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Original Paper

Preparation and Optimization Formula of Whey Protein-Pectin Complex As Nanocapsules for Lemon (*Citrus limon*) Juice

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***Abstract*—Lemon (*Citrus limon*) juice was nanoencapsulated in whey protein concentrate (WPC)-pectin complex coacervates, and then dried using freeze-drying to produce solid nanocapsules. To optimize the nanoencapsulation condition, central composite design (CCD) of response surface methodology (RSM) was implemented. WPC (%), pectin (%) and pH level were taken as variables, while antioxidant activity and D-limonene content as responses. The optimized condition was in 6.0% WPC and 3.0% pectin at pH 3.1 by maximize the responses. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) images showed the morphology of lemon juice nanocapsules as spherical nanoparticles with an average size of 22.3 nm.**

***Keywords*— optimization, lemon juice, WPC-pectin, complex coacervates, antioxidant activity, D-limonene,**

I. INTRODUCTION

Lemon (*Citrus limon*) fruit provide abundant health benefit because of rich in antioxidant compounds such as phenolic, flavonoid, and vitamin C [1]. It has also a good flavor composed by many volatile compounds in which D-limonene is the most abundant [2]. Unfortunately, lemons have limited shelf-life that highly affected by environment (such as pH, temperature, light, and oxygen). Many technologies have been used to maintenance the quality and minimize the losses of lemons from any damage, during processing, storage, and distribution, such as packaging technology (modified atmosphere packaging and low-density polyethylene packaging), drying method and processing in the form of juice [3,4].

Encapsulation is one of important technology in food industry, which can help to prevent off-flavors and off-taste, undesirable texture, and protect chemical and biological degradation of food during processing and storage caused by moisture and heat. This technology can also achieve controlled release of encapsulated nutrients to a specific rate [5,6]. Capsule material for food must be certified as generally recognized as safe (GRAS), food-grade, biodegradable, non-toxic and able to form a barrier between sensitive bioactive compounds and its environments [7]. Almost of capsule material used for food ingredients are biopolymers (polysaccharides, protein, and lipid).

Different types of biopolymers are capable of binding and encapsulating food bioactive, and creating molecular complexes including polysaccharide-polysaccharide, protein-protein, and protein-polysaccharides (nano) complexes. Polysaccharide-protein nanoparticle has synergistic combination between the functional group of various biopolymers and has higher chemical and colloidal stability than pure single biopolymer nanoparticles [8]. Previous research reported that whey protein-pectin complex has been applied to encapsulate D-limonene, a major compound of lemon essential oil [9].

Whey protein consists mainly of several globular proteins, α-lactalbumin (α-la), β-lactoglobulin (β-lg), bovine serum albumin (BSA), immunoglobulin and also several protein/peptide component comprising lactoperoxidase, lysozyme, and lactoferrin. As a natural protein, whey protein exhibit positive charge below its isoelectric point (IP) and negative charge above its isoelectric point [10]. Pectin is a soluble dietary fibers (SDF) that has complex polysaccharides structural, mainly composed of α (1,4)-D-galacturonic residues, with various degrees of methyl esterification. The electrical charge of pectin is negative over a wide of pH value. Complex coacervation of WPC-pectin occurs when pH solution reduces below the protein IP. If the pH was reduced too far below the protein IP, extensive complex formation will occur, and this eventually leads to precipitation [11,12]. The complex formation also depends on other factors such as protein-polysaccharide ratio, temperature, ionic strength, and charge density [10,13]. The nanocomplexes prepared by 4% whey protein and 1% pectin at pH 3.0 is the best treatment for D-limonene [9].

Research on nanoencapsulation of lemon juice has not been carried out. In this research, physical and chemical parameters of lemon juice nanocapsules were studied from various formulas of pectin and whey protein concentrate (WPC) at different pH values. Response surface methodology (RSM) was implemented to get the optimum conditions based on the response of antioxidant activity and D-limonene content. The morphology properties was analysed using scanning electron

microscopy (SEM), while the topography and particle size distribution were analysed using atomic force microscopy (AFM).

II. Materials and Methods

A. Materials

Domestic lemon *(Citrus limon*) fruits were obtained from the market in Hiroshima Prefecture, Japan. Lemon juice was obtained by squeezing the lemons by hand. Whey protein concentrate (WPC; protein 78.00 %, lipid 6.50 %, and carbohydrate 7.00 %) was purchased from Alpron, Japan. Pectin from apple was purchased from Fujifilm Wako Pure Chemical Corporation, Osaka, Japan. Maltodextrin (MD; DE = 16.5 – 19.5) was purchased from Sigma-Aldrich, St.Louis, USA. Tween 80 (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), a non-ionic surfactant, was applied as the emulsifying agent. All the solutions for nanocapsules prepared by deionized water.

B. Methods

Preparation of Lemon Juice Nanocapsules

The scheme of the preparation is shown in Fig.1 as described in the previous research [9] with modification. WPC was dissolved into deionized water to obtain 100 mL solutions. At the same time, pectin was dissolved in hot deionized water (70 ^{o}C) to prepare 100 mL solution. Maltodextrin (50 g) was dissolved in deionized water to prepare 100 mL solutions. Maltodextrin is used to increase the total soluble solids of the samples for obtaining the higher powders. These solutions were slightly stirred on magnetic stirrer for at least 30 min until homogenous and then stored at 4 ^{o}C to complete hydration of biopolymers. All the solutions then filtered through 0.45 μm pore size to be used for further preparation. The designed formula of WPC-pectin nanocapsules for lemon juice at various pH values can be seen in Table I.

The WPC, pectin, and maltodextrin (50 %) solution at ratio 1:1:1 (v/v/v) were mixed and stirred on a magnetic stirrer at 1,000 rpm, 37 ^{o}C for 45 min to be used as capsule polymer. Then tween 80 at ratio 5 % of the total solids was added into the solution and stirred until homogenous. Lemon juice as core material was filtered through 0.45 μm pore size and then added into this solution gradually. The pH of solution was adjusted based on the designed formula and then stirred at 1,000 rpm, 37 ^{o}C for 30 min.

The lemon juice nanocapsules solution was centrifuged at 13,000 rpm, 4 ^{o}C for 30 min. Pellet was be collected as lemon juice nanocapsules and then dried using freeze-drying. (Fig. 1.) The dried powder was collected and stored in the freezer for further characterization.

TABLE I. Response Surface Methodology (RSM) Designed Formula of WPC-Pectin Nanocapsules for Lemon Juice

Std	Run	Factor 1: WPC (%)	Factor 2: Pectin (%)	Factor 3: pH
16	1	4.0	2.0	3.5
14	2	4.0	2.0	4.3
2	3	2.0	1.0	3.0
30	4	4.0	2.0	3.5
20	5	7.4	2.0	3.5
11	6	4.0	0.3	3.5
2	7	6.0	1.0	3.0
6	8	6.0	1.0	4.0
3	9	2.0	3.0	3.0
19	10	4.0	2.0	3.5
8	11	6.0	3.0	4.0
4	12	6.0	3.0	3.0
13	13	4.0	2.0	2.7
12	14	4.0	3.7	3.5
9	15	0.6	2.0	3.5
17	16	4.0	2.0	3.5
5	17	2.0	1.0	4.0
15	18	4.0	2.0	3.5
18	19	4.0	2.0	3.5
7	20	2.0	3.0	4.0

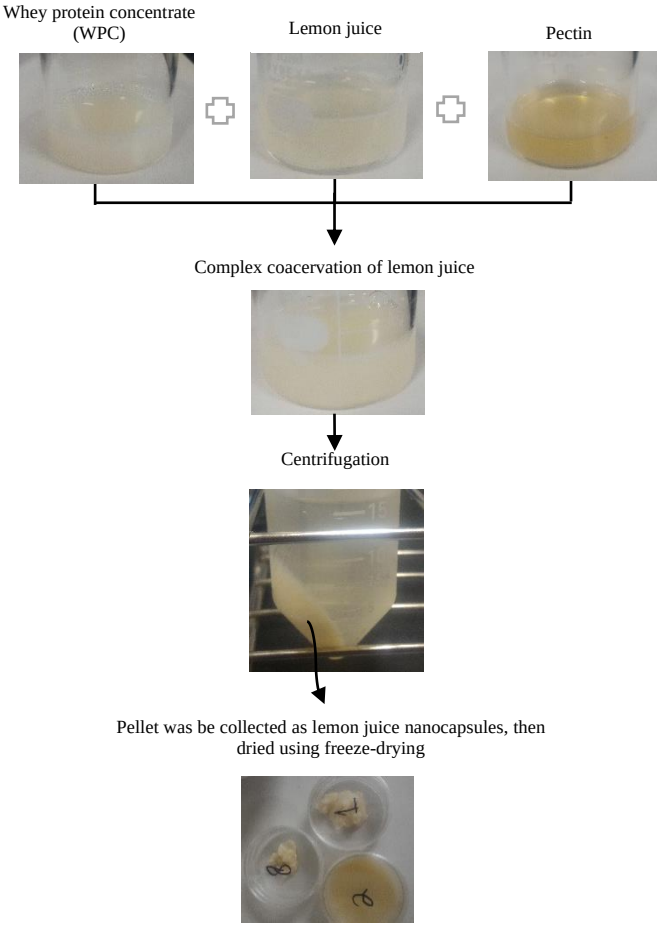


Fig. 1. Schematic preparation of WPC-pectin complex for lemon juice

Morphology, Topography and Particle Size Analysis and Distribution

The morphology of samples was examined using scanning electron microscopy (SEM, Miniscope TM3000, Hitachi High-technologies Corp, Tokyo) at 5,000; 10,000; and 30,000 x magnifications. Samples were placed on a sample stage, and then coated with gold before was observed. Topographical surface and particle size distribution estimation were analyzed using atomic force microscopy (AFM, SNOAM, Hitachi-Tech

Science Corp, Tokyo) with Dynamic Force Mode in air. One drop of dilution samples were placed on cover glass. A silicon cantilever (OMCL-AC160TS-C3, Olympus Corp., Tokyo) with an oscillation frequency of 300 kHz and a spring constant of 42 Nm^{-1} was used.

Quantification of D-Limonene as Flavour Compounds

Headspace gas chromatography-mass spectrometer (HS-GC-MS, QP 5050, Shimidzu) has been used for qualitative and quantitative analysis of D-limonene compounds. The operational condition: column DB-WAX, using He as gas carrier with a flow of 0.9 mL min^{-1}. The vaporization chamber temperature was 200 ^oC and heart temperature was 230 ^oC. The column inlet pressure was at 100 kPa; column flow rate was 0.9 ml min^{-1}; constant linear velocity was 24.7 cm sec^{-1}. The evaporated room temperature control condition: the start temperature was 30 ^oC for 5 min, and later increased 3 ^oC min^{-1} up to 160 ^oC. After that, increased 20 ^oC min^{-1} up to 200 ^oC. The program time was at 50.33 min. Mass spectrometer followed the operational condition: elution time of 3 min, scan rate of 500 fragments sec^{-1}, mass range (m/z) from 50 to 250, data sampling time from 5-43 min, interval at 0.5 sec^{-1} and threshold at 2000. D-Limonene from Fujifilm Wako Pure Chemical Corporation was used as standard.

Antioxidant activity

The antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as described earlier with slight modification [14]. Briefly, 200 µL of sample extract in methanol 80 % were mixed with 320 µL citrate buffer solution (pH 2.5 in water), 100 µL ethanol and 280 µL DPPH solution (0.5 mM in ethanol). The mixture was homogenized by vortex and incubated at room temperature for 5 min. The optical density (OD) was measured at 524 nm using ultraviolet (UV)/visible spectrophotometer (U-1900, Hitachi). Ethanol served as standard OD. The antioxidant activity was expressed as % radical-scavenging activity (RSA) and was calculated as follow:

RSA (%) = (standard OD-sample OD) / standard OD x 100

Analysis of Data

The study was designed and analyzed by central composite design (CCD) of response surface methodology (RSM) through design expert software (State-Ease Co., version 12). The effect of three independent variables of WPC (%), pectin (%) and pH on antioxidant activity and D-limonene content of lemon juice nanocapsules was studied to determine the optimum conditions.

III. Results and Discussions

A. *Effect of WPC Concentration, Pectin Concentration, and pH levels on D-Limonene Content of Lemon Juice Nanocapsules*

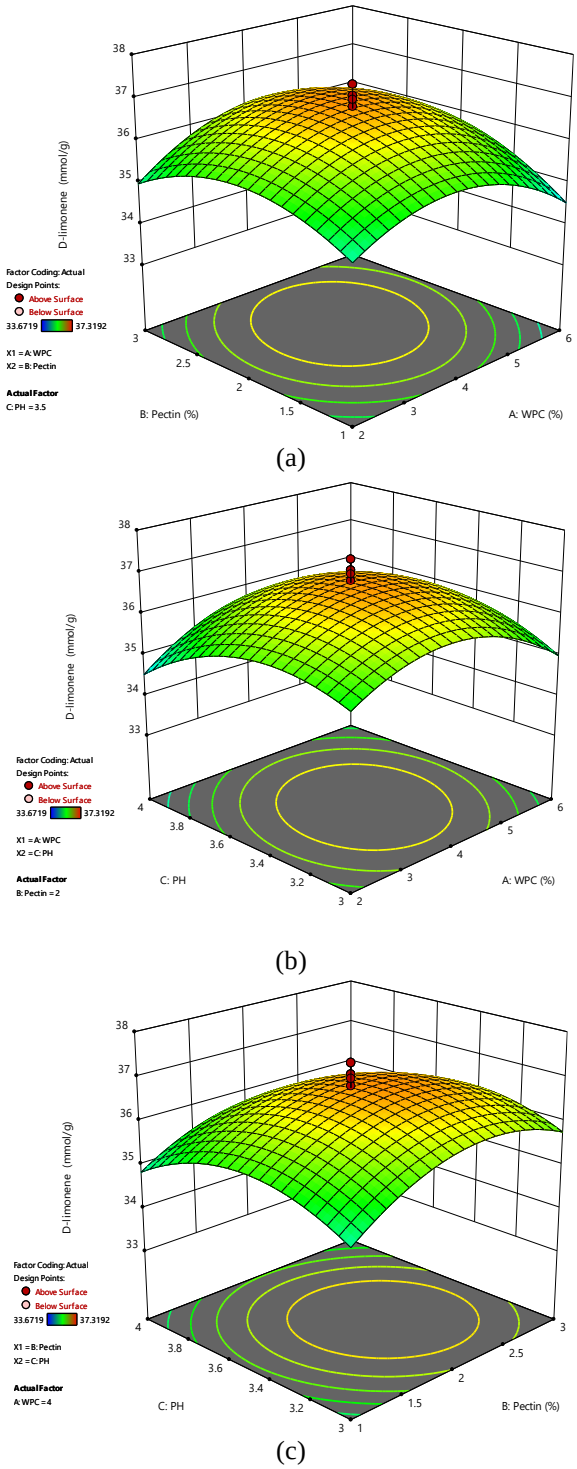


Fig. 2. RSM plots of the interaction of WPC, pectin and pH variables on D-limonene content in lemon juice nanocapsules

Limonene is the most abundant compound of lemon essential oil [15]. In this research, D-limonene was found in small amounts in lemon juice obtained from a hand squeezing process. As can be seen from Fig. 2, the D-limonene content of lemon juice nanocapsules was influenced by ratio of biopolymers and pH value. The highest (37.32 mmol/g) and the lowest (33.67

mmol/g) D-limonene was obtained in sample number 10 (4 % WPC, 2 % pectin at pH 3.5) and sample number 15 (0.6 % WPC, 2 % pectin at pH 3.5), respectively. In previous research found that the optimum nanoencapsulation condition of D-limonene from orange peel oil was in 4 % WPC and 1 % pectin at pH 3.0 [9].

The ratio of biopolymers (protein and polysaccharide) in the solution strongly influences the charge balance of poly-ions and consequently change their complexation behavior [16]. The highest D-limonene content of lemon juice nanocapsules was observed at a ratio WPC-pectin of 2:1 and the lowest of 2:3, respectively. Higher and lower the ratio of WPC-pectin than 2:1 decreased the D-limonene content (Fig. 2a). The ratio should be balanced with each other in terms of their surface charges. Imbalance charge formed a soluble complex with weaker electrostatic interaction and caused lower coacervate yield [14]. Another study on formation pectin (low methoxyl) and whey protein complex containing thiamine (a water-soluble vitamin) in acidic foods showed similar results that the best ratio of whey protein and pectin was 2:1 [17].

Protein and polysaccharide form electrostatic complexes when they have opposite electrical charges. The electrical charge on pectin that contains acidic groups is negative at over a wide range of pH values. WPC is negatively charged at pH above the protein isoelectric point, but the net electrical charge changes to positive at pH below the isoelectric point [8,9]. The highest D-limonene content of lemon juice nanocapsules is at pH 3.5. Fig. 2c and 2d shown that at higher and lower of pH than 3.5, lemon juice nanocapsules has lower D-limonene content. The complex formation will occur at pH below the protein isoelectric point. If the charge on the protein is not to high, the complexes are soluble. If pH is too far below the isoelectric point, extensive complex formation occurs and this eventually leads to precipitation [10,11].

B. Effect of WPC Concentration, Pectin Concentration, and pH levels on Antioxidant Activity of Lemon Juice Nanocapsules

Antioxidant activity of lemon juice nanocapsules was expressed as a radical-scavenging activity (RSA) of DPPH. Lemon fruit extract is rich in proanthocyanidins, phenolic, flavonoids, hesperidin, eriocitrin, and vitamins E and C [1-3]. Many of these are good source as dietary antioxidants that prevent and treatment of various chronic and degenerative diseases. The highest (15.5%) and the lowest (5.1%) RSA of lemon juice nanocapsules (20 mg/ml) are in treatment number 5 (7.4% WPC, 2% pectin at pH 3,5) and treatment number 2 (4% WPC, 2% pectin at pH 4), respectively.

Antioxidant activity of lemon juice nanocapsules was influenced by WPC-pectin concentration and pH value. Higher concentration of WPC and pectin, higher the antioxidant activity (Fig. 3a). Study of grape pomace polyphenols showed that at WPC-maltodextrin ratio 60:40 has the highest microencapsulation efficiency and at 100 % of WPC had better release of 83 % of phenolic compounds [18]. As can be seen in Fig. 3b and 3c, at higher pH have lower antioxidant activity. Protein has lower positive charge at higher pH might be caused an imbalanced charge in solution. In this condition will produce weaker electrostatic interactions [16].

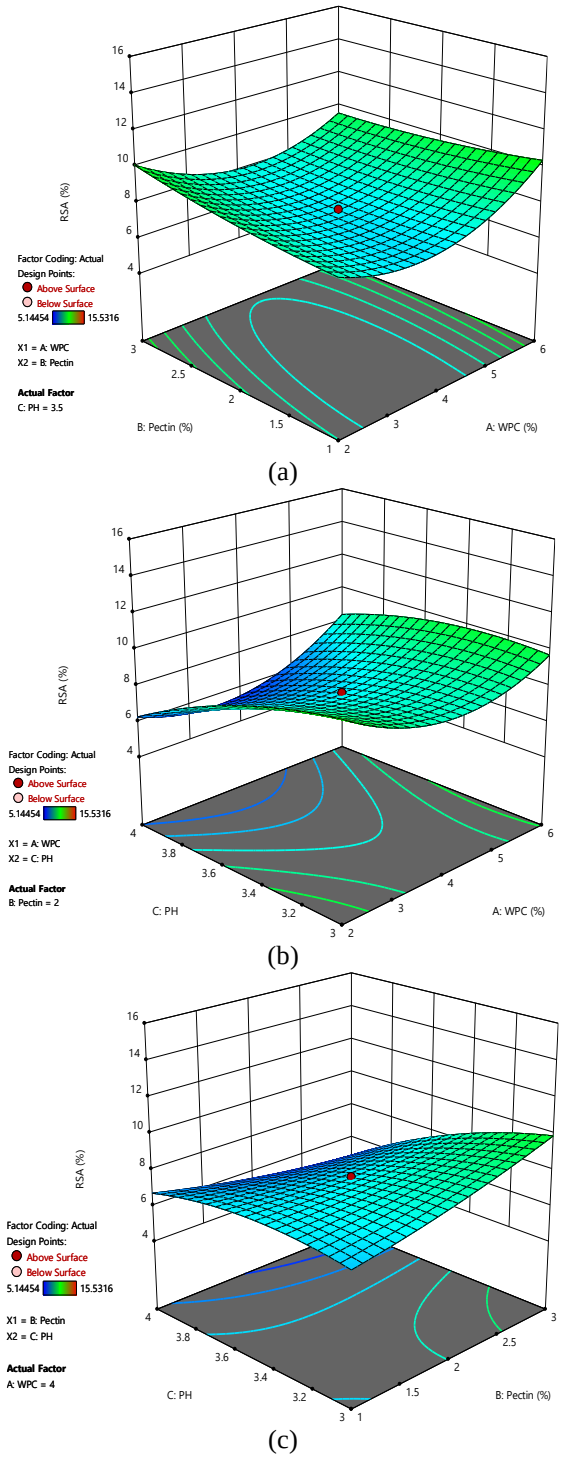


Fig. 3. RSM plots showing the interaction of WPC, pectin and pH variables on the antioxidant activity of lemon juice nanocapsules

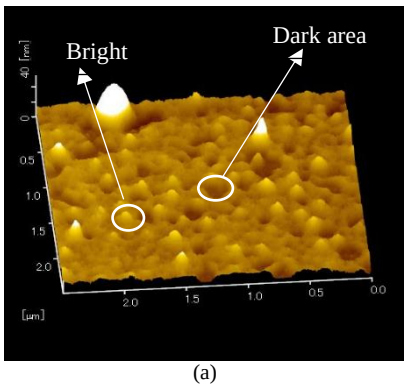
C. Optimization and Confirmation Formula of Pectin-WPC Complex as Nanocapsules for Lemon Juice

Table II showed the optimized component, goals, limits, and level of importance at the optimization stage of formula. Antioxidant activity and D-limonene were optimized responses

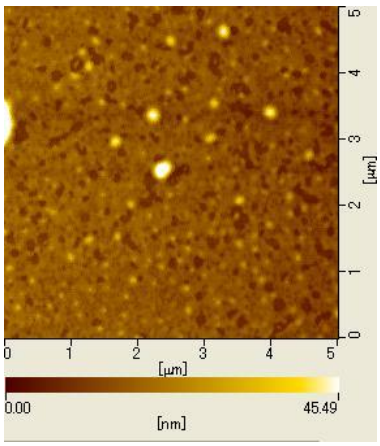
with maximizing goal andlevel importance of 5. This is related to the fact that lemon juice is rich in phenolics, flavonoids, and vitamins C [1], and D-limonene is the most abundant volatile compounds of lemons [2].

The optimum formula solution to obtain the highest antioxidant activity was recommended at 6.0% WPC, 3.0% pectin, at pH 3.1 (Table II). To confirm the prediction of optimum condition, an experiment with 3 replications was prepared based on RSM suggestion (6.0 % WPC, 3.0 % pectin, at pH 3.1). The antioxidant activity (RSA) and D-limonene value was 12.38 % and 36.00 mmol/g, respectively. Compared with predicted value (Table III), the verification results are in the range of 95 % predicted interval (PI) low and 95 % PI high which means that the optimum formula is consistent.

D. Morphology, Topography, and Particle Size Analysis and Distribution



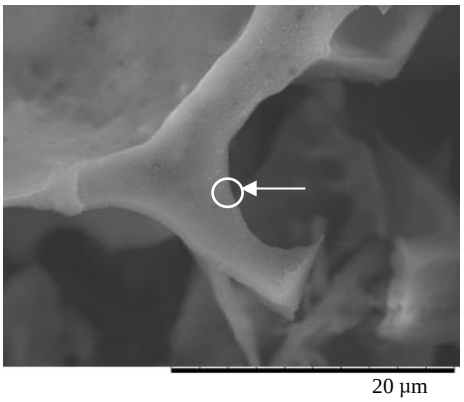
(a)



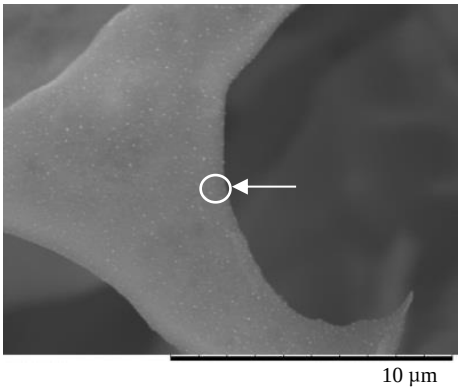
(b)

Fig. 4. AFM images of lemon juices nanocapsules: (a) the brighter area indicated higher structure, and (b) particle size distribution

Morphological and topographical surface of lemon juice nanocapsules are shown at Fig. 4 and Fig. 5. The SEM photograps showed that the form lemon juices nanocapsules prepared by 6.0% WPC, 3.0% pectin at pH 3.1 was spherical. The bright area in AFM images indicated high structure and the dark area indicated low structure. So, the brighter area demonstrated the higher samples. Particle size distribution frequency was analyzed by AFM and the results showed that lemon juice nanocapsules has a particle size average of 22.3 nm ± 7.5.



(a)



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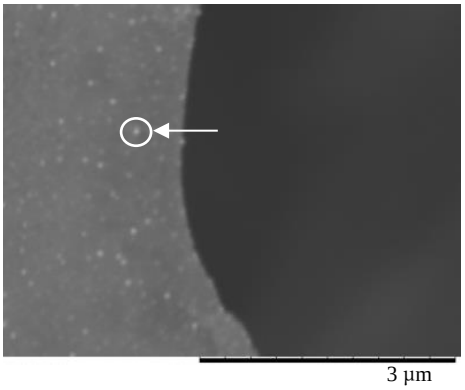

(c)

Fig. 5. SEM photographs of lemon juices nanocapsules by SEM at (a) 5,000; (b) 10,000; and (c) 30,000 of magnification

TABLE II. OPTIMIZATION PROCEDURE, FORMULA SOLUTION AND CONFIRMATION STAGE OF PECTIN-WPC NANOCEMPLEXES FOR LEMON JUICE

Name	**Goal**	**Lower Limit**	**Upper limit**	**Lower weight**	**Upper weight**	**Importance**
WPC	In range	2	6	1	1	
Pectin	In range	1	3	1	1	
pH	In range	3	4	1	1	
Antioxidant activity	Maximize	5.14	15.53	1	1	+++++
D-limonene	Maximize	33.67	37.32	1	1	+++++
Formula Solution	**WPC (%)**	**Pectin (%)**	**pH**	**Antioxidant activity (% RSA)**	**D-limonene (mmol/g)**	
1	6.0%	3.0%	3.1	10.54	35.22	

TABLE III. CONFIRMATION OF OPTIMUM FORMULA BASED ON RSM SUGGESTION

Response	**Predicted mean**	**Data Mean**	**95%PI low**	**95% PI high**
Antioxidant activity (% RSA)	10.54	12.38	7.62	13.45
D-limonene (mmol/g)	35.22	36.00	34.19	36.24

IV. CONCLUSIONS

The optimized condition for nanoencapsulation of lemon juice was found in 6.0 % WPC and 3.0 % pectin at pH 3.1 since it maintains the optimum antioxidant activity (12.38% of RSA) and D-limonene value (36.00 mmol/g). Lemon juice nanocapsules have a spherical form and an average size of 22.3 nm. Further studies will be focused on the characterization of the physical and chemical properties of lemon juice nanocapsules. In addition, the potential anticancer activity of lemon juice nanocapsules on colon-26 cells by *in vitro* method will also be studied.

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