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Nano-encapsulation of olive leaf phenolic compounds through WPC–pectin complexes and evaluating their release rate

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ABSTRACT

In this study, W/O micro-emulsions as primary emulsions and a complex of whey protein concentrate (WPC) and pectin in the external aqueous phase were used to produce W/O/W emulsions. Average droplet size of primary W/O emulsion and multiple emulsions stabilized by WPC or WPC–pectin after one day of production was 6.16, 675.7 and 1443 nm, respectively, which achieved to 22.97, 347.7 and, 1992.4 nm after 20 days storage without any sedimentation. The encapsulation efficiency of phenolic compounds for stabilized W/O/W emulsions with WPC and WPC–pectin were 93.34% and 96.64%, respectively, which was decreased to 72.73% and 88.81% at 20th storage day. The lowest release of phenolics observed in multiple emulsions of WPC–pectin. These results suggest that nano-encapsulation of olive leaf extract within inner aqueous phase of W/O/W emulsions was successful, and there could be a high potential for the application of olive leaf extract in fortification of food products.

1. Introduction

Encapsulation of bioactive compounds by protecting and controlling their release can improve their functional properties such as antioxidant activities. Water-in-oil-in-water ($W_1/O/W_2$) multiple emulsions have been proposed as a major method for encapsulation of water soluble bioactive compounds [1–3,37]. W/O/W emulsions have many applications in the food industry, such as reducing fat content, coating flavor-producing compounds, controlling and targeting release and protection of bioactive hydrophilic materials [4–7,38]. W/O/W emulsions are consisted of W/O and O/W emulsions and because there are two interfaces, thermodynamic instability and release of the inner aqueous phase compounds into the outer aqueous phase are the main barriers for practical usage of these emulsions. The characteristics of primary W/O emulsion (dispersed phase) and secondary emulsion are very important in terms of the stability and release of multiple emulsions [8,9,39,40].

Selection of a stable W/O primary emulsion can increase the stability of W/O/W multiple emulsions. Increased antioxidant properties of alpha-tocopherol, flavanol, and polyphenols of green tea; increased stability of beta carotene against oxidation, and bio beta-carotene activity; increased lycopene solubility and its resistance against light; increased lutein and phytosterols' solubility, are among the modern applications of nano-emulsions in the food industry [10]. Also, Faridi et al. [3] used nano-emulsions as a primary emulsion in W/O/W encapsulation of saffron bioactive components.

Emulsification of W/O emulsions in an aqueous phase containing hydrophilic emulsifiers results in the production of the W/O/W multiple emulsions and as a consequence, the release rate of inner aqueous phase compounds is controlled. Biopolymers such as milk proteins, gums and polysaccharides can be used as a hydrophilic emulsifier in the external aqueous phase of W/O/W emulsions. Recent researches have shown that whey protein isolate has a good emulsifying potential for use in food emulsions [11–15]. Emulsions stabilized with proteins are more stable than those stabilized by low molecular weight emulsifiers due to formation of a flexible film by proteins which can prevent coagulation of particles in the dispersed phase [16,17]. Also, using a complex of whey protein isolate and pectin in external aqueous phase can improve the stability and release of double emulsions [18].

In W/O/W emulsions, two general mechanisms occur for the release of hydrophilic compounds: (a) the release through interlinking between the internal aqueous phase droplets and the surface globules which results in rupture of the membrane between

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the internal aqueous phase and the surface cell; (b) release of soluble materials trapped in the internal aqueous phase, by diffusion from internal aqueous phase to outer aqueous phase, not rupture of the membranes; because of the driving force resulted from the density difference and the Laplace pressure of droplets [19,37].

Olive leaf (*Olea europaea*) is one of the herbs which is useful in treating many diseases. It reduces fever, malaria, and prevents high blood pressure [20]. Among different parts of olive tree, the leaf is one of the richest sources of phenolic compounds [21]. Oleorupein and its derivatives such as hydroxyl-tyrosol and tyrosol is the most abundant phenolic compound within olive leaf [22,23]. It has been found that there is a linear relationship between the amount of Oleuropein in olive leaves and their antioxidant activity [24,25].

When phenolic compounds as natural antioxidants are added into oil, the release control of them during storage probably has a major role on their antioxidant activity. The aim of the present study was to protect and control the release of phenol compounds of olive leaf to increase their antioxidant property by nano-encapsulation of olive leaf extract in the internal aqueous phase of W/O/W emulsions, which were formulated by W/O micro-emulsions as primary emulsions and applying a complex of biopolymers as hydrophilic emulsifiers in the external aqueous phase.

2. Materials and methods

Refined soybean oil without any antioxidant additive was purchased from a local oil refining factory (Alia Golestan Co., Iran). Olive leaf of Mission variety was collected from Gorgan, Iran, and citrus pectin (GA: > 65%, DE: 71.1%) was purchased from MP biomedical (Netherlands). Whey protein concentrate (80% protein) and sorbitan monooleate (span 80) was obtained from Sapoto cheese (USA) and Merck (Germany), respectively. Sodium azide was supplied by Sigma–Aldrich (St. Louis, USA) and other chemicals were of analytical grade.

2.1. Microwave assisted extraction of phenolic compounds from olive leaf

Olive leaves were dried in an oven (Memmert, ULM 400, Germany) at 40 °C for two days; then powdered by a Sunny model SFP-820 laboratory mill (40 mesh). For extraction of phenolic compounds, the procedure of Taghvaei et al. [26] was adopted by some modifications. A microwave oven (Samsung, model: CF3110N-5, Korea) was modified for extraction which was consisted of a volumetric flask (500 ml) coupled with a condenser at the top and a magnetic stirrer beneath. The microwave output was 900 W with 2450 MHz frequency and its inner cavity dimensions were 400 mm × 300 mm × 250 mm. For each extraction, 1 g of dried leaves was blended with 50 ml of methanol solvent in a 500 ml volumetric flask and was placed in microwave oven; while magnetic stirring, 6 min of irradiation was performed (8 s power on and 15 s power off in order to prevent super-boiling of solvent). After that, the methanol extract was filtered and solvent removed under reduced pressure at 40 °C by means of a rotary evaporator (IKA RV 10 basic, Germany) and remaining dried by means of a freeze-drier model Operon FDB-5503 (Korea) at −20 °C. The total phenolic content of OLE was 206.81 mg/g.

2.2. Total phenolic content

Total phenolic content of olive leaf extract was determined by the Folin–Ciocalteu micro-method [21]. Briefly, 20 μl of extract solution was mixed with 1.16 ml distilled water and 100 μl of Folin–Ciocalteu reagent, followed by addition of 300 μl of Na_2CO_3 solution (20%) after 1 min and before 8 min. Subsequently, the mixture was incubated in a shaking incubator at 40 °C for 30 min and its absorbance was measured at 760 nm (PG Instruments T80, UK). Gallic acid was used as a standard for calibration curve. The phenolic content was expressed as Gallic acid equivalents using the following linear equation based on the calibration curve:

$$Y = 0.0019X + 0.0306, \quad R^2 = 0.9986,$$

where Y is the absorbance and X is concentration as Gallic Acid equivalents (μg/ml).

2.3. Preparation of biopolymer solution

The powdered citrus pectin was dispersed in distilled water and stirred at 50 °C for 30 min; then it was kept at room temperature overnight for complete hydration. WPC was dissolved in distilled water and kept refrigerated for 24 h for complete hydration. The pH of solution was adjusted on 6.0 with phosphate buffer and was heat-treated at 70 °C for 20 min, and cooled down quickly. The complex solution of WPC and pectin was also prepared by adding pectin solution into the solution of WPC, and stirring at room temperature for an hour. Then, the pH of solution was set on 6 by phosphate buffer and was kept in a refrigerator overnight. Regulating the percentage of biopolymers in solutions was done as shown in Table 1. 0.004% of sodium azide was included in the solutions as an antimicrobial substance. All multiple emulsions composed of 30% primary emulsion and 70% outer aqueous phase.

2.4. Preparation of emulsions

W/O/W multiple emulsions were made in a two-step procedure.

2.4.1. Preparation of the primary micro-emulsion

First, W/O micro-emulsion was produced according to the method of Faridi et al. [3]. The mixtures of soybean oil and span 80 were prepared with different ratios and, were stirred in 300 rpm to become transparent. Then, the dropwise addition of aqueous phase containing olive leaf extract into the different ratios of soybean oil and span 80 while stirring (300 rpm). After each addition, the system was given enough time to become transparent and isotropic. The addition of aqueous phase continued until the system did not become transparent in spite of mixing for a long time. The volumes of added water for each of mass ratios were recorded. Eventually, weight percentage was considered for aqueous phase, span 80 and soybean oil for micro-emulsion preparation, 7:31:62% (w:w:w). The produced micro-emulsion contained 142.69 mg total phenol.

2.4.2. Preparation of W/O/W multiple emulsions

The second step of emulsification was performed at 10 °C. The pre-double emulsions were formed by gradually adding the primary W/O micro-emulsions into the continuous aqueous phase containing biopolymers during homogenization with rotor-stator homogenizer (8000 rpm for 5 min, T25 IKA, Germany). Two types of biopolymer solutions were applied: (1) single layer whey protein concentrate, and (2) two layer whey protein and pectin. These W/O/W emulsions were then further emulsified using the mentioned homogenizer (15,000 rpm for 8 min). The multiple emulsions were composed of 30% w/w dispersed micro-emulsion and 70% w/w continuous phase of biopolymers solution.

2.5. Microstructure of the emulsions

The microstructure of nano-emulsions was investigated by electron microscopy. The multiple emulsion structure and its evolution

Table 1
Experimental design of different multiple emulsions: percentage content of components.

Multiple emulsion[a]	Dispersed (inner) phase composition: micro-emulsion				Continuous aqueous phase (biopolymers)			Preparation method
	Water + olive leaf extract (% w/w)	Span 80 (% w/w)	Soybean oil (% w/w)	Total phenolic (mg)	Water (% w/w)	WPC (% w/w)	Pectin (% w/w)	
WPC	7	31	62	142.69	62	8	–	Single layer
WPC–P	7	31	62	142.69	61.8	8	0.2	Two layer

[a] Treatment abbreviations employed: WPC (whey protein concentrate), WPC–P (whey protein–pectin).

with time was directly observed by photo microscopy, ×100 objective (Micros Austria, MCX 100, Austria). Multiple emulsions were diluted with deionized water by 1:5 ratios, and one drop of it was inserted between two glass microscope slides and observed by placing a camera (Canon A550, Kuala Lampur, Malaysia) on the eyepiece, and the shooting took place.

2.6. Droplet size analysis of emulsions

Size distribution of water droplets in W/O micro-emulsions and oil droplets in W/O/W multiple emulsions were determined by the laser light scattering method using Zetasizer (Malvern Instruments, Worcestershire, UK). Before measuring the size of oil droplets, the W/O/W emulsions were diluted in deionized water by 1:5 ratios.

2.7. Sedimentation analysis

Freshly-made emulsions were poured into 10 ml glass tubes to a height of 8 cm and stored at 30 °C. The height of the opaque emulsion phase was measured every four days during 20 days (h_t) and compared with the initial emulsion height (h_0) to determine sedimentation stability (S):

$$S = (h_0 - h_t/h_0) \times 100\% \quad (1)$$

2.8. Viscosity measurement

The viscosity of emulsions was measured using a programmable viscometer (model LVDV-II + Pro, Brookfield Engineering Laboratories, USA) and by a ULA spindle. Approximately 16 ml of emulsions were poured into the concentric cylinder of the device, and the apparent viscosity of emulsions was determined [36].

2.9. Release properties

The method of [27] was used for determination of release properties, which is the percentage of compounds trapped within the inner aqueous phase during W/O/W emulsification. Thus, 3 g of multiple emulsions was mixed with 3 g of phosphate buffer (pH 7) and centrifuged (Centurion k2042, USA) in 4500 rpm at room temperature for 90 min. Then, the lower phase was collected carefully, and total phenolic content was analyzed according to Folin–Ciocalteu method using tannic acid as a standard for calibration curve. Phenol content was expressed as mg equivalent as gallic acid per gram of extract [28]. Using the following formula, the percentage of encapsulated compounds was identified:

$$E(\%) = 100 - (C_2 \times 100/C_1) \quad (2)$$

where C_2 is the percentage of encapsulated compound (total phenolics) in outer aqueous phase and C_1 equals to the percentage of compounds in inner phase.

2.10. Color

In order to investigate the effect of storage on the color of emulsions, during 20 days storage at 30 °C, color was measured using the method of Mahadavee Khazaei [29] by some modification. The system Included of a digital camera (Canon A550, Japan), an image-capturing box and image analysis software (Image j 1.47v, National Institutes of Health, USA). 8 ml of the emulsions were transferred into a glass tube and holder was placed at the bottom of the box and the digital camera was fixed 25 cm far from the sample.

2.11. Statistical analysis

The experiments (Table 1) were carried out in a completely randomized factorial design. The collected data were analyzed by ANOVA; the means were compared by the Duncan's multiple range tests at the 5% level through SPSS version 21 (IBM, USA).

3. Results and discussion

Multiple emulsions were prepared using the "two-step" procedure described above. Food grade materials were used for stabilization of all emulsions. Stability (droplet size, sedimentation, viscosity and, morphology) and color indices changes of primary and multiple emulsions were investigated. Also the release of phenolic compounds from inner aqueous phase of multiple emulsions was studied over time.

3.1. Stability of primary W/O micro-emulsions

The size distribution of droplets in the primary W/O micro-emulsions containing olive leaf extract were measured based on laser light scattering as given in Fig. 1A. Average droplet size of dispersed water droplets one day after production was 6.16 nm which achieved to 22.97 nm 20 days after storage (Table 2). The increase in the droplet size observed after 20-day storage probably reflects some chemical aging of the surfactant molecules (usual for this type of surfactant in presence of water). According to our results, primary nano-emulsion maintained its stability during storage and no sedimentation was observed (Fig. 2c). Using high levels of a lipophilic surfactant (span 80) and also, the small size of droplets (5–100 nm) caused the high thermodynamic stability of obtained nano-emulsions [30]. The viscosity of W/O micro-emulsion was 58.8 and 60.1 m Pa s at 6.1 s^{-1} shear rate after 1st and 22th day of production, respectively. The increases of viscosity over time could be the result of surfactant micelle formation and the increase in droplet size [30].

In general, very small changes observed in the viscosity and droplet size of micro-emulsions after 20 days storage (Fig. 3a), revealing high stability of these emulsions over time.

3.2. Stability of W/O/W multiple emulsions

The size distribution of droplets in multiple emulsions is given in Fig. 1B. The average droplet size of W/O/W emulsions stabilized with a complex of WPC and pectin were larger than those emulsions stabilized only with WPC (Table 2). Given the same conditions and parameters involved in the production of double emulsions, it could be concluded that the simultaneous use of two

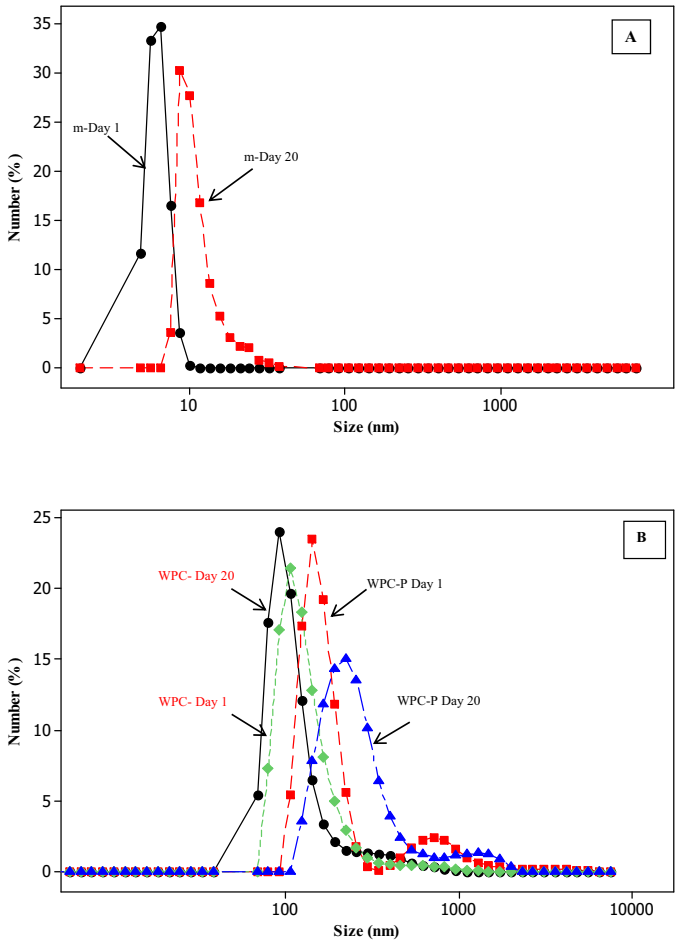

Fig. 1. Particle size distribution of (A) W/O micro-emulsions and (B) W/O/W emulsions of single layer WPC and two layer WPC–pectin.

biopolymers (WPC and pectin) increases the thickness of emulsifier layer around droplets and increases their size. The droplet size of W/O/W emulsions stabilized only with WPC, one day after preparation was 675 nm which decreased during storage to 347.7 nm (Table 2). This phenomenon could be explained by thinning the liquid film between the internal droplets and the globule interface followed by its rupturing and releasing entire droplets [2]. In the WPC–pectin stabilized double emulsions, a small increase in particle size was observed over time, which could represent a release or penetration of outer water phase into the inner phase without film rupturing, because the soluble complex of WPC and pectin is more elastic and creates more aggregates [2,19].

We observed creaming in double emulsions stabilized only by WPC over time (Fig. 2b). But no sedimentation was observed when combined pectin and WPC used in external aqueous phase of multiple emulsions. The soluble complex of WPC–pectin is more elastic

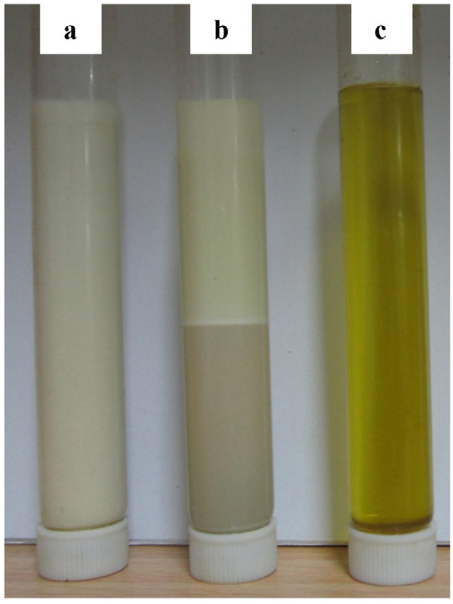

Fig. 2. The sedimentation of the multiple emulsions: (a) stabilized by complex of WPC and pectin, (b) stabilized by WPC alone; and (c) W/O micro-emulsion. All emulsions are 20 days old.

and creates more aggregates than WPC alone. Thus, the effective WPC–pectin soluble complex creates a stiff interface which improved the stability of emulsions [2].

The viscosity of WPC stabilized emulsions was 16.4 m Pa s at 6.1 s^{-1} shear rate after one day of production, which increased during 20 days storage to 24.4 m Pa s (Table 2). This could be possibly due to the jelly network formation. WPC has more hydrophilic groups compared with pectin, which will absorb more water and could form a jelly structure [31]. On the contrary, viscosity of WPC–pectin stabilized double emulsions was 128 m Pa s after one day of the production and declined during 20 days of storage to 114.1 m Pa s at 6.1 s^{-1} shear rate. In fact, the viscosity of W/O/W emulsions depends on some parameters such as creaming, nature and behavior of biopolymers inside the continuous phase, release of inner aqueous phase into outer aqueous phase, and also the type and intensity of interaction between droplets, the size and size distribution of droplets, which are among those important parameters affecting the rheological features of these emulations. Therefore, due to small changes observed in the viscosity of multiple emulsions stabilized by a complex of WPC and pectin, it can be concluded that characteristics of these emulsions have been remained stable over time.

Eventually, the morphology of droplets within multiple emulsions in Fig. 3 shows the full coverage and stability of oil droplets (W/O micro-emulsions) after 20 days storage. As expected, inner droplets of the double emulsions were too tiny to be observed under the light microscope.

Table 2
Physical properties of primary and multiple emulsions stabilized by WPC/pectin at pH 6, by two methods of single layer coating, and simultaneously double layer coating, loaded with olive extract in the inner phase.

Sample code	Particle size *Z*-average, (nm)[1]		Viscosity at 6.1·10 s^{-1} (m Pa s)		Sedimentation stability (S), %		EE% of phenolic compound	
	Day 1	Day 22	Day 1	Day 22	Day 1	Day 22	Day 1	Day 22
W/O (primary micro-emulsion)	6.16^{Aa}	22.97^{Ba}	58.8	60.1	100	100	–	–
WPC	675^{Ab}	347.7^{Bb}	16.4	24.4	100	100	93.34^{Aa}	72.73^{Ba}
WPC–P	1443^{Ac}	1992^{Bc}	128	114.1	100	100	96.64^{Ab}	88.81^{Bb}

[1] Values with different letters (A–B) within the same column differ significantly ($P < 0.05$) and values with different letters (a–c) within the same row differ significantly ($P < 0.05$).

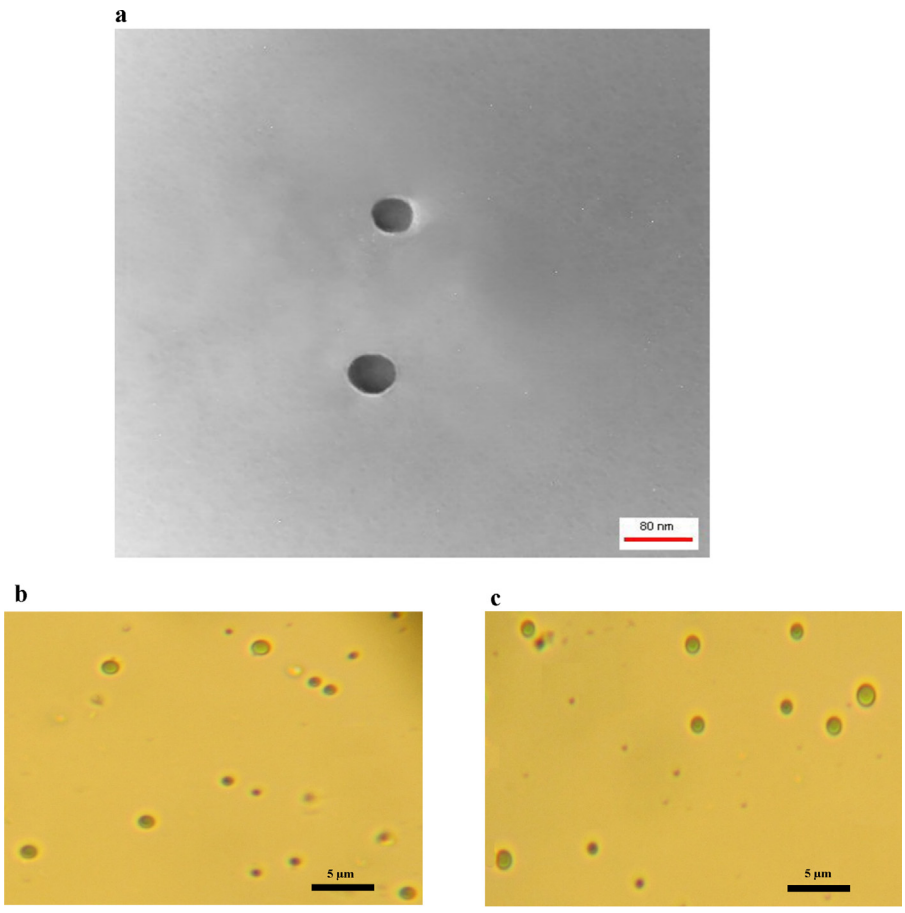

Fig. 3. TEM of (a) W/O micro-emulsion; Photomicrographs of (b) multiple emulsions stabilized with WPC; (c) stabilized with complex of WPC and pectin. All emulsions are 20 days old.

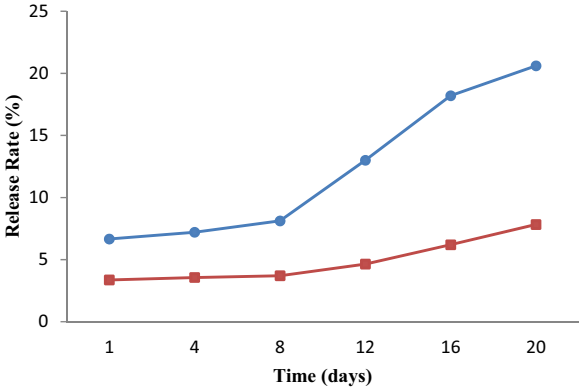

Fig. 4. Release trend of phenolic compounds from single layer WPC multiple emulsions (●), and two layer WPC–pectin multiple emulsions (■) for 20 days at 30 °C.

3.3. Release properties

Release control of the phenolic compounds has a major effect on their antioxidant activity. In this study, the release of phenolic compounds from inner aqueous phase of W/O/W emulsions during 20 days storage at 30 °C was monitored by evaluating total phenolic content analysis (Fig. 4).

After 20 days, double emulsions stabilized only with WPC and containing olive leaf extract as the dispersed phase of their primary emulsions released approximately 22% of their total phenolics. This could be due to slow and very limited concentration driving force of phenolic compounds between inner and outer aqueous phases. While those multiple emulsions stabilized with WPC–pectin released just about 8.1% of their phenolic compounds at the same time, which was significantly ($P < 0.05$) lower. These results clearly show that a complex of WPC and pectin as hydrophilic emulsifier in the external aqueous phase of double emulsions increased the encapsulation efficiency and reduced the release of phenolic compounds during storage. In general, properties of layers around oil particles have a significant influence on the release of inner components. The walls of oil particles coated by double layers are elastic and could have more aggregations than single-layers, therefore, the stability increases and the release is decreased for double-layer stabilized emulsions [18].

To establish an electrostatic absorption force between the protein and polysaccharide, the pH of biopolymer solutions should be adjusted. The pH of external aqueous phase was adjusted to 6 by using buffer phosphate. At pH 6, the soluble complex is easily adsorbed onto the interface, and there is an increasing steric stabilization against coalescence. But at pH above 6, WPC would not be able to absorb pectin any more [2,32].

Polysaccharides with carboxyl groups such as pectin are able to interact with strong electrostatics at pH > P_I up to pH 6 to formulate colloidal particles stabilized by negative charges [33].

Two main mechanisms are posed for the release of hydrophilic compounds from inner phase of W/O/W emulsions: (i) The instability of primary emulsion and multiple emulsions leading to spilling of the inner phase content into the outer phase (breakdown mechanism), (ii) hydrophilic compounds are transferred from the inner aqueous phase into the external aqueous phase because of Laplace pressure and osmotic pressure [35].

Table 3
The color changes of micro-emulsions, and multiple emulsions with single layer, or double layer, during 20 days storage at 30 °C.

	Day 1			Day 4			Day 8			Day 12			Day 16			Day 20		
	L	*a*	*b*	*L*	*a*	*b*	*L*	*a*	*b*	*L*	*a*	*b*	*L*	*a*	*b*	*L*	*a*	*b*
Micro-emulsion	53.5	−5.2	47.6	52.8	−5.0	47.6	51.6	−5.8	47.3	47.3	−5.4	48.1	46.8	−5.12	47.8	43.0	−5.6	48.4
WPC	67.5	−4.4	7.9	66.2	−4.4	8.2	65.7	−4.2	8.7	64.8	−4.9	10.5	57.2	−5.0	12.2	56.4	−4.6	13.0
WPC–P	66.9	−4.5	7.3	67.4	−4.3	7.5	64.7	−4.3	7.8	62.4	−4.6	8.3	60.8	−4.79	9.6	58.3	−4.9	10.9

Decrease in droplet size of W/O/W emulsions stabilized with WPC alone indicates the occurred rupture of film in these emulsions. But, the release mechanism in WPC–pectin stabilized double emulsions was occurred without film rupturing, because the soluble complex of WPC and pectin is more elastic and particle size is not decreasing over time [32]. Morphology observations of these emulsions over time indicated that the structure of droplets has been preserved and also, the size distribution results represent a small change in size of droplets over time. Thus, it can be concluded that film rupturing was not occurred in these emulsions and the diffusion has had a facilitated transport by the lipophilic surfactant across the oil membrane.

Garti and Aserin [9] studied the release of electrolytes in the presence of monomeric emulsifiers. They have indicated that the transport takes place mostly with diffusion by a reverse micelle mechanism, because even if the droplets are very stable to coalescence. Lutz et al. [32] confirmed the release of inner electrolytes for W/O/W emulsions by transport of the entire inner droplets and diffusion mechanism. Bonnet et al. [35] studied the release of magnesium in multiple emulsions, and indicated that coalescence was not a major event, and magnesium release preferentially occurred without film rupturing.

3.4. Color analysis

Results of color indices (a^*, b^*, L^*) in emulsions during storage at 30 °C have been presented in Table 3. We observed that blueness/yellowness (b^*) and redness/greenness (a^*) of W/O micro-emulsions did not change significantly ($P > 0.05$) during storage, but a decreasing trend in lightness value (L^*) was measured during storage in W/O micro-emulsion. With increased the droplet size of micro-emulsions (Table 2), the light which could pass through them is decreased, therefore a decreasing trend in "*L*" value over time is reasonable. "*b*" value of W/O/W emulsions was lower than primary W/O micro-emulsions. This could indicate the coverage of W/O micro-emulsion by biopolymers in W/O/W emulsions. Also, "*a*" value of W/O/W emulsions did not change significantly ($P > 0.05$) during storage, but a decreasing trend in "*L*" value and an increasing trend in "*b*" value was measured during storage in W/O/W emulsions. Indeed, the increase in release of W/O/W emulsions causes an increase in the concentration of color which increases the "*b*" value and decreases the lightness. The lightness of emulsions with increasing the concentration of color is reduced, because molecules absorb light, thus, less light could passes through the emulsions [34].

4. Conclusion

Our results revealed that the W/O/W emulsions formulated by a primary W/O micro-emulsion and a complex of biopolymers within the external aqueous phase could result in a high stability and controlled release of the encapsulated compounds. Multiple emulsions stabilized only with WPC had the fastest release rate. When pectin was added to WPC to form a soluble interfacial biopolymer complex, an increase in stability and a slower release was observed. The multiple emulsions stabilized by a complex of WPC and pectin would release the entrapped molecules by transport of the entire inner droplets, while the same emulsions stabilized only by WPC would release the phenolic compounds by a film rupturing mechanism. It was found that with increasing the release of W/O/W emulsions, the concentrated of color was changing over time, and the "*b*" value increased and the lightness decreased. To summarize, we can formulate stable multiple emulsions which are capable of releasing the entrapped molecules in a controlled pattern.

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