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Encapsulation of Vitamins E and C in Whey Protein Concentrate-Chitosan Emulation: Physicochemical Properties

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Abstract

This study investigated the use of different ratios of whey protein concentrate (WPC) and chitosan (Cs) in encapsulating vitamins C and E. The aim of this research is the stability of vitamins C and E in environmental conditions. In this study, experimentally, the effect of different amounts of WPC and Cs on the chemical and physical properties of samples containing vitamin C and E is evaluated. The chemical and physical structures of the emulsions including (apparent viscosity, zeta potential, particle size, emulsion stability at pH 4.3 and 6.3 and the encapsulation efficiency of vitamins C and E) were investigated. The data was analyzed using Design Expert software. The main finding this study, the particle size, zeta potential, viscosity, emulsion stability in pH 4.3 and 6.3, and encapsulation efficiency of vitamins E and C varied from 255 to 595 nm, +20.11 to 31.77 mV, and 28.45 to 45.67 cP, 90 to 100% and 60 to 90%, and 93.83 to 93.36% and 93.60 to 77.33% respectively($p<0.05$). In general, based on the results obtained from the response level, treatment 9 (WPC: Cs, 2: 0.25) at pH 4.3 had the lowest viscosity, particle size, and the highest particle size and encapsulation efficiency.

Keywords: *Encapsulation, Emulsion, whey protein concentration, chitosan*.

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1. Introduction

Vitamin E (α-tocopherol), a fat-soluble antioxidant, plays an essential role in the human body (Budinčić *et al*., 2021; H. Chen *et al*., 2020). Nevertheless, Vitamin E similar to other lipophilic nutraceuticals has low solubility in water and is biologically unstable when exposed to harsh environmental conditions like oxygen, temperature, and light (H. Chen *et al*., 2020; Öztürk, 2017).

Vitamin C or ascorbic acid (AA) is water-soluble (Katouzian & Jafari, 2016). It is broadly applied in foods as an antioxidant agent and vitamin supplement. Due to neutralizing the free radicals, vitamin C can decrease the cancer risk (Katouzian & Jafari, 2016; Kheynoor *et al*., 2018). However, it is sensitive to oxygen, heat, light, moisture, and air, limiting its applications.

Encapsulation is an approach that has been widely used to stabilize nutraceuticals against harsh conditions (Ghobadi *et al*., 2021). Encapsulation can either reduce the oxidation rate of bioactive components or reduce their sensitivity to light and oxygen (X. Liu *et al.,* 2020). Encapsulation also supports the entrapment of hydrophobic compounds which are insoluble in aqueous phases (Hassane Hamadou *et al.*, 2023). Carriers from proteins and polysaccharides have been predominantly used to embed the bioactive components (Khan *et al.,* 2019; Wusigale *et al.,* 2020; Yousefi *et al.*, 2023). Proteins are incorporated into the encapsulation system mainly due to their strong interfacial properties. They can act as emulsifiers and reduce the interfacial tension to produce more stable emulsion or delivery systems (X. Chen *et al.,* 2018; Soleimanifard *et al.,* 2021). Polysaccharides are used in delivery systems mainly due to their stabilizing and thickening effects, leading to more thermodynamically stable emulsion systems (H. Chen *et al.,* 2020; Ge *et al.*, 2018).

Chitosan (CS) is a polysaccharide with cationic charge that can be highly considered in the pharmaceutical and food industry applications owing to its specific attributes such as nontoxicity, biocompatibility, biodegradability, and productibility from an economical and abundant source (Rajabi *et al.,* 2019). When CS is subjected to the acidic media, its amine groups are ionized, leading to water solubility and positively charged. (Rutz *et al.,* 2017; Yousefi *et al.*, 2023).

Whey protein concentrate (WPC) is a natural amphiphilic biopolymer, derived from milk processing (Jamshidi *et al.,* 2018). It can act as a hydrophilic emulsifier and widely used to stabilize the bioactive components (Assadpour & Jafari, 2017; Mohammadi *et al.,* 2016; Santana *et al.,* 2016).

The complexes containing proteins and polysaccharides provide efficient entrapment of bioactive cores through the electrostatic interactions, maintain the nutraceuticals against the harsh conditions (Khan *et al.,* 2019; Niu *et al.,* 2023). The polyelectrolyte complexes which are dependent on electrostatic interactions have shown trigger-controlled release mechanisms predominantly based on the pH alterations (Q. Liu *et al.,* 2022).

According to the literature, WPI and CS have been used to produce polyelectrolyte nanoparticles-loaded doxorubicin as a positively charged chemotherapeutic agent, indicating improved encapsulation efficiency, enhanced stability, controlled release, and biodegradability compared to whey protein or CS encapsulation alone (Yadollahi *et al.,* 2023).

Co-encapsulation of hydrophilic and hydrophobic compounds can extend their applications (X. Chen *et al.,* 2018; X. Liu *et al.,* 2020). Vitamin C and vitamin E are intrinsically hydrophilic and hydrophobic, respectively. When they are encapsulated simultaneously, it would be possible to incorporate them in aqueous and/or fatty matrixes, exerting antioxidant effects in both hydrophobic and hydrophilic media (X. Chen *et al.,* 2018; Rashid *et al.,* 2022).

The objective of the present study was the encapsulation of vitamins E and C using the combination of chitosan and whey protein concentrate. Regarding the liquid complexes, particle size, zeta potential, system stability, viscosity and encapsulation efficiency were determined. chemical and morphological analysis were determined.

2. METHODOLOGY

Materials

Vitamin E (α-Tocopherol) with a purity of 96% (w/w), vitamin C (L-Ascorbic acid) with a purity of 99% (w/w), and medium molecular weight (MW) CS (deacetylation degree of 85%) were obtained from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). WPC 80% protein (w/w) was purchased from Fonterra Co., Ltd. (Auckland, Australia). All other reagents were of analytical grade and obtained from Merck Co., Ltd. (Darmstadt, Germany).

Preparation of CS-WPC complex loaded with vitamin E and vitamin C

Preparation of vitamin E/WPC aqueous solution

WPC aqueous solutions (2, 3 and 4% w/w) were prepared using magnetic stirring for 2 h at 1000 rpm, followed by homogenization at 14000 for 3 min using Ultra-Turrax (T 25 digital ULTRA-TURRAX®, IKA, Germany). A mixture of vitamin E (0.16%, w/w), sunflower oil (4%, w/w), and tween 80 (1%, w/w) was prepared using the homogenization procedure mentioned above.

Preparation of vitamin C/CS aqueous solution

CS aqueous solutions (0.25, 0.375, and 0.5%, w/w) were prepared by dissolving an appropriate amount of CS into 1% (w/w) acetic acid aqueous solution under magnetic stirring for 10 min at 1000 rpm and homogenized using Ultra-Turrax at 14000 rpm for 3 min. An appropriate amount of vitamin C (0.9 %, w/w) was added into the CS aqueous solutions, and homogenized as mentioned (Luo *et al.*, 2011; Wang *et al.*, 2016).

Final complex emulsion formation

The complex between the biopolymers was formed by dropwise addition of vitamin C/CS aqueous solution into the vitamin E/WPC aqueous solution while the pH was adjusted to 4.3 and 6.3 followed by homogenization at 14000 rpm for 2 min (Wang *et al.*,2016). The fresh complex emulsions were assessed for stability in pH 4.3 and 6.3, then measure the zeta potential, particle size, viscosity and encapsulation efficiency in 4.3.

Characteristics of complex emulsions

Emulsion stability

The emulsions' stability was determined using the accelerated method by centrifugation at 20000 g, 30 min, and 25 °C. For this purpose, 50 ml of each emulsions was centrifuged. Then remained stable complex was assessed using a graduated cylinder. Finally, the stability of the emulsion was calculated using Equation (1) (Ghasemi *et al.*, 2017).

Emulsion stability (%) = (The volume of emulsion remained stable)/The volume of primary emulsion)×100

Particle size and zeta potential

The particle size and zeta potential of produced complex emulsions were determined using dynamic light scattering (DLS) (Zeta sizer, Malvern Instruments, USA) (Ghasemi *et al.*, 2018). The samples were diluted 10-fold using distilled water and then particle size and zeta potential analysis were performed at 25 ℃

Viscosity

The viscosity of samples was measured according to Ghasemi *et al.* (2017). Briefly, 16 mL of produced complex emulsions were transferred into a cylinder, followed by determination of viscosity using a Brookfield viscometer (LVDV Pro II, Brook-field Engineering Laboratories, spindle S00, USA) at the ambient temperature, and the shear rate of 18.3 1/s.

Encapsulation efficiency

The encapsulation efficiency (EE) of vitamin C was determined according to Desai, Liu & Park's method (2006) with a slight modification. First, 100 ml sample was centrifuged at 4000×g for 30 min (Hettic, Universal 320, USA). The transparent part was passed through a 0.2 µm filter (Millipore, USA)and extracted by HCl (0.1 N). The vitamin C content was analyzed by measuring the absorbance at 244 nm (ʎmax of vitamin C in HCL 0.1 N) after appropriate dilution using a UV spectrophotometer (Perkin Elmer Lambda-2 UV–Visible Spectrophotometer, Germany). For the EE was calculated using the equation:

$$EE\ (\%) = \frac{Total\ Vitamin\ C - free\ Vitamin\ C}{Total\ Vitamin\ C} \times 100 \quad (2)$$

where Total Vitamin C indicate Theoretical vitamin C concentration in the emulsion and free vitamin C was obtained using a membrane separation method. (Desai, Liu & Park., 2006).

The EE of vitamin E was calculated according to Luo et al. (2011). 100 ml sample was centrifuged at 4000×g for 30 min (Hettic, Universal 320, USA). The transparent part was passed through a 0.2 µm filter and extracted by hexane. Then, it was analyzed by measuring the absorbance at 290 nm with a UV/Vis spectrophotometer described in previous studies. Vitamin E was calculated by an appropriate calibration curve of free tocopherol in hexane (Luo et al., 2011).

All measurements were performed in three replicates.

The EE was computed using the equation below:

$$EE\ (\%) = \frac{Total\ Vitamin\ E - free\ Vitamin\ E}{Total\ Vitamin\ E} \times 100 \quad (3)$$

Statistical analysis

Response surface methodology (*RSM*) was employed to investigate the effects of independent variables including

WPC and CS concentrations on particle size, zeta potential, emulsion stability, viscosity, and EE of produced complex emulsions as dependent variables or response factors. The concentration of WPC ranged between 2 to 4% w/w while concentrations of CS were in the range of 0.25 to 0.5% w/w. All experiments were performed at three replications and reported as means and standard deviations (*SD*). The mean difference was also carried out using the Duncan test by employing *SPSS* version 22 at the % confidence level of 95% ($p<0.05$).

1. Findings

Characteristics of emulsions

Emulsion stability at pH=4.3 and at pH=6.3

The relationship between emulsion stability and the independent stability variable is shown two by two at the central point of the third variable in Figure 1. In Figure (1-a), with the increase of WPC concentration from (2 to 4) and rise of CS concentration from (0.25 to 0.5) at pH 4.3, the stability decreases from 100% to 90%, and in Figure (1-b) at pH 6.3. Stability reduced from 90% to 60%.

Particle size and zeta potential

The results of particle size and zeta potential measurement are shown in Table 2. Based on the particle size model, the linear effects of WPC and Cs concentration on the average particle size and zeta potential were significant at the 95% confidence level ($P\geq0.05$). According to the sum of squares, it can be concluded that the WPC concentration had the greatest effect on the average particle size and zeta potential($p<0.05$). The surface plots of WPC and CS concentration effect on the particle size and zeta potential have been shown in Figure (4-a and 4-b).

Viscosity

According to the data in Table 1. WPC concentration significantly affected apparent viscosity ($P<0.05$). The relationship between the apparent viscosity and the independent variables of the extraction in two pairs in Figure (3) shows that the apparent viscosity increases with the increase concentration of WPC and chitosan. This increase in viscosity occurred more steeply with increasing WPC concentration.

Encapsulation efficiency

The surface plots of WPC and CS concentration effect on the encapsulation efficiency measurement have been shown in Figure 4. The results revealed that the encapsulation efficiency of vitamin C was significantly affected by WPC and CS concentration ($P\leq0.05$), while WPC and CS concentration had no significant effect on the encapsulation efficiency of vitamin E.

3. Discussion

Characteristics of emulsions

Emulsion stability at pH=4.3 and at pH=6.3

The results showed that at pH equal to 4.3, the emulsion stability ranged from 90 to 100 %, and the emulsion stability ranged from 60 to 90 % at pH equal to 6.3 (Figures (1-a & 1-b)). The interaction between protein and polysaccharide in the liquid medium can lead to the formation of a soluble complex or insoluble between two polymers. It depends on the pH, the molar ratio between the two polymers, and the ionic strength of the medium(Weinbreck *et al.*, 2004; Ye, A., 2008). The soluble complex forms when the number of charges in the complex is not equal, and as a result, the charged network causes more solubility of the complex. On the other hand, when the opposite charges of two polymers neutralize each other, an insoluble complex is created and leads to aggregation and co-precipitation (Weinbreck et al., 2004; Ye, 2008). Researches indicate the WPC-chitosan complex is formed in the pH range of 5.4 to 5.7 (El-Sayed *et al.*, 2015). Table 2 shows that the emulsion stability at pH equal to 4.3 is more stable than at pH equal to 6.3. The reason can be the steric repulsion between the positive charges of chitosan and whey protein concentrate at a pH equal to 4.3. Ghasemi *et al.* (2018) showed that maximum (98.5 %) and minimum (83 %) stability were achieved for (WPC= 4 wt%, pectin =1 wt% and pH= 9) and (WPC =4 %, pectin=1 % and pH= 3), respectively. Hosseini *et al.* (2015) stated that polysaccharides are often introduced to increase the stability of protein-based aqueous delivery systems, and they can interact with proteins electrostatically. Therefore, the electrostatic interactions depend on their ionic strength, pH, and charge properties (Hosseini *et al.*, 2015).

Particle size and zeta potential

It can be seen in Figure (2-a & 2-b) that by increasing the concentration of WPC and CS, the particle size increases, and the zeta potential decreases. Although the previous studies focused on the effect of chitosan on the zeta potential and stability of emulsions, the simultaneous effect of WPC and chitosan on the zeta potential of emulsions is not

investigated. The results of the present study revealed that the chitosan and WPC concentrations significantly affected the zeta potential of emulsions (P≤0.05). In general, a higher zeta potential is associated with smaller particle sizes and greater emulsion stability. This proposes that there might be an inverse correlation between zeta potential and particle size in emulsions (Wang, C. et al., 2022). Therefore, sample 9, which had the highest zeta potential, had the lowest particle size (259 nm).

Hu *et al.* (2020) showed that chitosan addition improved the positive zeta potential, especially when WPF-18 was combined with curcumin (at pH 3.5), and chitosan had the maximum potential (~54.6 mV). It indicates relatively stable dispersion under acidic conditions regions on its surface (Hu *et al.*, 2020). Wang *et al.* (2021) reported anthocyanins (ACNs) were encapsulated in nano-complexes with chitosan hydrochloride (CHC), carboxymethyl chitosan (CMC), and whey protein isolate (WPI). The ACN-loaded CHC/CMC-WPI nano-complexes (ACN-CHC/CMC-WPI) showed a preferred particle size (332.20 nm) and zeta potential (+23.65 mV) (Wang, S. *et al.*, 2021). De Queiroz *et al.* (2018) demonstrated that the trypsin inhibitor isolates encapsulated in Chitosan-whey protein. The values obtained for the EWPI (encapsulated in whey protein isolate), ECH (encapsulated in chitosan), and ECW (encapsulated in chitosan and whey protein isolate) (at pH=7.5) were +0.3 mV, +23.31 mV, and -38.66 mV, respectively (De Queiroz *et al.*, 2018)

Viscosity

Figure (3) shows that the emulsion viscosity ranges from 28.45 to 45.67 cP. It was generally owing to the biopolymers' concentration and molecular weight in the solutions. The low molecular weight biopolymers were used to limit the biopolymers' chain entanglement and consequently acquire non-agglomerated particles. The statistical analysis revealed the significant effect of WPC and CS concentration on the viscosity of the emulsions. It was increased with increasing WPC and CS concentration (P≤0.05). Ghasemi *et al.* (2018)showed the minimum and maximum viscosities, which were associated with values of acidic pH with different proportions of WPC-pectin complex, had the highest and lowest viscosity equal to 35 and 16 mPa.s (Ghasemi *et al.*, 2018).

Encapsulation efficiency

The results revealed that the encapsulation efficiency of vitamin C was significantly affected by WPC and CS concentration (P≤0.05), while WPC and CS concentration had no significant effect on the encapsulation efficiency of vitamin E. It can be seen in Figure (٤-a & 4-b) that by increasing the concentration of WPC and CS, the encapsulation efficiency of vitamin C and Vitamin E decrease. Previous studies reported that the wall material type used in encapsulation significantly affects the encapsulation efficiency (Ramakrishnan et al., 2018; Zahran *et al*., 2022). Hinnenkamp *et al.* (2021) was found whey proteins as wall material in the encapsulation process form intermolecular voids in the microcapsule wall due to their size and globular nature which cause a reduction in encapsulation efficiency (Hinnenkamp *et al.*, 2021). Also, previous studies demonstrated that the chitosan concentration could have different effect on the encapsulation efficiency of different substances (Alishahi, 2014; Li *et al.*, 2022). However, the pH of emulsions could be affected the relationship between chitosan concentration and encapsulation efficiency (Laila & Pudjiraharti, 2019).

The high encapsulation efficiency could be obtained when the surface material is in the lower content. Also, better encapsulation efficiency will be achieved when the ratio of the core-to-wall material is high. Barbosa *et al.* (2005) reported that the ideal core-to-wall materials ratio is between 2:1 and 4:1. The ratio below 2 could result in a rise in the surface material content, while a ratio equal to 4 could result in the creation of low-content powders (Barbosa *et al.*, 2005). The vitamins-to-wall material ratio applied in sample 9 was approximately 2:1. It may be attributed to the high encapsulation. In a similar study, Wang et al. (2021) showed that the CAN (Anthocyanin)-CHC (Chitosan hydrochloride) / CMC (Carboxymethyl chitosan)-WPI (whey protein isolate) nano-complexes had a maximum efficiency of encapsulation (efficiency 60.70 %) than the ACN-CHC/CMC (49.20 %), probably since the WPI addition outcomes in a structure of complex double layer embedded (Wang, S. *et al.*, 2021).

In the other study, De Queiroz *et al.* (2018) reported the ECW (encapsulated in CS and WPC isolate) exhibited excellent incorporation efficiency (98.5 %), also those encapsulated with individual chitosan and isolated protein exhibited 98.53% and 97.34 %, respectively (De Queiroz *et al.*, 2018). Lekshmi et al. (2019) reported the efficiency of encapsulation of squalene encapsulated with WPC and chitosan was 75.40±0.22 % (Lekshmi *et al.*, 2019).

4. Conclusion

This study evaluated the utilization of WPC/CS combinations as wall materials for vitamin C and E encapsulation. The WPC: CS ratio equal to 2:0.25 performed the minimum emulsion viscosity, particle size, maximum emulsion stability at 4.3 pH and zeta potential, and encapsulation efficiency of encapsulated vitamins. The results confirmed that vitamin C and E encapsulation in emulsion CS-WPC is a potential process for producing stable encapsulates for functional food development.

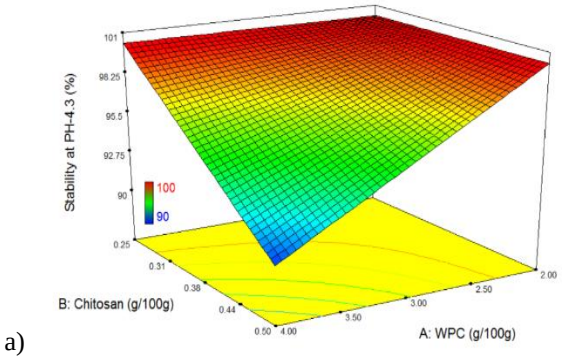
Table 1. Orthogonal array based on central composite design.

No.	Cs%	WPC%	Mixing 1:1 Cs and WPC
1	1	8	**0.5:4**
2	0.75	8	**0375:4**
3	0.5	8	**0.25:4**
4	1	6	**0.5:3**
5	0.75	6	**0.375:3**
6	0.5	6	**0.25:3**
7	1	4	**0.5:2**
8	0.75	4	**0.375:2**
9	0.5	4	**0.25:2**

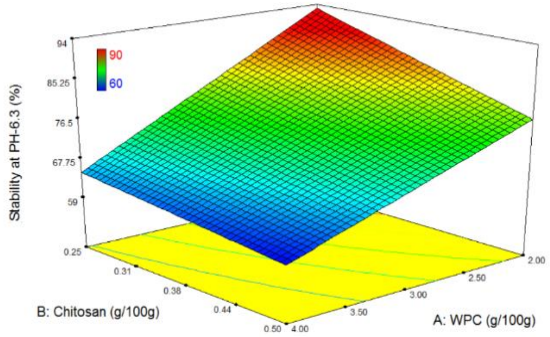
Table 2. Effect of WPC and CS concentration on emulsions properties

No.	Particle size(nm)	Z-Potential (mv)	Viscosity (cP)	Stability in pH 4.3 (%)	Stability in pH 6.3 (%)	EE% Vitamin E	EE% Vitamin C
1	595±0.57^{a}	20.11±0.82^{g}	45.67±0.06^{a}	90±2.51^{b}	60±1.15^{f}	93.43±0.70^{a}	77.33±0.70^{i}
2	569±2.33^{b}	20.55±0.57^{g}	43.25±0.03^{b}	95±1.15^{b}	60±1.15^{f}	93.36±0.06^{a}	85.6±0.06^{f}
3	545±2.30^{c}	23.12±0.66^{e}	41.76±0.03^{c}	100±0.00^{a}	65±0.01^{e}	93.76±0.33^{a}	91.13±0.33^{c}
4	498±1.05^{d}	22.52±0.11^{f}	39.55±0.26^{g}	95±1.15^{b}	70±3.60^{d}	93.73±0.30^{a}	79.26±0.30^{h}
5	342±1.45^{f}	24.33±0.08^{d}	37.62±0.13^{h}	100±0.00^{a}	75±1.00^{c}	100.00±0.00^{a}	88.3±0.26^{e}
6	255±2.57^{h}	31.14±0.37^{a}	35.83±0.11^{i}	100±0.00^{a}	80±2.00^{b}	100.00±0.00^{a}	92.4±0.27^{b}
7	421±1.66^{e}	25.68±0.03^{b}	34.39±0.18^{d}	100±0.00^{a}	75±1.73^{c}	100.00±0.00^{a}	81.63±0.31^{g}
	289±0.33^{g}	28.4±0.17^{b}	31.93±0.13^{e}	100±0.00^{a}	90±1.00^{a}	100.0±0.00^{a}	90.33±0.46^{d}
	259±2.02^{h}	31.77±0.14^{a}	28.45±0.31^{f}	100±0.00^{a}	90±0.01^{a}	100.00±0.00^{a}	93.6±0.53^{a}

Different letters in each column show a significant statistically difference (P<0.05)

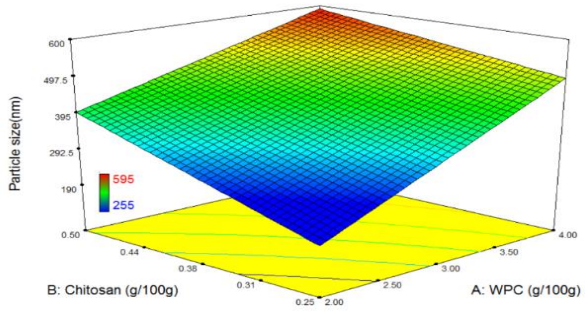


a)

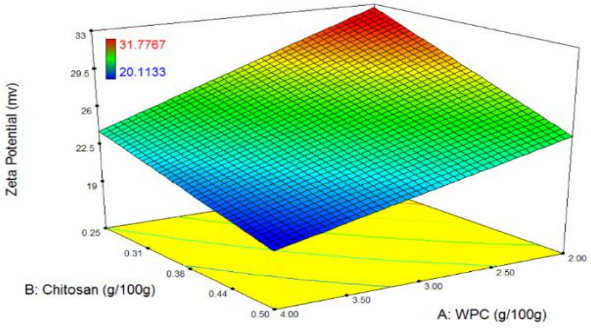


b)

Fig 1. Surface plots of the effect of WPC and CS concentration on stability in pH 4.3(1-a)and 6.3(1-b) of emulsions



a)



b)

Fig 2. Surface plots of the effect of WPC and CS concentration on particle size (2-a)and potential zeta(2-b) of emulsions

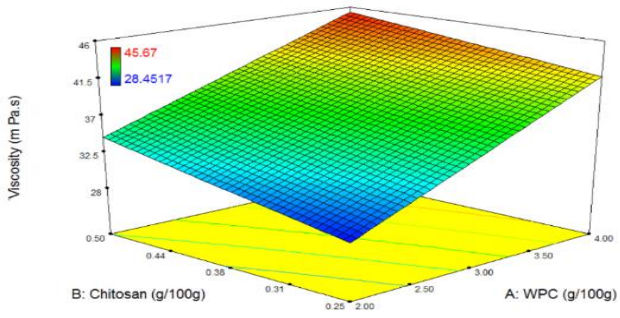
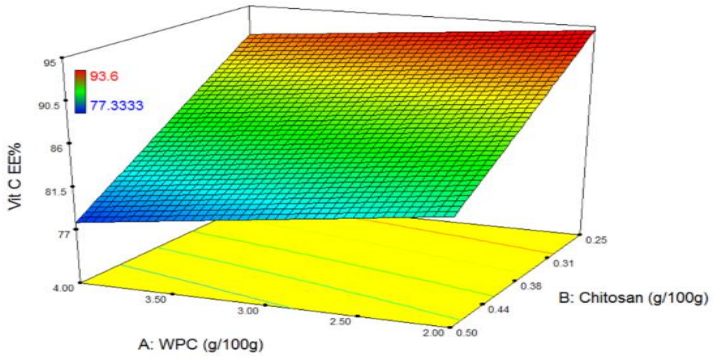
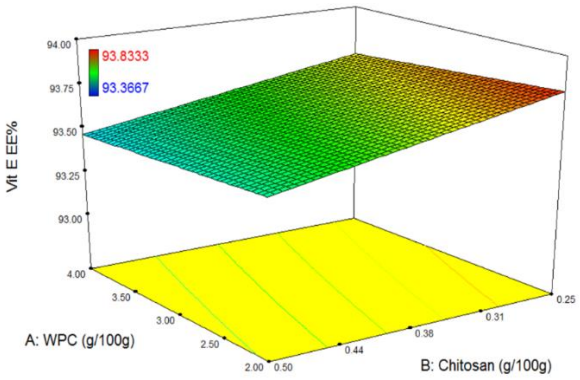


Fig 3. Surface plots of the effect of WPC and CS concentration on viscosity of emulsions



a)



b)

Fig 4. Surface plots of the effect of WPC and CS concentration on EE% Vitamin C (4-a) and Vitamin E (4-b) of emulsions

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