Structure and stability of \(W_1/O/W_2\) emulsions as influenced by WPC and NaCl in inner aqueous phase

Research Highlights

- \(W_1/O/W_2\) double emulsions were successfully prepared using the phase ratio of 9:21:70
- WPC and NaCl in internal aqueous phase had a great impact on emulsion characteristics
- Increase in salt content in \(W_1\) phase resulted in decrease in final emulsion droplet size
- WPC @ 6% and salt @ 2 or 4% in \(W_1\) phase obtained maximum stability to double emulsion

Abstract

Effect of WPC and NaCl in internal aqueous phase (\(W_1\)) of \(W_1/O/W_2\) type double emulsions was studied. Pre-emulsion and final emulsion were prepared using microfluidizer and Ultra-Turrax high shear mixer, respectively. The emulsions prepared using salt exhibited uniform droplet size distribution and structural integrity. WPC at 6% and NaCl at 2 or 4% levels demonstrated better sedimentation stability (>99%) and encapsulation stability (>95%) during preparation and storage of double emulsions. Samples without added NaCl showed poor emulsion stability and structural integrity. Higher level of WPC i.e. 8% resulted in poor stability and encapsulation efficiency of double emulsions at all salt levels. Combined use of optimum levels of WPC and NaCl along with processing interventions resulted in stable double emulsions even after storage at room temperature for 10 days. This study highlights the fact that structural integrity of internal aqueous phase (\(W_1\)) depends upon presence of osmotic agent i.e. salt and stabilising proteins i.e. WPC.

Keywords: Double emulsion; Whey protein concentrate; Sodium chloride; Stability
1. Introduction

Double emulsions (DE) or multiple emulsions have been used as structured delivery system for encapsulation of a wide range of bioactive compounds. They are used in food, pharmaceutical and cosmetic industries (Tekin Pulatsü et al. 2018). These multiphase systems majorly comprise of water-in-oil-in-water (W/O/W) emulsions although there are applications wherein oil-in-water-in-oil (O/W/O) emulsions have been used (Lamba et al. 2015). W/O/W emulsions can be created by dispersing minute water droplets (W$_1$) inside fat globules (O) which in turn are then dispersed in a continuous water phase (W$_2$). In the first stage, the homogenization is carried out in the presence of a lipophilic emulsifying agent to obtain W$_1$/O emulsion whereas in the second stage, W$_1$/O emulsion is homogenized with the new aqueous phase (W$_2$) in the presence of a hydrophilic emulsifying agent to obtain W$_1$/O/W$_2$ DE (Lamba et al. 2015). As W$_1$/O/W$_2$ emulsions have two interfaces, they are thermodynamically not so stable and their practical utility is limited on account of release of the inner aqueous phase into the outer aqueous phase (Mohammadi et al. 2016).

The type and concentration of the surfactants and the intensity of shear being created during homogenization significantly determine the characteristics of primary and double emulsions (Ding et al. 2019). For DEs non-ionic surfactants are generally preferred. Monomeric emulsifiers are simple molecules that align at interface but do not provide desired stability in DEs because they have a tendency to migrate from one phase to another (Dickinson 2011). On the contrary, polymeric surfactants produce more stable emulsions due to formation of a more sterically bulky viscoelastic layer around the emulsified droplets which in turn result in less droplet flocculation and coalescence (Leong et al. 2017). Whey proteins are such important food grade hydrophilic emulsifiers used in aqueous phase of the DEs (Mantovani et al. 2016). They exhibit excellent
surface activity, which can avoid the coalescence or creaming process of dispersed phase, giving
stability to oil/water interface (Hu et al. 2003). Electrolytes such as salt have been recommended
for not only regulating osmotic gradient between inner and outer aqueous phase of the emulsion but
also increasing efficiency of emulsifiers. Salt maintains a balance between Laplace and osmotic
pressure and thus improves the emulsification efficiency (Scherze et al. 2006) and prevents Ostwald
ripening thereby improving long term emulsion stability (Dickinson 2011). Electrolytes also
strengthen the interfacial film around droplets in the internal aqueous phase (Foudazi et al. 2015)
and reduce coalescence between droplets. Concentration of salts have effect on the stability of
emulsion and higher salt concentration increases osmotic pressure gradient resulting in leakage of
water molecules from external to the internal aqueous phase thereby leading to swelling and
expansion of W1/O emulsion droplets and destabilization of emulsion (Evageliou et al. 2019).

DEs can be created by several techniques to ensure a stable emulsion that retains the inner
water (W1) droplets within the oil droplets. Two-stage high-pressure homogenization is often
recommended for each of the stage, with lower pressures being exercised in the second step to
avoid releasing W1 into W2 (Ding et al. 2019). Of the several options of devices available for
creating emulsion, Rotor-stator type mixers such as Silverson and Ultra Turrax are generally used
for creation of micro-emulsions from two immiscible phases. Polydispersity of dispersed particles
because of shear inhomogeneity is the major concern while using these devices (Dickinson 2011).
Microfluidization and ultrasonication techniques are more energy efficient mixing processes that
can yield finer emulsions (nanoemulsions) with narrow particle size distribution. Uniform droplet
size offer consistent encapsulation efficiency and emulsion stability (Khalid et al. 2017). Formation
of nanoemulsions would necessitate first preparation of coarse or pre emulsion using rotor stator
type mixer followed by passing it through either microfluidization or ultrasonication. However, most of the work reported in the past on DEs focussed on single aspect of the emulsion preparation method and formulation of individual phases. Further, more studies are needed on combined use of different homogenising and dispersing devices.

The present study was therefore aimed to create stable W₁/O/W₂ by combined use of microfluidizer and high shear mixer Ultra-Turrax and optimize ingredients mainly in internal aqueous phase (W₁). Specifically current work investigates the effect of whey protein and salt levels in inner aqueous phase on stability and structure of DEs during processing and storage.

2. Materials & Methods

2.1. Materials

WPC 70 was procured from Modern Dairy, Karnal, India. Sodium alginate was purchased from Loba Chemie Pvt. Ltd (Mumbai, India). Rice Bran Oil was purchased from A. P. Organic Pvt. Ltd. (Ludhiana, India). PGPR 90 was procured from Danisco India Pvt. Ltd. (Gurgaon, India) as free sample. Tween 20 and Span 85 were purchased from Sigma-Aldrich Inc (St. Louis, USA.). Tween 80 was sourced from Amresco (Ohio, Japan). Nile blue A and Immersion oil were purchased from Sigma-Aldrich Inc (St. Louis, USA.).

2.2. Preparation of W/O/W DE

Water-in-oil-in-water (W₁/O/W₂) emulsions were prepared using a two-step emulsification method described by Fechner et al. (2007) with slight modifications. The inner aqueous phase (W₁) was prepared with dextrose (5%, w/w) or sodium alginate (3%) or WPC-70 (0-8%), NaCl (0-6%, w/w), sodium azide (0.02%, w/w) in distilled water with sodium phosphate buffer (0.1 M, pH 7.0)
at 20 °C for 5 min using moderate magnetic stirring. Dextrose or WPC-70 was used for binding the active component and sodium azide was used as a preservative. Oil phase (O) was rice bran oil containing 2% (w/w) of the oil soluble emulsifier PGPR. The outer aqueous phase (W₂) was composed of milli-Q water and hydrophilic emulsifier i.e. Tween 20 or Tween 80 or a combination of Span 80 + Tween 80 added at a level of 1-3%.

Water-in-oil (W₁/O) emulsion was prepared by mixing the inner aqueous phase (W₁) (30%, w/w) with the oil phase (O) (70%, w/w) and allowing the mixture to temper at 60 °C for 25 min; the mixture was then homogenised at 60 °C using a hand blender (Dx-505, Lee Handy, Leema Industries, India) operating at 15,000 rpm for 2 min. After mixing, coarse emulsion was passed through Microfluidizer (M-110P, Microfluidics, USA) at a pressure of 20,000 psi to obtain W₁/O emulsion. The W₁/O emulsion (30%, w/w) was then gradually added to the outer aqueous phase (W₂) (70%, w/w), mixed using a hand blender and the final W₁/O/W₂ DE was prepared by homogenizing the mixture at 12,000 rpm for 10 min using Ultra-Turrax T-25 high shear mixer (IKA-Laboratory equipment Ltd., Germany). Ratio of different phases in the prepared DE were W₁:O:W₂ = 9:21:70. Experimental emulsions produced were stored at 5-7 °C.

2.3. Sedimentation Stability

Freshly-made DEs were poured into 10 mL graduated glass cylinders (Borosil glass works, India) and stored at room temperature (30±2°C). Height of the opaque emulsion phase was measured daily over a week (hₜ) and compared to the initial emulsion height (h₀) to determine sedimentation stability (S):

\[
S = \frac{h₀-hₜ}{h₀} \times 100
\]
2.4. Particle Size Distribution

Droplet size distribution was measured by dynamic light scattering using a Horiba Particle Size Analyser (LA-950, Laser Scattering Particle Size Distribution Analyzer, HORIBA, Horiba Instruments Ltd., Japan) equipped with a He-Ne laser ($\lambda = 623$ nm). The optical parameters selected were, a dispersed phase refractive index of 1.462, a droplet absorbance of 0.001 and a continuous phase refractive index of 1.333. Droplet size measurements were carried out in triplicate on each emulsion and the results are reported as the typical droplet size distribution, and the typical volume weighted mean droplet size $d_{43}$.

$$d_{43} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3}$$

(2)

Where $n_i$ is the number of droplets of diameter $d_i$.

2.5. NaCl Release and Encapsulation Efficiency

NaCl release from the inner aqueous phase towards the external aqueous phase was measured as per the method recommended by Sapei et al. (2012) using a conductivity meter (Fisher Scientific, India). Conductivity meter was dipped into a 50 mL aliquot of W$_i$/O/W$_2$ DE. The conductivity values were converted into NaCl concentration using a calibration curve. The fraction of NaCl released (FR) in the external aqueous phase was defined as the ratio of NaCl released into W$_2$ at a specific time ($M_t$) relative to the total amount present in the external aqueous phase if all NaCl were released ($M_\infty$)

$$\text{FR \%} = \frac{M_t}{M_\infty} \times 100 \%$$

(3)

2.5.1. Encapsulation efficiency
Encapsulation efficiency (EE) is defined as percentage of NaCl still entrapped within the inner aqueous phase (W₁):

\[
\text{EE (\%)} = 100 - FR\% \quad (4)
\]

2.6. Microscopic Examination

2.6.1. Light microscopy

Simple light microscope (Labo microscope, Ambala, India) was used to observe the structure of W₁/O/W₂ DEs. The sample was prepared by diluting the W₁/O emulsion with rice bran oil and DE with sodium phosphate buffer (0.1 M, pH 7.0) in the ratio of 1:3. Microscopic examination was carried out by taking 10 µL of sample on a plain glass slide covered with a cover slip and examined under oil immersion through 100x objective. The image of each sample was captured by using digital camera (Nano Tech Imaging, China).

2.6.2. Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) (Olympus Flview 1000, Olympus, Japan) was used to confirm the structure of W₁/O/W₂ DEs. The samples for CLSM was prepared by adding Nile Red to oil phase (O) phase and Nile blue to outer aqueous phase (W₂). CLSM was carried out by taking 1 mL of (i) the W₁/O emulsion and mixing it with 2 mL of rice bran oil or (ii) the W₁/O/W₂ DE and mixing it with 2 mL of sodium phosphate buffer (0.1 M, pH 7.0). All samples were stained for both protein and fat by thoroughly mixing with 5 µL Nile Blue (1% w/v) and 5 µL Nile Red (1% w/v), respectively; 10µL of sample was then dropped on a plain glass slide, covered with a cover slip and examined under oil immersion using 60x objective, with a zoom factor of 5x. The W₁, O and W₂ phases were confocally illuminated simultaneously using Argon laser 488 nm
excitation for oil phase and 633 nm excitation for water phase. Emissions were collected in the wavelength range of 510-590 nm and 650-759 nm, respectively.

2.7. Viscosity measurement

The viscosity of emulsion were measured at 20 °C using coaxial cylindrical rotational viscometer VISCO STAR plus (FUNGILAB, S.A.) having digital display and fitted with probe TL5 to the spindle at 50 rpm, LCP to the spindle at 100 rpm and L2 to the spindle at 5 rpm.

2.8. Statistical Analysis

All experiments were carried out in triplicate and results are expressed as means ± standard error of mean (SEM). Statistical analyses were performed using SPSS 13.0 package. The means of values were analysed by using one-way Analysis of Variance (ANOVA) followed by Tukey’s b comparison test to establish the significance of differences among the mean values at p<0.05.

3. Results and discussion

3.1. Optimisation of matrix materials and processing conditions for preparation of DEs.

Matrix materials and processing conditions for the preparation of DEs (W₁/O/W₂) were selected based upon the preliminary experiments. Sedimentation stability and visual observations were considered the selection criteria for ingredients and processing conditions. The levels of ingredients in the inner aqueous phase (W₁) i.e. hydrated sodium alginate, dextrose (D-glucose), and whey protein concentrate (WPC-70) were 3%, 5%, and 1%, respectively. During the initial experimental phase, DEs were prepared using microfluidizer (20,000 psi) for W₁/O emulsion and homogenizer (2500 psi) for W₁/O/W₂ DE.
Emulsions made with WPC-70 showed the highest stability followed by the emulsions prepared with dextrose whereas, sodium alginate in inner aqueous phase showed the lowest sedimentation stability (Supplementary Table ST1). In contrast to the literature suggesting that solidification of internal aqueous phase improves emulsion stability (O’Regan and Mulvihill 2010), our finding suggests that solidified sodium alginate resulted in poor sedimentation stability which may be attributed to lower viscosity of outer aqueous phase allowing settling of heavy inner aqueous phase. Therefore, WPC was chosen as major construct material for W\textsubscript{1} phase for further studies as it showed better sedimentation stability (>80%). For the better stability of the double emulsions non-ionic surfactants are preferred. Higher molecular weight emulsifiers e.g. proteins improve stability of DEs (Leong et al. 2017). Based on literature review (Sapei et al. 2012; Choudhary et al. 2018) and our previous experiences, rice bran oil (RBO) and the oil soluble emulsifier with low HLB value PGPR (HLB=3) were selected for the middle oil phase (O).

A criterion of selecting optimum level of PGPR was again based upon the sedimentation stability of the DEs. Sedimentation stability of DE having 4\% PGPR in the oil phase were found better than those with 2\% PGPR. Addition of polymeric viscous material to inner aqueous phase requires higher concentration of surfactant for stability of W/O emulsions (Dickinson 2011). A further increase of the concentration of PGPR (6\%) did not improve much the stability of the emulsions. Therefore, 4 \% level of PGPR addition was selected for further studies. Su et al. (2006) also found that 4\% level of PGPR in the oil phase was sufficient enough to prepare the stable emulsions and no significant advantage was observed above 4\% level. In similar studies, Artiga-

Artigas et al. (2019) reported that increase in PGPR concentration above certain level led to increase in emulsion droplet size and decrease in emulsion stability.
As reported in the literature (Leong et al. 2017), stability of the DE also depends on the emulsifier added to the outer aqueous phase (W₂). Low molecular weight emulsifiers with high HLB (Tween-20, Tween-80 and combination of Span-80 + Tween-80) were added to the outer aqueous phase (W₂) of DEs as secondary emulsifiers for stabilization of the (water-containing) oil droplets (W₁/O). For this purpose trials were conducted using dextrose in the inner aqueous phase and 4% PGPR in the oil phase. Increase in the levels of Tween-20, Tween-80 and combination of Tween 80 and Span 80 from 1% to higher levels (2% or 3%) in the outer aqueous phase after fixing levels of all other ingredients, resulted in considerable decrease in sedimentation stability (Data not shown). However, the emulsion with 1% Tween-80 showed significantly higher stability (p<0.05) and was considered optimum for further trials. These results corroborate well with the findings of Sapei et al. (2012) who noted that addition of 1% level of Tween-80 increased the stability of the DE. The decrease in the sedimentation stability of the DE at higher levels of the emulsifier addition may be attributed to the increase in the hydrophobicity of the outer aqueous phase (W₂). There should be proper HLB balance for the stability of the emulsion.

As an alternative to homogenizer, Ultra-Turrax T-25 was used for the preparation of stabilized W₁/O emulsion while optimising the processing conditions. At 20,000 rpm, the sedimentation stability was found highest i.e. >80% (Supplementary Table ST1). In contrast, creation of W₁/O/W₂ emulsion shear conditions of 15,000 rpm for 5 minute was found to be optimum (data not shown). O’Regan and Mulvihill (2009) also used Ultra-Turrax T-25 homogenizer for the preparation of W₁/O emulsion and shear condition used was 20,000 rpm for 4 minute and the stability and encapsulation efficiency of the emulsion was found satisfactory. Sedimentation stability of DE prepared using homogenizer and Ultra-Turrax was different from
each other (Supplementary Table ST1). Emulsion samples prepared using Ultra-Turrax high shear mixer showed higher stability than that those using homogenizer. In Ultra-Turrax, shear applied is very high as compared to the homogenizer; so the particle size obtained is expected to be smaller as compared to homogenizer. Smaller particles contribute to emulsion stability. Trujillo-Cayado et al. (2018) also reported that emulsions with monomodel particle size distribution were obtained when higher homogenization energy applied using Ultra-Turrax high shear disperer.

3.2. Effect of WPC and salt addition as the construct material for inner aqueous phase ($W_1$) of DEs

DEs prepared using varying levels of WPC alone or in combination with salt in the inner aqueous phase, were subjected to evaluation for sedimentation stability, encapsulation efficiency, particle size distribution and morphology both for fresh and stored samples. DEs were stored for 10 days at 30 °C

3.2.1 Sedimentation stability

Fresh emulsions containing WPC (2% to 8%) in the inner aqueous phase (Fig.1) were reasonably stable exhibiting sedimentation stability >70%. However, the addition of salt (2 and 4%) further increased sedimentation stability (>80%) significantly at all levels of WPC except 8% concentration in the inner aqueous phase. Maximum stability (100%) was observed at 6% WPC level with 2% or 4% salt levels. Salt or any other electrolyte when added to internal aqueous phase enhances emulsifier efficacy by increasing emulsifier adsorption density at oil/water interface and reduces interfacial tension in W/O emulsions (Foudazi et al. 2015). Further, the salt prevents water droplets coalescence in W/O emulsions by counter balancing laplace pressure difference between droplets, thus preventing Ostwald ripening (Scherze et al. 2006), it also balances osmotic pressure difference between $W_1$ and $W_2$ phases (Kanouni et al. 2002). Therefore, the presence and
concentration of NaCl in \( W_1 \) played a critical role in balancing these effects, with excess NaCl resulting in water migration from \( W_2 \) to \( W_1 \) and subsequent swelling of the \( W_1/O \) droplets (Evageliou et al. 2019).

An overall decrease was observed in the sedimentation stability of the DEs upon storage for 10 days at various levels of WPC and salt in \( W_1 \) phase. However, the extent of this decrease was less pronounced in emulsion samples having at 6% level of WPC with 2% or 4% level of salt (Fig. 2) indicating more stable emulsion. As is evident in Fig. 1(b), emulsions prepared with 2% NaCl and various levels of WPC showed some decrease in the sedimentation stability during storage. The decrease in stability of DE during storage can be attributed to either of the two instability mechanism i.e. coalescence of the small inner water droplets (\( W_1 \)) with the globule interface (O) or coalescence of the small inner water droplets within the oil globule itself as proposed by Ficheux et al. (1998). Over the 10 days, sedimentation stability of DEs containing 2% WPC decreased from 99 to 95%. At the end of storage period, maximum stability (99.41%) was observed in the samples having 6% WPC and 4% NaCl (Fig. 2) while the lowest (79%) was found in case of 8% WPC and 4% NaCl. Based on these results, the emulsion with 6% WPC in combination with 2% or 4% salt can be recommended for the preparation of stabilized delivery system for water soluble bioactive substances.

3.2.2. Encapsulation efficiency

Encapsulation efficiency (EE) also known as ‘yield’ is an important property which denotes the proportion of marker present intact in the inner aqueous phase (\( W_1 \)) after secondary emulsification and during storage. EE is a key stability parameter that characterizes effectiveness of DE. Higher value of EE would indicate more stability and retention of internal aqueous phase after
secondary emulsification (Dickinson 2011). In this study NaCl not only acted as stabilizing factor for W<sub>1</sub>/O emulsion but also acted as a marker to characterize EE.

The encapsulation efficiencies (EE) of DE at different levels (2-8%) of WPC in combination with 0, 2 or 4 % salt levels in the inner aqueous phase are shown in Fig.1. Encapsulation efficiency of all the fresh samples was > 87% regardless of the differences in internal aqueous phase (W<sub>1</sub>). EE of the DEs decreased with increasing concentration of WPC in the W<sub>1</sub> phase both with and without salt addition. However, a small but definite improvement in EE was observed when salt was added at 2 % and 4% level in W<sub>1</sub> especially at 4% and 6% level of WPC. These results corroborate well with the findings of earlier researchers. Srinivasan et al. (2000) noted that addition of NaCl to the emulsion stabilized by sodium caseinate decreased the flocculation rate and improved the creaming stability of the emulsion. Increase of the ionic strength of W<sub>1</sub> phase upon NaCl addition leads to balancing Laplace pressure across the intermediate oil layer, decreases surface tension between W<sub>1</sub>/O and improves adsorption of emulsifier (WPC 70 in this study) at the interface (Sapei et al. 2012).

A decrease in the encapsulation efficiency of the WPC stabilised DE was observed upon storage for 10 days at room temperature (Fig. 1). This indicated release of NaCl from inner aqueous phase (W<sub>1</sub>) to the outer aqueous phase (W<sub>2</sub>) with time and, therefore, destabilization. The maximum drop in the efficiency (89 to 75%) was observed in the samples containing 8% WPC and 2% NaCl. This indicates that excessive amount of WPC results in poor functionality (i.e. EE). This may be attributed to increased volume of hydrated WPC particles of W<sub>1</sub> phase to the extent that the amount of PGPR may have become less effective in maintaining oil droplets intact during storage, therefore, loss of EE (Dickinson 2011). Alternatively this may be associated with thinning of outer
aqueous phase because of the release of the inner water droplets (Ficheux et al. 1998) into the external phase (W₂). At the lower levels of WPC (2-6% WPC at 2% NaCl), comparatively very less decrease in the encapsulation efficiency was observed.

At 4% level of NaCl content in W₁ (Fig. 1c) higher values of encapsulation efficiency of the emulsions were noticed at all levels of WPC as compared to the emulsions prepared at lower salt levels. Maximum retention of encapsulation efficiency during storage was seen in emulsion with 2% WPC with a decrease from ~99% to about ~92% after storing at room temperature for 10 days. Optimum level of WPC may have formed viscoelastic layer (Leong et al. 2017) at the interface of W₁/O which could have synergistically made complexes with PGPR to prevent droplet coalescence and improved emulsion stability further (Knoth et al. 2005; Hemar et al. 2010). Lowest encapsulation efficiency value was observed in case of emulsion having 8% WPC and 4% NaCl as it dropped from initial value of about ~92% to about ~80% in 10 days. However, the results revealed that addition of 4% salt to the W₁ phase resulted in better emulsion stability as it maintained higher encapsulation efficiency during storage as compared to 2% salt levels. Electrolyte balance in inner aqueous phase (W₁) is critical for achieving optimum encapsulation efficiency and stability of emulsions (Lamba et al. 2015).

3.2.3. Microstructure of DEs

Photomicrographs and Confocal Laser scanning microscopy confirmed the presence of intact DEs both in fresh and stored samples. Confocal scanning microscopic images showed the structure of fresh DEs consisting of internal aqueous phase W₁ (Fig. 3A, row X and column 1; red droplets) dispersed in continuous oil phase (Fig. 3A, row X and column 2; green background) for making, W₁/O emulsion, which is further dispersed (Fig. 3A, row Y and column 1; green particles)
in continuous water phase (W₂) (Fig. 3A, row Y and column 2; red background in). In row Y, the reverse colour scheme (red dispersion medium as the aqueous phase and green as oil droplets) validates the presence of intact and stable DEs (W₁/O/W₂). It is also apparent from the images that the size of primary emulsion droplets (W₁/O) is much smaller than final emulsion (W₁/O/W₂). Presence of salt (4% NaCl) appears to have resulted in finer emulsion, which support the basis for sedimentation stability reported in section 3.2.1. Smaller size W₁ or W₁/O droplets increase stability of DEs (Leong et al. 2017).

Figure 3B shows the microscopic images of the DE captured with the help of simple bright field microscope at 100X oil immersion lens. All the fresh WPC stabilized emulsions exhibited relatively large size, more rigid and intact structure including those without salt in W₁ phase. No visible difference was observed in the structure of freshly prepared DEs after increasing concentration of WPC or salt in W₁ phase. Structural integrity of DEs decreased upon storage as evidenced by partial fragmentation of DEs in Figure 3B (row Y). This suggests that destabilisation of matrix took place over the storage period which resulted in heterogeneity in the size and structure of DEs. Samples without salt and lower levels of WPC (i.e. 2%) in W₁ phase exhibited maximum level of destabilization. It suggests importance of optimum level of salt and WPC for structural stability of DEs. WPC 70 may act as high molecular weight emulsifier and can form viscoelastic film at the W₁/O interface (Dickinson 2011). Presence of WPC, therefore in inner aqueous phase (W₁) may increase emulsion stability. Maximum stability upon storage for 10 days was observed for the DEs containing 6% WPC and 2% salt in W₁ phase (Fig. 3B row Y). Coalescence of DE droplets was also observed during storage which is a common phenomenon for DEs.

3.2.4. Particle size analysis
An increase in particle size ($d_{43}$) of stored emulsions (W$_1$/O/W$_2$) was observed with an increase in WPC concentration and salt concentration in W$_1$ phase (Table 1). Particle size distribution of DEs (Fig. 4) also indicated that with an increase in the concentration of WPC at both the salt levels i.e. 2 and 4% in the inner aqueous phase (W$_1$) of DEs, there was an increase in the particle size of the emulsions. The maximum mean particle size (15.33 µm) was observed in the samples containing 8% WPC and 2% NaCl (Table 1). Slight increase in particle size of W$_1$/O droplets was observed upon storage as seen through microscopy (Fig. 3B). An overall increase in the particle size of the DE is expected in the stored samples due to coalescence of the oil droplets.

Unlike previous reports (Su et al. 2006; O’Regan and Mulvihill 2010), the droplet size distribution (Fig. 4A & 4B) of DEs was almost monomodal indicating monodisperse emulsions. Use of microfluidizer for preparation of W$_1$/O emulsion and Ultra-Turrax for final W$_1$/O/W$_2$ emulsion resulted in relatively uniform droplet sizes when compared with data available in literature (O’Regan and Mulvihill 2010). Trujillo-Cayado et al. (2018) also observed monomodal droplet size distributions when Ultra-Turrax was employed for thyme essential oil based emulsion preparation.

At all the levels of WPC, increase in the concentration of NaCl (2 to 4%) resulted in decrease in mean particle size. The particle size of WPC stabilized double emulsions with 2% NaCl were in the range of ~ 0.17 to 22.80 µm and thus very small as compared to those with 4% NaCl levels which were in the range of ~ 0.88 to 40 µm. On the contrary in nano emulsions (O/W), increasing amount of NaCl didn’t influence the particle size, rather the zeta potential (Sharma et al., 2017) which may be attributed to lower volume of dispersed phase compare to double emulsions. Addition of salt to inner aqueous phase helps in diffusion of emulsifier to the interface thus reducing interfacial tension which in turn results in smaller emulsion droplets (Sapei et al. 2012).
Presence of smaller emulsified droplets both in inner (W$_1$ in O phase) and outer (W$_1$/O in W$_2$ phase) phase improves overall emulsion stability (Leong et al. 2017). This may be one of the reasons for obtaining better encapsulation efficiency and sedimentation stability for the samples containing 6% WPC and 2% salts in the W$_1$ phase (Fig. 1; Fig. 2 and Fig. 3). On the other hand, excessively smaller particle size may lead to loss of encapsulation efficiency because of shear induced breakdown of intermediate oil phase causing mixing of both the aqueous phases (Dickinson 2011).

3.2.5. Viscosity

With an increase in the level of WPC (from 2 to 8%) in the inner aqueous phase of W$_1$/O/W$_2$ emulsion, the viscosity of DE decreased at both salt levels i.e. 2 and 4% (Fig. 5). At 4% salt content in W$_1$, DEs exhibited higher viscosity ~3000 cP (~1700 cP at 2% salt level). At lower levels of WPC (<6%), the effect of salt addition had dominating effect on the viscosity of DEs. Salt in inner aqueous phase (W$_1$) balances Laplace pressure and osmotic pressure. Higher level of salt may increase the viscosity of DE because of swelling of W$_1$/O emulsion droplets due to water migration from outer aqueous phase (W$_2$) to inner phase (W$_1$) (Lutz and Garti 2006; Sapei et al. 2012). At higher levels (>6%) of WPC addition, the viscosity of DE decreased significantly and was found to be minimum for the samples with 8% WPC at both salt levels i.e. 2 and 4 % salt. This may be a because of structural breakdown of the DEs. Carrillo-Navas et al. (2012) reported a decrease in viscosity of DEs at higher levels WPC additions.

4. Conclusion

W$_1$/O/W$_2$ type DEs were successfully prepared using the phase ratio of 9:21:70 and by using microfluidizer for W$_1$/O and Ultra-Turrax for final DE. DEs prepared by using optimised formulation and processing conditions were stable for 10 days at room temperature. This ensured
relatively monodisperse emulsion droplets and better encapsulation efficiency. WPC and NaCl levels in internal aqueous phase of W₁/O/W₂ emulsions had a great impact on emulsion characteristics. Increase in salt content in W₁ phase resulted in decrease in final emulsion droplet size. WPC 6% and salt levels of 2 or 4% in W₁ phase showed maximum stability (>99%), structural integrity, encapsulation efficiency (>95%) and sedimentation stability during processing and storage of the final emulsions. A further systematic study is recommended to study the effect of change in matrix composition of the outer aqueous phase of DEs.

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References


Fig. 1 Sedimentation stability (left) and encapsulation efficiency (right) of fresh and stored W₁/O/W₂ double emulsions prepared using different WPC concentrations at various salt levels 0% (a), 2% (b), 4% (c) in the inner aqueous phase (W₁). Error bars represent standard error of mean of three replications. Error bars represent standard error of mean of three replications.

Fig. 2 Photographs of double emulsions (W₁/O/W₂) prepared using different matrix materials in inner aqueous phase after storage for 10 days at room temperature. 1: 6% WPC + 4% NaCl; 2: 8% WPC + 4% NaCl; 3: 2% WPC + 4% NaCl, arrow showing the separation.

Fig. 3 A: Confocal Scanning Laser Microscopic (CSLM) images of water-in-oil (W₁/O) emulsion (X) and water-in-oil-in-water (W₁/O/W₂) double emulsions (Y) (1: 8% WPC + 0% NaCl; 2: 6% WPC + 4% NaCl). Scale bar indicates 10 µm size. B: Microscopic images of double emulsion prepared using various levels of WPC and NaCl in internal aqueous phase (1: 2% WPC + 0% NaCl; 2: 2% WPC + 2% NaCl; 3: 6% WPC + 2% NaCl). Row X) Fresh sample; Row Y) Stored sample. Scale bar indicates 1 µm size.

Fig. 4 Effect of NaCl (A: 2% NaCl; B: 4% NaCl) on particle size distribution of WPC stabilized double emulsions after storing for 10 days at room temperature. Samples viz., 8% WPC + 2% NaCl (■); 6% WPC + 2% NaCl (▲); 4% WPC + 2% NaCl (X); 2% WPC + 2% NaCl (♦). Values are d₄₃ means ± SE of triplicate.

Fig. 5 Effect of NaCl concentration on viscosity of double emulsion stabilized by whey protein concentrate. 2% NaCl (♦); 4% NaCl (■). Values are means ± SE of triplicate.
Table 1. Effect of NaCl concentration on particle size of stored W1/O/W2 double emulsion stabilized by WPC

<table>
<thead>
<tr>
<th>WPC level (%)</th>
<th>Particle Size (d_{4,3}, \mu m)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2% NaCl</td>
</tr>
<tr>
<td>2.00</td>
<td>5.23±0.10^aX</td>
</tr>
<tr>
<td>4.00</td>
<td>6.96±0.00^bX</td>
</tr>
<tr>
<td>6.00</td>
<td>8.00±0.03^cX</td>
</tr>
<tr>
<td>8.00</td>
<td>15.33±0.02^dX</td>
</tr>
</tbody>
</table>

Note: All the values expressed as Mean ± SE (n=3). Means with different superscripts in the same row (X, Y) or column (a, b, c, d) are significantly different (p<0.05).
**Fig. 1** Sedimentation stability (left) and encapsulation efficiency (right) of fresh and stored W1/O/W2 double emulsions prepared using different WPC concentrations at various salt levels 0% (a), 2% (b), 4% (c) in the inner aqueous phase (W1). Error bars represent standard error of mean of three replications. Error bars represent standard error of mean of three replications.

**Fig. 2** Photographs of double emulsions (W1/O/W2) prepared using different matrix materials in inner aqueous phase after storage for 10 days at room temperature. 1: 6% WPC + 4% NaCl; 2: 8% WPC + 4% NaCl; 3: 2% WPC + 4% NaCl, arrow showing the separation.

**Fig. 3** A: Confocal Scanning Laser Microscopic (C SL M) images of water-in-oil (W1/O) emulsion (X) and water-in-oil-in-water (W1/O/W2) double emulsions (Y) (1: 8% WPC + 0% NaCl; 2: 6% WPC + 4% NaCl). Scale bar indicates 10 µm size. B: Microscopic images of double emulsion prepared using various levels of WPC and NaCl in internal aqueous phase (1: 2% WPC + 0% NaCl; 2: 2% WPC + 2% NaCl; 3: 6% WPC + 2% NaCl). Row X) Fresh sample; Row Y) Stored sample. Scale bar indicates 1 µm size.

**Fig. 4** Effect of NaCl (A: 2% NaCl; B: 4% NaCl) on particle size distribution of WPC stabilized double emulsions after storing for 10 days at 37 °C. Samples viz., 8% WPC + 2% NaCl (■); 6% WPC + 2% NaCl (▲); 4% WPC + 2% NaCl (X); 2% WPC + 2% NaCl (♦). Values are d43 means ± SE of triplicate.

**Fig. 5** Effect of NaCl concentration on viscosity of double emulsion stabilized by whey protein concentrate. 2% NaCl (♦); 4% NaCl (■). Values are means ± SE of triplicate.
(A)  
![Image of microscopic images](image1.png)  

(B)  
![Image of microscopic images](image2.png)
Fig 5

![Graph showing the relationship between WPC (%) and Viscosity (cP) for 2% NaCl and 4% NaCl solutions. The graph indicates a decrease in viscosity as WPC increases for both concentrations.](image)