhance the whey protein content of cheese by the incorporation of whey protein-enriched double emulsions.

Put simply, a double emulsion is an emulsion in which the emulsion droplets contain an inner emulsion of the opposite phase (Leong, Zhou, Kukan, Ashokkumar, & Martin, 2017). The inclusion of water-in-oil-in-water (W/O/W) double emulsions in cheese making is a relative new idea, which has so far been used to reduce the fat content (Felfoul, Bornaz, Baccouche, Sahli, & Attia, 2015; Leong, et al., 2020; Lobato-Calleros, et al., 2007; Lobato-Calleros, Rodriguez, Sandoval-Castilla, Vernon-Carter, & Alvarez-Ramirez, 2006; Lobato-Calleros, et al., 2008) or to encapsulate nutrients such as vitamin B-12 into cheese (Giroux, et al., 2013). In addition, the use of double emulsions allows substitution of anhydrous milk fat (rich in saturated fatty acids (Chouinard, Girard, & Brisson, 1998; DiNicolantonio & O'Keefe, 2017)) with less expensive and potentially healthier fat alternatives such as sunflower, canola or olive oil, which are rich in poly-unsaturated fatty acids (including ω-3 and ω-6) (Chouinard, et al., 1998; Ramos, Fernández, Casas, Rodríguez, & Pérez, 2009), further improving the nutritional value of cheese analogues.

It is possible to produce W/O/W double emulsions with a similar apparent volume fraction and size distribution to native milk fat globules, which can be used to make reduced-fat cheese with a fat microstructure and cheese texture comparable to full-fat cheese (Leong, et al., 2020). For incorporation of whey proteins into cheese, it is proposed here that W/O/W double emulsions can be prepared with an inner aqueous phase containing a high concentration of whey protein. Such a double emulsion can be formulated as the cheese milk, enabling the whey protein to be entrapped within the fat globules of the cheese. The double emulsion can be produced by first preparing a stable water-in-oil emulsion (W/O) with a high proportion of aqueous phase containing whey protein. This single emulsion can be emulsified into a whey protein stream to form a W/O/W double emulsion, which can then be mixed with skim milk to produce a cheese milk that will form a whey-protein fortified cheese.

Emulsions are inherently thermodynamically unstable, and forming stable double emulsions is challenging. Producing a stable inner W/O emulsion is particularly challenging (Leong, et al., 2017), typically relying on the addition of large amounts of surfactants to confer kinetic stability (Matsumoto, Kita, & Yonezawa, 1976) and the production of small droplets that can be accommodated within the outer oil droplets. The emulsification of both the primary and secondary emulsions requires high shear forces that can be applied using high-shear devices such as high-pressure homogenisers (Leong, Zhou, Zhou, Ashokkumar, & Martin, 2018; Stang, Schuchmann, & Schubert, 2001) and high-power low-frequency (~20 kHz) power ultrasound (Leong, et al., 2017; Leong, Zhou, et al., 2018). Power ultrasound has been successfully used

to produce stable micron and submicron sized fat/oil droplets using milk proteins as the emulsifier (Kaltsa, Michon, Yanniotis, & Mandala, 2013; Leong, et al., 2017; Mahdi Jafari, He, & Bhandari, 2006; Shanmugam & Ashokkumar, 2014) and has been effectively applied to both the primary and secondary emulsification stages of double emulsion production (Leong, et al., 2017; Leong, Zhou, et al., 2018). The ability of the strong physical effects of cavitation to induce structural changes in milk proteins that enhance their emulsifying properties can reduce the need for non-dairy emulsifiers in dairy based applications (Shanmugam, et al., 2014).

To date, dairy-based double emulsions have only been produced with low protein concentrations (<10% w/w) in the inner aqueous phase (Felfoul, et al., 2015; Leong, et al., 2020; Leong, et al., 2017; Leong, Zhou, et al., 2018; Lobato-Calleros, et al., 2007; Lobato-Calleros, et al., 2006; Lobato-Calleros, et al., 2008). The ability to encapsulate significant quantities of whey protein inside the internal aqueous phase of double emulsions does not appear to have been investigated. For example, Leong and co-workers formed dairy-based W/O/W double emulsions using skim milk (with a total protein content of ~4% w/w) as the inner aqueous phase, using food-grade surfactants to stabilise the droplets (Leong, et al., 2017). This single emulsion was then emulsified into the secondary aqueous phase using skim milk proteins to stabilise the outer secondary emulsion. While this formulation resulted in stable double emulsions, it did not result in a practically significant encapsulation of whey protein. More recently Silva, Bui, Dharmadana, Zisu, and Chandrapala (2020) demonstrated that whey proteins could provide greater stability and encapsulation efficiencies for double emulsions than caseins, due to the electrostatic repulsion provided by the higher negative charge on the whey protein-stabilised emulsion droplets. However, according to the data provided, the maximum protein encapsulation was only ~1.4 $g_{protein}/L_{double\ emulsion}$.

The aim of the present study was to develop the fundamental understanding needed to produce whey-protein enriched cheese via double emulsion encapsulation. The initial objective was to produce stable water-in-oil single emulsions containing high loading fractions of concentrated whey protein solutions, and requiring minimal amounts of food-grade emulsifiers (lecithin and polyglycerol polyricinoleate (PGPR)). Subsequently, the effect of ultrasonication and formulation parameters on droplet properties, encapsulation efficiency and stability were investigated to understand how to maximise the loading of whey-protein-enriched single emulsions into double emulsions. The final objective was to incorporate these double emulsions into cheese milk and to study their effect on protein coagulation and protein retention

in model cheddar curd matrices. By developing an understanding of how to successfully formulate and incorporate whey protein-enriched double emulsions into cheese matrices, this study will help develop technologies to reintegrate whey streams into cheese production to produce nutritionally enhanced products.

2. Materials and Methods

2.1.Skim milk and protein powders

Whey protein concentrate (WPC) powder ('Select whey protein concentrate', Professional Whey, Erina NSW, Australia) was used as the whey protein source for encapsulation into the inner emulsions and for stabilising the oil-in-water single and double emulsions. Skim milk was purchased from a local supplier ("Pauls Skinny Milk", produced by Lactalis Australia, South Brisbane, Australia). The protein content of the WPC powder and skim milk were measured to be 73% w/w (4.9% casein and 68.1% whey protein) and 4.1% w/w (3.3% casein and 0.8% whey protein) using a previously reported method (Gamlath, et al., 2020).

2.2.Formation of primary water-in-oil emulsions

Primary water-in-oil emulsions were produced using a combination of food-grade emulsifiers. Soy lecithin (1% w/w of the oil phase) and PGPR (4% w/w, 3% w/w or 2% w/w of the oil phase) were added to sunflower oil (O) (Woolworths Home brand, Bella Vista, Australia) and stirred at 40 °C until fully dissolved. As previously reported by Leong, Zhou, Zhou, Ashokkumar, and Martin (2018), a combination of lecithin and PGPR was more effective for producing stable W/O/W double emulsions than either surfactant used alone. The aqueous phase (W1) of the single emulsions was prepared by dissolving varying amounts of WPC powder in distilled and deionised water to achieve final powder concentrations of 20%, 30%, or 40% w/w. The viscosities of these systems were measured to be 6.8 mPa.s, 29.6 mPa.s and 109.1 mPa.s in the order of increasing solid loading. These protein solutions also contained 2% w/w NaCl (Sigma Aldrich, St Louis, Missouri, USA) as an entrapment marker (Leong, et al., 2017). The solutions' densities were measured to be 1.05 mg/mL, 1.08 mg/mL and 1.14 mg/mL in order of increasing solid loading.

To produce primary water-in-oil emulsions (W1/O), protein solutions and oil were loaded into a sonication cell cooled with water circulation at W1:O volume ratios of 3:7 or 4:6 and a total liquid volume of 25 mL. Emulsification was carried out for 3 minutes at an applied calorimetric power of 33.8 W, by placing the tip of a 20 kHz, 11 mm ultrasonic horn (Model 102C (CE), Branson Ultrasonics, St. Louis, Missouri, USA) at the oil-water interface. The applied

calorimetric power intensity and energy density were 1.35 W/mL and 243 J/mL respectively. The bulk temperature of the sample was maintained below 50 ºC in all emulsification trials.

2.3. Double emulsion formation

For the initial formulation study, the outer aqueous phase (W2) of the double emulsions was prepared by dissolving 5% w/w WPC powder in distilled and deionised water. Pre-formed W1/O emulsions were emulsified into the outer aqueous phase at volume ratios of 2:8, 3:7, and 4:6 to produce whey-protein enriched double emulsions. Emulsification was performed in the same sonication cell as used to produce the W1/O emulsion, with room temperature cooling water circulated and a total sample volume of 25 mL. The samples were emulsified for 90 s at various calorimetric power levels (6.8 W 20.3 W and 33.8 W) by placing the 20 kHz, 11 mm ultrasonic horn at the emulsion-water interface. The power intensities and energy densities corresponding to the different applied powers were 0.27 W/mL, 0.81 W/mL and 1.35 W/mL; and 24.5 J/mL, 73.1 J/mL and 121.7 J/mL, respectively. The bulk temperature of the sample remained below 50 ºC in all emulsification trials.

A summary of the emulsion and cheese milk formulations used in the rennet gelation and cooked curd trials are presented in Table 1. For the rennet gelation study, 20 mL of double emulsion cheese milk and control cheese milk were formulated by mixing a double emulsion or a whey protein stabilized single emulsion (SE) with skim milk, 5% WPC solution and 40% WPC solution to reach a final fat and protein content of 2.8% w/w and 4.3% w/w. For comparison, a cheese milk with fat emulsified in whey protein was included as a control to maintain similar interactions between the fat globule membrane and the casein matrix, as these interactions could affect curd formation and fat loss.

Table 1: Formulations of emulsions and cheese milks used in rennet gelation kinetics measurement and cooked curd trials.

Emulsions		
Emulsion type	Compositions and ratios of emulsion components	Power intensity of ultrasonic emulsification
Double emulsion	W1: 40% w/w WPC solution with and 2% w/w NaCl O: Sunflower oil with 1% lecithin and 3% PGPR W2: 5% w/w WPC solution W1:O ratio = 3:7, SE:W2 ratio = 4:6	Primary emulsion = 1.35 W/mL Secondary emulsion = 0.81 W/mL

Single emulsion	W: 5% w/w WPC solution O: Sunflower oil with 1% lecithin and 3% PGPR O:W ratio = 4:6				0.81 W/mL	
Cheese milks						
Experiment	Cheese milk type	Double emulsion (% w/w)	Single emulsion (% w/w)	Skim milk (% w/w)	5% WPC solution (% w/w)	40% WPC solution (% w/w)
Rennet gelation	DE cheese milk	10	0	90	0	0
	Control cheese milk	0	7	90	1.8	1.2
Cooked curd trial	DE cheese milk	15	0	85	0	0
	Control cheese milk	0	10.5	85	2.7	1.8

W1 = double emulsion inner aqueous phase; O = oil phase; W2 double emulsion outer aqueous phase; W = single emulsion aqueous phase.

2.4.Rennet gelation and kinetics

20 mL of double emulsion cheese milks and control cheese milks (prepared according to Table 1 in Section 2.3) were subjected to rennet gelation at 31 °C by adding 0.2 mL of 3.5 IMCU/mL rennet solution. Gelation kinetics were measured according to a previously reported method (Gamlath, et al., 2020) using an AR-G2 rheometer (TA Instruments) using a controlled shear strain of 2.5% at 1 Hz and a standard concentric cylinder (996284) tool for 1 hr.

2.5.Formation of cooked curds

Double emulsion cheese milk and control cheese milks were formulated by mixing a double emulsion or a single emulsion (formulated as presented in Table 1) with skim milk, 5% WPC solution and 40% WPC solution to reach a final fat and protein content of 4.2 ± 0.1% w/w and 4.4 ± 0.1% w/w (2.9% w/w casein 1.5% w/w whey protein). These cheese milks had a higher fat content compared to the rennet gelation trials, as more double emulsion was included during formulation in order to entrap a more internal aqueous phase containing WPC in the curd.

Cooked curds were prepared according to a previously reported method (Cipolat-Gotet, Cecchinato, Stocco, & Bittante, 2016) with some modifications. In brief, 10 g aliquots of cheese milk were loaded into 50 mL yellow capped bottles and warmed to 31 ºC. Mesophilic starter culture (Cheeselinks, Lara, Australia) containing a mix of *Lactococcus lactis* subsp. lactis and *Lactococcus lactis* subsp. cremoris was added at a concentration of 1 x 10^{-5} % w/w and ripened for 45 min. Rennet was added to reach a final concentration of 1 x 10^{-4} % w/w and gelled for 2 hours. The curds were then cut into ~ 1 cm^3 cubes using a spatula. The water bath

temperature was set to 70 ºC, allowing the sample temperature to gradually increase to 56 ºC within 30 min. The curds were strained through a 1 mm x 1 mm sieve for 30 min. Final curd and whey masses were measured and refrigerated overnight before compositional analysis.

2.6. Optical and fluorescence microscopy

An emulsion sample of approximately 3 μL was transferred onto a glass slide and covered with a cover slip. An optical microscope (Olympus, Tokyo, Japan) fitted with a 60× oil immersion optical lens was used to visualise the emulsions.

The same optical microscope was used to visualise fluorescence emission. Nile red (2 mg/mL in ethanol) (Sigma Aldrich, Missouri, USA) was added to the oil phase of the double emulsion to reach a concentration ~ 0.02% w/w. An excitation wavelength was used in the range 460–495 nm, from which Nile red emits in the yellow (>565 nm) range of the visible spectrum.

2.7. Confocal laser scanning microscopy

Nile red and Fast green were used to stain the oil and proteins respectively. While Fast green participates in ionic interactions with basic amino acid residues in proteins (Tas, Ploeg, Mitchell, & Cohn, 1980) Nile red dissolves in lipids and is fluorescent in the presence of a hydrophobic environment (Greenspan, Mayer, & Fowler, 1985). Nile red (1 mg/mL in ethanol) and Fast green (2 mg/mL in water) (Sigma Aldrich, Missouri, USA) prepared according to Leong, Walter, et al. (2018) were added to the oil and aqueous phase (containing dissolved whey proteins) of the emulsions, to reach concentrations of ~10^{-3} mg/ mL and ~20^{-3} mg/ mL, respectively. Emulsions were made with stained oil and aqueous phases as described in sections 2.2 and 2.3. Emulsions were diluted 10 times using simulated milk ultrafiltrate (Martin, Williams, & Dunstan, 2007) before imaging. Cooked curds were also prepared with stained oil and protein dissolved aqueous phases as described in section 2.5.

A thin slice from the cooked curd or ~5 μL of diluted emulsions were transferred to a glass slide and carefully covered with a cover slip. A confocal microscope (Leica SP5, Leica Microsystems, Wetzlar, Germany) was used to image the samples, with the laser lines set at 488 and 633 nm for excitation. The fluorescence emission was captured through a 63× lens using 2 photomultiplier tubes set at 500–620 nm (Nile red emission) and 660–710 nm (Fast green emission).

2.8.Droplet size measurements

The droplet size of the initial W1/O single emulsion was estimated by analysing the optical microscopy images using ImageJ software (open source). In the software, a bandpass filter was used to enhance the edges of the emulsion droplets. A threshold of <0.1 μm^2 was used to avoid the inclusion of noise in the counting. The software was used to automatically determine the area of each droplet, and the corresponding diameter assuming spherical droplets. A volume-based particle size distribution was then derived, again assuming spherical droplets. Emulsion droplet size data collected from images taken from at least duplicate samples from the same emulsion were pooled together to assemble a size distribution. Each image had >150 counted droplets.

The particle size of the W1/O/W2 double emulsion droplets was measured using a Malvern Mastersizer 2000 (Malvern Panalytical Ltd, Malvern, UK) according to a previously reported method (Gamlath, et al., 2020). Double emulsion droplets consist of an outer sunflower oil layer covered by a whey protein emulsifier layer. Given that the thickness of the emulsifier layer made of whey protein (~ 5nm in size) is significantly small compared to the wavelength of light (~750 nm), the outer oil phase which is a few microns in thickness is the likely contributor for refracting light. Therefore, the refractive index of sunflower oil (1.462) and an absorption value of 0.001 (Chen, Chen, Ren, & Zhao, 2011) were fed into the software for droplet size determination. Water was selected as the bulk medium. Using different a refractive index or an absorption value may result in small changes in mean diameter measurements, but will not affect the trends observed in this study.

2.9.Conductivity measurements

Sodium chloride was added into the inner aqueous phase of the double emulsion as the entrapment marker, serving to indicate the amount of inner aqueous phase encapsulated within the double emulsion. The release of inner phase material increases the concentration of the salt in the outer aqueous phase, resulting an increase in conductivity that can be corresponded to a decrease in encapsulation yield. A series of standard solutions representing 0%, 25%, 50%, 75% and 100% of salt release were prepared to quantitatively relate the changes in conductivity to salt released from the emulsions. Standards for each specific W1/O/W2 emulsion formulation were prepared by including the same concentration of aqueous and oil phases with salt added to the external aqueous phase after emulsification. The standards were prepared by sonication using a 20 kHz horn at 33.8 W power for 90 seconds, corresponding to a power density of 1.35 W/mL. Sonication at these conditions was sufficient to completely homogenise

fat droplets in the standards. The conductivity of all systems was measured within ~15 min of formation, using a k = 1.0 laboratory conductivity probe (TPS, Brendale, Australia) calibrated against a 2.76 mS standard solution and connected to TPS LabCHEM-Cond conductivity meter (TPS, Brendale, Australia).

The measured conductivities of the emulsions were correlated to the percentage of salt released using the standard curves and the encapsulation yield calculated as the proportion of total salt that remained inside the oil droplets. Then, the proportion of encapsulated WPC in the inner aqueous phase as a percentage of the total double emulsion was calculated as:

$$Proportion\ of\ encpasulated\ WPC\ \left(\frac{g}{L_{of\ DE}}\right) = 1000\ \times W1\ loading\ in\ primary\ emulsion(\frac{mL}{mL}) \times SE\ loading\ \ in\ secondary\ emulsion(\frac{mL}{mL}) \times WPC\ concentration\ of\ W1\ (\frac{g}{g}) \times Density\ of\ W1(\frac{g}{mL})\ \times Encapsulation\ yield\%$$

The encapsulation yield was calculated using the conductivity vs salt release standard curve according to the following equation:

$$\%\ Encapsulation\ yield = 100 - \%\ salt\ release\ that\ corresponds\ to\ the\ measured\ conductivity\ value$$

It should be noted that quantification of encapsulation using conductivity measurements may be subject to the relative diffusion rates of whey protein, water and sodium chloride through the oil and surfactant boundaries. As the concentrations of NaCl and whey protein are higher in the inner aqueous droplet than the external aqueous phase, there is a driving force for diffusion out of the encapsulated droplets. In general, water molecules will diffuse across semipermeable membranes (oil and surfactant boundaries in this case) faster than Na^+ or Cl^- ions that diffuse together to maintain charge neutrality (Hancock & Cath, 2009). Although the relative rate of whey protein diffusion across oil and surfactant boundaries has not been previously reported, it is likely to diffuse much more slowly than water or NaCl due to its much higher molecular weight. Although the conductivity measurements were applied reasonably soon after emulsification (~15-30 minutes), there may have been sufficient time for preferential diffusion to lead to an underestimation of the whey protein encapsulation.

2.10. Moisture quantification

The moisture content of curd and whey samples was measured using gravimetry. Samples were oven dried overnight at 105 °C and the moisture content and total solids were determined using their wet and dry masses according to the following equations:

$$Moisture\ content = \frac{Weight\ of\ the\ sample\ before\ drying\ (g) - Weight\ of\ the\ sample\ after\ drying\ \ (g)}{Weight\ of\ the\ sample\ before\ drying\ (g)} x100$$

$$Total\ solids = 100 - Moisture\ content$$

2.11. Protein quantification

Protein analysis of curd and whey samples was performed based on the Dumas combustion method using a LECO Trumac CNS analyser (LECO Corporation, Michigan, USA), according to a previously reported method (Gamlath, et al., 2020). The protein content of cheese milk was calculated using the protein contents of skim milk and WPC (Section 2.1) and their relative amounts used during emulsion and cheese milk formulation (Table 1). The following formulae were used to calculate curd yield, protein retention and loss:

$$Curd\ yield = \frac{Mass\ of\ curd\ (g)}{Mass\ of\ cheese\ milk\ (g)} x100$$

$$Protein\ retention\ in\ curd = \frac{Protein\ content\ of\ curd\ (\frac{g}{g}) \times Mass\ of\ curd\ (g)}{Protein\ content\ of\ cheese\ milk\ \left(\frac{g}{g}\right) \times Mass\ of\ cheese\ milk(g)} \times 100$$

$$Protein\ loss\ in\ whey = \frac{Protein\ content\ of\ whey\ (\frac{g}{g}) \times Mass\ of\ whey(g)}{Protein\ content\ of\ cheese\ milk\ (\frac{g}{g}) \times Mass\ of\ cheese\ milk(g)} \times 100$$

2.12. Sodium dodecyl sulphate polyacrylamide-gel electrophoresis (SDS-PAGE)

SDS-PAGE was used to determine the molecular weight profile of the proteins present in the curd and whey. It was carried out using Bio-Rad (Bio-Rad Technologies, Gladesville, Australia) pre-cast 4-20% Criterion TGX 18 well gel cassettes in a Bio-Rad Criterion cell. For sample preparation, 22 mg of cooked curd or 143 µL of whey were mixed with 978 µL and 143 µL of distilled and deionised water, respectively. 10 µL of 0.4 M ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, Missouri, USA) was added to the sample and mixed in an orbital shaker for 1 hr. Subsequent SDS-PAGE analysis of these samples was performed using a previously reported protocol (Gamlath, et al., 2020) to achieve a clear separation of protein bands based on their molecular weight. Milk proteins were

identified based on the location of each protein band in relation to the standard molecular weight marker. The relative quantities of proteins in each sample were qualitatively determined by visual observation of band intensities.

2.13. RP-HPLC

The relative abundance of β-lactoglobulin and α-lactalbumin in cooked curds was measured by reverse phase high performance liquid chromatography (RP-HPLC) (Agilent technologies, binary pump 1260, vial sampler 1260, degasser 1260 HiP, diode array detector 1290) with eluents A (water: Acetonitrile: trifluoro acetic acid 950:50:0.7) and B (acetonitrile: trifluoro acetic acid 1000:0.5). The gradient was 0% of B for the first 5 min, then increased to 31% over the next 9.8 min, to 40% over the next 17.1 min, to 100% over the next 6.7 min, and then kept at 100% for 3.7 min and returned to the starting condition within 2.7 min (Creusot & Gruppen, 2007). A C18 column (Jupiter 5 µm, 300 A^0, 250 x 4.6 mm) was used with a flow rate of 0.8 mL (at 40 °C) and injection volume 50 µL. 110 mg of curd or 714 µL of whey was mixed with 889 µL or 286 µL of distilled and deionised water. 10 µL of 0.4 M Ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich, Missouri, USA) was added to the mixture, shaken for 1 hr and centrifuged at 2717 g in a Thermofisher Hereus Megafuge 8 benchtop centrifuge (Thermofisher, Scoresby, Australia) fitted with a HIGHConic III fixed angle rotor for 20 minutes. The supernatants were further diluted (5 times in cooked curd samples and 10 times in whey samples) with eluent A and filtered with a 0.45 µm syringe filter before injection.

2.14. Statistical analysis

Results from replicate measurements of curd composition were analysed with Minitab 17 (Minitab LLC, State College, Pennsylvania, USA) using a one-way ANOVA (with a 95% confidence interval), followed by a Tukey's pairwise comparison.

3. Results and Discussion

3.1. Formulation of whey-protein enriched water-in-oil primary emulsions

The relative size of the primary W1/O emulsion droplets and the secondary W1/O/W2 double emulsion droplets is key to both encapsulation efficiency and double emulsion stability. The W1/O single emulsion (SE) droplets should be small enough to avoid creaming or flocculation and to allow multiple inner emulsion droplets to be encapsulated within oil droplets of similar size to native milk fat globules (i.e. approximately 5-10 µm in diameter). As such, submicron-sized droplets of whey protein solutions were targeted for inclusion in the double emulsion

formulation. To maximise the amount of whey protein encapsulated inside the double emulsion droplets, W1/O emulsions were formed i) using high aqueous phase loading rates of 30% or 40% v/v (i.e. aqueous phase-to-oil ratios of 3:7 or 4:6), and ii) with high concentrations of WPC in the inner aqueous phase (20%, 30% and 40% w/w) (preliminary trials showed that 40% w/w was the maximum possible WPC concentration that could be completely solubilised). Lipophilic emulsifier concentrations were kept constant at 4% w/w and 1% w/w respectively. The size of W1/O emulsion droplets produced by ultrasonication as function of both loading rate and whey protein concentration was estimated by optical microscopy and image analysis using ImageJ software (Figure 1).

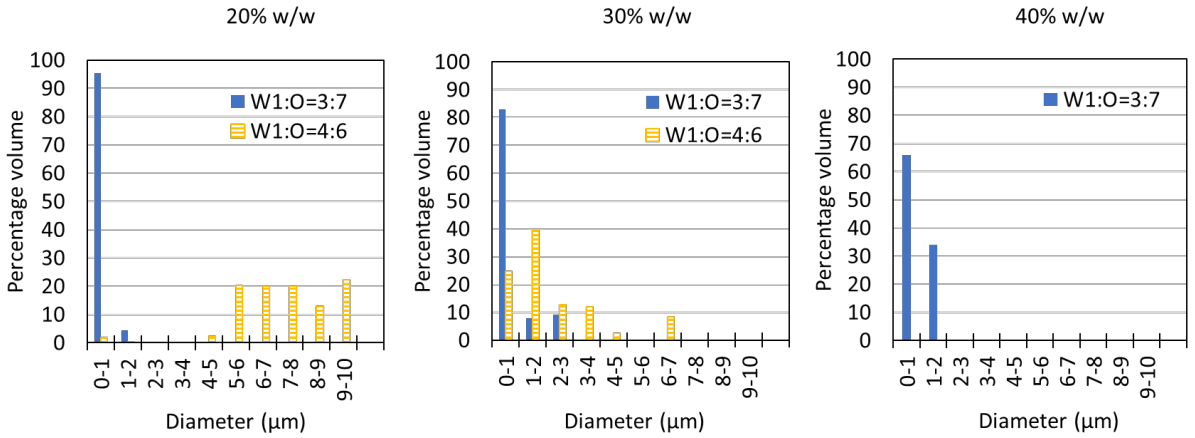


Figure 1: Volumetric droplet size distributions of W1/O emulsions formed by ultrasonication using 20% w/w (left), 30% w/w (middle) and 40% (right) WPC solutions and sunflower oil at W1:O ratios of 3:7 and 4:6. Data collected from multiple images (at least 2) were pooled to determine the size distributions. Note that there was significant variability in the size distribution of W1:O = 4:6 systems due to incomplete emulsification. Microscopic images representing each emulsion are provided in Figure A1.

At a W1:O ratio of 3:7, >95% v/v of the emulsion droplets with 20% WPC in the aqueous phase were in the desired submicron range (<1 µm) (Figure 1). At higher WPC concentrations (30% and 40%) there were more droplets in the micron range (1-3 µm), likely too large for double emulsion encapsulation. The volumetric proportion of droplets >1 µm was 4.5%, 17.0% and 34.1% in the emulsions with 20%, 30% and 40% WPC, respectively.

The size of droplets resulting from ultrasonic emulsification can be affected by the interfacial tension, the viscosities of the dispersed and bulk phases, the W:O ratio, and the intensity and duration of the applied sonication (Gaikwad & Pandit, 2008). As the type of oil, the W:O ratio,

the emulsifier (lecithin and PGPR) concentration, and ultrasonic parameters were kept constant in this study, the increase in the droplet size can be attributed to the increase in the dispersed phase viscosity at higher WPC concentrations (the viscosities of the 20%, 30% and 40% WPC solutions were measured to be 6.8 mPa.s, 29.6 mPa.s and 109.1 mPa.s, respectively). Although the effect of dispersed phase viscosity on the droplet size resulting from ultrasonic emulsification has not been reported for water-in-oil emulsions, it has previously been shown that an increase in the dispersed phase viscosity increased the droplet size in ultrasonically generated oil-in-water emulsions (Gaikwad, et al., 2008). This is also consistent with an increase in droplet size with increased dispersed phase viscosity in O/W emulsions produced by homogenisation, with the higher viscosity resisting droplet break up by shear and turbulent forces (Pandolfe, 1981).

When the W:O loading was increased to 4:6, the emulsion droplets were much larger than those at a 3:7 W:O ratio (Figure 1). Beyond this however, complete emulsification could not be achieved at any of the WPC concentrations at 4:6 W:O loading. Although the aqueous phase could be seen to immediately mix into the oil phase upon ultrasonication of the 20% and 30% WPC solutions, it was visibly evident that the emulsification was inhomogeneous within the sample. The microscopy images indicated that although sub-micron sized droplets were formed in some parts of the sample (Figure A1), the majority of the droplets were in the micron range (Figure 1 and Figure A1). Further, gel-like regions were formed in the samples. In the system with 40% WPC the aqueous phase could not be mixed into the oil phase as a gel was rapidly formed in the region of the horn.

At higher aqueous phase ratios, more cavitation may occur due to the presence of more dissolved air pockets for bubble nucleation, which in turn reduces the cavitation threshold pressure (Crum, 1982). This leads to less violent individual bubble collapse that may reduce the magnitude of the shear forces generated. These weaker shear forces may not be adequate to break the closely packed larger droplets (that occupy a higher volume fraction compared to a system with a lower W:O loading) that are formed as the aqueous phase is dispersed into the oil. Further, at higher water fractions, the overall lipophilic emulsifier concentration in the systems is lower; hence the ability to stabilise a large number of smaller droplets is limited. Finally, as the emulsion forms, the bulk fluid becomes less mobile due to droplet packing, and this will occur preferentially near the horn tip. As such, the physical effects of cavitation may become restricted to regions near the surface of the probe, leading to an increase in the localised temperature, denaturation and subsequent gelation of whey proteins.

3.2. Reducing the emulsifier concentration

At a W1:O ratio of 3:7, emulsions could be produced with the majority of the droplets in the desired submicron range using a PGPR and lecithin concentrations of 4% w/w and 1% w/w, respectively, in the oil phase. Attempts were made to reduce the amount of emulsifier, to reduce the formulation cost. Here, the PGPR concentration was reduced from 4% w/w in the oil phase to 3% or 2% w/w, while keeping the lecithin concentration constant at 1% w/w. With a PGPR concentration of 3%, the droplet size distributions (Figure 2) were comparable to those at 4% PGPR (Figure 1), with 3.1%, 25.6% and 44.3% of the dispersed phase volume occupied by droplets exceeding 1 μm in diameter, for the 20%, 30% and 40% WPC solutions, respectively. However, a further reduction in the PGPR concentration to 2% resulted in a dramatic change in the droplet size distributions, with micron-sized droplets occupying the majority of the volume (Figure 2). This suggested that the combination of 2% PGPR and 1% lecithin was not sufficient to stabilise the large interfacial area associated with the sub-micron sized oil droplets created during ultrasonication. As a result of poor stabilisation, smaller droplets coalesced into larger droplets.

There was no apparent effect of the WPC concentration on the droplet size of systems containing 2% PGPR and 1% lecithin, presumably as hydrophilic whey protein molecules (Galus & Kadzińska, 2016) do not have a suitable hydrophilic-lipophilic balance (HLB) to stabilise water-in-oil emulsions (Gülseren & Corredig, 2012). Attempts to create W/O emulsions without PGPR or lecithin were unsuccessful (with a 20% w/w WPC solution and sunflower oil at a W1:O loading of 3:7), as the oil and aqueous phases separated soon after sonication. Emulsions formed with 3% PGPR and 1% lecithin were observed by microscopy to be stable for at least after 3 days of storage in ambient conditions (data not shown). Therefore, these emulsifier concentrations were used to stabilise the internal aqueous phase during double emulsion formation.

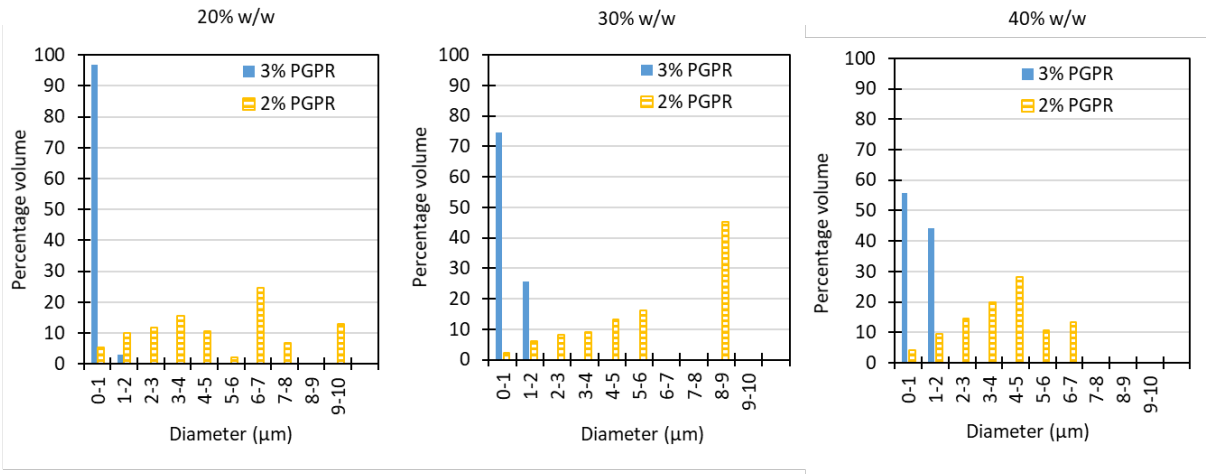


Figure 2: Droplet size distributions of W1/O emulsions formed using 20% w/w (left), 30% w/w (middle) and 40% (right) WPC solutions and sunflower oil at W1:O ratios of 3:7, using 3% w/w and 2% w/w PGPR in the oil phase while maintaining a lecithin concentration of 1%. Data collected from multiple images were pooled together to determine the size distributions. Microscopic images representing each emulsion are provided in Figure A2.

3.3.Double emulsion formation

To maximise whey protein encapsulation, multiple W1/O droplets should be encapsulated within a secondary oil droplet during double emulsion formation. Rather than requiring low HLB surfactants such as lecithin and PGPR, the secondary (or outer) oil-in-water emulsions can be stabilised by milk proteins (Leong, et al., 2017). As the minimum required lipophilic emulsifier concentration to produce the submicron size W1/O droplets was used, the amount of free emulsifier in the oil phase is low, meaning the secondary oil droplets are likely to be emulsified predominantly by the protein in the secondary, outer aqueous phase. In this formulation, whey protein was used as the emulsifier, as a lower cost alternative to casein. Whey protein has been used successfully to form stable oil-in-water emulsions (Desrumaux & Marcand, 2002) (Kaltsa, et al., 2013) and has desirable nutritional properties such as the presence of branched-chain amino acids (leucine, isoleucine, and valine), solubility in gastric pH (Walzem, Dillard, & German, 2002) and proportionately more sulphur containing amino acids (cystine, methionine) than casein (Witard, et al., 2013). In addition to the emulsion formulation and the size of the internal droplets, the ultrasonication parameters are critical to the secondary emulsification stage of double emulsion production. The intensity and duration of ultrasonication must be balanced to ensure oil droplets of the desired size are produced, without breaking and releasing the water-in-oil emulsion. To investigate the effect of ultrasonic parameters on the encapsulation efficiency, double emulsions were formed with W1/O:W2

loadings of 2:8, 3:7 and 4:6 using the same ultrasound horn for 90 s at power intensities of 0.27 W/mL, 0.81 W/mL and 1.35 W/mL, corresponding to specific energy values of 24.5 J/mL, 73.1 J/mL and 121.7 J/mL, respectively.

W1/O:W2 loading did not have a major effect on the oil droplet size of the double emulsions (Figure 3 A). Higher W1/O:W1 ratios have previously been reported to result in larger secondary double emulsion droplets at lower energy densities (~ 1.3 J/mL) (Leong, et al., 2017), which was attributed to the applied ultrasound energy being diluted in relation to volume of material to be emulsified (Leong, et al., 2017). However, at the much higher specific energy densities used in this study (>24.5 J/mL), the size of the droplets was not affected, with the applied energy being sufficient to cause similar size reductions at all loadings tested.

In contrast, the power intensity had a considerable effect on the oil droplet size of the double emulsions, with the increased shear forces leading to smaller droplets. A power intensity of 0.27 W/mL applied for 90 s was insufficient for this formulation, producing double emulsions with a mean oil droplet size >200 μm. Sonication at 1.35 W/mL produced double emulsion oil droplets with a mean size close to that of the native milk fat globules, however the size distribution was bimodal, with peaks around 0.1-1 μm and 1-10 μm. The presence of submicron size droplets in the double emulsion may affect the textural properties of cheese if they were to be used as a milk fat replacer. Small fat droplets are believed to act as inert weak points in the otherwise strong protein matrix, reducing curd firmness (Michalski, et al., 2003). In comparison, at the intermediate power intensity of 0.81 W/mL, the majority of the oil droplets in the double emulsion were ~10 μm, with a few larger droplets inflating the volume mean droplet size. Increasing the sonication time from 90 s to 120 s at 0.81 W/mL produced more uniformly sized droplets, ~10 μm in diameter, but a further increase to 150 s resulted in submicron sized droplets (data not shown).

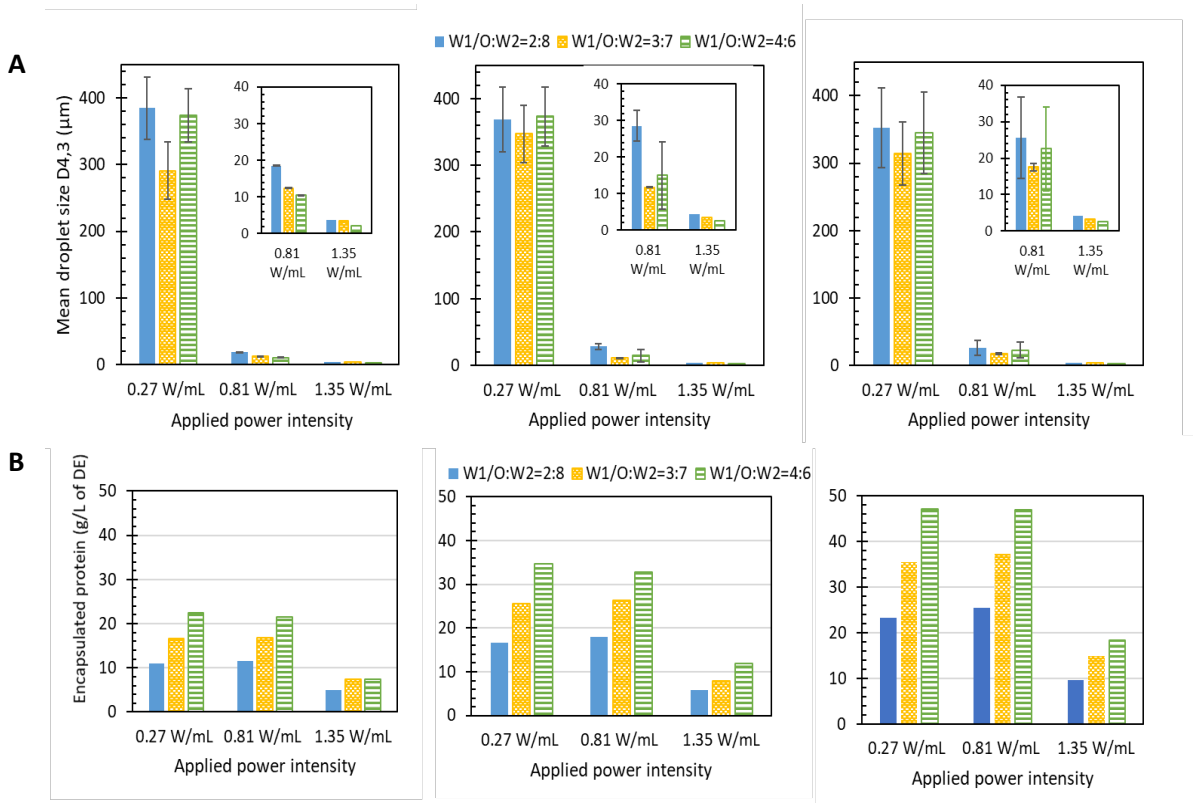


Figure 3: (A) Volume mean droplet size and (B) the amount of whey protein encapsulated in the inner aqueous phase of the double emulsions (in g of encapsulated protein per L of double emulsion) formed by loading the W1/O single emulsion made with 20% w/w (left), 30% w/w (middle) and 40% (right) WPC solutions into the secondary aqueous phase (containing 5% w/w WPC) at W1/O:W2 ratios of 2:8, 3:7 and 4:6. Ultrasound (20 kHz) was applied at the stated power intensity for 90 s.

Optical microscopy images of double emulsions formed at 0.81 W/mL confirmed the presence of inner droplets encapsulated within the double emulsion oil droplets (Figure 4A). When the oil soluble fluorescent dye, Nile red, was dissolved in the sunflower oil, the inner droplet did not emit fluorescence, confirming that the double emulsion droplet contained internal aqueous regions (Figure 4B). Contrary to previous observations of irregularly shaped internal aqueous droplets of skim milk (Leong, et al., 2017), the internal aqueous droplets were spherical in this study. This could be due to the different emulsifiers used, with Span 80 employed in the study by Leong and co-workers, a surfactant which has been reported to interact with milk proteins such as BSA to form gel like complexes (Gaiti, Aserin, & Cohen, 1994). The irregular shape of the internal aqueous phase of skim milk reported by Leong and co-workers was believed to be a result of interactions between Span 80 molecules at the oil-water interface and whey proteins in the aqueous phase (Leong, et al., 2017). They later reported that using PGPR (1-5%

w/w in the oil phase) as a lipophilic emulsifier could mitigate such aggregation of the internal skim milk droplets (Leong, Zhou, et al., 2018), consistent with the use of PGPR in the current study and the observed spherical shape of the inner aqueous droplets.

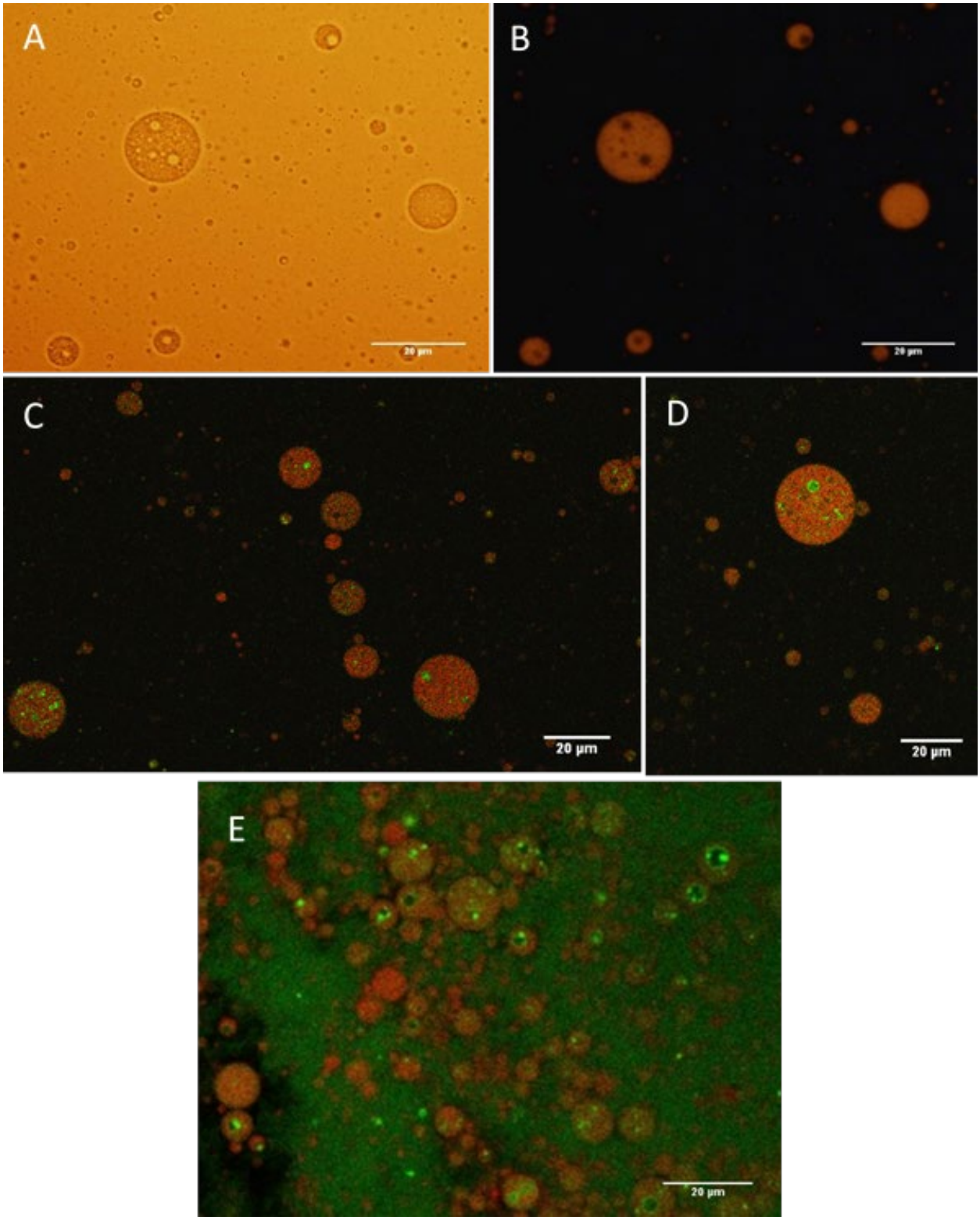


Figure 4 – Optical (A), fluorescent (B) and confocal laser scanning (C, D) microscopy images of W1/O/W2 double emulsions prepared with 40% w/w WPC solution, sunflower oil and 5%

w/w WPC solutions as W1, O and W2 phases, respectively. E is a confocal laser scanning microscopy image of a skim milk rennet gel filled with a W1/O/W2 emulsion. The orange areas in (B) represent the oil phase containing fluorescent Nile red, while the dark areas represent the aqueous phase. In the confocal laser scanning microscopy images proteins and fat are stained in green (Fast green) and red (Nile red), respectively. The scale bars are 20 μm.

When proteins in the internal aqueous phases were stained with Fast green, the confocal microscopy images revealed that the internal aqueous phase was fluorescent, confirming that proteins were indeed encapsulated within the double emulsion droplets (Figure 4 C, D). However, surface active whey proteins were concentrated near the oil-water interface of the internal emulsion droplets (Figure 4 E), suggesting that although whey proteins alone are incapable of stabilising water-in oil emulsions, they can perturb the emulsifier layer formed by PGPR and lecithin. Aggregates were also present near the oil-water interface of the internal emulsion droplets (Figure 4 E). As the bulk temperature during primary emulsification (< 50 °C) did not exceed the denaturation temperature of whey proteins (~65 °C (Boye, Alli, Ismail, Gibbs, & Konishi, 1995)), their aggregation could be due to denaturation in the presence of an interface, shear induced disruption of intramolecular forces in proteins (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011), or a combination of both phenomena.

Understanding how to maximise the amount of whey protein encapsulated in the double emulsions is a primary aim of this study. To quantify the amount of WPC solution encapsulated within the double emulsion droplets, the extent of sodium chloride released from the inner aqueous phase was determined by measuring the conductivity of the double emulsion. Based on this, the amount of whey protein encapsulated in the double emulsion was calculated and compared in relation to the ultrasonic emulsification parameters used. The results revealed a reduction in the proportion of encapsulated whey proteins at higher ultrasonic power intensities (Figure 3B). This reduction in encapsulation can be correlated to the reduced size of the oil droplets obtained from emulsification at higher power intensities, indicating that coarse double emulsion droplets are very effectively broken down leading to the escape of the internal water droplets from the W1/O emulsion during secondary emulsification. While the amount of whey protein encapsulated varied significantly between different formulations, from ~5 to almost 50 $g_{encapsulated}$ protein/mL of double emulsion, even the lowest value obtained here (5 g/L of double emulsion) is significantly higher than the maximum previously reported protein encapsulation rate (calculated to be ~1.4 g protein /L of double emulsion of double emulsion) achieved by Silva, et al. (2020). Given the formulation tested here had an aqueous outer phase containing

50 g/L protein, the highest encapsulation rate (of ~ 47 g/L for the W1/O:W2 = 4:6, 40% WPC) represents almost a doubling in the total protein content due to the protein encapsulated within the double emulsion. However, it should be noted that the conductivity measurements used to determine encapsulation yield may have led to an underestimation of the encapsulation yield as explained in section 2.9. and the actual encapsulation yield would be be higher.

In addition, the osmotic pressure in the internal droplet is higher than that of the external aqueous phase due to the higher concentrations of whey protein and NaCl, which may lead water to diffuse into the internal aqueous phase. Transfer of water from the external aqueous phase to the internal droplet could lead to swelling of the internal droplets (Wen & Papadopoulos, 2001). This could be a reason for the larger internal droplet size of the double emulsion (Figure 4E) compared to the droplet size of W1/O single emulsion (Figures 1 and 2). Excessively swelled internal droplets may no longer be retained in the oil droplets resulting in the collapse of the secondary droplet over time (Wen, et al., 2001). This would reduce encapsulation over time; however it would be largely an artefact resulting from the use of NaCl as a marker, which would not be used in real formulations. Regardless, as conductivity was measured at similar times after emulsion preparation in all the systems studied, it is possible to attribute the reduction in whey protein encapsulation with sonication power in systems with similar WPC concentrations and W1/O:W2 loading to shear-induced disintegration of the internal aqueous phase.

3.4. Formation of whey protein-enriched cheese curds containing double emulsions

The overall aim of the study was to enable whey protein enrichment of cheese via double emulsion encapsulation. To investigate whether whey protein encapsulated double emulsions could be successfully incorporated into cheese making, cooked curds were produced from cheese milk containing a whey protein-stabilised and whey-protein encapsulated double emulsion in place of the milk fat. A control curd was also prepared which contained a single emulsion of sunflower oil, stabilised with whey protein. While both cheese milks had similar fat and protein contents, ~ 42% w/w of the whey protein in the double emulsion cheese milk was encapsulated inside the oil droplets.

The effect of including a double emulsion in the cheese milk on rennet gelation was examined using oscillatory rheometry on milk systems containing similar protein contents to cooked curd trials, however with a lower fat content (2.8% w/w). The results did not reveal any significant differences in either the rate of rennet gelation or the gel strengths between the double emulsion and single emulsion formulations (Figure A3).

The retention of the encapsulated whey protein through processing to a cooked curd was evaluated. It was also compared to the whey protein retained in the control single emulsion, in which the whey protein was present in the external aqueous phase. Compositional analysis of the cooked curds showed that the double emulsion curds had 14% and 15.5% increases in the yield and protein content, respectively, compared to the control (Table 2).

Table 2: Results of compositional analysis of double emulsion and control curds based on methods described in Sections 2.10 and 2.11.

Curd system	DE curd	Control curd
Protein content in cheese milk (% of wet mass)	4.37 ± 0.00 [a]	4.34 ± 0.00 [b]
Casein content in cheese milk (% of total protein)	64.46 ± 0.01 [a]	65.15 ± 0.09 [b]
Whey protein content in cheese milk (% of total protein)	35.54 ± 0.01 [a]	34.85 ± 0.09 [b]
Moisture content in cooked curd (% of wet mass)	71.48 ± 0.30 [a]	70.14 ± 0.03 [b]
Protein content in cooked curd (% of dry matter)	40.0 ± 1.9 [a]	34.3 ± 2.6 [b]
Total solids in drained whey (% of wet mass)	7.57 ± 0.25 [a]	7.94 ± 0.31 [a]
Protein content in drained whey (% of wet mass)	1.23 ± 0.09 [a]	1.74 ± 0.08 [b]
Curd yield (% of cheese milk)	32.8 ± 0.3 [a]	28.7 ± 0.9 [b]
Protein retention in curd (% of protein in cheese milk)	85.8 ± 4.3 [a]	68.1 ± 3.6 [b]
Protein loss in whey (% of protein in cheese milk)	16.9 ± 1.5 [a]	25.5 ± 0.6 [b]

*Standard deviation of replicate samples measured from duplicate systems are indicated next to the mean. **Significantly different values ($p < 0.05$) across a row are denoted using superscript letters.

The protein retention in the control cheese was 68.1 ± 3.5 % w/w, similar to the casein content in cheese milk, reflecting that the majority of free whey protein was drained out during syneresis. Note that the casein content in cheese milk was lower than the casein content in skim milk (~80%) as whey protein was added to achieve a casein:whey protein ratio of ~2:1. SDS-PAGE analysis confirmed there was an insignificant amount of casein lost in the whey (Figure 5). Protein retention was much higher in the double emulsion curds, at 85.8 ± 4.3 % w/w. This rate of protein retention was greater than the amount of casein in the cheese milk. This demonstrates that whey protein encapsulated within the oil was retained, representing a major increase in whey protein retention over the control. The protein concentration in the double emulsion whey was in fact lower (1.23 ± 0.09% w/w) than in the whey of the control (1.74 ± 0.08% w/w), with a corresponding equal reduction in the intensity of all SDS-PAGE protein bands (Figure 5). However, a significant increase in the individual whey protein components

in the curds could not be detected by the densitometry of SDS-PAGE gel images (data not shown), possibly due to the comparatively low increase in the intensity of the whey protein contents relative to the high concentration of casein. Although there was some variability between the analyses, RP-HPLC analysis of the curds provided direct evidence of an increase in the β-lactoglobulin and α-lactalbumin contents (Figure A4).

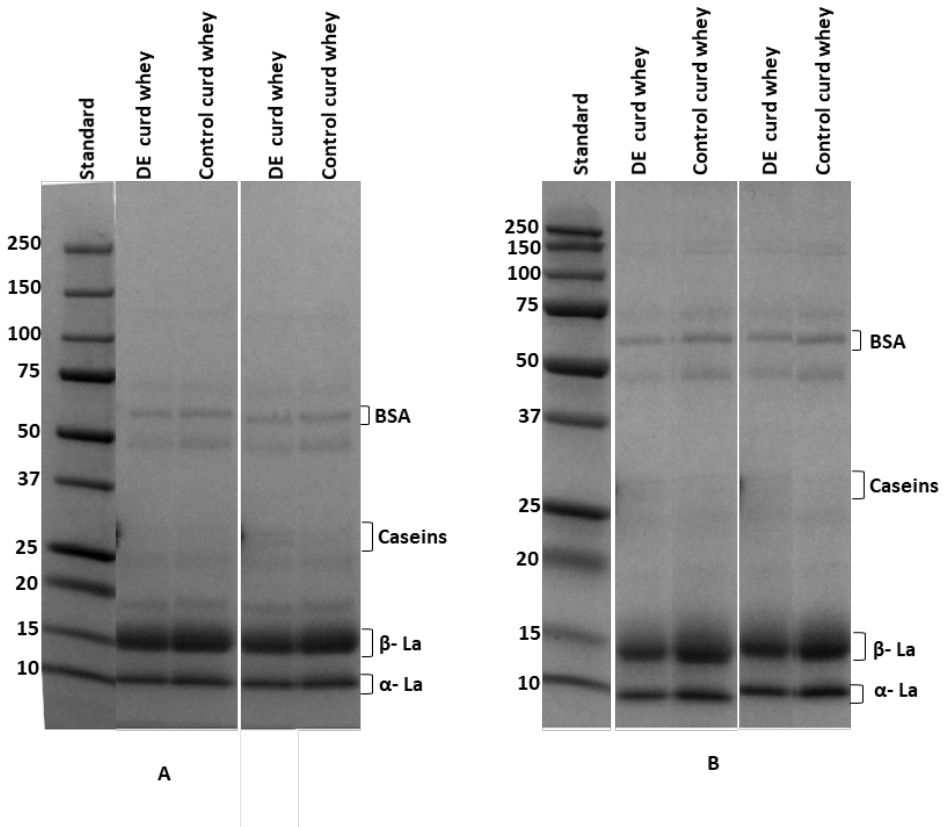


Figure 5: SDS-PAGE profiles of whey drained from double emulsion and single emulsion control curds. A and B are lanes from two separate gels used during repeat experiments. Note that lanes extracted from each gel image were rearranged in the figures to facilitate visual comparison.

CLSM images (Figure 6) also confirmed the presence of protein encapsulated within oil droplets in the cooked curds. Oil droplets were segregated into pockets in the protein matrix, suggesting that using whey proteins as an emulsifier, which unlike casein cannot integrate directly into the para-casein matrix, could reduce previously reported active interactions between the casein network and the oil droplets when casein-rich skim milk was used (Leong, et al., 2020). This increase in the mobility of fat droplets within the casein matrix, could improve the textural properties of the cheese.

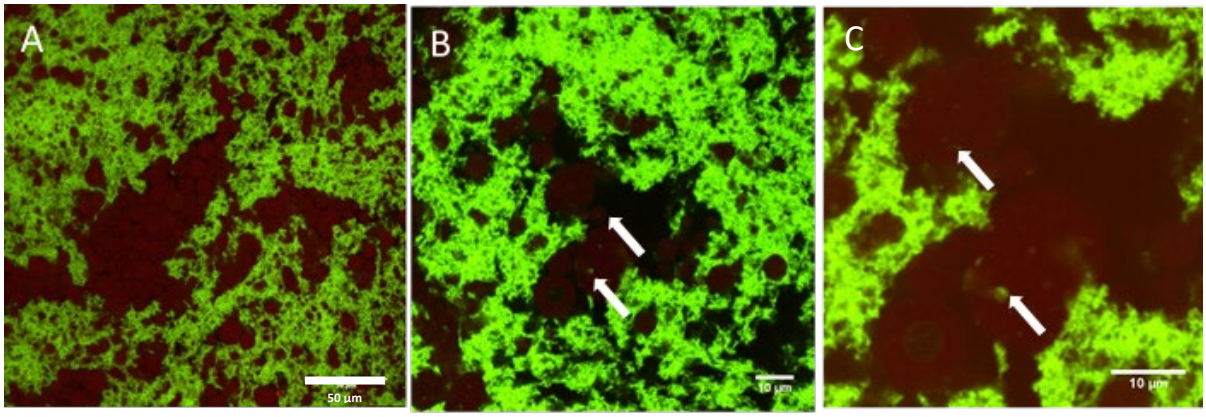


Figure 6: Confocal laser scanning microscopy images of cooked curds prepared using whey protein-enriched double emulsions at lower (A) and higher (B, C) magnifications. Areas of green florescence inside the red oil droplets (indicated by arrows) represent encapsulated whey protein. Note that higher laser gains were applied in order to visualise encapsulated proteins inside the oil droplets (B, C), which were low in concentration compared to the protein-rich casein matrix.

Although the current study demonstrates the possibility of forming whey enriched double emulsions and emulsion filled cheeses using ultrasonic emulsification, further studies are required to investigate the impact of ultrasound on their sensory attributes. High-power, low-frequency ultrasound (400 W and 24 kHz) was previously reported to cause an increase in 'burnt', 'metallic', and 'rubbery' flavours in milk, when the sonication time was increased from 50 s to 300 s (Marchesini, et al., 2012). Development of off flavours was attributed to heat-induced oxidation of lipids into volatile compounds and denaturation of proteins when exposed to extreme localised temperature zones formed during sonication bubble collapse (Carrillo-Lopez, et al., 2021). An alternative could be to form the double emulsions using high-pressure homogenisation instead of ultrasound (Leong, Zhou, Zhou, Ashokkumar, & Martin, 2018; Stang, Schuchmann, & Schubert, 2001), which could avoid off flavours and be easier to scale up for industrial production. In addition, future investigations into the stability of the double emulsions during cheese maturation and storage will be of interest.

4. Conclusions

In this study, ultrasound was successfully applied in the formation of whey protein-enriched water-in-oil-in-water double emulsions using minimal amounts of food-grade emulsifiers. These emulsions had a markedly higher rate of protein encapsulation than previously reported studies. The results also demonstrate the possibility of manipulating emulsion formulation and

ultrasonic emulsification parameters to form double emulsions of varying size distributions and protein contents. Whey protein-rich double emulsions were successfully incorporated into cooked curds formed by rennet gelation to increase the retention of whey proteins which are otherwise lost during syneresis. The understanding developed in this study provides a novel means of utilising whey protein and increasing the nutritional content of dairy products including cheese.

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Appendices

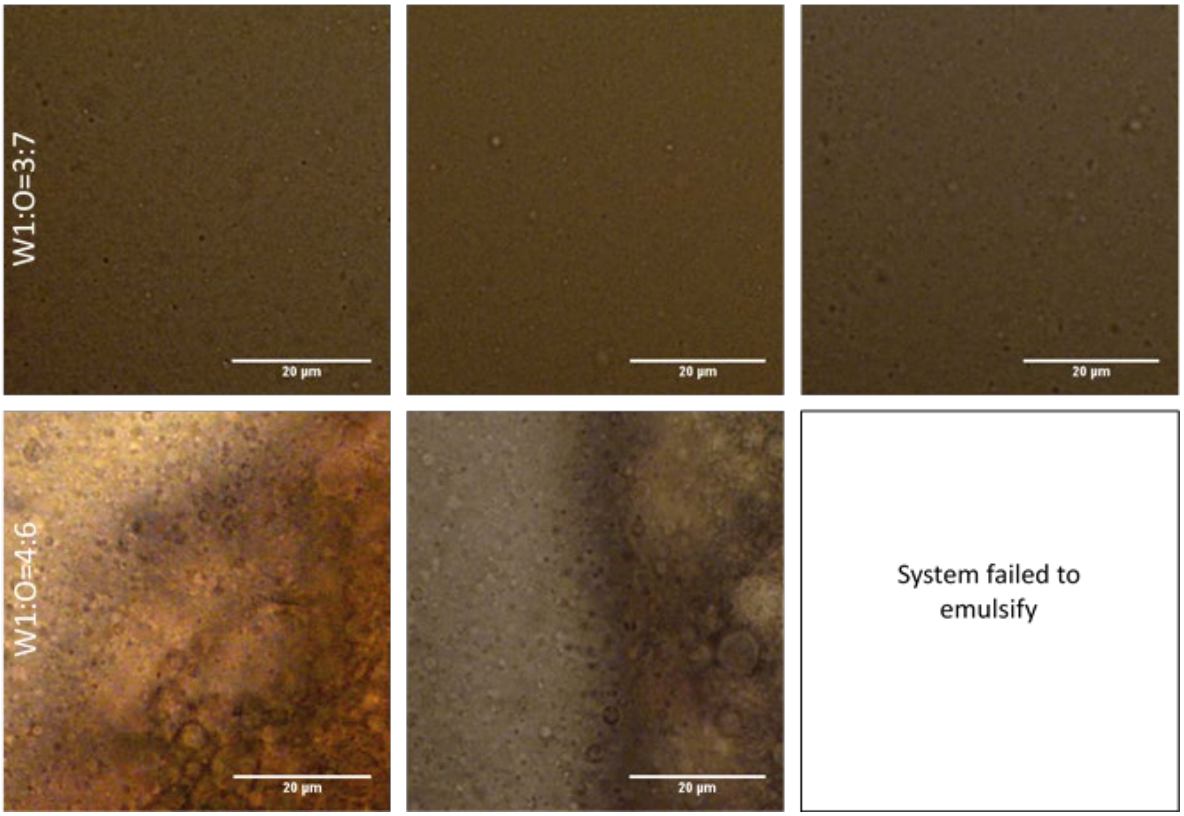


Figure A1: Optical microscopy images representing of the W1/O emulsions formed using 20% w/w (left), 30% w/w (middle) and 40% (right) WPC solutions and sunflower oil at W1:O ratios of 3:7 (top) and 4:6 (bottom). In the microscopy images, emulsion droplets can be identified as white spots in the dark background. Data collected from multiple images (at least 3) were pooled together to determine the size distributions.

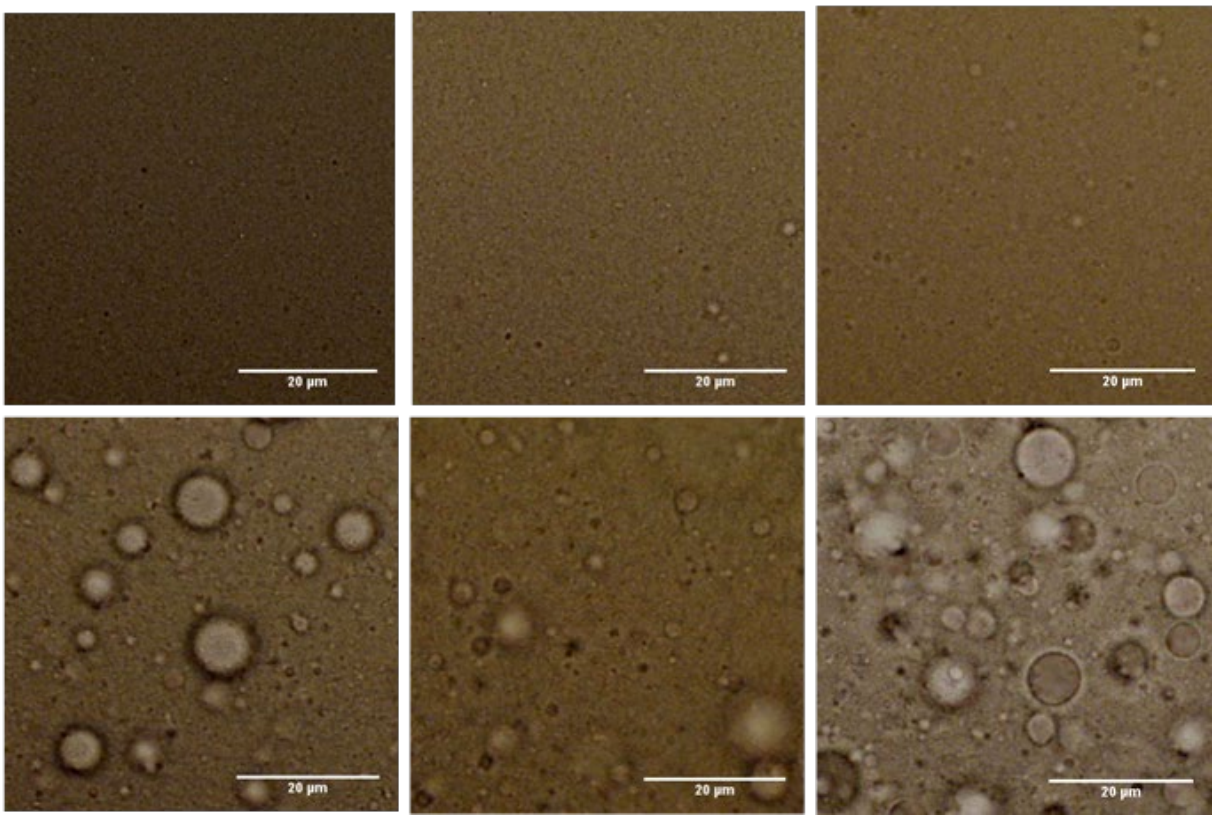
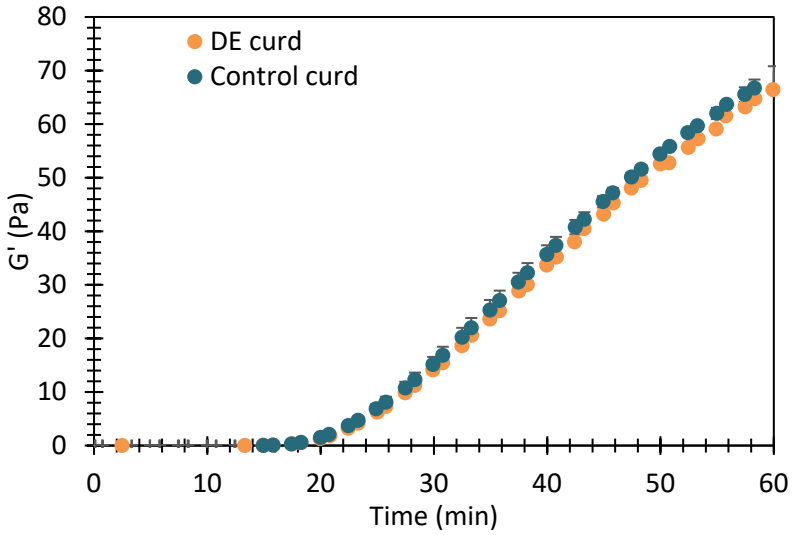
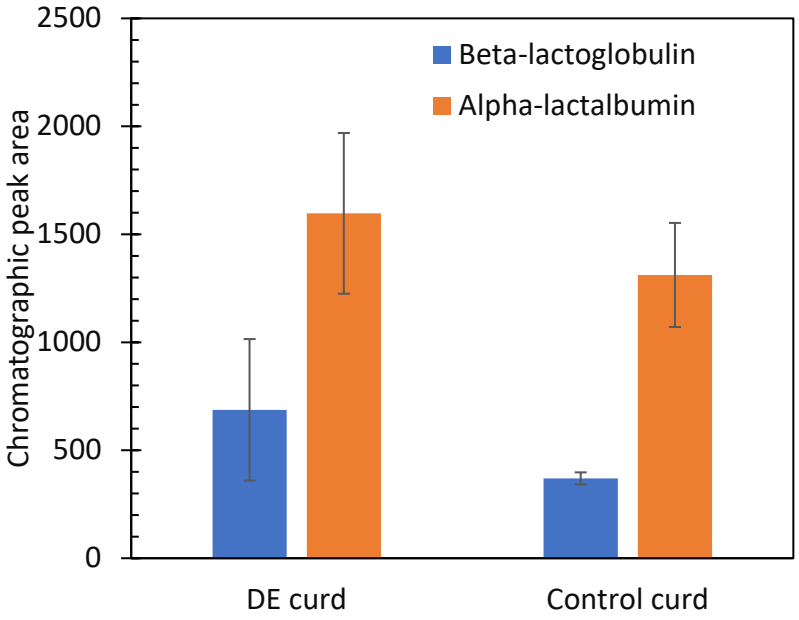


Figure A2: Optical microscopic images representing the W1/O emulsions formed using 20% w/w (left), 30% w/w (middle) and 40% (right) WPC solutions and sunflower oil at W1:O ratios of 3:7, using 3% w/w (top) and 2% w/w PGPR (bottom) in the oil phase while maintaining a lecithin concentration of 1%. In the microscopy images, emulsion droplets can be identified as white spots in the dark background. Data collected from multiple images were pooled together to determine the size distributions.



833

834 Figure A3: Comparison of the rate of increase in the storage modulus with time between
835 rennetted double emulsion cheese milk ( ) and a control single emulsion cheese milk ( ).



836

837 Figure A4: HPLC peak areas of β-lactoglobulin and α-lactalbumin in double emulsion and
838 control single emulsion curd samples.

839