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Research Article

Effect of Selected Stabilizers and Processing Aids on the Stability of a Double Emulsion Encapsulating Bitter Gourd Extract

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Abstract: Effect of three variables in differing concentrations [NaCl (3-5%), polyglycerol polyricinoleate (PGPR) (2-4%) and dairy protein-polysaccharide complexes (Whey protein concentrate(WPC-80)-gum Arabic(GA) and sodium caseinate(SC)-gum Arabic in 1:2 ratio)] on the stability of $W_1/O/W_2$ emulsion matrix that was used to encapsulate bitter gourd extract was evaluated. The double emulsion matrix was characterized by apparent viscosity, zeta potential, turbidity and sedimentation stability by visual appearance. The physical parameters of the double emulsion matrix were very highly significantly ($p < 0.001$) affected by all variables such as the concentration of salt, PGPR and complex (WPC-GA and SC-P) as well as their interactions. The double emulsions prepared with WPC-GA became unstable immediately after preparation or after one day of preparation. SC-GA stabilized double emulsions were found more stable than WPC-GA stabilized emulsions. A double emulsion containing 5% NaCl, 2% PGPR and 16.5% SC-GA was found most stable (10 days at 37°C) in comparison to other combinations used.

***Keywords*:** dairy protein-polysaccharide complex, NaCl, PGPR, bitter gourd extract, stability

1. Introduction

An emulsion consists of one immiscible liquid dispersed in another, and stabilized using a third component such as emulsifiers or stabilizers [1]. Instead of a single emulsion, a double emulsion is a multi-compartmentalized dispersion system in which the emulsion itself is dispersed into another immiscible liquid [2]. Water-in-oil-in-water ($W_1/O/W_2$) double emulsion have immense potential for application in various fields such as pharmaceuticals, cosmetics and the food industry, for formulation of healthier foods including nutraceutical and functional foods by reducing oil (calories) content, salt (sodium) concentrations, masking unacceptable flavors/odors through compartmentalization and as vehicles for encapsulation and controlled release of bioactive compound i.e. vitamins, carotenoids, ω-3 fatty acids, phytochemicals, microorganisms, lactoferrin and minerals [3-4]. Beside these applications, double emulsions ($W_1/O/W_2$) have thermodynamic instability such as coalescence, flocculation, Ostwald ripening and phase separation and are subjected to leakage of the encapsulant either from the internal aqueous phase or outer aqueous phase during processing and storage due to increased interfacial area [3, 5]. The stability of double emulsion can be affected by their composition comprising of type and concentration of surfactants, electrolytes, the nature of encapsulants, ratio of dispersed emulsion to continuous phase, osmotic balance between inner and outer phase, various processing regimes such as formulation

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methods, shear applied, temperature of operation and conditions, final desired droplet size and storage conditions [6]. There are two different types of surfactants usually required to stabilize the double emulsion ($W_1/O/W_2$) i.e. primary emulsion surfactant for the inner aqueous droplets and secondary emulsion surfactant for intermediate oil phase droplets. Polyglycerol polyricinoleate (PGPR) is considered as a highly effective oligomeric emulsifier for W/O emulsions [7-8]. It is used as an oil-soluble surfactant, which is able to prevent coalescence of newly formed water droplets by the formation of tiny water droplets in the oil phase and facilitates droplet break-up by reducing the interfacial tension [9-10]. Salt (NaCl) is an electrolyte that generates the osmotic pressure to counterbalance the Laplace pressure and stabilizes W/O emulsions against Ostwald ripening (diffusional ripening) [10-12]. Therefore, addition of electrolytes along with appropriate hydrophobic emulsifiers and hydrophilic emulsifiers is essential to prepare coalescence-stable W/O/W double emulsion. Biopolymers like proteins and polysaccharides may improve the stability of double emulsions by increasing the viscosity of inner and outer water phase, which prevent loss of inner water droplets and W_1/O droplet motion, respectively [13-14], controlling particle size change through formation of interfacial layer surrounding droplets [15-16] and controlling osmotic pressure [5]. However, proteins are less effective emulsifiers near their iso-electric pH, which limits their application in acidic foods. Hence, electrostatic protein-polysaccharide complexes gave better functional properties: emulsification, stability, encapsulation efficiency, texture/rheology, surface hydrophobicity and mouth feel than that of the proteins and polysaccharides alone even near the iso-electric pH of the protein [17-20]. The objective of the present investigation was to study the effect of hydrophobic emulsifiers (PGPR as primary emulsifier) and salt as an electrolyte and dairy protein-polysaccharide complexes as secondary emulsifiers. NaCl and PGPR were used in varying ranges in the primary emulsion (W/O) and complexes [Whey protein concentrate-80-gum Arabic (WPC-GA) and sodium caseinate- gum Arabic (SC-GA)] in 1:2 ratio in the outer aqueous phase of the double emulsion (W/O/W) that would be applied for encapsulating bitter gourd extract. The emulsion was characterized on the basis of the parameters such as apparent viscosity, zeta potential, sedimentation stability (by visual appearance) and turbidity measurements.

2. Materials and methods

The raw materials required for the study were purchased from different manufacturers and suppliers as detailed in Table 1.

Table 1. Materials for formulating the double emulsion based functional mayonnaise and their analysis

No.	Item	Features	Procured from
1	Whey protein concentrate-80	79.86% protein, 8.09% lactose	Mahaan Proteins Ltd., New Delhi
2	Sodium caseinate (Protonate 8868)	88% protein	
3	β-Pectin	Degree of acetylation of about 18%, Degree of esterification (DE) > 50%	CPKelco, Huber India Company, Mumbai, Maharashtra
4	Gum Arabic (Fibre gum B)	-	KP Manish Global Ingredients Pvt. Ltd., Chennai
5	Polyglycerol polyricinoleate (PGPR) GRINSTED® PGPR 90	GRINSTED® PGPR 90	DuPont Danisco India Private Limited, Gurgaon, Haryana
6	Sodium chloride		
7	Refined rice bran oil (Fortune)	-	Local market, Karnal, Haryana
8	Vinegar (4% acetic acid)		
9	Reverse osmosis (RO) water	-	-
10	Bitter gourd extract	5.95%, w/w total solids content and 5% charantin content	Ambe Phytoextracts Pvt. Ltd., New Delhi

2.1 *Experimental factorial design*

In order to optimize the stable double emulsion ($W_1/O/W_2$) matrix, salt, PGPR and dairy protein-polysaccharide complexes and their interaction influence on the physical properties of the functional mayonnaise were evaluated using 3^3 full factorial design composed of the three variables at three levels each. The dairy protein-polysaccharide complexes were protein concentrate 80-gum Arabic (WPC-GA) and sodium caseinate-gum Arabic (SC-GA) in 1:2 ratio. Double emulsion matrix comprised of varied concentrations of salt in inner aqueous phase (W_1) containing bitter gourd extract (55.2%), PGPR in middle oil phase (O) and dairy protein-polysaccharide complexes in outer aqueous phase (W_2). All the variables were presented in percentage (% w/w) and their influence on the double emulsion matrix were evaluated with apparent viscosity, sedimentation stability (at 7°C and 37°C) by visual observations, zeta potential and turbidity measurements. The experimental design consisted of 54 formulations (27 formulations for each out of two (WPC: GA, SC: GA) dairy protein-polysaccharide complex) as detailed in Table 2. The low, medium and high levels were represented as (-1), (0), and (+1) for the minimum, medium and maximum values of each variable, respectively. A full factorial design approach was adopted to study the influence of the variables and their interaction on the physical properties of the double emulsion to optimize the double emulsion matrix.

Table 2. 3^3 full factorial design to investigate the influence of variables on double emulsion-based matrix

Variables (%, w/w)		Levels		
		Low level (-1)	Medium level (0)	High level (+1)
NaCl (%, w/w in W_1, inner aqueous phase)		3	4	5
PGPR (%, w/w in O, middle oil phase)		2	3	4
Dairy protein-polysaccharide complexes in 1:2 ratio (%, w/w in W_2, outer aqueous phase)	WPC: GA	18	19.5	21
	SC: GA	13.5	15	16.5

2.2 *Preparation of dairy protein-polysaccharide complexes*

The protein-polysaccharide complexes were formulated according to Salminen and Weiss [20] with slight modifications. Dairy protein (WPC-80 and SC) and polysaccharide (gum Arabic) were used in the ratios 1:2. The protein and polysaccharide were weighed into separate beakers for solubilization with RO water. Protein solutions were prepared by magnetic stirring constantly at ambient temperature for 1-2 h, whereas polysaccharide solutions were prepared by magnetic stirring for 6-10 h at moderate speed to ensure complete dissolution. Protein and polysaccharide solutions were initially adjusted to pH 7.0 using 1.0 N NaOH before further mixing. Then, they were mixed, the pH of the mixture reduced to 4.0 with 4% acetic acid (vinegar) and stored at ambient temperature overnight.

2.3 *Preparation of double emulsion ($W_1/O/W_2$) matrix*

The inner aqueous phase (W_1) was prepared with the aqueous soluble bitter gourd extract, (55.2%, w/w) and varying levels of NaCl (3.0-5.0%, w/w) in RO water. Middle oil phase (O) consisted of rice bran oil containing PGPR as hydrophobic emulsifier (2-4%, w/w). Outer aqueous phase (W_2) was the protein-polysaccharide complex prepared as explained in the previous section. Both W_1 and O were pasteurized at 72°C for 15 sec, while outer aqueous phase heated at 85°C for 20 min. Salminen H and Weiss J [20] followed by cooling to 4-7°C in ice water bath.

The primary water-in-oil (W_1/O) emulsion was premixed by mixing the inner aqueous phase (W_1) (30%, w/w) with the oil phase (O) (70%, w/w) at room temperature, using a magnetic stirrer at moderate speed for 5 min. The mixture was then homogenized using an Ultra-Turrax [IKA Ultra-Turrax T25 (IKA®) India Pvt. Ltd. (Bangalore, India)] operating at 22,000 rpm for 5 min to form primary W_1/O emulsion. The primary (W_1/O) emulsion (30%, w/w) was gradually added to the outer aqueous phase (W_2) (70%, w/w) and mixed with magnetic stirring at moderate speed for 5

min. The pre-mix was finally homogenized using Ultra-Turrax at 15,000 rpm for 5 min to produce final double emulsion ($W_1/O/W_2$) (9:21:70:$W_1/O/W_2$).

2.4 *Apparent viscosity*

A controlled stress rheometer RHEOPLUS/32 (M/s Anton Paar, GmbH, Ostfildern, Germany), using a cone and plate geometry (CP-75, D = 0.149 mm) was used for the measurement of apparent viscosity the double emulsions, which was characterized in the shear rate range 0.01-100 s^{-1} [21].

2.5 *Zeta potential*

The zeta potential of the double emulsions was measured using Zetasizer Nano-ZS90 (Malvern Instrument Ltd., Malvern, Worcestershire, UK) to determine the stability of the emulsion. The emulsions were diluted 100 times with RO water and the experiment was carried out at 25°C. Zeta potential measurements were carried out in triplicate for each emulsion and the results were expressed in mV.

2.6 *Turbidity*

The turbidity of the double emulsions was determined in triplicates according to Pearce and Kinsella [22]. Aliquots (1 mL) of the emulsion were diluted serially with water and sodium dodecyl sulfate (SDS) solution to give final dilutions in the range 1/1000 to 1/5000 and an SDS concentration of 0.17%. The absorbance of the diluted emulsion was then determined in a l-cm path length cuvette at a wavelength of 500 nm in a spectrophotometer (M/s Thermofisher Scientific Inc., Rochester, New York, USA). Identical cuvettes were used for all samples and were rinsed with a jet of distilled water between determinations. Absorbance of triplicate aliquots of each emulsion was measured in each case.

$$T = \frac{2.303A}{l}$$

where T is the turbidity, A is the observed absorbance and l is the path length of the cuvette.

2.7 *Sedimentation stability*

The sedimentation stability (by visual appearance) of the double emulsion was determined in triplicates according to Sapei et al. [23]. Freshly made double emulsions were poured into 40 mL glass vials [internal diameter = 21 mm; length = 90 cm; Corning India (Gurgaon, India)] to a height of 6 cm and stored at 37°C. The emulsion was observed daily for the occurrence of phase separation.

2.8 *Statistical data analysis*

The influence of the varying ranges of PGPR, NaCl and dairy protein-polysaccharide complex on properties of double emulsion (W/O/W) matrix was studied and the data were analyzed by two-way analysis of variance (ANOVA) using SPSS (IBM SPSS Statistics 21) software. A significant difference between variables and attributes (apparent viscosity, zeta potential, sedimentation stability and turbidity measurements) of the matrix was determined at $p < 0.001$ as shown in Appendix 1-4. All measurements of experimental attributes were done in triplicate.

3. Results and discussion

3.1 *Effect of different variables on physical properties of double emulsion*

54 formulations (27 each for WPC-GA and SC-GA complexes) were prepared using two-stage emulsification as detailed in Table 2.

3.2 *Apparent viscosity*

The influence of varying concentrations of the three variables [NaCl (3-5%, w/w) in inner aqueous phase (W_1), PGPR (2-4%, w/w) in middle oil phase (O), two different (WPC-GA and SC-GA) complexes in a 1:2 ratio in outer aqueous phase (W_2) of the double emulsion ($W_1/O/W_2$)] on the apparent viscosity (at 100 s^{-1} shear rate) just after the emulsions preparation is shown in Figure 1 (a-b).

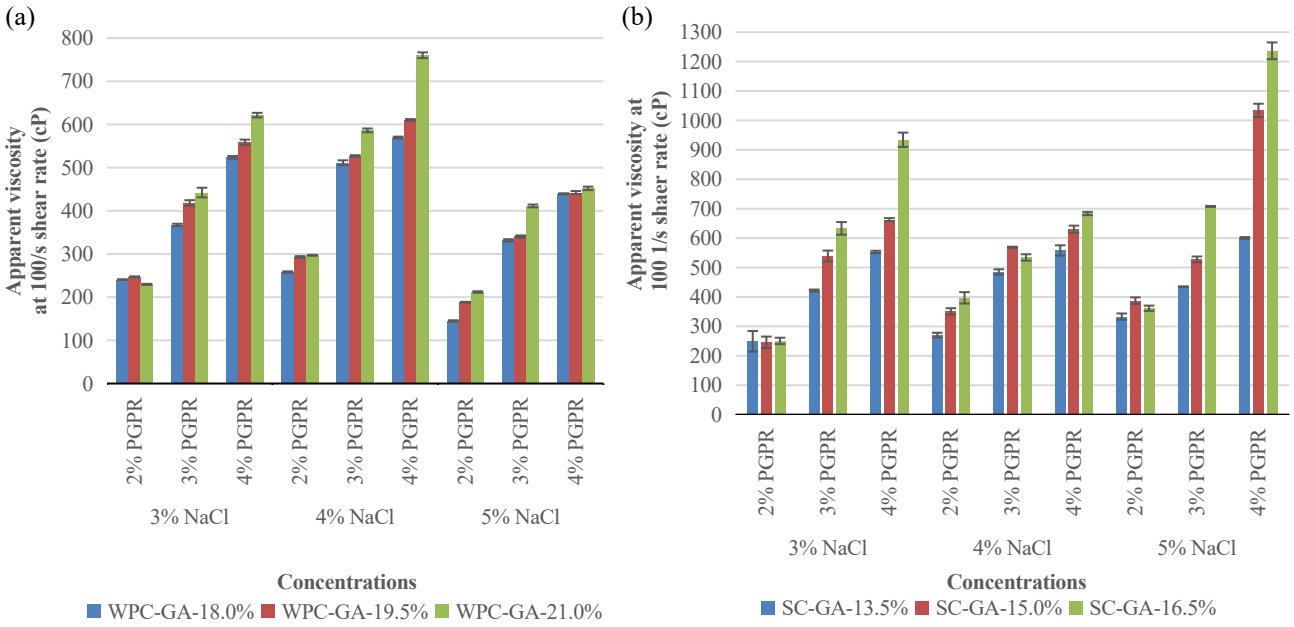

Figure 1. Effect of salt, PGPR and dairy protein-polysaccharide complexes [(a): WPC-GA complex, (b): SC-GA] on apparent viscosity of double emulsion-based matrix; n = 3

Freshly prepared double emulsion with SC-GA complex had a very highly significant ($p < 0.001$) value of apparent viscosity compared to WPC-GA stabilized double emulsion (Appendix 1). It increased with increasing concentration of the complexes. The apparent viscosity of double emulsion stabilized with both complexes (WPC-GA and SC-GA) increased with increasing concentration of PGPR ($p < 0.001$). The average values of apparent viscosity increased up to 4% NaCl concentration, and decreased thereafter at 5% NaCl in WPC-GA stabilized double emulsion, while in case of SC-GA stabilized double emulsion, it decreased with NaCl up to 4% and thereafter increased at 5% NaCl. The difference in the values of apparent viscosity with varying range of NaCl was very highly significant ($p < 0.001$). Interactions of all the variables exhibited a very highly significant ($p < 0.001$) effect on apparent viscosity of double emulsion (Appendix 1).

The viscosity of a double emulsion is influenced by the nature and behavior of the biopolymers and their ratio, weight ratio and concentrations in the continuous phase solution, the arrangement of the biopolymers at the oil-water interface and the degree of mobility of the water in the external water phase [24-25]. When the amount of water in external water phase was large, the emulsion droplets were apart from each other and so, the entanglement between the droplets reduced, resulting in low viscosity of the double emulsions. On the other hand, the droplets came closer and increased the entanglement between emulsion droplets by reducing the amount of water in external aqueous phase. Thus, the viscosity of the double emulsions increased. Viscosity of the double emulsions would be increased because water can penetrate into the inner phase to equalize the osmotic pressure of the double emulsion during the second step of their preparation [26]. In the present study, the viscosity of the double emulsions was influenced mainly by relationships between the concentrations of the protein-polysaccharide complexes and NaCl in the continuous phase and inner aqueous phase, respectively, and the degree of mobility of the water in the external aqueous phase. The effect of concentration of the protein-polysaccharide complexes on the apparent viscosity of the double emulsions (W/O/W)

was the same as observed by Lutz et al. [25]. In addition, apparent viscosity of the primary emulsion (W/O) increased with increasing PGPR concentration [27]. Hence, the increased apparent viscosity of the final double emulsion (W/O/W) may be due to increased resistance to shearing during secondary emulsification. Our results agreed with the studies reported by Bahtz et al. [9] and Su et al. [8] who found that the viscosity of the W/O emulsion increased with increasing concentrations of PGPR. This may have led to a subsequent reduction in rate of coalescence and Ostwald ripening of the water droplets by decreasing the droplet diameter. On the other hand, though with initial increase in NaCl concentration (up to 4%) the apparent viscosity of primary emulsion increased, it reduced at higher NaCl concentration (5%) [27]. This may be because NaCl plays an important role in formation of double emulsion by matching the osmotic and Laplace pressures between the two aqueous phases (W_1 and W_2) of the double emulsion. Presence of excess NaCl concentration in inner aqueous phase causes migration of the water droplets from outer aqueous phase (W_2) to inner aqueous phase (W_1), leading to subsequent swelling and sometimes bursting of the W_1/O droplets in the presence of concentration gradient between the two aqueous phases [28].

3.3 *Zeta potential*

The effect of the variables on ζ-potential of the double emulsion are shown in Figure 2.

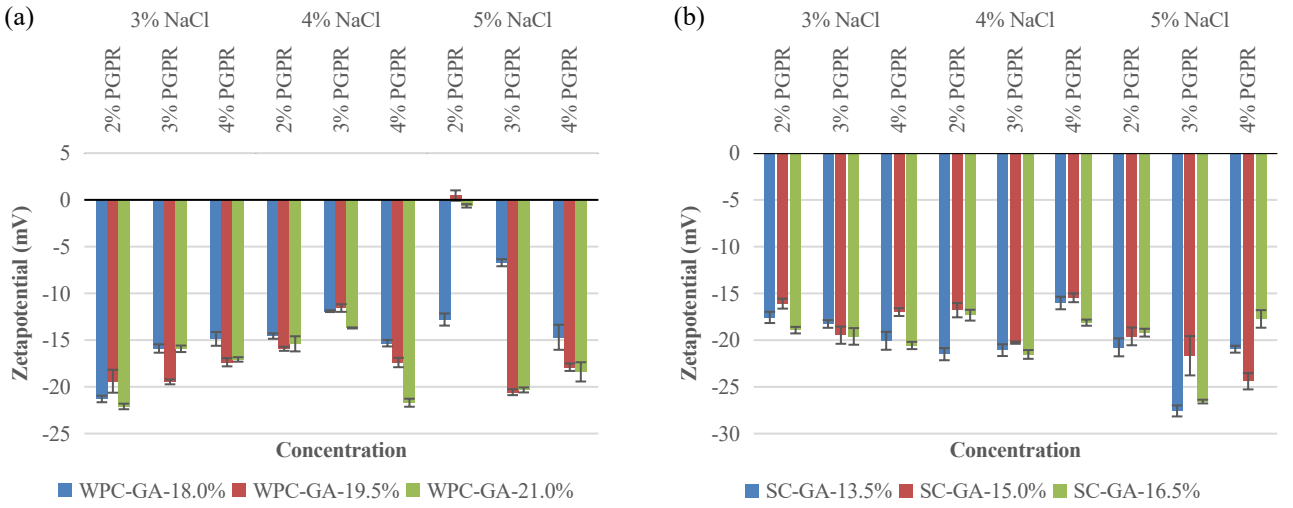

Figure 2. Effect of salt, PGPR and dairy protein-polysaccharide complexes [(a): WPC-GA complex, (b): SC-GA] on zeta potential of double emulsion-based matrix; n = 3

The ζ-potential of all the combinations of the variables were negative for all the emulsion formulations immediately after preparation, except in the double emulsion composed of 5% NaCl, 2% PGPR and 19.5% WPC-GA complex. WPC-GA stabilized double emulsion showed higher ζ-potential values in the range of -0.64 ± 0.18 mV to +0.46 ± 0.56 mV, than SC-GA stabilized double emulsion having ζ-potential values from -15.47 ± 0.47 mV to -27.57 ± 0.59 mV. The range was -16.03 to -27.57 mV at 13.5% SC-GA, which increased to -15.47 to -24.40 mV at 15.0%, but it again decreased to -17.33 to -26.57 mV at 16.5% SC-GA concentration (Figure 2b). WPC-GA stabilized double emulsion also showed the same trend of ζ-potential values as shown by SC-GA stabilized double emulsion with varying range of WPC-GA concentration (18-21%) in outer aqueous phase (W_2) as presented in Figure 2(a). From Figure 2(a), it can be seen that the ζ-potential values of WPC-GA stabilized double emulsion increased with increasing concentration of NaCl (3-5%) while it decreased with increasing concentration of PGPR (2-4%). However, the ζ-potential of double emulsion stabilized with SC-GA was found to increase up to 3% PGPR as well as 4% NaCl, after which it decreased at 4% PGPR and 5% NaCl concentrations (Figure 2b). It is evident from Appendix 2, that the zeta potential of double emulsion was very highly and significantly ($p < 0.001$) affected by the variables such as salt, PGPR, complexes (WPC-GA and SC-GA) and their interaction.

According to Mirhosseini et al. [29] an emulsion is considered as stable, if the zeta potential value is more than ± 25. Hence, ζ-potential is considered a key indicator for the stability of double emulsion (W/O/W) based functional mayonnaise because the stability is affected by surface charge (ζ-potential) of the emulsion droplets. ζ-potential values below 25 indicated flocculation, while above 25 mV they indicated deflocculating emulsions [30]. In the presence of anionic dairy protein-polysaccharide complex, the surface charge on the droplets of the double emulsion was negative except in the combination of emulsions constituted with 5% NaCl, 2% PGPR and 19.5% WPC-GA complex. This may be due to the electrostatic repulsion between the adjacent WPC-GA complex molecules, which decreased at higher NaCl concentration and consequently reduced the surface charge on the oil/water interface. The negative surface charge of the double emulsion based functional mayonnaise increased with increase in concentration of the complexes in the external aqueous phase (W_2) of the double emulsions ($W_1/O/W_2$). This can be attributed to the increase in electrostatic repulsion between the protein-polysaccharide complex molecules associated with their increased electrical charge, which prevented the aggregation and flocculation of the droplets. Gulseren and Corredig [31] evaluated that dairy proteins (β-lactoglobulin/ sodium caseinate) decline the interfacial tension at the soy oil-water interface in the presence of PGPR, without displacement of PGPR by protein particles. They concluded that the PGPR interacts with the hydrophobic moieties of the proteins, causing changes in the viscoelastic properties of the interface. Besides, the presence of proteins restricts the ability of PGPR to pack effectively at the interface.

Prichapan and Klinkesorn [32] reported that zeta potential was found higher in W/O/W emulsions prepared with sodium caseinate than those with WPC. Therefore, it had more electrostatic repulsive force between droplets which prevented droplet flocculation and coalescence. In presence of high NaCl concentration, the magnitude of ζ-potential decreased, probably due to the screening effect of electrically charged ions. This can be interpreted in terms of the counter-ions (Na^+) in inner aqueous phase, which accumulated loosely around the negatively charged -COO^- groups on the protein-polysaccharide complex surface due to the electrostatic interaction between the emulsion droplets and ion-binding effect. Low ionic (NaCl) concentration facilitated a strong electrostatic repulsion between the emulsion droplets, which prevented ions coming into close contact. However, once a critical ion (NaCl) concentration was reached, electrostatic repulsion was no longer sufficiently strong to overcome the attractive forces such as van der Wall and hydrophobic forces acting between the emulsion droplets, thus causing the emulsion droplets to aggregate [33]. As a result of NaCl addition, the negative surface charge (ζ-potential) of the double emulsions shifted towards zero (slightly positive). Raviadaran et al. [34] studied the effect of hypotonicity, isotonicity and hypertoxicity on the stability of $W_1/O/W_2$ multiple nanoemulsion by measuring the conductivity (concentration of NaCl) in external water phase of the nanoemulsion. They found that isotonic stabilized nanoemulsion gave the lowest change in mean droplet diameter, NaCl concentration and water content by 1.5%, 2.6% and 0.4%, respectively as compared to hypotonic and hypertonic stabilized nanoemulsion due to reduced water movement. These results support the finding of Liu et al. [35] and Onsaard et al. [36] that the magnitude of the electrical charge (ζ-potential) of emulsions prepared with SC-carboxymethylcellulose and WPC-maltodextrin/carrageenan was negative at all NaCl concentration range (0-500 mM). But the magnitude of surface charge decreased and the NaCl concentration increased due to reduction in the electrostatic repulsion between oil-water emulsion droplets.

3.4 *Turbidity*

The effect of the variables on the turbidity of the double emulsion based functional mayonnaise is shown in Figure 3. The differences in turbidity values of double emulsions stabilized with each of the three variables i.e., salt, PGPR and complexes (WPC-GA and SC-GA) were very highly significant ($p < 0.001$). Their interaction also showed a very high significant ($p < 0.001$) effect on turbidity values of the double emulsion (Appendix 3). SC-GA stabilized double emulsions showed a very highly significant ($p < 0.001$) lower mean turbidity values (3.70 ± 0.20 cm^{-1} to 4.57 ± 0.25 cm^{-1}) than those (7.51 ± 0.36 cm^{-1} to 8.97 ± 0.39 cm^{-1}) of WPC-GA stabilized double emulsions (Figure 3 a-b and Appendix 3).

There was a very highly significant increase in average turbidity values of double emulsions with increasing concentration of both WPC-GA (18-21%) and SC-GA (13.5-16.5%) complexes. The average turbidity values of SC-GA stabilized double emulsion decreased with increase in concentrations of PGPR as well as NaCl, while the mean turbidity values of WPC-GA stabilized double emulsion decreased up to 3% PGPR. After this, it increased at 4% PGPR concentration and also it increased with increasing concentration of NaCl (3-5%) (Figure 3 a-b).

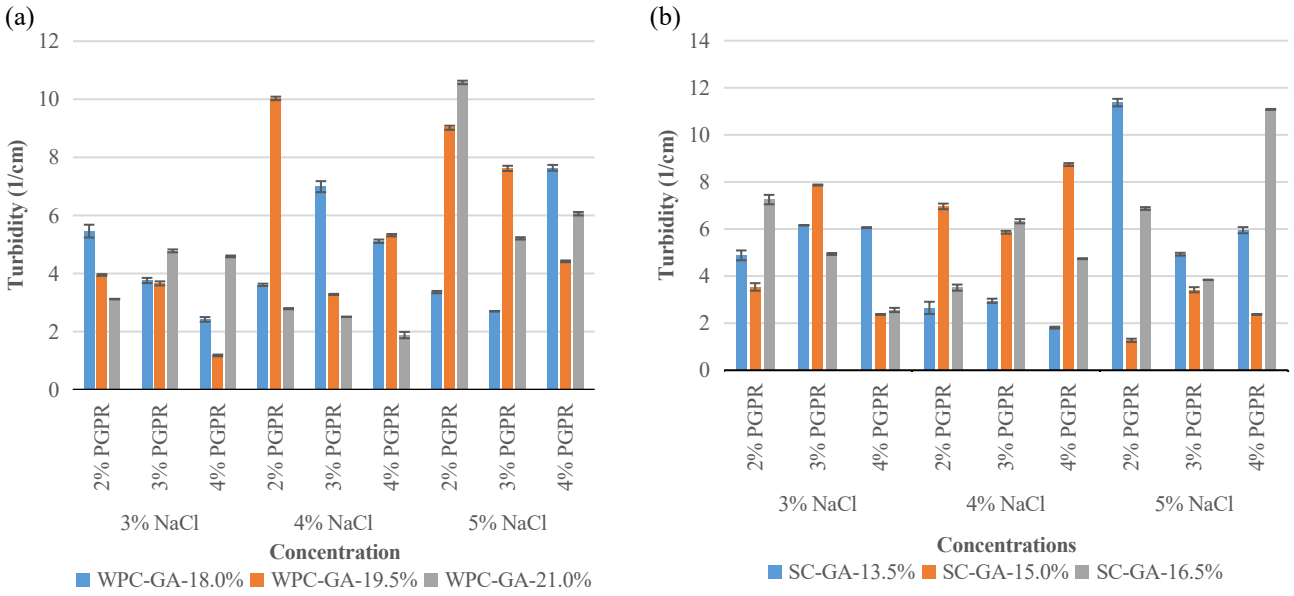

Figure 3. Effect of salt, PGPR and dairy protein-polysaccharide complexes [(a): WPC-GA complex, (b): SC-GA] on turbidity measurements of double emulsion-based matrix; n = 3

Protein-polysaccharide complexes enhance emulsifying and functional properties of the double emulsion in comparison to the proteins and polysaccharides alone, probably due to the simultaneous presence of the two biopolymers as well as the complexes' structure. Several factors were reported to influence the emulsifying properties of protein-polysaccharide complexes such as protein-to-polysaccharide molar mass ratio, their total concentration, polymer charge density, pH, ionic strength, temperature and method of mixing [37-38].

Frentcel et al. [39] studied the stability of water-in-paraffinic oil (W/O) emulsion using turbidity measurement and has found most suitable for determination of the required HLB, amount and type of emulsifier, and the inner water phase fraction. This method correlates the particle size distribution and turbidity of the colloidal systems [40-42]. Emulsion activity (EAI) and emulsion stability (ESI) of double emulsion can be enhanced by covering the emulsion droplets by surfactants. The biopolymers form a bulky and flexible film that may be strongly anchored on the O/W interface [37, 43-44]. Double emulsion- based functional mayonnaise exhibited higher optical turbidity with increasing concentration of the dairy protein-polysaccharide complexes, probably because increasing the concentration of the complexes led to higher viscosity of the complexes by inducing strong electrostatic interaction. Weinbreck et al. [45] reported that Whey protein-GA had maximum viscosity at pH 4.0 due to decoupling the effect of biopolymer concentration and electrostatic interactions, as more Whey protein was bound with the GA. Turbidity of the samples decreased with increasing the NaCl concentration. Weinbreck et al. [46] observed that the Whey protein-GA complex coacervates got shrunk and ionic strength increased from 0-100 mM, may be because the micro-ions led to screening of the charges of the polymers. Su et al. [47] reported that the ionic environment reduced the emulsifying ability of the hydrophobic emulsifier (PGPR) from 4 wt% PGPR in a buffered system to 2 wt% PGPR in distilled water systems and with an encapsulation efficiency > 90% of W/O/W emulsions prepared with SUPER GUM™.

3.5 *Sedimentation stability*

The double emulsion based functional mayonnaise samples were kept at 37 ± 1°C and 7 ± 1°C and observed daily for phase separation. It was observed that the sedimentation stability (by visual separation) is a function of storage temperature. The double emulsions stabilized with SC-GA showed higher sedimentation stability (by visual observation) as compared to WPC-GA stabilized emulsions at both temperatures (7°C and 37°C). They were stable up to 10 days and 119 days of the emulsions preparation at 37°C and 7°C, respectively, whereas in samples in which WPC-GA was used as a secondary surfactant destabilized on the same day or after one day (37°C) and eleven days (7°C) of

preparation. It could be seen from Figure 4 (a, c) that the WPC-GA complex stabilized double emulsions with varying ranges of all the variables (salt, PGPR, and WPC:GA complex) were destabilized on 1st day or 2nd day of the preparation. The sedimentation stability increased with increasing NaCl concentration as well as SC-GA complex concentration while it decreased with increase in PGPR concentration (Figure 4b, d). It is evident from Appendix 4 (a, b), that the sedimentation stability of double emulsion was very highly and significantly affected by the variables such as salt, PGPR, complexes (SC-GA and WPC-GA) and their interactions.

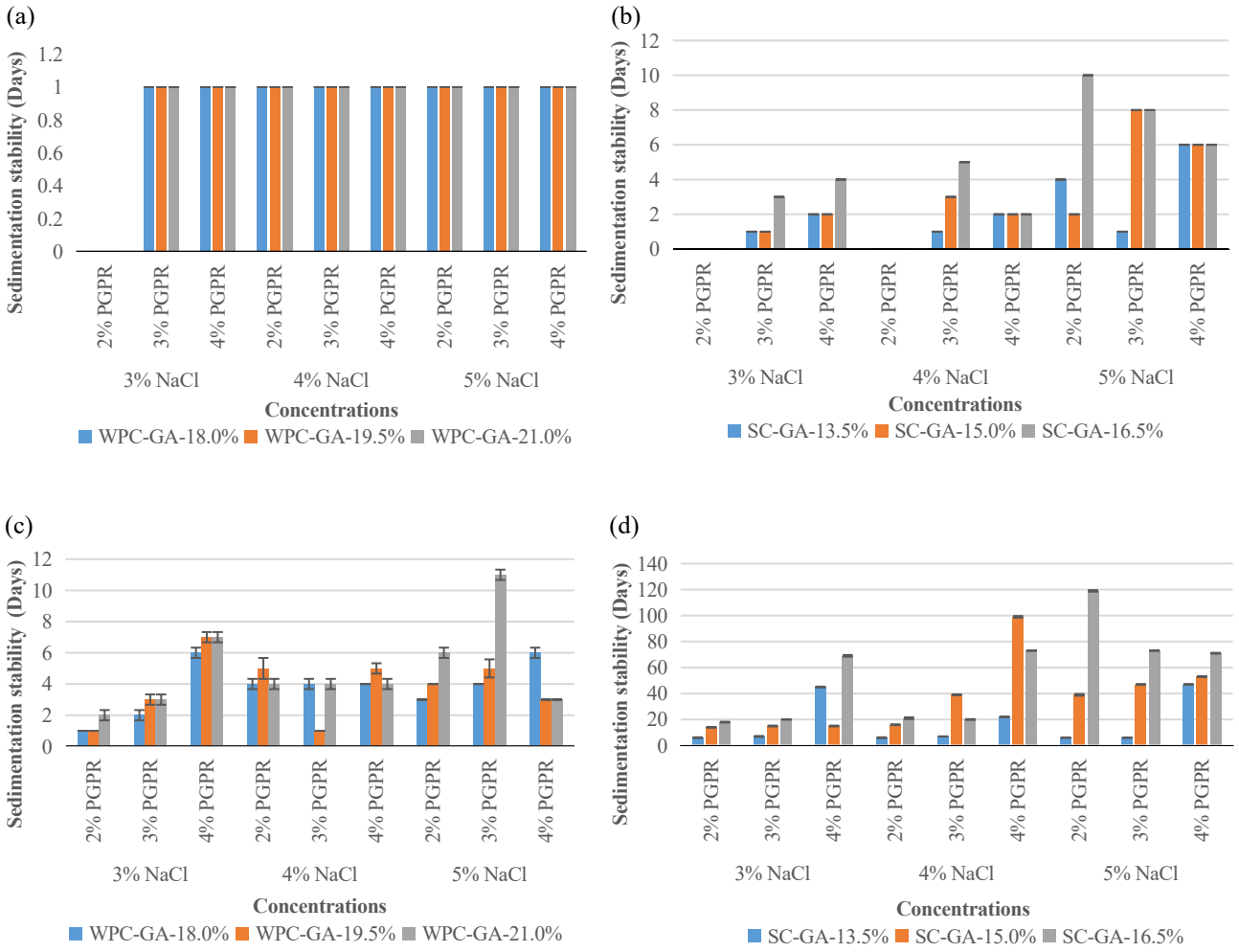

Figure 4. Effect of salt, PGPR and dairy protein-polysaccharide complexes [(a): WPC-GA, (b): SC-GA at 37°C], [(c): WPC-GA, (d): SC-GA at 7°C] on sedimentation stability of double emulsion-based matrix; n = 3

Sedimentation stability in terms of creaming depends on the various parameters such as viscosity, density differences between phases and radii of droplets [44]. Lutz et al. [25] observed no coalescence in double emulsions stabilized with soluble WPI/modified pectin complex for almost one month because oil droplets were fully covered by external oil-water interfacial (O/W_2) film comprising of the WPI/modified pectin. The soluble complexes of WPI/pectin also reduced the interfacial tension on surface of the emulsion droplets. Further they reported that the level of creaming also depended on the NaCl concentration (1.0, 1.5 and 4.4%, w/w) in the inner aqueous phase. Lutz et al. [15] claimed that the stability of the double emulsions was affected by various conditions *viz*., pH, WPI/pectin ratio, weight ratio and pectin type. They found a most stable double emulsion at pH 6.0 with small droplet size (15 µm), low creaming, high yield and minimum water transport because at this pH, WPI and pectin formed a negatively charged complex which

acted as a good emulsifier and enhanced emulsion stability by adding repulsion forces between the emulsion droplets. Salminen and Weiss [20] studied the electrostatic adsorption of heat-treated biopolymer complexes [Whey protein isolate (WPI)-apple pectin at 85°C for 20 min] and stability of theses complexes on emulsion interfaces. They found that the emulsions covered with the complexes showed good stability to NaCl (up to 200 mM) and heat (up to 90°C) by creating a thick polymer layer. Muschiolik et al. [48] investigated the effect of addition of NaCl in inner aqueous phase of multiple emulsions prepared with 4% PGPR during storage. They found that presence of the NaCl in the inner aqueous phase (W_1) of multiple emulsions is essential to carry out coalescence free W/O emulsions prepared with PGPR. Addition of NaCl increased the encapsulation stability and oil droplet stability of multiple emulsions. Landfester [10] reported that PGPR facilitates droplet break-up by reducing the interfacial tension, and prevents coalescence of newly formed water droplets via the Gibbs-Marangoni effect (Walstra) and by steric stabilization. Diffusional degradation remains the destabilizing mechanism, which has to be precluded by osmotic pressure regulation. Bahtz et al. [9] reported that PGPR used as an oil-soluble surfactant was able to spontaneously form tiny water droplets in oil phase that brought structural changes in the oil layer. Further, they described that the overall water transport rate increases and water transport stagnates because of maximized structure formation at below and above a critical concentration of the oil-soluble surfactant, respectively.

4. Conclusions

The study of the factors influencing the formulation of bitter gourd encapsulated double emulsion matrix established the appropriate concentrations of NaCl, PGPR and dairy protein-polysaccharide complexes for ensuring high stability of the double emulsion matrix. Our results revealed that WPC-GA complex stabilized double emulsions were destabilized immediately after preparation or after one day of preparation, while SC-GA stabilized double emulsions were more stable ($p < 0.001$). Since apparent viscosity, zeta potential, sedimentation stability and turbidity are the limiting factors, the main aim of this factorial design was to optimize a formulation with a maximum stability of the emulsions for the incorporation of aqueous bitter gourd extract. Thus, from the obtained results, the double emulsion containing 55.2%, bitter gourd extract, 5% NaCl, 2% PGPR and 16.5% SC-GA complex showed very highly significant ($p < 0.001$) stability as compared to other combinations.

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Declaration of competing interest

On behalf of both authors, the corresponding author states that there is no conflict of interest.

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