NANOENCAPSULATION STUDY OF LEMON (*Citrus limon*) EXTRACTS IN WHEY PROTEIN-PECTIN COMPLEX

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Abstract

Lemon juice (LJ) and lemon essential oil (LEO) have been reported to have many benefits for human health, but they have problems of high reactivity, low stability, and volatile nature causing many difficulties in the inclusion in the food matrix. Nanoencapsulation in whey protein concentrate (WPC) - pectin complex was studied to evaluate their ability to mask the bioactive compounds, increase the stability, and maintain the biological activities of LJ and LEO.

Lemons were collected from farmers in Hiroshima Prefecture, Japan in the 1st stage of harvesting (green color, immature). LJ was obtained by squeezing lemons by hand, while LEO was extracted by steam distillation technique. Nanoencapsulation of LJ and LEO in WPC-pectin was performed by complex coacervation technique. Characterization of LJ nanocapsules (LJNCs) and LEO nanocapsules (LEONCs) including particle size (AFM), morphology (SEM), encapsulation efficiency (HPLC and spectrometry), molecular (ATR-FTIR), volatile composition (HS-GC-MS), antioxidant activity (DPPH assay), and in vitro anticancer activity (CCK-8 assay) were evaluated.

Part 1: Production of LJNCs. The optimum encapsulation condition of LJ was found at 6 % WPC, 3 % pectin, and at pH 3.1. At this condition obtained the LJNCs with optimum antioxidant activity and D-limonene content. LJNCs have a spherical shape with an average size of 22.3 nm, and an encapsulation efficiency of 66.07 %. Molecular analysis showed that after the complex coacervation, there were found several shifts and changes in the peak of the functional groups associated with the synthesis of NCs and LJNCs. Interaction of carboxyl groups of pectin and amino groups of WPC was clearly demonstrated by FTIR spectra.

Loaded LJ in the WPC-pectin complex as indicated by the formation of a new peak at 1230 cm^{-1}, the sharper region between 1750-1700 cm^{-1}, and the shifted of peak 1632 to 1630 cm^{-1}. The encapsulation efficiency and FTIR analysis proved the success of encapsulation of LJ in the WPC-pectin complex (formation of LJNCs).

Encapsulation in the WPC-pectin complex showed the protection function of bioactive compounds in LJ. The encapsulation increased the retention of ascorbic acid after storage for 30 days at 4 ^{o}C and 40 ^{o}C (about 29.5 % and 27.8%, respectively). Thus, will increase the shelf life of LJ. LJ and LJNCs were reported to have the anticancer activity against colon-26 cells with IC_{50} at 1.12 mL/100 mL and 13.13 mg/mL, respectively. They also showed antioxidant activity against DPPH radicals. The results indicated that the nano-system of the WPC-pectin complex increased the stability of LJ during storage without removing the biological activities. Thus, it's potential to develop as a functional food in the future.

Part 2: Production of LEONCs. LEO was encapsulated by oil-in-water emulsification followed by complex coacervation. The main component of LEO was dominated by 4 volatile compounds consisting of D-limonene, β-pinene, γ-terpinene, and α-pinene. The encapsulation efficiency of LEO in the WPC-pectin complex was 77.37 %, which means that was efficient absorption of LEO in the complex. LJNCs have a spherical shape with an average size of 80.91 nm. The encapsulation significantly changed the composition of LEO by increasing the D-limonene, and γ-terpinene and decreasing β-pinene, and α-pinene. From molecular analysis by ATR-FTIR, it was found that there were shifts in the peak of the functional groups after the encapsulation process which indicated the success of the loading of LEO in NCs.

The in vitro assessment of anticancer activity of LEO and LEONCs

showed have a significant cytotoxic effect against colon-26 cells, which IC_{50} value was 105.88 µg/mL and 2.26 mg/mL, respectively. They also showed antioxidant activity against DPPH radicals. This proved that encapsulation did not remove the biological activities of LEO. Encapsulation in WPC-pectin complex increased the stability of volatile compounds composition of LEO during storage period at different temperatures (4 ^{o}C and 40 ^{o}C). It becomes interesting for the further development of LEONCs in food product applications.

Encapsulation of LJ and LEO in WPC-pectin complex can increase the stability without removing the biological activities. Thus, it`s potential to develop as a functional food in the future.

List of Abbreviations

ATR-FTIR	= Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy
CCK-8	= Cell Counting Kit-8
DPPH	= 2,2 diphenil-1-pycrylhydrazyl
HPLC	= High-Performance Liquid Chromatography
HS-GC-MS	= Headspace-Gas Chromatography-Mass Spectrometry
LEO	= Lemon Essential Oil
LEONCs	= LEO Nanocapsules
LJ	= Lemon Juice
LJNCs	= LJ Nanocapsules
NCs	= Nanocapsules
WPC	= Whey Protein Concentrate

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Interest of Study

In recent years, global food industries give priority to the development of health-promoting foods because of increasing in diet-related chronic diseases such as coronary heart diseases, type 2 diabetes mellitus, stroke, cancer, and obesity in the world. The function of food is no longer seen only as a hunger reliever, but also as the health benefits that can be obtained from its consumption. Plant-derived bioactive compounds including polyphenols, vitamins, bioactive peptides, flavonoids, dietary fiber, and essential oils have been reported in many clinical studies possess tangible health benefits for human health. However, their high reactivity, easy degradation by unfavourable environmental conditions after extraction from plant tissue, and / or low solubility in water caused many difficulties in their inclusion in the food matrix. Incorporation of bioactive compounds in food aims to improve the quality of food (taste, aroma, and shelf life) and improve the functional properties of food ingredients in terms of health promotion (antioxidant, and anticancer abilities). This has prompted the application of edible delivery

systems capable of protecting and releasing bioactive compounds in the production of functional foods.

The development of a delivery system for bioactive compounds in the food matrix focuses on increasing absorption and bioavailability while minimizing the effect on changes in quality and organoleptic properties of food ingredients. This encourages the involvement of nanotechnology in the application of encapsulation systems for foods. Nanoencapsulation is a technology to entrap bioactive compounds into the wall material or shell to form a capsule in the nanoscale range. The market of nanotechnology products in the food industries, including the mainly nanometric delivery system of bioactive compounds (as packaging coating and health-promoting foods) is about 7 billion USD in 2015 and is expected to increase to 20.4 billion USD in 2020 (Cerqueira and Pastrana, 2017; Chau et al., 2007).

This study focused on the production of the nanometric size delivery system of lemon juice (LJ) and lemon essential oil (LEO) in a whey protein-pectin complex. Lemons are a source of high functional values bioactive compounds (such as vitamin C, phenolic compounds, and essential oils) that exhibit great features ranging from nutritional to medicinal properties (Klimek-Szczykutowicz, 2020) so that they can be used in the production of functional foods. However, the bioactive

compounds in LJ and LEO have problems of high reactivity, volatile nature, low solubility, and poor stability causing many difficulties in their inclusion in the food matrix, and causing many losses during processing and storage (Burdulu et al., 2005; Turek and Stintzing, 2013). Those make a challenge for the application of LJ and LEO to food products. It was also studied the nanoencapsulation`s ability to increase the stability of bioactive compounds in LJ and LEO during the storage period and their potential antioxidant and anticancer activities.

1.2 Lemon Fruit: Nutritional and Functional Properties

Lemon (*Citrus limon)* is an important medicinal plant contributing essential nutrients such as polyphenol, vitamin C, and minerals to the human diet. Lemon fruit is an elongated, oval, pointed green berry that turns yellow during ripening (Klimek-Szczykutowicz, 2020). The schematic parts of citrus fruit can be seen in **Fig. 1**. The peel or rind consists of an epidermis (a leathery and waxy layer), the flavedo (a subepidermal layer that contains oil vesicles and carotenoid dyes), the albedo (spongy layer below the flavedo, a source of flavanones), and vascular bundles (a network of thin threads along the flesh). The inner flesh has segments, usually aligned, and situated around the soft central core of the fruit and wrapped

by a thin segment membrane called the septum. Small and densely packed sacs containing juice and seeds in most varieties fill the segments, and the citric acid contained in the juice together with a complex mix of other acids, oils, and sugars, give the characteristic flavor (Albrigo et al., 1977; Ranganna et al., 1983).

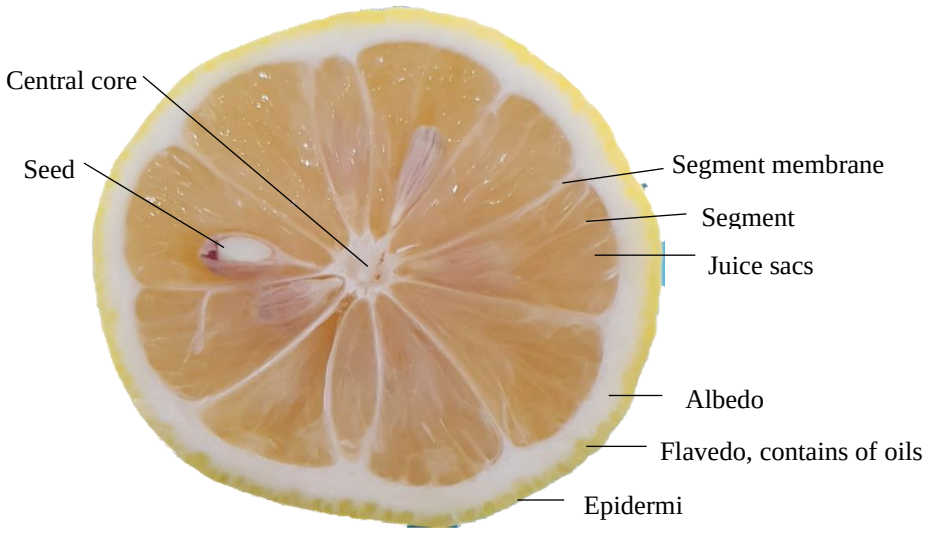


Fig. 1. Schematic part of citrus fruit

1.2.1 Nutritional values

Lemon fruit is rich in macronutrients (such as simple sugars, protein, and dietary fiber), and micronutrients (including folate, niacin, pantothenic acid, pyridoxine, riboflavin, thiamin, vitamin (C, A, and E), sodium, potassium, calcium, copper, iron, magnesium, manganese, zinc, α and ß-carotene, ß-cryptoxanthin and

xantophylls) which are essential for maintaining health body and supporting growth. It also has a low energy density and free of cholesterol (**Table 1**) (USDA, 2011).

Table 1. Nutritional values of lemon, raw, without peel

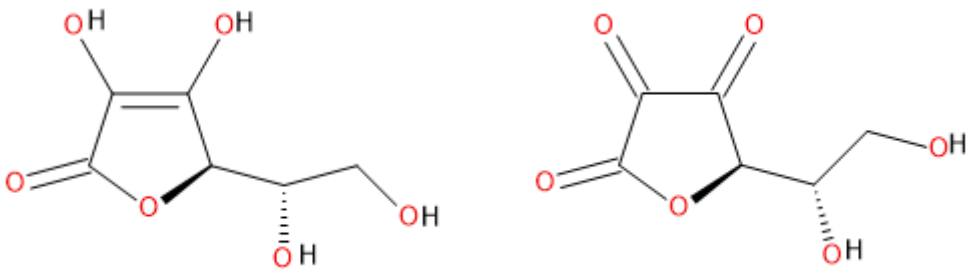
	per 100g fruit		per 100g fruit
Energy (kcal)	29	Vitamin E (mg)	0.15
Carbohydrate (g)	9.32	Sodium (mg)	2
Protein (g)	1.10	Potassium (mg)	138
Total fat (g)	0.30	Calcium(mg)	26
Cholesterol (g)	0	Copper (µg)	37
Dietary fiber (g)	2.80	Iron (mg)	0.60
Folate, total (µg)	11	Magnesium (mg)	8
Niacin (mg)	0.100	Manganese (mg)	0.030
Pantothenic acid (mg)	0.190	Zinc (mg)	0.06
Pyridoxine (mg)	0.080	ß-carotene (µg)	3
Riboflavin (mg)	0.020	alfa-Carotene (µg)	1
Thiamin (mg)	0.040	ß-Cryptoxanthin (µg)	20
Vitamin C (mg)	53	Xanthophylls (µg)	11
Vitamin A (IU)	22		

Source: USDA, 2011

The maturation and ripening stage of lemon was indicated by an increase in total sugar, a decrease in acidity and ascorbic acid level, and a change in the color of berries from green to yellow. The sweetness depends on the sucrose, glucose, and fructose compositions as major component of lemon carbohydrates. Sugar composition in LJ are glucose (1.40 %), fructose (1.35%) and sucrose (0.41%). The

ratio of sucrose to other reducing sugars is fluctuating depend on the stages of maturity, varieties, and decreases in the acidic environment with long-term storage (Ting and attaway, 1971; Kuraoka et al., 1976). Mostly organic acid composition in LJ are citric acid (4 – 4.38 g/100 ml) and malic acid (0.07 – 0.26 g/100 ml) (Vandercook, 1977).

1.2.2 Functional properties



(a) (b)

Fig. 2. Molecular structure of (a) l-ascorbic acid, and (b) dehydroascorbic acid

The contribution of lemon and other citrus fruits to human health is probably because of high concentration of vitamin C (ascorbic acid). In lemon fresh, vitamin C consists of l-ascorbic acid (50.4 mg/100g) and dehydroascorbic acid (23.9 mg/100g) (**Fig. 2.**) (Mitchell et al., 1992). The edible portion contains about 25%

of the total vitamin C in the whole fruit. The peels (flavedo and albedo), although generally known as the inedible parts, contain a higher concentration than other components (Nagy and Attaway, 1980).

Table 2. Flavonoids and phenolic acids composition of lemon extracts and LJ

Group of compounds	Part of fruit	Metabolites
Flavonoids	Whole fruit (pulp, seed, and peel)	Flavonones: eriocitrin, eriodiktyol, hesperidin, naringin, neoeriocitrin, neohesperidin. Flavones: apigenin, diosmetin, diosmin, homoorientin, luteolin, orientin, vitexin. Flavonols: isoramnethin, quercetin, limocitrin, rutoside, spinacetin.
	LJ	Flavonones: hesperidin, naringin. Flavones: apigenin, chrysoeriol, diosmetin, luteolin. Flavonols: isoramnethin, quercetin, rutoside dihydroxyflavonols: dihydroxyisoramnethin-7-O-rutinoside.
Phenolic acids	Whole fruit (pulp, seed and peel)	Dihydroferulic acid, p-hydroxybenzoic acid, 3-(2-hydroxy-4-methoxyphenyl)propanoic acid, synapic acid.
	LJ	Ferulic acid, synapic acid.

Source: Klimek-Szczykutowicz et al., 2020.

Flavonoids and phenolics acid are other important group of bioactive compounds in lemon determining their biological activity. In lemon fruit and LJ, flavonoids are detected: flavanones—eriodictyol, hesperidin, hesperetin, naringin; flavones—apigenin, diosmin; flavonols—quercetin; and their derivatives (**Table 2.**). In the whole fruit, other flavonoids are additionally detected: flavonols—limocitrin and spinacetin, and flavones—orientin and vitexin. Naringin, neohesperidin, and hesperidin are characteristics of lemon fruit). Phenolic acids in LJ mainly ferulic acid and synapic acid, and their derivatives. In the whole fruit, p-hydroxybenzoic acid and propanoic acid are additionally detected (Klimek-Szczykutowicz et al., 2020).

1.3 Lemon Essential Oil (LEO)

According to the Food and Agriculture Organization of the United Nations and United Nations Industrial Development Organization (FAO UN-UNIDO, 2005), essential oils (EOs) are defined as liquid products of steam or water distillation of plant parts (leaves, stems, bark, seeds, fruits, roots, and plant exudates). Expression is used exclusively for the extraction of citrus oil from the fruit peel because the chemical components of the EOs are easily damaged by heat.

As defined by the International Organization of standardization (ISO), the term EOs is reserved for the product obtained from a natural raw material of plant origin, by steam distillation, by mechanical processes from the epicarp of citrus fruits, or by dry distillation, after separation of the aqueous phase-if any- by physical processes (ISO 9235, 2021).

Fig. 3. Lemon fruits peel

LEO, the most important byproduct of lemon processing, is obtained by mechanical processes (cold pressing) or distillation (steam or water distillation) from the fruit peel, particularly in the flavedo part (**Fig. 3**). LEO is colorless or yellow and has a characteristic, strong lemon scent. The main components of LEO are monoterpenes, with the major volatile compounds are D-limonene (56.6-76.6 %), ß-pinene (6.0-17 %), γ-terpinene (3.0-13.3 %), and α-pinene (1.1-1.4 %) (**Fig. 4**.)

(a) (b) (c) (d)

Fig. 4. Molecular structure of (a) D-limonene, (b) ß-pinene, (c) γ-terpinene, and (d) α-pinene

Table 3. Compositions of LEO obtained from the lemon peel

Compounds	Extraction methods	
	Distilled (%)	Expressed (%)
Volatiles		
D-limonene	64.0-70.5	56.6-76.0
ß-pinene	8.2-14.0	6.0-17.0
γ-terpinene	8.4-10.7	3.0-13.3
α-pinene	1.1-2.1	1.3-4.4
ß-myrcene	1.4-1.6	Tr-2.2
α-terpineol		0.1-8.0
Sabinene	0.8-1.7	0.5-2.4
Neral	0.5-1.5	0.4-2.0
Geranial	0.7-2.2	0.5-4.3
p-cymene		Tr-2.3
Neryl acetate		0.1-1.5
Terpinen-4-ol		Tr-1.9
Non-volatiles		
Bergamottin	0.16-0.54	
Bergapten	0.0001-0.035	
Oxypeucedanin	0.09-0.82	
Geranloxy-7-methoxycoumarin	0.18-0.28	
Citropten	0.05-0.17	
Byakangelicol	0.006-0.16	
8-geranyloxypsoralen	0.01-0.045	
isopimpinellin	0-0.011	

Source: Tisserand and Young, 2014.

There are different compositions of LEO due to differences in origin, genetic background, season, climate, age, ripening stage, method of extraction, etc. The volatile and non-volatile components of LEO have been showing in **Table 3** (Tisserand and Young, 2014). LEO, having pleasant refreshing smell and rich aroma, has been classified as GRAS (FDA, 2020). It used as food additives in several food and beverage product, body care products (skin and hair scare), emotional care through aromatherapy, and preservatives due to their broad spectrum of biological activities including antimicrobial and antioxidant effect (Dosoky and Setzer, 2018).

1.4 Safety and Biological Activities of LJ and LEO

Two main parts of lemon fruit are LJ (edible part) and LEO, and both are categorized as generally recognized as safe (GRAS). LEO are non-toxic, non-mutagenic, and non-carcinogenic, but there is a possible skin sensitization issue if old or oxidized oil is used (Tisserand and Young, 2014). The distilled oils are not phototoxic, while the expressed oils carry a low to moderate risk of phototoxicity due to the presence of furanocoumarins (Opdyke, 1974).

Lemon fruit extract and LEO have abundant biological activities, such as anticancer activity, anti-inflammatory activity, antibacterial activity, antifungal activity, antiviral activity, prevention of diabetes, effect on the nervous system, and anti-obesity. Lemon fruit extract are additionally with antioxidant activity, hepatoregenerative activity, anti-allergic activity, effect on cardiovascular system, effect on respiratory system, effect on skeletal system and treatment of menstrual disorders. LEO is additionally with antiparasitic activity, anticaries activity, hepatoprotective and detoxification activity, effect on digestive system, and lipolytic and cholesterol-lowering activity (Klimek-Szczykutowicz et al., 2020).

1.5 Nanoencapsulation As a Valuable Option for LJ and LEO

The main challenges faced while the inclusion of LJ in food products are related to the high reactivity and low stability properties, while the inclusion of LEO faces the problems of volatile nature and low water solubility properties as mentioned previously. Encapsulation of LJ and LEO into nano-carriers can be an answer to availability and degradation issues.

Encapsulation is a process to enclose material into a capsule before delivery into a system (**Fig. 5.)**. It also has been defined as the technology of packaging

solids, liquids, or gaseous materials in small capsules that release their contents at controlled rates over prolonged periods and under specific conditions (Zuidam and Shimoni, 2010). Nanoencapsulation is encapsulation in the nanoscale range. Based on the size, materials are classified into macro (>5000 μm), micro (1.0 – 5000 μm), and nano (< 1.0 μm) (Jafari et al., 2008). Nanoscale dimension based on The US Food and Drug Administration (USFDA) and EU Commission is approximately 1 – 100 nm (BSI, 2011). However, a general accepted definition does not exist. Nanoencapsulation gives better performances than macro encapsulation system – and in the food field it can help to prevent off-flavors, off-taste, and undesirable texture of food, protect chemical and biological degradation during processing and storage caused by moisture and heat, and can achieve controlled release of encapsulated nutrients to a specific rate (Paredes et al., 2016).

(a) (b) (c)

Fig. 5. Encapsulation of bioactive compounds (a) capsule wall, (b) bioactive compounds, and (c) encapsulated bioactive compounds

Nanomaterial have unique properties unlike bulk materials in physicochemical (e.g., porosity), optical, mechanical, and catalytic properties. Its differences are also observed in the absorption, function, stabilization, strength, and weight of materials. However, when this generic technology is applied to foods, the changed properties of the nanomaterials may also affect the behavior and properties of the foods (Cockburn et al., 2012). Nanomaterial has tremendous advantages such as improved bioavailability of functional compounds; improved color, flavor and texture that enhancing the sensory acceptance; and also improved the delivery and absorption of active ingredients and nutrients. Furthermore, the benefits include targeted delivery, enhanced absorption and stability of the bioactive compounds, along with improved antimicrobial effects against pathogens (Chaundry and Castle, 2011; Duncan, 2011).

1.6 Complex Coacervation Technique

One of the nanoencapsulation methods of bioactive compounds is complex coacervation, which is based on the ability of cation and anionic water-soluble polymers to interact in water to form a liquid, neutral, polymer-rich phase called coacervate. The complex coacervation techniques involve the separation phase of a

mixture of polyelectrolyte from a solution and the subsequent deposition of the newly formed coacervate phase around the active ingredient (Zuidam and Shimoni, 2010). Coacervation of complexes can occur spontaneously by mixing oppositely charged polyelectrolytes in an aqueous medium (Priftis and Tirrell, 2012). Complex coacervation is known for its simplicity, low cost, scalability, and reproducibility in encapsulation of food ingredients that yields high encapsulation efficiency even at very high payload (up to 99%) (Timilsena et al., 2019).

1.6.1 Process and mechanism

A three-phase system involving complex encapsulation are solvent, bioactive compounds, and wall material. There are four main steps in this process: (a) preparations of an aqueous solution of two or more polymers. The solution prepared above the gelling agent temperature for carbohydrate and above the isoelectric point of pH of protein; (b) mixing and homogenized of core material (bioactive compounds) and aqueous solution; (c) change pH and temperature to a certain required level to induce coacervation and phase separation; and (d) hardening of the polymer matrices using heating, desolvation, or cross-linking for the

solidification of capsules (Timilsena et al., 2019). The process illustration can be seen in **Fig. 6**.

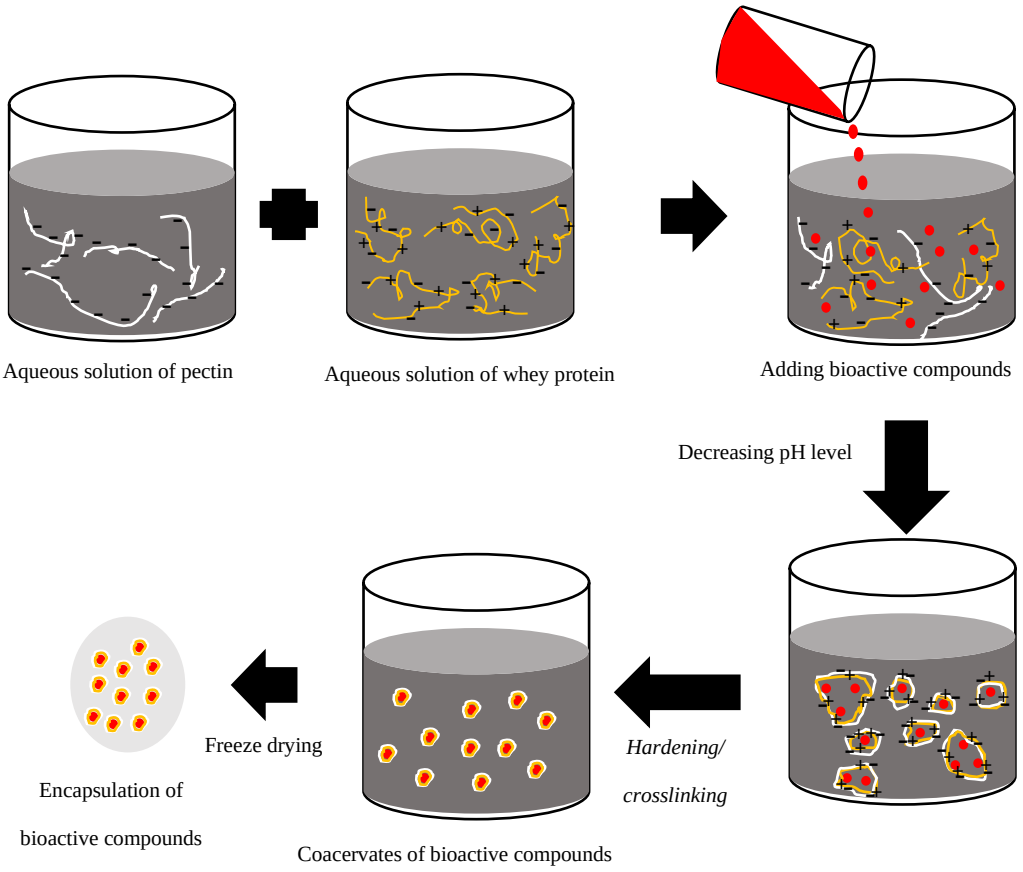


Fig. 6. Coacervation process mechanism of bioactive compounds in whey protein-pectin complex

The coacervation is initiated by either altering pH or by lowering the temperature or diluting or addition of a salt/coacervation agent. At whey protein-pectin complexes, the electrical charge of pectin is negative at all pH range. The net electrical charge of whey protein is negative at pHs above the isoelectric point, but changes to positive at below the protein isoelectric point. So, complex formation

will occur at pHs below the isoelectric point (Turgeon et al., 2007; De Kruiff and Tuiner, 2001); and Turgeon et al., 2003). The morphology and size, generally either mononucleated or multinucleated, are affected by the processing conditions.

1.6.2 Factors that influence the formation of protein/polysaccharide electrostatic complexes and coacervated

The factors that influence in conplex coacervation are the pH level, the ionic strength, the temperature, the molecular weight and the concentration of polymer (Priftis and Tirrell, 2012).

a. pH level

The interactions between whey proteins and polysaccharides in complex coacervation are directly controlled by the degree of ionization of the biopolymers, and thus pH level of the medium is important in electrostatic control (Turgeon et al., 2007). In general, proteins possess negative charge above their isoelectric point (pI) but become positively charged as soon as the pH is dropped below their pIs. This is caused by the protonation of amino groups. When an anionic polysaccharide solution is mixed with a protein solution and pH is reduced below pI of the protein,

the extent of electrostatic attraction increases due to increased charge difference between the reacting protein and polysaccharide molecules (Siow and Ong, 2013). Decrease pH level too far will lead precipitation because of too large positive charges in solutions. The optimum pH range for highest degree of complex coacervation is different for each biopolymer system and there is a very narrow pH range where coacervation is stable between any polymer systems (Ru et al., 2012).

b. Ionic strength

The quantity of salt present in the medium, generally verified by the addition of NaCl, affects the ionic strength of the solution, which in turn, influences the complex coacervation process. The increase in ionic strength reduced the enthalpy contribution of the interaction, concluding that the addition of NaCl inhibits the electrostatic interactions fundamental to complexation (Sperber et al., 2010). de Kruif (2004) reported that even a small amount of salt weakens the electrostatic binding between polymers. However, another research showed that at a low concentration of salt, there is no effect, but at high concentration of salt interfered with the complexation by substantial quantity of protein isolate aggregation (Liu et al., 2009).

c. Ration of biopolymers and molecular weight

Optimum pH and ionic strength is also dependent on mixing ratio of biopolymers, nature of core material and other experimental condition. The same combinations of biopolymers give optimum condition at slightly different pH level and mixing ratio, depending on the core. Studied the coacervation between bovine serum albumin (BSA) and pectin at various biopolymer ratios and pH levels found that higher concentrations of protein fraction leads to higher yield of coacervate (Ru et al., 2012). Shifting of ratio of cationic and anionic polymers from their optimum mixing proportion makes one component deficit and other component in excess, leaving some molecules of excess polymers unreacted. These unreacted molecules are left over in the equilibrium phase when the coacervates are recovered. Thus, optimisation of mixing ratio is also an important consideration from an economic point. The molecular weight of the participating biopolymers has also been reported to play an important role in complex coacervation. High molecular weight materials form gels or self-precipitates, whereas low molecular weight materials interact with each other by ion pairing that inhibits coacervation process (Burgess, 1994).

d. Temperature

The temperature may alter the conformation of proteins and polysaccharides, favoring non-electrostatic interactions. At low temperatures, hydrogen bonds are favored, whereas at higher temperatures hydrophobic interactions take precedent due to the exposure of the binding sites after denaturation of the globular proteins, as well as conformational rearrangement of the structure of polysaccharides. Hydrophobic interactions became more significant than hydrogen bonds with increased temperature (Schmitt et al., 1998; Philips and Williams, 2009).

e. Concentration of biopolymer

A high concentration of biopolymers also exhibits a negative effect on coacervation. Burgess (1994) indicated that high concentration does not allow the free movement of the molecules and restricts them in close proximity, reducing the energy gain during coacervation. This restricted movement suppresses interactions between macromolecules (Burgess, 1994).

f. Other factors

Previous studies reported that processing parameters (shear, temperature, and residence time) and techno-functional aspects (interfacial tension, viscosities and densities) of the system affect the coacervation conditions and coacervate phase properties (such as rheology and yield), and encapsulation efficiency of specific compounds (Xing et al., 2003). However, it is poorly reported in literature and have not been well described.

1.6.3 The post-processing: freeze-drying

Freeze-drying or lyophilization is one commonly used in the food industries after spray-drying to transfer the nanoparticles into dried powder. Freeze-drying consists of three stages: (1) solidification by freezing of the liquid-suspensions, (2) ice sublimation (primary drying), and (3) desorption of unfrozen water (secondary drying). In the solidification stage, pure water in liquid-suspension is converted into ice crystal resulting in an increase in the suspension concentration and viscosity that inhibits further crystallization. Next is ice sublimation, applying vacuum which leads to the depression of the boiling point that in turn induces ice sublimation stages. At the end of the stages, porous pellet is formed due to sublimation of ice

crystal. The final stages, desorption of unfrozen water is characterized by removal of water that did not come out during first and second stage. Freeze drying has been reported successfully for polymeric nanoencapsulation for both hydrophilic and lipophilic entrapped compounds (Mohammady et al, 2000).

1.7 Biodegradable Polymeric Nanocapsules

Both natural and synthetic polymer can be used as capsule materials in encapsulation, but for food application, it must be certified as GRAS, food-grade, biodegradable, non-toxic, and able to form a barrier between sensitive bioactive compounds and their environments (Nedovic et al., 2011). Whey proteins-pectin complex has got attention as ideal materials to produce encapsulation system in food due to its safety, high nutritional value, low cost, and various functional properties.

1.7.1 Whey protein

Whey protein is composed of various globular proteins, including α-lactalbumin (α-la), β-lactoglobulin (β-lg), bovine serum albumin (BSA), immunoglobulins, and lactoferrin (**Table 5**). In particular, β-lg comprises about

50%–60% of whole whey proteins in cow milk and contains one free thiol residue and two disulfide bonds. The most widely produced whey protein products are whey protein concentrates (WPC) and whey protein isolate. WPChas contained about 50%–75% total proteins, whereas whey protein isolateis composed of higher protein concentrations exceeding 90% (Livney, 2010; Gunasakeran et al., 2007)).

Whey protein as delivery material in the form of capsules has several functional properties, such as the ability to bind to hydrophobic bioactive compounds and other compounds, gel-forming capacities, emulsifying properties, and barrier effects. The binding ability to bioactive compounds is the most important factor for the ideal wall material in the delivery system. The binding ability to target bioactive compounds is the most important factor for ideal delivery material. β-lg is known as a member of the lipocalin protein family and has three potential binding sites for hydrophobic compounds: the inner hollow of the β-barrel, the surface cavity in a channel between the α-helix and β-barrel, and the external surface near Trp19–Arg124 (Roufik et al., 2006; Liang et al., 2007). Numerous studies suggest that whey protein, mainly β-lg, could bind various hydrophobic bioactive compounds such as vitamin D (Forrest et al., 2005), D-limonene (Ghasemi et al., 2018), retinol (Wang et al. 1997), and polyphenols (Shpigelman, 2010). Due

to the ability to bind hydrophobic flavor compounds, whey proteins have also been used as a delivery material for enhancing flavor or masking off-flavor. The composition of whey protein can be seen in **Table .4**.

Table 4. Composition of major proteins in whey, molecular mass (Mm), isoelectric point (pI), temperature and denaturation (Td), and number of amino acid residues

Whey protein	Mm (kDa)	pI	Td (°C)	Number of amino acid residues
β-lactoglobulin	18.3	5.2	71.9	162
α-lactalbumin	14.2	4.8	64.3	123
Immunoglobulins	150-900	5.5-6.8	-	-
Serum albumin	66.4	4.7-4.9	72.0-74.0	583
Protease peptones	<12	3.3-3.7	-	-
Lactoferrin	80.0	8.0-8.5	63.0 and 90.0	700
Lactoperoxidase	78.5	9.8	70.0	612

Source: Ramos et al., 2017.

Gelation, during heat treatment, whey proteins can be partially or fully unfolded, contributing to the exposure of hydrophobic groups and free sulfhydryl groups. This may enhance hydrophobic interactions and the formation of disulfide bonds between whey protein molecules, resulting in gel networks, including nano-delivery systems of various sizes (Chen and Subirade, 2005). Whey proteins have been considered to be good emulsifiers because whey proteins can adsorb at oil–

water interfaces and produce thick layers, contributing to the stabilization of emulsion droplets and preventing lipid separation which works against coalescence (Lee et al., 2013). Since whey proteins have antioxidant activity, metal chelating ability, and gel-forming capability, whey proteins used as delivery material may provide barrier effects for enhancing the encapsulation efficiency of bioactive compounds and preventing bioactive compound oxidation. It has been reported that β-lg acts as a free radical scavenger, since it has free sulfhydryl residues and aromatic amino acids leading to antioxidant activity (Hu et al. 2003; Yi et al, 2014. It can thus be used to enhance the oxidative stability of bioactive compounds (Lekshmi et al. 2019; Mehrad et al., 2018). In addition to the antioxidant activity and metal chelating property of whey proteins, whey proteins used as materials in nanoemulsion delivery systems can act as barriers on fat–water interfacial areas (Chen et al., 2018; Hou et al., 2019). They can contribute to a decrease in the access of transition metals to lipid droplets, resulting in a reduction in lipid oxidation (Hwang et al., 2017).

1.7.2 Pectin

Pectin is a complex polysaccharide, non-random structural heteropolysaccharide comprising of four distinct substructures: homogalacturonan, xylogalacturonan, and rhamnogalacturonan I and II. Pectin mainly consists of galacturonic acid units being linked by α-(1→4) linkages (Naqash et al., 2017). Molecular weight of pectin is in the range of 50,000-180,000 Da. Based on the esterification of the carboxyl group with methanol, pectin be classified into two groups: high methoxy pectin (HM) which has esterified carboxyl group higher than 50% and low methoxy pectin (LM) which esterified carboxyl group lower than 50%. The degree of esterification, molecular weight, and extraction condition determine the properties of pectin (gelling, textural properties, and stability) and its ample use in food formulation. Pectin is negatively charged molecules; the charge can interfere with gelation. Pectin is soluble in water and insoluble in most of the organic solvents. pectin exhibit Newtonian behavior at lower concentration and pseudoplastic behavior at higher concentration (Mellinas et al., 2020).

Many studies have been conducted on pectin and its combination with other hydrocolloids to act as nano-encapsulating agent for bioactive compounds. Combination pectin with other hydrocolloids forms smaller particles as compared

to sole biopolymer. Oxidized pectin was used to encapsulate doxorubicin for enhanced biological and anticancer effects (Takei et al., 2020). The anionic pectin in combination with β-lactoglobulin for encapsulation of ergocalciferol resulted in increased shelf-life stability of bioactive as compares to uncomplexed β-lactoglobulin. Pectin-whey protein complex was used to encapsulated anthocyanin (Arroyo-Maya and McClements, 2015), saffron (Esfanjani et al., 2015), phenolic extract (Mohammadi et al., 2016), and D-limonene (Ghasemi et al., 2018).

1.8 Aims and Scope

Nanoencapsulation of LJ and LEO in WPC-pectin by complex coacervation could solve the problems of difficulties inclusion in the food matrix for further application in foods. **Chapter 1**: presented the interesting study, and some proof of the possibility of encapsulated LJ and LEO in WPC-pectin complex. **Chapter 2**: focuses on the optimization formula of WPC-pectin complex as nanocapsules for LJ. WPC and pectin concentration at different concentrations and different level of pH were studied to decide the optimum coacervation technique in the production of LJNCs based on the responses of antioxidant activity and D-limonene content. In this chapter not only focused on the production of LJNCs but also aimed at

characterization of the LJNCs in terms of their morphological properties (Scanning Electron Microscopy/SEM), encapsulation efficiency (High Performance Liquid Chromatography/HPLC), particle size distributution (Atomic Force Microscopy/AFM), molecular (Fourier Transform Infrared Spectroscopy/FTIR) properties. The biological activities of LJNCs were also evaluated including antioxidant activity by DPPH assay and anticancer activity by *in vitro* method against murine colon carcinoma (colon-26) cells line.

Chapter 3: focused on fabrication of LEO nanocapsules (LEONCs) by complex coacervation using WPC-pectin as wall material. LEO was extracted from the lemon peel by steam distillation technique. Characterization of LEONCs in terms of their particle size (AFM), morphology (SEM), encapsulation efficiency (spectrophotometry), molecular (ATR-FTIR), and volatile composition (HS-GC-MS) properties was done. The fabricated LEONCs were also tested in the form of their antioxidant activity by DPPH assay and anticancer activity by *in vitro* methods against murine colon carcinoma (colon-26) cells line.

Finally, **Chapter 4** summarizes the advantages and limitations of each nano-formulations (LJNCs and LEONCs) as well as their biological activities potential, followed by recommendation for further exploration of these nanoencapsulation.

References

Albrigo LG, Carter RD. Structure of citrus fruits in relation to processing. In: Nagy S, Shaw PE, Veldhuis MK, editors. *Citrus science and technology*. Vol. 1. Westport, Conn.: AVI Publishing Company. 1977, 33-73.

Arroyo-Maya IJ, McClements DJ. Biopolymer Nanoparticles as Potential Delivery Systems for Anthocyanins: Fabrication and Properties. *Food Research International* 2015, 69, 1-8.

BSI (British Standard Institution). 2011. *PAS 71: Nanoparticles*, Vocabulary. BI: London, United Kingdom.

Burdulu HS, Koca N, Karadeniz F. Degradation of vitamin C in citrus juice concentrates during storage. *Journal of Food Engineering.* 2006, 74, 211-216.

Burgess DJ. 1994. *Complex coacervation: microcapsule formation, in Macromolecular complexes in chemistry and biology*. Springer. p. 285-300.

Cerqueira MA, Pinheiro AC, Ramos OL, Silva H, Bourbon AI, Vicente AA. Advances in food nanotechnology, in *Micro and Nano Technologies*. In editor Busquets, R. 2017. *Emerging Nanotechnologies in Food Sciences*. Boston, MA: Elsevier, 11-38.

Chau CF, Wu SH, Yen GC. The development of regulations for food nanotechnology. *Trends in Food Science and Technology*. 2007. 18(5), 269–280.

Chaundry Q, and Castle L. Food applications of nanotechnologies: An overview of opportunities and challenges for developing countries. *Trends Food Sci. Technol.* 2011, 22, 595-603.

Chen E, Cao L, McClements DJ, Liu S, Li B, Li Y. Enhancement of Physicochemical Properties of Whey Protein-Stabilized Nanoemulsions by Interfacial Cross-Linking using Cinnamaldehyde. *Food Hydrocoll.* 2018, 77, 976–985.

Chen L, Subirade M, Chitosan/β-Lactoglobulin Core-Shell Nanoparticles as Nutraceutical Carriers. *Biomaterials*. 2005, 26, 6041–6053.

Cockburn A, Bradford R, Buck N, Constable A, Edwards G, Haber B, Hepburn P, Howlett J, Kampers F, Klein C, Radomski M, Stamm H, Wijnhoven S, and Wildeman T. Approach to the safety assessment of engineered nanomaterials (ENM) in food. *Food Chem. Toxicol*. 2012, 50, 2224-2242.

de Kruif CG, Tuinier R. Polysaccharide Protein Interactions. *Food Hydrocolloid*. 2001, 15, 555–563.

de Kruif CG, Weinbreck F, de Vries R. Complex coacervation of proteins and anionic polysaccharides. *Current opinion in colloid & interface science*. 2004, 9, 5, 340- 349.

Dosoky NS, Setzer WN. Biological activities and safety of citrus spp. Essential oils. *Int. J. Mol. Sci.* 2018, 19, 1966, 1-25.

Duncan TV. Applications of nanotechnology in food packaging and food safety: Barrier materials, antimicrobials, and sensors. *J. Colloid Interface Sci*. 2011, 363, 1-24.

Esfanjani AF, Jafari SM. Biopolymer nano-particles and natural nano-carriers for nano-encapsulation of phenolic compounds. *Colloids Surf. B: Biointerfaces*. 2016,146, 532-543.

FAO UN-UNIDO. 2005. *Herbs, Spices, and Essential Oils: Post -harvest operations in developing countries*. UNIDO and FAO, http://www.fao.org/3/ad420e/ad420e.pdf.

FDA (U.S. Food and Drug Administration). 2020. *CFR-Code of Federal Regulation Title 21*.https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?cfrpart=182&showfr=1, accessed on April 19, 2021.

Forrest SA, Yada RY, Rousseau D. Interactions of Vitamin D3 with Bovine β-Lactoglobulin A and β-Casein. *J. Agric. Food Chem*. 2005, 53, 8003–8009.

Ghasemi S, Jafari SM, Assadpour E, Khomeiri. Nanoencapsulation of D-limonene within nanocarriers produced by pectin-whey protein complexes. *Food Hydrocolloids*. 2018, 77, 152-162.

Gunasekaran S, Ko S, Xiao L. Use of Whey Proteins for Encapsulation and Controlled Delivery Applications. *J. Food Eng*. 2007, 83, 31–40.

Hou P, Pu F, Zou H, Diao M, Zhao C, Xi C, Zhang, T. Whey Protein Stabilized Nanoemulsion: A Potential Delivery System for Ginsenoside Rg3 Whey Protein Stabilized Nanoemulsion: Potential Rg3 Delivery System. *Food Biosci*. 2019, 31, 100427.

Hu M, McClements DJ, Decker EA. Impact of Whey Protein Emulsifiers on the Oxidative Stability of Salmon Oil-in-Water Emulsions. *J. Agric. Food Chem*. 2003, 51, 1435–1439.

Hwang J, Ha H, Lee M, Kim JW, Kim H, Lee W. Physicochemical property and oxidative stability of whey protein concentratemultiple nanoemulsion containing fish oil. *J. Food Sci.* 2017, 82, 437,444.

ISO 9235:2021. *Aromatic natural raw materials*. https://www.iso.org/obp/ui/#iso:std:iso:9235:ed-3:v1:en , accessed on June 15, 2021.

Jafari SM, Assadpoor E, He Y and Bhandari B. Encapsulation efficiency of food flavours and oils during spray drying. *Drying Technol*. 2008, 26(7), 816-835.

Klimek-Szczykutowicz M, Szopa A, Ekiert H. *Citrus limon* (lemon) phenomenon—a review of the chemistry, pharmacological properties, applications in the modern pharmaceutical, food, and cosmetics industries, and biotechnological studies. *Plants*. 2020, 9 (119), 1-24.

Kuraoka T, Iwasaki K, Hino A, Kaneko Y, Tsuji H. Studies on the peel puffing of Satsuma mandarins, 4: Changes in sugar content during the development of the fruit rind. *J Japanese Soc Hort Sci*. 1976, 44, 375–380.

Lee M, Choi H, Ha H, Lee W. Production and characterization of beta-lactoglobulin/alginate nanoemulsion containing coenzyme Q10: Impact of heat treatment and alginate concentrate. *Korean J. Food Sci. An*. 2013, 33, 67-74.

Lekshmi RGK, Rahima M, Chatterjee NS, Tejpal CS, Anas KK, Vishnu KV, Sarika K, Asha KK, Anandan R, Suseela M. Chitosan–Whey Protein as Efficient Delivery System for Squalene: Characterization and Functional Food Application. I*nt. J. Biol. Macromol.* 2019, 135, 855–863.

Liang L,Tajmir-Riahi H, Subirade M. Interaction of β-Lactoglobulin with Resveratrol and its Biological Implications. *Biomacromolecules.* 2007, 9, 50–56.

Liu S, Low NH, Nickerson MT. Effect of pH, Salt, and Biopolymer Ratio on the Formation of Pea Protein Isolate−Gum Arabic Complexes. *Journal of Agricultural and Food Chemistry*. 2009, 57, 4, 1521-1526.

Livney YD. Milk Proteins as Vehicles for Bioactives. *Curr. Opin. Colloid Interface Sci*. 2010, 15, 73–83.

Mehrad B, Ravanfar R, Licker J, Regenstein JM, Abbaspourrad, A. Enhancing the Physicochemical Stability of β-Carotene Solid Lipid Nanoparticle (SLNP) using Whey Protein Isolate. *Food Res. Int.* 2018, 105, 962–969.

Mellinas C, Ramos M, Jimenez A, Garrigos, MC. Recent trends in the use of pectin from agro-waste residues as a natural-based biopolymer for food packaging applications: Review. Materials. 2020, 13, 673, 1-17.

Mitchell GE, McLauchlan RL, Isaacs AR, Williams DJ, Nottingham SM. Effect of low dose irradiation on composition of tropical fruits and vegetables. *J Food Comp Anal*. 1992, 5, 291–311.

Mohammadi A, Jafari SM, Esfanjani AF, Akhavan S. Application of nano-encapsulated olive leaf extract in controlling the oxidative stability of soybean oil. *Food Chem*. 2016, 190, 513–519.

Mohammady M, Mohammadi Y, Yousefi G. Freeze-drying of pharmaceutical and nutraceutical nanoparticles: the effects of formulation and techniques parameters on nanoparticles characteristics. *Journal of Pharmaceutical Sciences*. 2000, 109, 11, 3235-3247.

Nagy S, Attaway JA. 1980. *Citrus nutrition and quality*. Washington, D.C.: American Chemical Society.

Naqash F, Masoodi FA, Rather SA, Wani SM. Emerging concepts in the nutraceutical and functional properties of pectin − A Review. *Carbohydr Polym.* 2017, 168, 227-239.

Nedovic V, Kalusevic A, Manojlovic V, Levic S, Bugarski B. An overview of encapsulation technologies for food applications. *Procedia Food Sci*. 2011, 1, 1806-1815.

Opdyke D. L. J. Monographs on fragrance raw materials. *FoodCosmet.Toxicol.* 1974, *12*, 807–1016.

Paredes A, Asensio CM, Llabot J, Allemandi D. Nanoencapsulation in the food industry: manufacture, applications and characterization. *J. Food Bioengineering and Nanoprocessing*. 2016, 1, 1, 56-79.

Phillips GO, Williams PA. 2009. *Handbook of hydrocolloids*. Cambridge, U.K.: Woodhead Publishing Ltd.

Priftis D, Tirrell M. Phase behaviour and complex coacervation of aqueous polypeptide solutions. *Soft Matter*. 2012, 8, 9396-9405.

Ramos OL, Pereira RN, Martins A, Rodrigues R, Fucinos C, Teixeira JA, Pastrana L, Malcata FX, VEcente AA. Design of whey protein nanostructures for incorporation and release of nutraceutical compounds in food. *Critical Reviews in Food Science and Nutrition*. 2017, 57, 7, 1377-1393.

Ranganna S, Govindarajan VS, Ramana KVR. 1983. Citrus fruits—varieties, chemistry, technology, and quality evaluation. Part II. Chemistry, technology, and quality evaluation. A. Chemistry. *Crit Rev Food Sci Nutr*. 1983, 18, 313–386.

Roufik S, Gauthier SF, Leng X, Turgeon SL. Thermodynamics of Binding Interactions between Bovine Beta-Lactoglobulin A and the Antihypertensive Peptide Beta-Lg f142-148. *Biomacromolecules*. 2006, 7, 419–426.

Ru Q, Wang Y, Lee J, Ding Y, Huang Q. Turbidity and rheological properties of bovine serum albumin/pectin coacervates: effect of salt concentration and initial protein/polysaccharide ratio. *Carbohydrate Polymers*. 2012, 88, 3, 838-846.

Schmitt C, Sanchez C, Desobry-Banon S, Hardy J. Structure and technofunctional properties of protein-polysaccharide complexes: A review. *Critical Reviews in Food Science and Nutrition*. 1998, *38*, 689–753.

Shpigelman A, Israeli G, Livney YD. Thermally-Induced Protein–Polyphenol Co-Assemblies: Beta Lactoglobulin-Based Nanocomplexes as Protective Nanovehicles for EGCG. *Food Hydrocoll*. 2010, 24, 735–743.

Siow LF, Ong CS. Effect of pH on garlic oil encapsulation by complex-coacervation. *J. Food Process. Technol*. 2013, 2, 4, 199.

Takei T, Sato M, Ijima H, Kawakami K. In Situ Gellable Oxidized Citrus Pectin for Localized Delivery of Anticancer Drugs and Prevention of Homotypic Cancer Cell Aggregation. *Biomacromolecules*. 2020, 11(12), 3525-3230.

Timilsena YP, Akanbi TO, Khalid N, Adhikari B, Barrow CJ. Complex coacervation: principles, mechanisms and applications in microencapsulation. *Int J Biol Macromol.* 2019, 121, 1276-1286.

Ting SV, Attaway JA. 1971. *Citrus fruits*. In: Hulme AC, editor. The biochemistry of fruits and their products. London: Academic Press. p 107–179.

Tisserand, R., Young, R. *Essential Oil Safety*, 2nd ed.; Elsevier: New York, NY, USA, 2014.

Turek C, Stintzing FC. Stability of essensial oils: a review. *Comprehensive reviews in food science and food safety*. 2013, 12, 40-53.

Turgeon SL, Beaulieu M, Schmitt C, Sanchez C. Protein-Polysaccharide Interactions: Phase-Ordering Kinetics, Thermodynamic and Structural Aspects. *Curr. Opin. Colloid Interface Sci. 2003*, 8, 401–414.

Turgeon SL, Schmitt C, Sanchez C. Protein-Polysaccharide Complexes and Coacervates. *Curr. Opin. Colloid Interface Sci.* 2007, 12, 166–178.

USDA (United States Department of Agriculture), Agricultural Research Service. USDA National Nutrient Database for Standard References, Release, 2011. In Liu YQ, Heying E, Tanumihardjo SA. History, global distribution, and nutritional importance of citrus fruit. *Comprehensive Reviews in Food Science and Food Safety*. 2012, 11, 530-545.

Vandercook CE. 1977. *Organic acids*. In: Nagy S, Shaw PE, Veldhuis MK, editors. *Citrus science and technology*. Vol. 1. Westport, Conn.: AVI Publishing Company. p 209–27.

Wang Q, Allen JC, Swaisgood HE. Binding of Retinoids to β-Lactoglobulin Isolated by Bioselective Adsorption1. *J. Dairy Sci*. 1997, 80, 1047–1053.

Xing F, Cheng G, Yang B, and Ma YL. Microencapsulation of capsaicin by the complex coacervation of gelatin, acacia and tannins. *J. Appl. Polym.Sci*. 2003, 91 (4), 2669-2675.

Yi J, Zhu Z, McClements DJ, Decker EA. Influence of Aqueous Phase Emulsifiers on Lipid Oxidation in Water-in-Walnut Oil Emulsions. *J. Agric. Food Chem*. 2014, 62, 2011–2104.

Zuidam NJ, Shimoni E. 2010. Overview of microencapsulates for use in food products or processes and methods to make them. In: Zuidam NJ, and Nedovic VA. Ed. *Encapsulation technologies for active food ingredients and food processing*. Chapter 2. New York: Springer Science + Business Media, LLc., 3-29.

CHAPTER 2: OPTIMIZATION AND CHARACTERIZATION OF NANOENCAPSULATION OF LEMON JUICE IN WHEY PROTEIN-PECTIN COMPLEX

2.1 Introduction

Lemon (*Citrus limon*) fruit provides abundant health benefits because of rich in bioactive compounds such as vitamins, phenolics, flavonoids, dietary fiber, minerals, and essential oils. These phytochemicals are generally consumed through fresh fruit or juices and have been suggested to have a wide variety of biological functions including antioxidant, anticancer, anti-inflammatory, antibacterial, and hepato-regenerative activity (Klimek-Szczykutowicz, 2020). However, studies reported that bioactive compounds in lemon juice (LJ) are easily degraded by unfavourable conditions during processing and storage. Citrus juice preservation free reported have short-term storage (5-10 days) depending on the industrial processing (Nicoli et al., 1999).

Encapsulation is one of the important technologies in the food industry, which can help to prevent off-flavors, off-taste, undesirable texture, and protect chemical and biological degradation of food during processing and storage. This technology

can also achieve controlled release of encapsulated nutrients at a specific rate (Weiss et al., 2006). One of the nanoencapsulation techniques of bioactive compounds is complex coacervation, which is based on the ability of cation and anionic water-soluble polymers to interact in water to form a liquid, neutral, polymer-rich phase called coacervate. The complex coacervation techniques involve the separation phase of a mixture of polyelectrolyte from a solution and the subsequent deposition of the newly formed coacervate phase around the active ingredient (Zuidam and Shimoni, 2010). Complex coacervation can occur spontaneously by mixing oppositely charged polyelectrolytes in an aqueous medium (Priftis and Tirell, 2012). Previous research reported that a whey protein-pectin complex has been applied to encapsulate D-limonene, a major compound of orange peel oil (Ghasemi et al., 2018).

Whey protein consists mainly of several globular proteins, α-lactalbumin (α-la), β-lactoglobulin (β-lg), bovine serum albumin (BSA), immunoglobulin, and also protein/peptide components comprising lactoperoxidase, lysozyme, and lactoferrin. As a natural protein, whey protein exhibit a positive charge below its isoelectric point (IP) and a negative charge above its isoelectric point (Turgeon et al., 2007). Pectin is a soluble dietary fiber 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 pH level. 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, an extensive complex formation will occur, and this eventually leads to precipitation (Turgeon et al., 2003; de Kruif, 2001). The complex formation also depends on other factors such as protein-polysaccharide ratio, temperature, ionic strength, and charge density (Priftis and Tirell, 2012). The nano-complex prepared by 4% whey protein and 1 % pectin at pH 3.0 is the best treatment for D-limonene (Ghasemi et al., 2018).

Research on nanoencapsulation of LJ has not been carried out. In this research, the physical and chemical parameters of LJ nanocapsules (LJNCs) were studied from the various formulas of whey protein concentrate (WPC) and pectin at different pH levels. Response surface methodology (RSM) was implemented to get the optimum conditions based on the responses of antioxidant activity and D-limonene content. This study not only focused on the production of LJNCs but also aimed at the characterization of the LJNCs in terms of their morphological properties (Scanning Electron Microscopy), encapsulation efficiency (High-Performance Liquid Chromatography), particle size distribution (Atomic Force

Microscopy), molecular (Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy) properties. The antioxidant activity of LJNCs was evaluated by 2,2-diphenyl-1-picrylhydrazyl assay and the anticancer activity by *in vitro* method against murine colon carcinoma (colon-26) cells (Cell Counting Kit-8 assay).

2.2 Materials and Methods

2.2.1 Materials

Domestic lemon (*Citrus limon*) fruits were obtained in 1st stage of harvesting (green color, immature) in October – December period from farmers in Hiroshima prefecture, Japan. LJ was obtained by squeezing the lemons without peel by hand (**Fig.7**).

(a) (b)

Fig. 7. (a) Lemons at the 1st stage of harvesting and (b) lemons without peel

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.

2.2.2 Preparation of LJNCS

The scheme of LJ nanocapsules (LJNCs) preparation is shown in **Fig.8**. as described in the previous research (Ghasemi et al., 2018) 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 was used to increase the total soluble solids of the samples for obtaining the higher powders. These solutions were slightly stirred on a magnetic stirrer for at least 30 min until homogenous and then stored at 4 ^{o}C to complete hydration of biopolymers. All the solutions were then filtered through 0.45 μm pore

size to be used for further preparation. The designed formula of WPC-pectin nanocapsules for LJ at various concentrations of WPC and pectin at different pH levels were shown in **Table 5**.

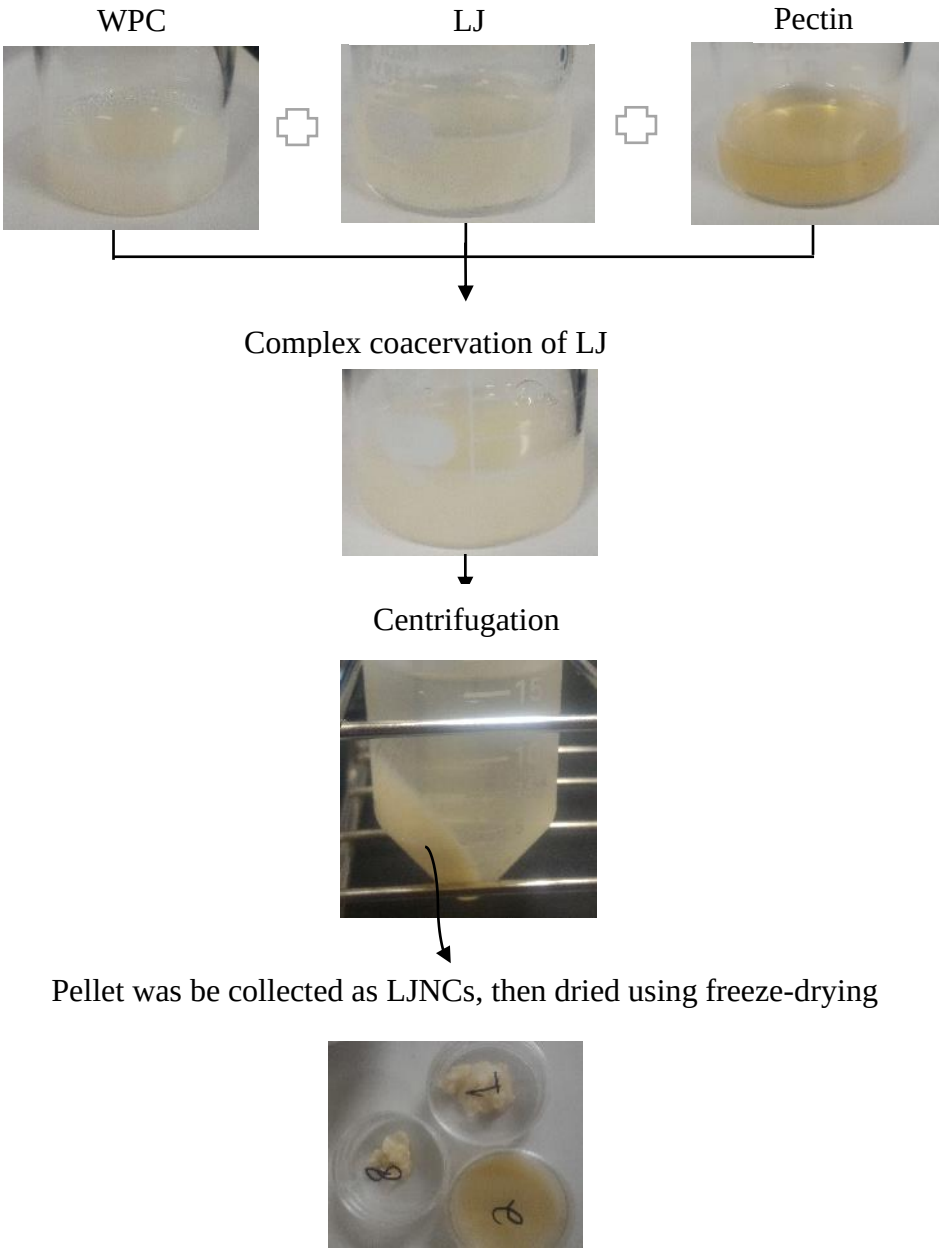


Fig. 8. Schematic process of LJNCs preparation

Table 5. Response surface methodology (RSM) designed formula of WPC-pectin nanocapsules for LJ

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

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 a ratio 5 % of the total solids was added into the solution and stirred until homogenous. LJ 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 LJNCs solution was centrifuged at 13,000 rpm, 4 ^{o}C for 30 min. Pellet was be collected as LJNCs and then dried using freeze-drying. The dried powder was collected and stored in the freezer for further characterization.

2.2.3 Antioxidant activity by (DPPH) assay

The antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay as described earlier with slight modification (Hatano et al., 1988). 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 an 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:

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

DPPH assay principle: DPPH free radical, stable violet colour at room temperature, is reduced in the presence of an antioxidant molecule, giving rise to colourless ethanol solution (**Fig. 9**).

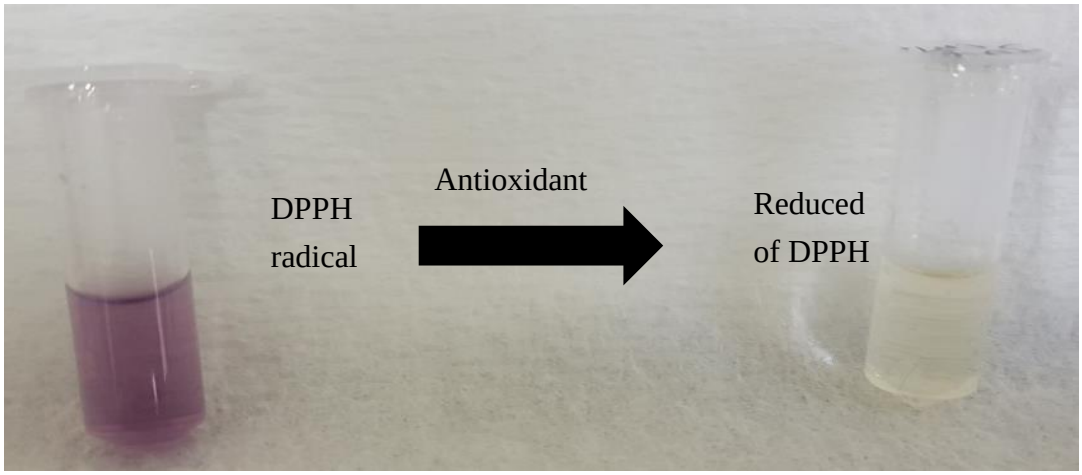


Fig. 9. Mechanism antioxidant activity analysis by DPPH assay

2.2.4 Quantification of D-limonene content by HS-GC-MS

Headspace gas chromatography-mass spectrometer (HS-GC-MS, QP 5050, Shimadzu) 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 ^{o}C and heart temperature was 230 ^{o}C. 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 ^{o}C for 5 min, and later increased 3 ^{o}C min^{-1} up to 160 ^{o}C. After that, increased 20 ^{o}C min^{-1} up to 200 ^{o}C. 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.

2.2.5 Morphology and particle size distribution

The morphology of the LJNCs was examined using scanning electron microscope (SEM) (Miniscope TM3000, Hitachi High-technologies Corp, Tokyo) at 5,000; 10,000; and 30,000 x magnifications. Samples were placed on sample stage, and then coated with gold before was observed. Topographical surface and particle size distribution estimation were analysed using atomic force microscope (AFM) (SNOAM, Hitachi-Tech Science Corp, Tokyo) with dynamic force mode in air. One drop of samples dilution was 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.

2.2.6 Molecular characterization by ATR-FTIR spectroscopy

To reveal possible chemical interactions in encapsulation, polymers (pectin, WPC, maltodextrin), NCs, LJ, and LJNCs were analyzed using Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy (ATR-FTIR). A Thermo Scientific Nicolet iS10 FTIR spectrometer (Thermo Fischer Scientific, USA), equipped with a KBr beam splitter, DTGS detector and diamond ATR cell was used to obtain the spectrum. OMNIC software from Thermo Scientific was used to provide instrument control and data acquisition. The ATR-FTIR spectra of samples were recorded from 4,000 to 400 cm^{-1}. As a background, air spectrum was scanned using the same instrumental conditions before all measurements.

2.2.7 Encapsulation efficiency

Encapsulation efficiency (%) of LJNCs was determined by evaluating the total amount of vitamin C using the High-Performance Liquid Chromatography (HPLC) method. The encapsulation efficiency was calculated as followed:

$$Encapsulation\ efficiency\ (\%) = \left(\frac{total\ vitamin\ C\ in\ LJNCs}{total\ vitamin\ C\ loaded}\right) x\ 100$$

HPLC condition for vitamin c analysis: HPLC-grade acetonitrile (≥ 99.9 %) from Fisher Scientific and ammonium acetate from Sigma-Aldrich (St. Louis, USA) were used as mobile phase (acetonitrile / 100 mM ammonium acetate = 80/20 (v/v)). Ascorbic acid in many concentrations was used as standard. All solutions and samples were filtered through 0.45 μm Nylon filter discs (Millipore, USA) prior to HPLC analysis. The analytical system consisted of an Alliance ® HPLC Waters 2695 Separation Module equipped with a UV detector (254 nm) (Fig.11). The HPLC column was Inertsil HILIC (size 4.6 x 250 mm, 5 μm) and the column temperature was maintained at 30 ^{o}C. The flow rate was 1 mL min^{-1}. The duration time was 15 min.

2.2.8 Anticancer activity of LJNCs on colon-26 cells

Cell line: murine colon carcinoma (Colon-26) cell line was purchased from RIKEN BRC CELL BANK (Tsukuba, Japan). Colon-26 cells (RCB2657, RM092147) were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10 % (v/v) fetal bovine serum (FBS). The cells were incubated at 37 ^{o}C in a 5 % CO_2 atmosphere. To investigate the cytotoxicity of samples, CCK-8 assay (Dojindo, Japan) was performed. Colon-26 cells were seeded in a 96-well cell

culture plate (Watson ® Bio Lab, Japan) at a density of 2 x10^3 cells $well^{-1}$ 100 μL^{-1} and incubated at 37 ^oC in a 5 % CO_2 incubator for 24 h. After incubation, each medium was replaced, and 100 µL of sample dissolved in the medium was added, and then the cells were incubated for 24 h under the same condition as above. Finally, 10 µL of the CCK-8 reagent was added into each well and incubated at 37 ^oC in a 5 % CO_2 incubator for 6 h. The fluorescent intensity (FI) of the samples was recorded on a microplate reader (Varioskan Flash from Thermo Scientific, Waltham, MA, USA) at 450 nm.

$$Cell\ viability\ (\%) = \frac{A\ sample - A\ color\ blank\ of\ sample}{A\ control - A\ color\ blank\ of\ control}\ x\ 100$$

Inhibition of proliferation activity (%) = 100 – cell viability (%)

The IC_{50} value was calculated by plotting the sample concentration and antiproliferative activity value. CCK-8 assay: allows sensitive colorimetric assays for the determination of cell viability in cell proliferation and cytotoxic assays. Dojindo's highly water-soluble tetrazolium salt, WST-8, is reduced by dehydrogenase activities in cells to give a yellow-color formazan dye, which is soluble in the tissue culture media **(Fig. 10.**). The amount of the formazan dye,

generated by the activities of dehydrogenases in cells, is directly proportional to the number of living cells. The detection sensitivity of CCK-8 is higher than the other tetrazolium salts such as MTT, XTT, MTS, or WST-1 (Dojindo, 2021).

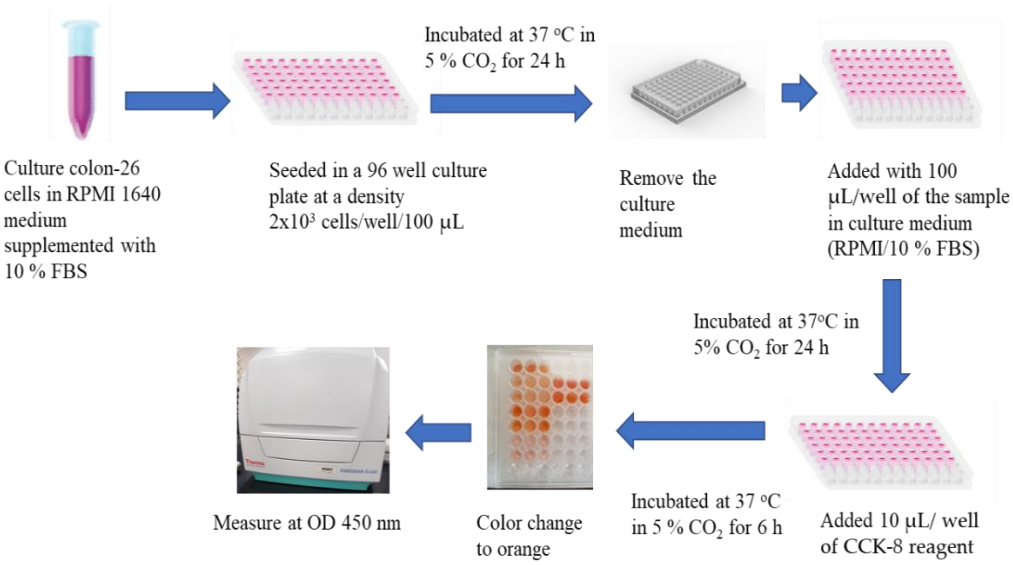


Fig. 10. *In vitro* antiproliferation activity against colon-26 cells by CCK-8 assay

2.2.9 Statistical analysis

All measurements were carried out in triplicate. The results were expressed as mean ± standard deviation (SD). Data obtained were analyzed by one way analysis of variance (ANOVA) followed by the *least significant differences* (LSD) at $p < 0.01$ using Microsoft excel. IC_{50} was calculated by plotting the results (antioxidant activity and anticancer activity) and the concentration of samples.

2.3 Results and Discussions

2.3.1 Effect of WPC concentration, pectin concentration, and pH levels on D-Limonene content of LJNCs

Limonene is the most abundant compound of LEO (Tisserand and Young, 2014). 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. 11**, the D-limonene content of LJNCs 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 (Ghasemi et al., 2018).

The ratio of biopolymers (protein and polysaccharide) in the solution strongly influences the charge balance of poly-ions and consequently change their complexation behaviour (Ye, 2008). The highest D-limonene content of LJNCs 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. 11a**). 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 (Ding et al., 2017. Another study on formation pectin (low methoxy) 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 (Sultana et al., 2012).

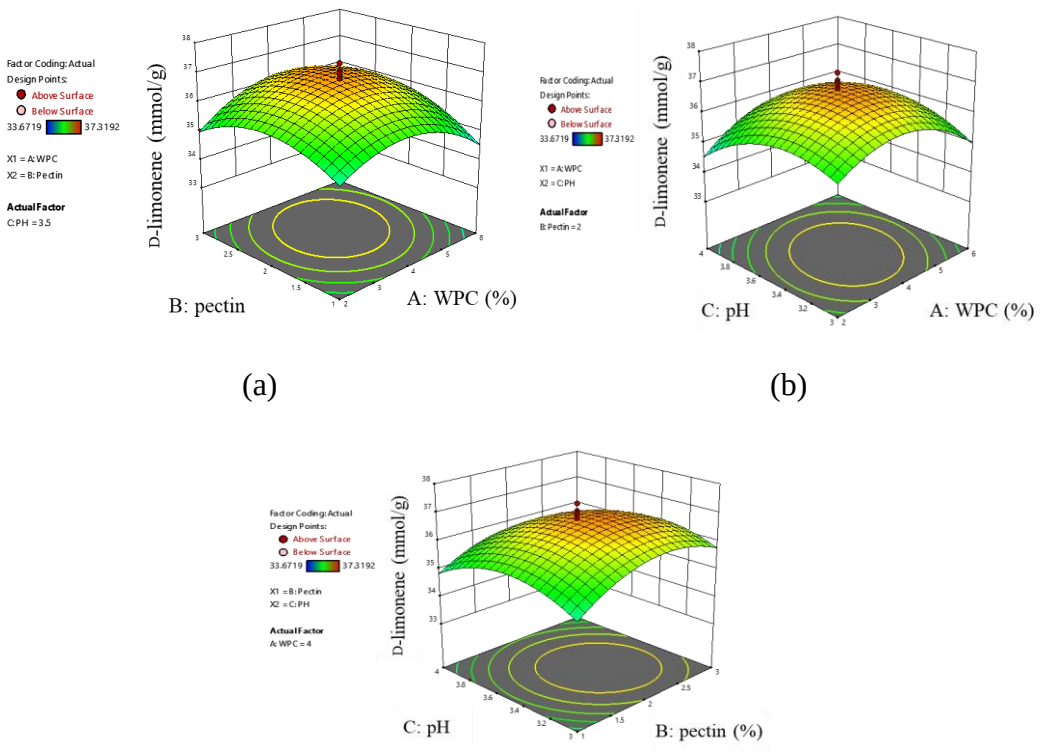


Fig. 11. RSM plots of the interaction of WPC, pectin, and pH variable on D-limonene content in LJNCs

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 (Ye, 2008; Turgeon et al., 2007). The highest D-limonene content of LJNCs is at pH 3.5. Fig. 11c and Fig. 11d shown that at higher and lower of pH than 3.5, LJNCs has lower D-limonene content. The complex formation will occur at pH below the isoelectric point of protein. If the charge on the protein is not too high, the complexes are soluble. If pH is too far below the isoelectric point, extensive complex formation occurs and this eventually leads to precipitation (Priftis et al., 2012; Tisserand and Young, 2014).

2.3.2 Effect of WPC concentration, pectin concentration, and pH levels on antioxidant activity of LJNCs

Antioxidant activity of LJNCs 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 (Klimek-Szczykutowicz et al., 2020). 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 LJNCs 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.

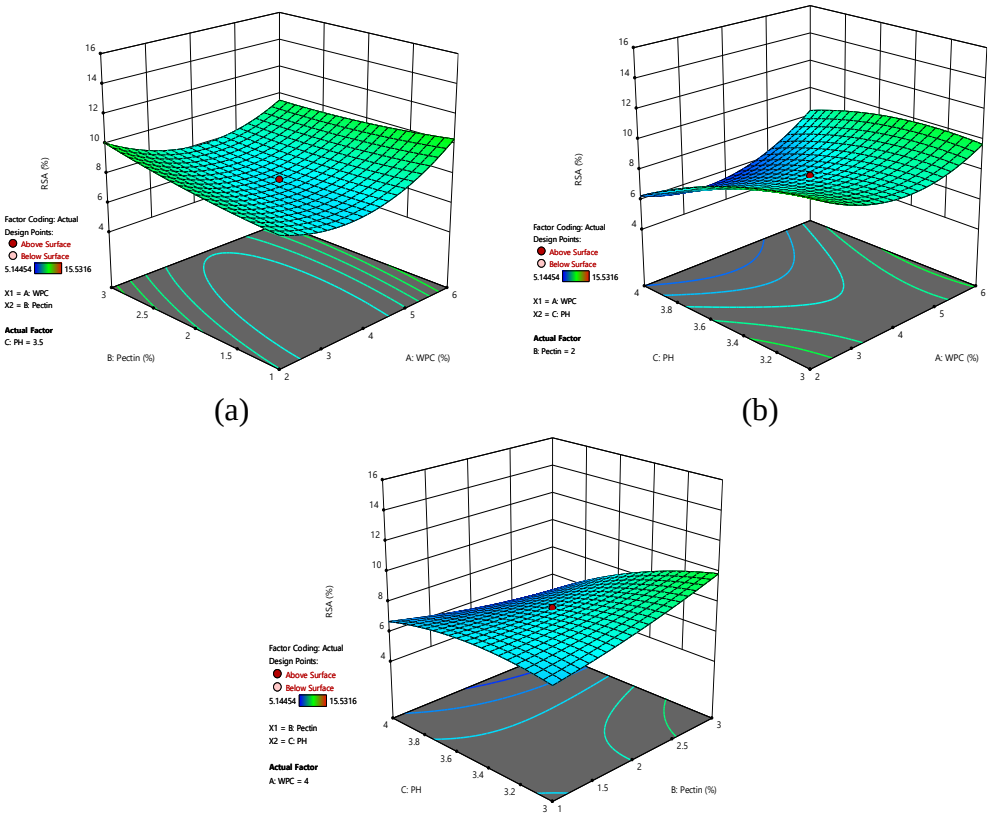


Fig. 12. RSM plots showing the interaction of WPC, pectin and pH variables on the antioxidant activity of LJNCs

Antioxidant activity of LJNCs was influenced by WPC-pectin concentration and pH value. Higher concentration of WPC and pectin, higher the antioxidant

activity (**Fig. 12a**). 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 (Farraq et al., 2018). As can be seen in **Fig. 12b** and **12c**, 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 (Ye, 2008).

2.3.3 Optimization and confirmation formula of WPC-pectin complex as NCs for LJ

Antioxidant activity and D-limonene were optimized responses with maximizing goal and the level importance of 5. This is related to the fact that lemon juice is rich in phenolics, flavonoids, and vitamins C (Klimek-Szczykutowicz et al., 2020), and D-limonene is the most abundant volatile compounds of lemons (Ghasemi et al., 2018).

The optimum formula solution to obtain the highest antioxidant activity was recommended at 6.0 % WPC, 3.0 % pectin, at pH 3.1. 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 7), the verification results are in the range of 9 5% predicted interval (PI) low and 95 % PI high which means that the optimum formula is consistent.

Table 6. Procedure, formula solution, and confirmation stage for the LJNCs formation

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 7. Confirmation of optimum formula based on RSM suggestion

Responses	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

2.3.4 Morphology, topography, and particle size analysis

Morphological and topographical surface of LJNCs was shown at **Fig. 13** and **Fig. 14**. The SEM photographs (**Fig. 13**) showed that the form of LJNCs prepared by 6.0% WPC, 3.0% pectin at pH 3.1 was spherical.

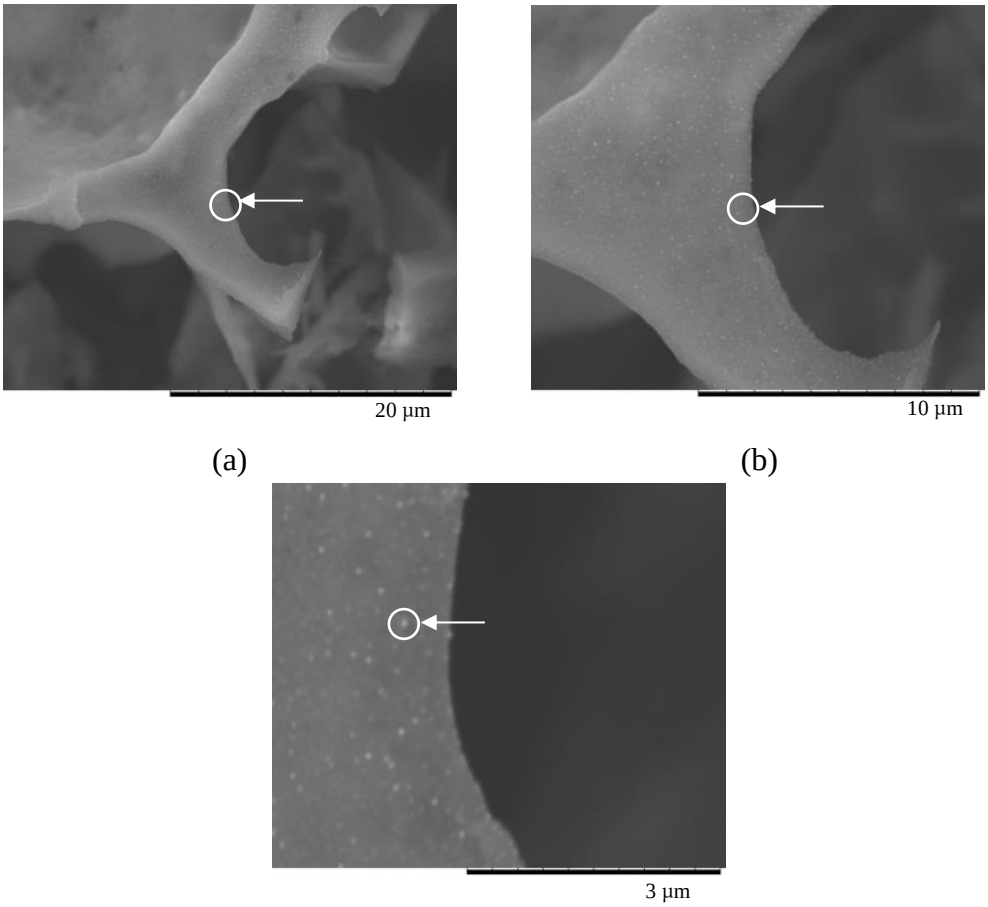
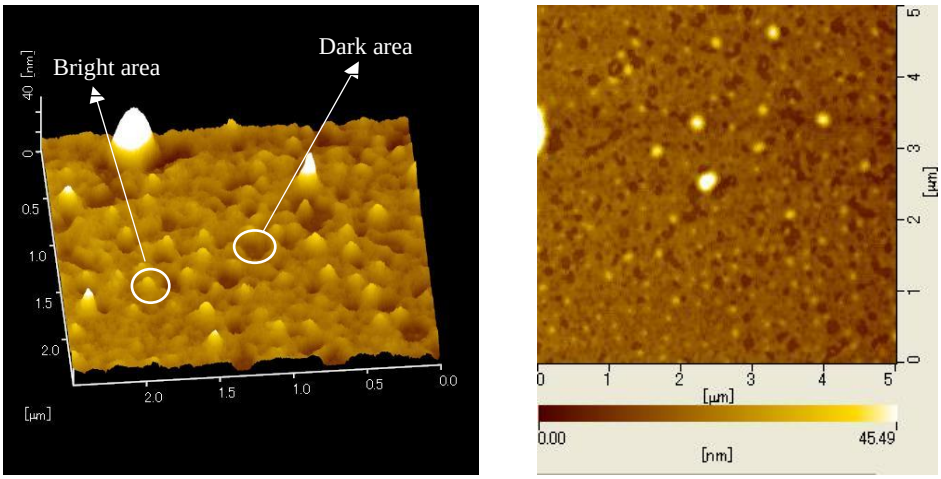


Fig. 13. SEM photographs of LJNCs at (a) 5,000, (b) 10,000 and (c) 30,000 of magnifications

The bright area in AFM images (**Fig. 14**) 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 LJNCs has a particle size average of 22.3 nm ± 7. 5.

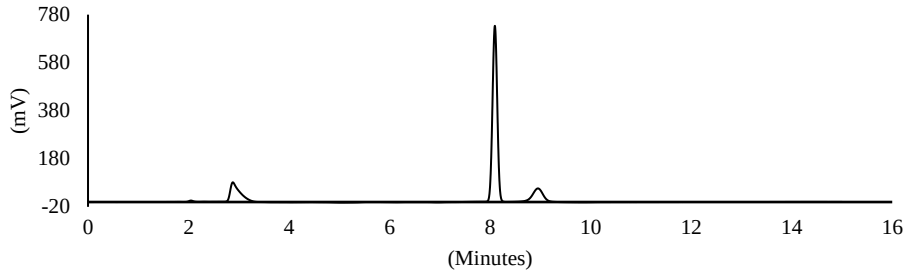


(a) (b)

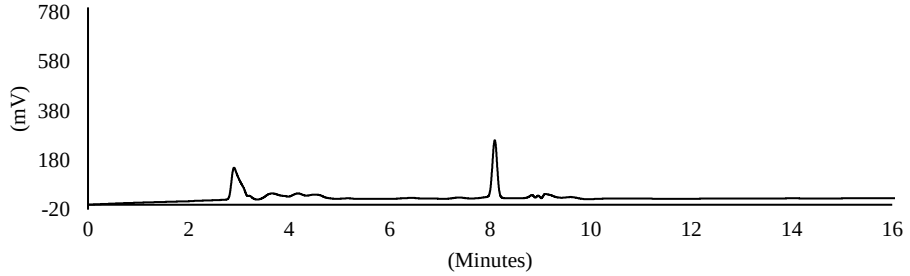
Fig. 14. AFM images of LJNCs: (a) the brighter area indicated higher structure, and (b) particle size distribution

2.3.5 Encapsulation efficiency

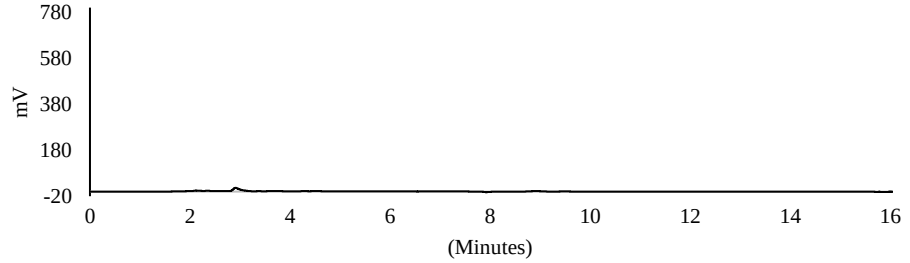
Encapsulation efficiency is used as indicator for the successfulness of encapsulation. Encapsulation efficiency was calculated as the ratio of entrapped vitamin C content to its total initial loaded (**Fig. 15**). Results indicated that whey protein-pectin complex showed encapsulation efficiency of 66.07 % ± 2.4.



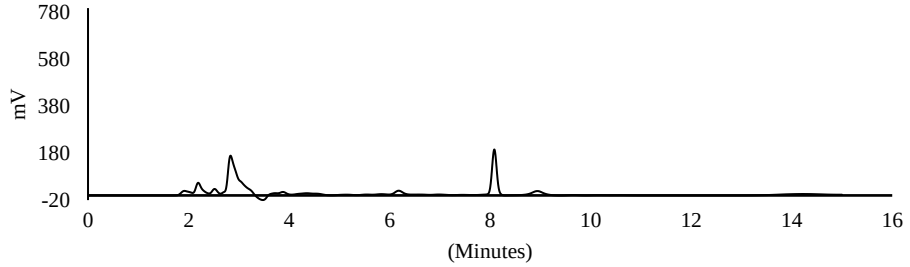
(a)



(b)



(c)



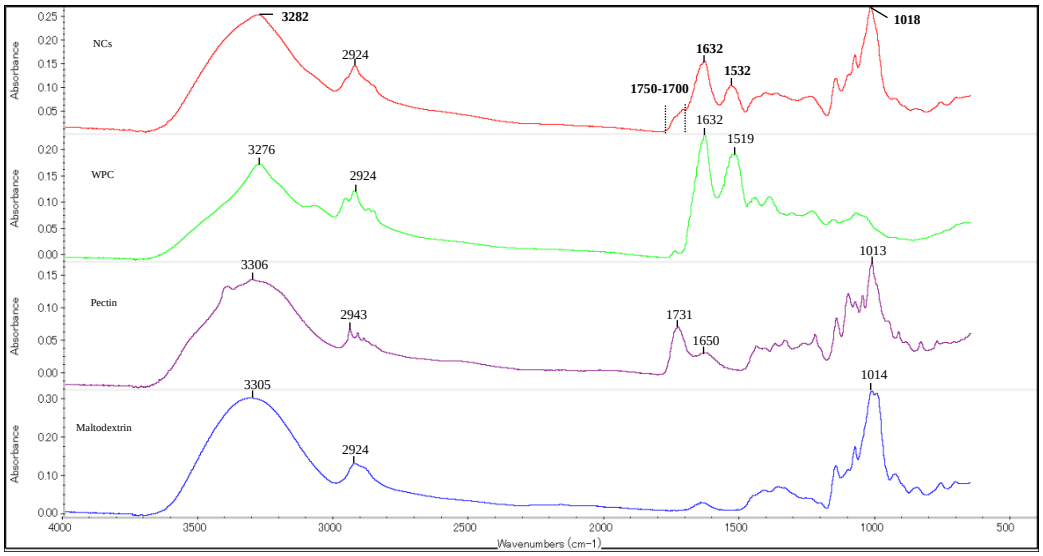
(d)

Fig. 15. Representative HPLC chromatogram of (a) ascobic acid standard, (b) LJ, (c) NCs, and (d) LJNCs

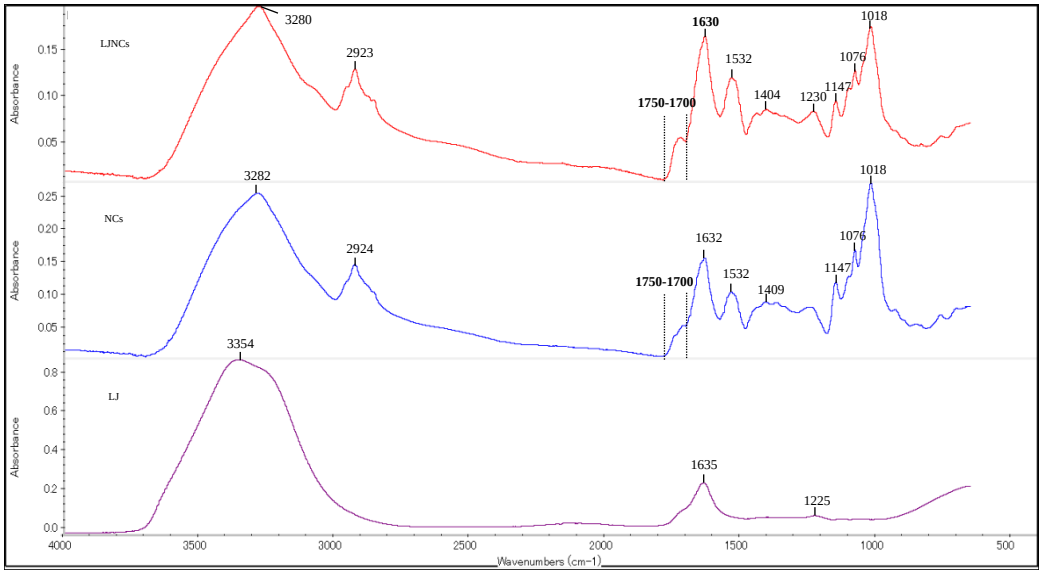
2.3.6 Molecular analysis of NCs, LJ and LJNCs

The ATR-FTIR spectroscopy was conducted to analyze the structural characteristics of WPC, pectin, maltodextrin, LJ, as well as their interactions during the formation of NCs (unloaded) and LJNCs (loaded) (**Fig.16**). In the spectral range studied of 4000 to 400 cm^{-1} there are numerous peaks which corresponds to the different molecular bonds of the sample components interacting with IR radiation. At shown in **Fig. 16a**, pectin showed its characteristic absorption at 1731 and 1650 cm^{-1} which were assigned to stretching of COOR (esterified carboxyl group), and COO^- (free carboxyl group), respectively (Gnanasambandan and Proctor, 2000). The absorbance at 1731 cm^{-1} is higher than 1650 cm^{-1} indicated high methoxyl pectin. In the spectrum of WPC showed characteristic absorption at 1632, 1519, 1446, 1392, 1236 and 1072 cm^{-1}, were attributed to the stretching vibration amide I (C=O, C-N), amide II (N-H, C-N), C-H, COO^-/COOH (fat related), and C-N/CO bonds, respectively (Assadpour et al., 2017, and Ghasemi et al, 2017). Amide I and amide II related to peptide bonds (CO-NH) (and Wang et al., 2018 and Andrade et al., 2018). Maltodextrin present featuring the band assigned to C-O at 1014 cm^{-1}. During the complex coacervation, carboxyl groups in pectin interact with amino groups in WPC. The NCs showed a broad band at 3282 cm^{-1}, shifted to another

position from 3276 and 3306 cm^{-1} in WPC and pectin respectively, attributed to NH2 and OH groups vibration (Espinosa-Andrews et al. 2010).



(a)



(b)

Fig. 16. FTIR spectra of (a) NCs formation from WPC, pectin and maltodextrin, and (b) LJNCs formation from LJ and NCs

After formation of NCs, the intermolecular interaction between WPC-pectin were explained by several major changes in FT-IR spectra. First, at peek 3000 to 3500 cm^{-1}, related to hydrogen bonding in WPC (3276 cm^{-1}) and amino groups in pectin (3306 cm^{-1}), was shifted to another position in 3282 cm^{-1} in the NCs which attributed to NH_2 and OH groups vibration (Espinosa-Andrews et al. 2010). These shifts clearly indicated the enhancements of hydrogen bonding is also involved in the interactions between polymers and the consequent formation of complex coacervates. The new region formed around 1750-1700 cm^{-1} which was connected to the band 1632 cm^{-1}, which could be the carboxyl groups of pectin between the amide groups of WPC. Furthermore, peak of amide II at 1519 cm^{-1} shifted to 1532 cm^{-1} in NCs indicated the interaction of amide groups and carboxyl groups. Addition, the peak at 1013 and 1014 cm^{-1} of pectin and maltodextrin respectively shifted to 1018 cm^{-1} indicated the interaction between polymers.

At shown in Fig. **16b**, the FTIR spectrum of pure LJ showed a characteristic absorption at 3354, 1635, and 1225 cm^{-1} which corresponding to the stretching of O-H, aromatic ring, and citric acid, respectively. The aromatic ring stretching vibration could be related to flavonoids (Mot et al., 2011). The region between 1474 and 119 cm^{-1} contains O-C-H, C-C-H, and C-OH bending vibrational modes

(Irudayaraj and Tewari, 2003). The intermolecular interaction after loaded LJ in LJNCs were explained by several major changes in FT-IR spectra. First, the peak at 3354 and 3282 cm^{-1} of LJ and NCs, respectively, were shifted to another position at 3280 cm^{-1} in LJNCs. The region between 1750-1700 cm^{-1} in LJNCs was sharper and higher than in NCs. The peak at 1632 cm^{-1} of NCs was shifted to 1630 in LJNCs. Its indicated interaction between LJ and NCs. New peak formation at 1230 cm^{-1} in LJNCs corresponded to the citric acid vibration from LJ (1225 cm^{-1}). The FTIR results showed the molecular interaction between LJ and NCs components in LJNCs formation.

2.3.7 Stability of vitamin C in LJ and LJNCs during storage

Vitamin C has the least stability among all kinds of vitamins and is easily destroyed during processing and storage because of many factors such as pH, temperature, the presence of enzymes, hydrogen peroxide, light, and metallic catalyzers. Vitamin C is a heat-sensitive substance that the higher processing temperature the higher losses in vitamin C in the products. To know the effect of encapsulation on the stability of LJ, the retention of vitamin C as ascorbic acid during storage for 30 days at different temperatures (4 ^{o}C and 40 ^{o}C) was analyzed.

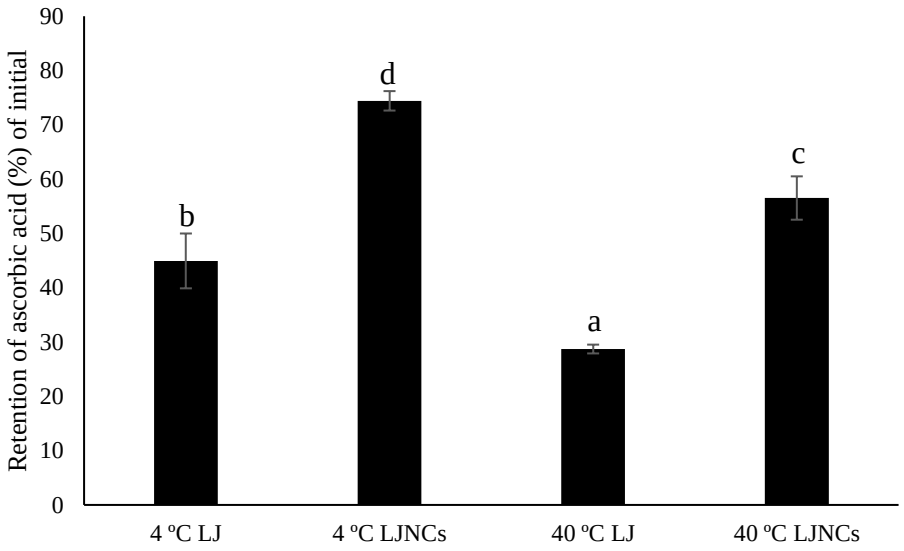


Fig 17. Stability of vitamin C in LJ and LJNCs during storage period for 30 days at different temperatures. Data represented the mean ± SD (n=3). The different letters indicated a significant difference by *the least significant difference* at $p < 0.01$.

There was a significant difference in the retention of ascorbic acid after storage (**Fig. 17)**. At low temperatures of storage, LJ and LJNCs have higher retention of ascorbic acid than in higher temperatures. Encapsulation increased the retention of ascorbic acid after the storage period, increased about 29.5 % at 4 ^{o}C and 27.8 % at 40 ^{o}C. A previous study reported a similar result, that the storage of lemon juice concentrate at 37 ^{o}C and 45 ^{o}C for 8 weeks decreased of vitamin C with the retention of ascorbic acid was 24.3 % and 20 %, respectively (Burdulu et al., 2006). At higher concentration is more losses in ascorbic acid. The retention of

vitamin C in LJ untreated after storage at 4 ^{o}C and 29 ^{o}C for 4 weeks reported decreases (Ajibola et al., 2009).

2.3.8 Anticancer activity

Colon cancer ranks fifth of the most cancer cases in worldwide in 2020 with about 1.15 million of new cases and 576,858 of new death (GLOBOCAN, 2000). In this study, the assessment of anticancer activity of LJ and LJNCs were performed by *in vitro* method based on the cell viability of colon-26 cells using CCK-8 assay. LJ and LJNCs exhibited significant cytotoxic effect against colon-26 cell line ($p < 0.01$), with IC_{50} was about 1.12 mL/100 mL in LJ and 13.13 mg/mL in LJNCs (**Fig. 18**).

Previously study reported that treatment of LJ at 1.4% (v/v) for 72 h inhibited 50 % of HL60 cells viability (Fernandez-Bedmar, 2011). Raimondo et al (2015) demonstrated that the nanovesicles extracted by ultracentrifugation from LJ might induce apoptosis in CML cells by activating TRAIL-mediated cell death. Previously study reported that treatment of LJ at 1.4 % (v/v) for 72 h inhibited 50 % of HL 60 cells viability (Fernandez-Bedmar, 2011). The antiproliferative activity of LJNCs may be correlated with bioactive compounds in LJ. LJ contains several

bioactive compounds including vitamin C, flavonoids (hesperidin and naringin), and phenolic acids (ferulic acids) which are reported to exhibit antiproliferative activity.

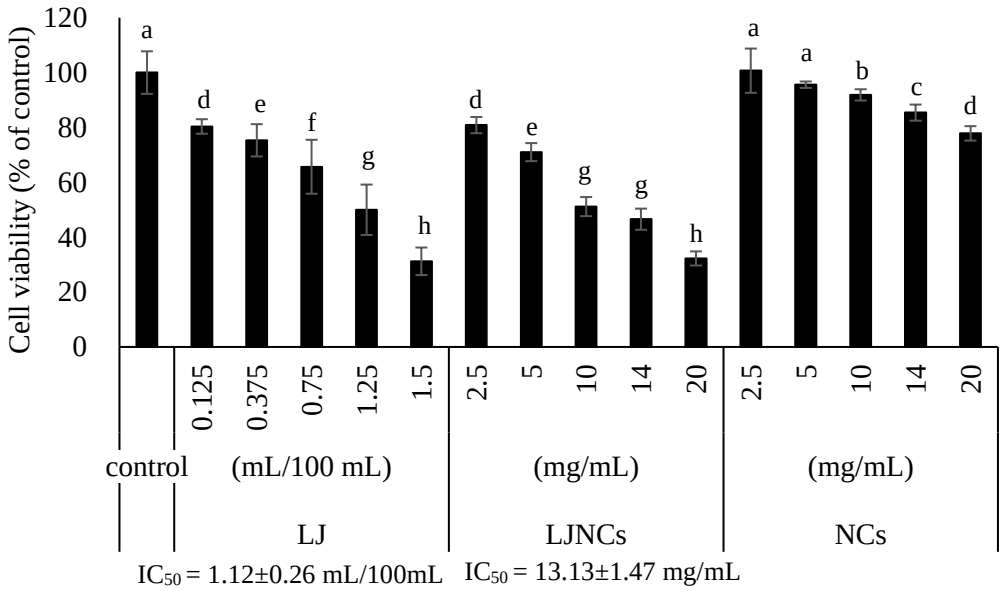


Fig. 18. Cell viability of colon-26 cells after treatment with LJ, LJNCs, and NCs. Data represented the mean ± SD (n=3). The different letters indicated a significant difference by *the least significant difference* at $p < 0.01$.

Vitamin C (ascorbic acid) have some anticancer mechanism including: (1) acts as an electron donor and as a reducing agent enabling it to have a cytotoxic effect; (2) induces pro-oxidant effects and inhibits energy metabolism, mainly mediated by H_2O_2; (3) increase in the calcium influx in the endoplasmic reticulum, the translocation of Bad to the mitochondria from the cytosol after dissociation from

14-3-3β and an increase in the expression of Bax; (4) serves as a cofactor for a large family of enzymes containing iron which are required for the regulation of HIF factors (required for tumour angiogenesis, treatment evasion and metastasis); and (5) at pharmacologic doses decrease the proliferation of RKO and SW480 colon cancer cells and induce their apoptosis and necrosis (Halabi et al., 2018).

Hesperidin and naringin are the main flavonoids present in LJ (**Table 2**). Both have been reported in many studies to have anticancer activity against colon cancer. Several anticancer mechanism of hesperidin: (1) exhibited cytotoxic and pro-apoptotic effects through activation of capase-3; (2) suppressed cell proliferation markers, and reduced ACF in AOH-induced colon carcinogenesis; and (3) induced apoptosis via targeted inhibition of constitutively activated Aurora-A mediated PI3 K/Akt/GSK-3band mTOR pathways coupled with autophagic stimulation against AOM-induced colon carcinogenesis (Yi et al., 2017). Naringin also prevented intestinal tumorigenesis likely through a collection of activities including anti-proliferation, induction of apoptosis, modulation of glycogen synthase kinase (GSK)-3b and APC/b-catenin pathways and anti-inflammation (Zhang et al., 2016).

Another bioactive compound in LJ that may play a role in anticancer activity is phenolic acid (ferulic acid). Alazouni et al. (2021) reported that ferulic acids has

significant anti cancer activity against colon cancer by inhibiting proliferation and promoting apoptosis. General anticancer mechanism of ferulic acid including the ability to scavenge reactive oxygen species, promote cytoprotective enzyme function, decreased lipid peroxidation, single-strand DNA breakdown, and inhibition of some proteins and rupture of biological membrane (Barone et al., 2009; Kumar and Pruthi, 2014).

In NCs unloaded LJ, anticancer activity may be caused by the WPC component. Whey protein components, β-lactoglobulin and α-lactalbumin reported have anticancer activity. α-Lactalbumin possesses anti proliferative effects on colon adenocarcinoma cell line (CaCo2 or HT-29 mono layers) (Sternhagen and Allen, 2001). α-LA can also be a potent calcium concentration-elevating and apoptosis-inducing agent. Multimeric form of α-LA was shown to promote apoptosis in transformed and immature cells while sparing mature epithelial cells. During this process calcium levels are elevated, allowing a connection between calcium levels and apoptosis (Hakansson et al., 1995). The mechanism of anticancer activity of β-Lg may be related to its sulphur aminoacid content. This suggests a possible role in protecting DNA in methylated form. Indeed, the aminoacid composition of β-Lg plays an important role in preventing oxidative damage. Particularly, β-Lg

influences tissue levels of the thiol-glutathione, a multifunctional tripeptide, that binds and eliminates endogenous and exogenous mutagens and carcinogens (Pepe et al., 2013).

2.3.9 Antioxidant activity

The DPPH assay was used to analysis antioxidant activities in LJ and LJNCs. The IC_{50} against DPPH radical of LJ and LJNCs was 64.29 µL/mL and 148.43 mg/mL, respectively. LJ is rich in bioactive compounds including vitamin C, phenolic acids and and flavonoids which have been reported to have good antioxidant activity. An antioxidant is a molecule that prevents oxidation of other molecules. Vitamin C is a powerful antioxidant because of (1) having ability to donate a hydrogen atom and form a relatively stable ascorbyl-free radical and (2) enhances iron absorption by reducing Fe^{3+} to Fe^{2+} from non-heme iron sources. As a water soluble vitamin, vitamin C is reported can neutralize reactive oxygen species and reduce the oxidative stress (Pehlivan, 2017).

Ferulic acid is main phenolic acids in LJ. The antioxidant mechanism of ferulic acid are (1) the ability to form stable phenoxyl radicals, by the reaction of the radical molecule with the molecule of antioxidant. This makes it difficult to

initiate a complex reaction cascade leading to the generation of free radicals. (2) act as hydrogen donor, giving atoms directly to the radicals. This is particularly important for the protection of cell membrane lipid acids, from undesired autoxidation processes. And (3) as a secondary antioxidant, ferulic acids and their related compounds are able to bind transition metals such as iron and copper (Kiewlicz et al., 2015). The main flavonoid in LJ are naringin dan hesperidin, which have been reported have antioxidant activity (Wilmsen et al., 2005) . Naringin and hesperidin contained 4′-OH and 3′-OH, respectively, which can probably increase the antioxidant power of flavonoids (Di Majo et al., 2005).

2.4 Conclusion

The optimum encapsulation condition of LJ was found at 6 % WPC, 3 % pectin, and at pH 3.1. At this condition obtained the LJNCs with optimum antioxidant activity and D-limonene content. LJNCs have a spherical shape with an average size of 22.3 nm, and an encapsulation efficiency of 66.07 %. Molecular analysis showed that after the complex coacervation, there were found several shifts and changes in the peak of the functional groups associated with the synthesis of NCs and LJNCs. Interaction of carboxyl groups of pectin and amino groups of WPC

was clearly demonstrated by FTIR spectra. Loaded LJ in the WPC-pectin complex as indicated by the formation of a new peak at 1230 cm^{-1}, the sharper region between 1750-1700 cm^{-1}, and the shifted of peak 1632 to 1630 cm^{-1}. The encapsulation efficiency and FTIR analysis proved the success of encapsulation of LJ in the WPC-pectin complex (formation of LJNCs).

Encapsulation in the WPC-pectin complex showed the protection function of bioactive compounds in LJ. The encapsulation increased the retention of ascorbic acid after storage for 30 days at 4 ^{o}C and 40 ^{o}C (about 29.5 % and 27.8%, respectively). Thus, will increase the shelf life of LJ. LJ and LJNCs were reported to have the anticancer activity against colon-26 cells with IC_{50} at 1.12 mL/100 mL and 13.13 mg/mL, respectively. They also showed antioxidant activity against DPPH radicals. The results indicated that the nano-system of the WPC-pectin complex increased the stability of LJ during storage without removing the biological activities. Thus, it's potential to develop as a functional food in the future.

References

Ajibola VO, Babatunde OA, Suleiman S. The effect of storage method on the vitamin C content in come tropical fruit juices. *Trends in Applied Sciences Research*. 2009, 4(2), 79-84.

Assadpour E, Jafari SM, Maghsoudlou Y. Evaluation of folic acid release from spray dried powder particles of pectin-whey protein nano-capsules. *International Journal of Biological Macromolecules*. 2017, 95, 238-247.

Burdulu HS, Koca N, Karadeniz F. Degradation of vitamin C in citrus juice concentrates during storage. *Journal of Food Engineering.* 2006, 74, 211-216.

de Kruif CG, Tuinier R. Polysaccharide Protein Interactions. *Food Hydrocolloid*. 2001, 15, 555–563.

Di Majo D, Giammanco M, La Guardia M, Tripoli E, Giammanco S, Finotti E. Flavanones in Citrus fruit: structure-antioxidant activity relationships. *Food Research International*. 2005, 38 (10), 1161-1166.

Espinosa-Andrews H,Sandoval-Castila O, Vazquez-Torez H, Vernon-Carter EJ, Lobato-Calleros C. Determination of the gum-Arabic-chitosan interactions by Fourier Transform Infrared Spectroscopy and characterization of the microstructure and rheological features of their coacervates. *Carbohydrate Polymers.* 2010, 79, 541-546.

Fernandez-Bedmar Z, Anter J, Cruz-Ares SdL, Munoz-Serrano A, Alonso-Moraga A, Perez-Guisado J. Roles of citrus juices and distinctive components in the modulation of degenerative processes: genotoxicity, antigenotoxixity, and longevity in *Drosophila*. *Journal of Toxicology and Environmetal Health.* 2011, 74, 1052-1066.

Ghasemi S, Jafari SM, Assadpour E, and Khomeiri M. Production of pectin-whey protein nano-complexes as carriers of orange peel oil. *Carbohydrate Polymers.* 2017, 177, 369-377.

Ghasemi S, Jafari SM, Assadpour E, Khomeiri. Nanoencapsulation of D-limonene within nanocarriers produced by pectin-whey protein complexes. *Food Hydrocolloids*. 2018, 77, 152-162.

GLOBOCAN (Global Cancer Observatory) Data Base. 2000. In Sung H, Ferlay J, Siegel RL. Laversanme M, Soerjomataram, I, Jemal A, Bray F. Global cancer

statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca Cancer J Clin*. 2021, 71, 209-249.

Gnanasambandam R, Proctor A. Determination of pectin degree of esterification by diffuse reflfectance fourier transform infrared spectroscopy. *Food Chemistry*. 2000, 68 (3), 327-332.

Hakansson A, Zhivotovsky B, Orrenius S, Sabharwal H, Svanborget C. Apoptosis induced by a human milk protein. *Proceedings of the National Academy of Sciences of the United States of America.* 1995, 92 (17), 8064–8068.

Halabi, IE, Bejjany R, Nasr R, Mukherji D, Temraz S, Nassar FJ, El Darsa H. Shamseddine A. Ascorbic acid in colon cancer: from the basic to the clinical applications. *Int. J. Mol.Sci*. 2018, 19, (2752), 1-13.

Irudayaraj J, Tewari J. Simultaneous monitoring of organic acids and sugars in fresh and processed apple juice by fourier transform infrared-attenuated refrlection spectroscopy. *Appl Spectrosc.* 2003. 57 (12), 1599-15604.

Kiewlicz J, Szymusiak H, Zieliński R: Symthesis. Thermal stability and antioxidant activity of long-chain alkyl esters od ferulic acid. *Zywn Nauk Technol Ja*. 2015, 4, 188–200.

Klimek-Szczykutowicz M, Szopa A, Ekiert H. *Citrus limon* (lemon) phenomenon—a review of the chemistry, pharmacological properties, applications in the modern pharmaceutical, food, and cosmetics industries, and biotechnological studies. *Plants*. 2020, 9 (119), 1-24.

Mot A, Silaghi-Dumitrecu R, Sarbu C. Rapid and effective evaluation of the antioxidant capacity of propolis extracts using DPH cleaching kinetic profiles, FT-IR and UV-vis spectroscopic data. *Journal of Food Composition and Analysis*. 2011, 24 (4-5), 516-522.

Nicoli MC, Anese M, Parpinel M. Influence of processing on the antioxidant properties of fruit and vegetables. *Trends in Food Science & Technology*. 1999, 10 (3), 94-100.

Pehlivan FE. 2017. *Vitamin C: An Antioxidant Agent.* In Book: Vitamin C, Chapter II, Edited by Hamza AH, 2017. Crotia: InTech Open, 23-34.

Pepe G, Tenore GC, Mastrocinque R, Stusio P, Campiglia P. Review article: potential anticarcinogenic peptides from bovine milk. *Journal of Amino Acids*. 2013, ID 939804, 1-7.

Priftis D, Tirrell M. Phase behaviour and complex coacervation of aqueous polypeptide solutions. *Soft Matter*. 2012, 8, 9396-9405.

Raimondo S, Naseli F, Fontana S, Monteleone F, Dico AL, Saieva L, Zito G, Flugy A, Manno M, Bella MAD, Leo GD, Alessandro. Citrus limon-derived nanovesicles inhibit cancer cell proliferation and suppress CML xenograft growth by inducing TRAIL-meditaed cell death. *Oncotarget*. 2015, 14 (23), 19514-19527.

Sternhagen LG, Allen JC. Growth rates of a human colon adenocarcinoma cell line are regulated by the milk protein alpha-lactalbumin. *Advances in Experimental Medicine and Biology.* 2001, 501, 115–120.

Turgeon SL, Beaulieu M, Schmitt C, Sanchez C. Protein-Polysaccharide Interactions: Phase-Ordering Kinetics, Thermodynamic and Structural Aspects. *Curr. Opin. Colloid Interface Sci. 2003*, 8, 401–414.

Turgeon SL, Schmitt C, Sanchez C. Protein-Polysaccharide Complexes and Coacervates. *Curr. Opin. Colloid Interface Sci.* 2007, 12, 166–178.

Weiss J, Takhistov P and McClements J. Functional materials in food nanotechnology. *J. Food Sci*. 2006, 71, 107-116.

Wilmsen PK, Spada DS, Salvador M. Antioxidant activity of the flavonoids hesperidin and naringin in chemical and biological systems. *J Agric Food Chem*. 2005, 53, 4757-4761.

Yi L, Ma S Ren D. Phytochemistry and bioactivity of *Citrus* flavonoids: a focus on antioxidant, anti-inflammatory, anticancer and cardiovascular protection activities. *Phytochem Rev*. 2017, 16, 479-511.

Zhang Y-S, Li Y, Wang Y, Wang Y, Sun S-Y, Jiang T, Li C, Cui S-X, Qu X-J. Naringin, a natural dietary compound, prevents intestinal tumorigenesis in Apc (Min/+) mouse model. *J Cancer Res Clin Onco*l . 2016, 142, 913–925

Zuidam NJ, Shimoni E. 2010. Overview of microencapsulates for use in food products or processes and methods to make them. In: Zuidam NJ, and Nedovic VA. Ed. *Encapsulation technologies for active food ingredients and food processing*. Chapter 2. New York: Springer Science + Business Media, LLc., 3-29.

CHAPTER 3. NANOENCAPSULATION OF LEMON ESSENTIAL OIL IN WHEY PROTEIN-PECTIN COMPLEX AND EVALUATION OF ITS POTENTIAL ANTIOXIDANT AND ANTICANCER ACTIVITIES

3.1 Introduction

Complex coacervation is one of the most practical techniques in micro and nanoencapsulation that is extensively used in food industries. In food applications, nanoencapsulation can be effectively used to protect the sensitive bioactive food ingredients from unfavourable environmental conditions, eradication of incompatibilities, solubilization, or masking of unpleasant taste or aroma as well as controlled release and absorption in the gastrointestinal tract (Fathi et al., 2012). Complex coacervation technique is based on the ability of cationic and anionic water-soluble polymers to interact in water to form a liquid, neutral, polymer-rich phase called coacervate. Complex coacervation technique is prompt nanoencapsulation preparation because of high payload and high encapsulation efficiency (up to 99 %), relatively lower cost processing, ability to use food-grade shell materials and synthesis at ambient temperature (Timilsena et al., 2019).

Lemon essential oil (LEO) is the most important by-product of lemon processing, obtained from the peel particularly in the flavedo part by cold-pressing or steam distillation technique. Volatile and semi-volatile compounds represent 85-99 % of the citrus oil fraction (Dugo and Mondello, 2011). LEO has generated considerable appeal from the food sector because of its GRAS (generally recognized as safe) status (USFDA, 2020). Previously study reported that LEO has many biological activities including antioxidant and anticancer activities (ChunYan, 2010). However, their high reactivity, easy degradation by unfavorable environmental conditions after extraction from plant tissue, and/or low solubility in water caused many difficulties in their inclusion in the food matrix and limit their further use in the food industry.

Colorectal cancer (CRC) is abnormal and uncontrolled cell growth with start in the colon or rectum. CRC is the second leading cause of cancer-related deaths and the third most new cancer in 2020 (GLOBOCAN, 2020). The increasing of chronic diseases including cancer prompts global food industries to give priority to the development of health-promoting foods. Therefore, it is possible to use an alternative active food ingredient such as LEO as a functional food to inhibit the proliferation of cancer cells.

So far, LEO has been encapsulated in different polymeric nanoparticles using different techniques, such as emulsion-complex coacervation in chitosan-cellulose (Jiang et al, 2021); encapsulation in chitosan-Hicap system (Hasani et al., 2018); emulsion-spray drying in mesquite gum and nopal mucilage (Cortes-Camargo et al., 2017); and encapsulation in gum Arabic, maltodextrin, and modified starch (Kausadikar et al., 2015). In the literature, LEONCs by emulsion-coacervation techniques were produced using the different polymers; however, to our best knowledge, so far there have been no studies on the use of WPC-pectin complex for LEO encapsulation, and no studies have been conducted on the effects of LEO encapsulation on the changes in volatile composition and biological activity (antioxidant and anticancer activities). This study focused on the fabrications of LEO nanocapsules (LEONCs) by complex coacervation using WPC-pectin as wall material, characterization of LEONCs in terms of their particle size (Atomic Force Microscopy), morphology (Scanning Electron Microscopy), encapsulation efficiency (spectrophotometry), molecular (Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy), and volatile composition (Headspace Gas Chromatography-Mass Spectrometry) properties. The fabricated LEONCs were also tested in terms of their antioxidant

activity by 2,2-diphenyl-1-picrylhydrazyl assay and anticancer activity by *in vitro* methods against murine colon carcinoma (colon-26) cells (cell counting kit-8 assay).

3.2 Materials and methods

3.2.1 Materials

Lemons were collected in the 1st stage of harvesting (green color, immature) from farmers in Hiroshima prefecture, Japan. 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 of biopolymers were prepared with deionized water.

3.2.2 Extraction of lemon essential oil (LEO)

The lemon peel including flavedo (epicarp) and albedo (mesocarp) layers were carefully peeled. Extraction of LEO was carried out from fresh lemon peel without prior processing by steam distillation using a special oil steam distiller (PureStiller type K-HJ200, Japan) for 2.5-3.0 h.

1. Hot plate / heater	6. Cold water (inlet)
2. Water	7. Hot water (outlet)
3. Steam	8. Water with LEO
4. Aromatic plants	9. LEO
5. Steam mixed with LEO	10. Aromatic water

Fig. 19. Schematic of LEO extraction from lemon peel by steam distillation technique

The mechanism of LEO extraction by steam distillation technique (**Fig. 19**): when the water boiled (about 100 ^{o}C), the steam passes through the fresh lemon peel. The combination of heated steam and gentle pressure causes the essential oil to be released from protective sacs (in the flavedo part). As the steam mixture with LEO flows through a condenser, the cold temperature changed the steam back into a liquid and collects into the reservoir. The aromatic water (hydrosol) and LEO separated naturally because LEO is immiscible with water and it has less density, so LEO was in the top layer, while water was in the bottom layer. The obtained LEO was dried over anhydrous sodium sulfate and stored at -20 ˚C in glass vials covered by aluminum foils until further analysis.

3.2.3 Volatile composition analysis

The volatile composition of the samples was analyzed using a headspace gas chromatography-mass spectrometer (HS-GC-MS, Shimadzu GCMS-QP-5050 series) auto-injector equipped with a DB-WAX column (60 m x 0.25 mm i.d. (inner diameter); film thickness = 0.25 mm). GC parameters were as follows: the carrier gas was helium and be set at a flow rate of 0.9 mL min^{-1}. The ion source temperature was 200 ˚C, the interface temperature was 230 ˚C, and the pressure

was 100 kPa. The column oven temperature was initially set at 50 °C for 5 min, and then ramped to 160 °C at 3 °C min^{-1}, and after that, it was warmed up to 200 °C at 20 °C min^{-1}. MS parameters were as follows: the interface temperature was 230 °C, elution time was 3 min, the full scan mode from *m/z* 50 to 250, the scan speed was 500, the threshold was 2,000, and the interval was 0.5 sec^{-1}. The identification of volatile compounds was based on a comparison of their GC retention time and mass spectra with the retention index of D-limonene (Wako, No. 124-03892), β-pinene (Aldrich Chemistry, No. 402753), and α-pinene (Aldrich Chemistry, 147524) standard, and the reference spectra from the US National Institute of Standards and Technology (NIST) data base library.

3.2.4 Production of LEONCs

Production of LEONCs based on the previous study by Ghasemi et al. (2018) with some modifications.

a. Preparation of biopolymers

WPC (4 g) was dissolved into deionized water to obtain 100 mL solutions. At the same time, 1 g of pectin was dissolved in hot deionized water (70 °C) to prepare 100 mL solution. Maltodextrin 50 % (w/v) was prepared in deionized

water. Maltodextrin is used to increase the total soluble solids of the sample for obtaining the higher powder. Each polymer solution was slightly stirred on magnetic stirrer for at least 30 min until homogenous and then stored at 4 ^{o}C for 24 h to complete hydration of biopolymers. All the solutions then filtered through 0.45 μm pore size to be used for further preparation.

b. Nanoemulsion

The biopolymer solution of WPC (4 % w/v), pectin (1 % w/v) and maltodextrin (50 % w/v) at ratio 1:1:1 were mixed and stirred on magnetic stirrer at 1,000 rpm, 37 ^{o}C for 30 min. polysorbate 80 was added at a ratio of 5 % (w/w) of the total solid. LEO as core material was added into this solution gradually (100 μL of LEO g^{-1} of biopolymers; molecular weight of LEO = 0.853g mL^{-1}). An ultrasonic homogenizer was used to produce oil-in-water nanoemulsions. The operational parameter established in a 5 min sonication time and 25 % ultrasonic power amplitude.

Ultrasonicator consists of an ultrasonic chamber having an ultrasonic probe. The disruptive forces created by the ultrasonic probe in combination with cavitation, turbulence, and interfacial waves breaks the course emulsions flowing

in the ultrasonic chamber to fine nanoemulsios (Kentish et al. 2008). Nanoemulsions production at small scale in the laboratory use bench-top sonicator (Fig. 20). The piezoelectric crystal probe in the sonicator generates intense pressure waves.

c. Complex coacervation and freeze drying

The pH level of the nanoemulsions was adjusted at 3.0 to initiate the complex coacervation and then stirred at 37 ^{o}C for 30 min. The solution centrifuged at 13,000 rpm, 4 ^{o}C for 30 min. Pellet was collected as LEONCs by filtration using Whatman paper number 4, and then frozen. To obtain the dried LEONCs, it was continued by freeze drying. The dried powder was collected and stored at -20 ^{o}C for further characterization.

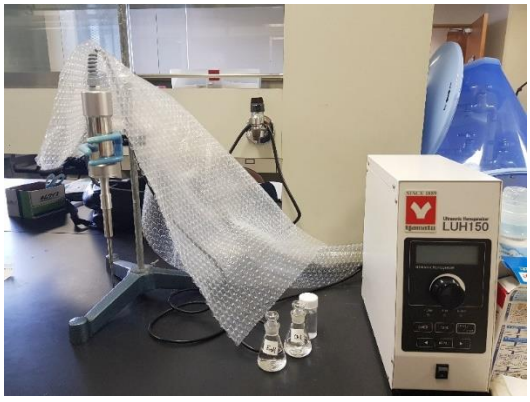


Fig. 20. Ultrasonic homogenizer, LUH 150-Yamato for laboratory scale

3.2.5 Morphology and particle size distribution

The morphology of the LEONCs was examined using scanning electron microscopy (SEM) (Miniscope TM3000, Hitachi High-technologies Corp, Tokyo) at 5,000, 10,000 and 20,000 x magnifications. Samples were placed on sample stage, and then coated with gold (Au) to have conducting samples.

Topographical surface and particle size distribution estimation were analysed using atomic force microscopy (AFM) (SNOAM, Hitachi-Tech Science Corp, Tokyo) with Dynamic Force Mode in air. One drop of samples dilution was 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 N m^{-1} was used.

3.2.6 Encapsulation efficiency

Encapsulation efficiency (%) of LEONCs was determined according to the method described by Bae and Lee (2008). Ten millimeters of hexane were added to 1.0 g of LEONCs powder in a glass jar with a lid, which was shaken gently by hand for the extraction of free oil during 2 min, at room temperature. The solvent was filtered through a Whatman paper filter number 4 and the powder collected

on the filter was rinsed three times with 20 mL of hexane. Then the solvent was left to evaporate at room temperature and after at 60 ^{o}C, until constant weight. The non-encapsulated oil (surface oil) was determined by mass difference between the initial clean flask and that containing the extracted oil residue (Jafari et al., 2008). Total oil was assumed to be equal to the initial oil, since the preliminary test revealed that all the initial oil was retained. Encapsulation efficiency (EE) was calculated as followed:

$$Encapsulation\ efficiency\ of\ LEONCs\ (\%) = \left(\frac{TO - SO}{TO}\right) x\ 100$$

Where TO is total oil initial loaded and SO is total oil in the surface

3.2.7 Molecular characterization by ATR-FTIR Spectroscopy

To reveal possible chemical interactions in encapsulation, polymers (pectin, WPC, maltodextrin), NCs, LEO and LEONCs were analyzed using Attenuated Total Reflectance – Fourier Transform Infrared Spectroscopy (ATR-FTIR). A Thermo Scientific Nicolet iS10 FTIR spectrometer (Thermo Fischer Scientific, USA), equipped with a KBr beam splitter, DTGS detector and diamond ATR cell was used to obtain the spectrum. OMNIC software from Thermo Scientific was

used to provide instrument control and data acquisition. The ATR-FTIR spectra of samples were recorded from 4000 to 400 cm^{-1}. As a background, air spectrum was scanned using the same instrumental conditions before all measurements.

3.2.8 Biological activities

a. Antioxidant activity

The antioxidant activity is estimated by 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma Aldrich). The DPPH assay was prepared according to the protocol described by Asikin et al. (2012) with modification. Briefly, 50 µL of samples at different concentrations was added with 150 µL of DPPH (0.1 mM). After incubation for 45 min in the dark at room temperature, the absorbance was measured at 517 nm using spectrophotometer. Ethanol served as negative control. Antioxidant activity is indicated by the percentage of inhibition of DPPH and calculated using following equation:

$$Inhibition\ (\%) = \frac{(Abs.control - Abs.sample)}{Abs.control} x\ 100$$

b. Anticancer activity of samples against colon-26 cells

Cell line: murine colon carcinoma (colon-26) cell line was purchased from RIKEN BRC CELL BANK (Tsukuba, Japan). Colon-26 cells (RCB2657, RM092147) were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10 % (v/v) fetal bovine serum (FBS). The cells were incubated at 37 ˚C in a 5 % CO_2 atmosphere. To investigate the cytotoxicity activity of samples, a CCK-8 (cell counting kit-8) assay (Dojindo, Japan) was performed. Colon-26 cells were seeded in a 96-well cell culture plate (Watson ® Bio Lab, Japan) at a density of 2 $x10^3$ cells $well^{-1}$100 μL^{-1} and incubated at 37 ˚C in a 5 % CO_2 incubator for 24 hours. After incubation, each medium was replaced, and 100 µL of sample dissolved in the medium was added and then the cells were incubated for 24 h under the same condition as above. Finally, 10 µL of the CCK-8 reagent was added into each well and incubated at 37 ºC in a 5% CO_2 incubator for 6 h. The fluorescent intensity (FI) of the samples was recorded on a microplate reader (450 nm, Varioskan Flash from Thermo Scienctific, Waltham, MA, USA).

$$Cell\ viability\ (\%\ of\ control) = \frac{A\ sample - A\ color\ blank\ of\ sample}{A\ control - Acolor\ blank\ of\ control}\ x\ 100$$

Antiproliferative activity (%) = 100 – cell viability. The IC_{50} value was calculated by plotting the sample concentration and antiproliferative activity.

3.2.9 Statistical analysis

All measurements were carried out in triplicates. The results were expressed as mean ± standard deviation (SD). Data obtained were analyzed by one way analysis of variance (ANOVA) followed by *least significant differences* (LSD) at $p < 0.01$ using Microsoft excel. IC_{50} was calculated by plotting the results (antioxidant activity and anticancer activity) and the concentration of samples.

3.3 Results and Discussions

3.3.1 The yield of LEO and entrapment efficiency of LEONCs

In the present study, the steam distillation technique of fresh lemon peel obtained a colorless and transparent LEO with a yield of 1.18 ± 0.06 % (dry matter) (**Fig. 21**). Bourgou et al. (2012) reported that the yield was 1.30 %, while Moosavy et al. (2017) reported a yield of 1.33 % using dried peel for extraction. The yield of LEO is depended on many factors, including plant variety, age of plant, stage of ripening, and the extraction method (Bagamboula et al. 2004).

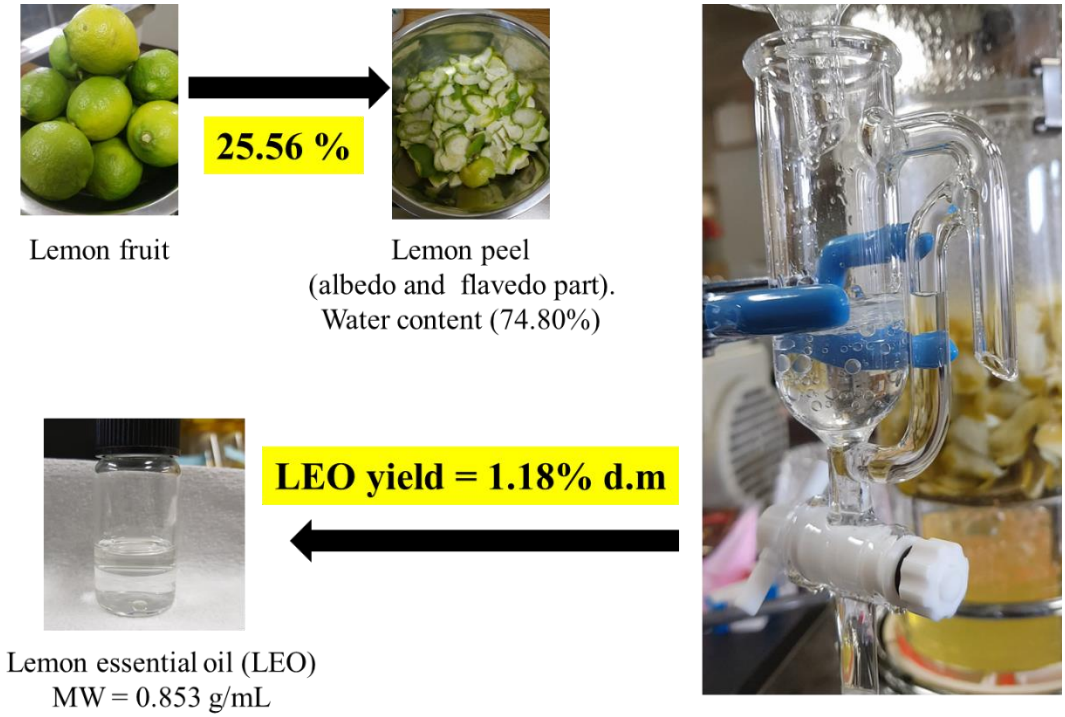
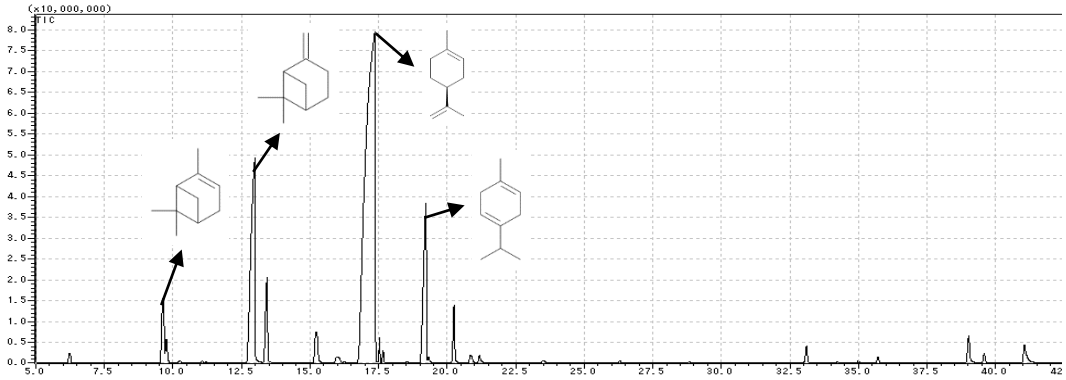


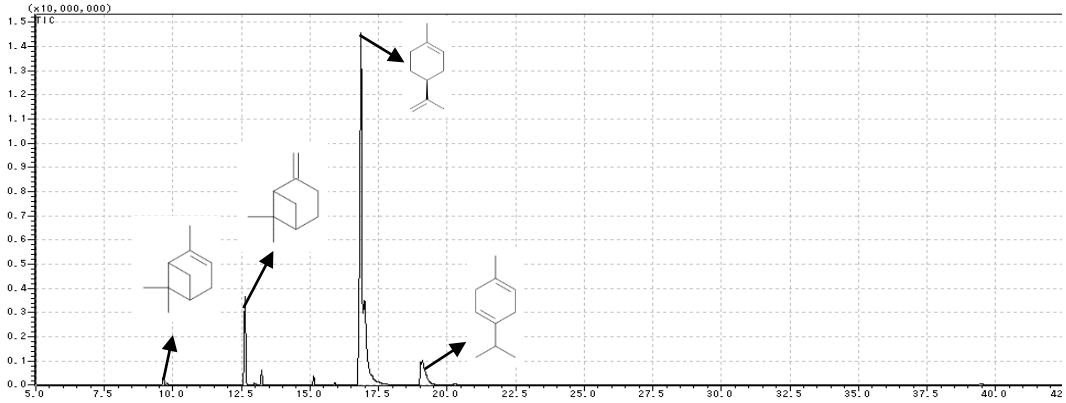
Fig. 21. The LEO extraction from lemon peel by steam distillation

The encapsulation efficiency of LEO in whey protein-pectin complex was 77.37 ± 1.95 %. This result means that there was efficient adsorption of the essential oil in whey protein-pectin complex. Previously research showed that the encapsulation efficiency of orange peel oil in whey protein-pectin complex was about 88 % (Ghasemi et al., 2018). Encapsulation efficiency by complex coacervation depend on the pH levels, polypeptide mixing ratio, ionic strength, temperature, molecular weight, and the concentration of polymer (Priftis and Tirell, 2012).

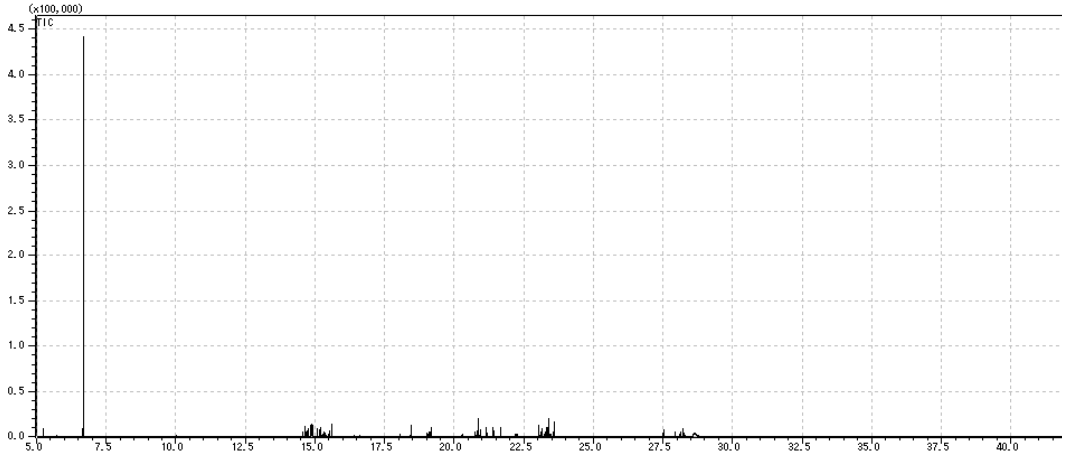
3.3.2 Volatile compound composition



(a)



(b)

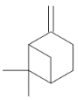
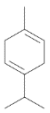
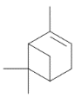


(c)

Fig. 22. Chromatogram from GC-MS of (a) LEO, b) LEONCs, and (c) NCs

The volatile compounds composition of LEO and LEONCs was analyzed using HS-GC-MS. Four compounds (D-Limonene, β-pinene, γ-terpinene, and α-pinene) were identified as the major compounds of LEO and LEONCs, represented 86.51 % and 93.79 % of all fractions, respectively (**Fig. 22** and **Table 8**). No volatile compounds were detected in NCs indicated of unloaded LEO.

Table 8. The predominant volatile compounds of LEO and LEONCs

Compound names	RT (min)	Molecular structure	Area (%)	
			LEO	LEONCs
D-limonene	16.858		59.80 ± 1.35[a]	74.01 ± 0.84[b]
β-pinene	12.717		15.31 ± 0.15[a]	9.61 ± 0.14[b]
γ-tepinene	18.975		8.43 ± 0.20[a]	9.26 ± 0.1 [b]
α-pinene	9.542		2.97 ± 0.27[a]	0.91 ± 0.01[b]
			86.51 [a]	93.79[b]

Data represented the mean ± SD (n=3). The different letters in one row indicated the significant differences in one row by *the least significant differences* at $p < 0.01$.

D-Limonene was the most abundant volatile compounds in LEO (59.80 %) which increased significant in LEONCs (74.01 %). In many studies of essential oil from lemon peel, D-limonene was found to be the most prominent, same as our

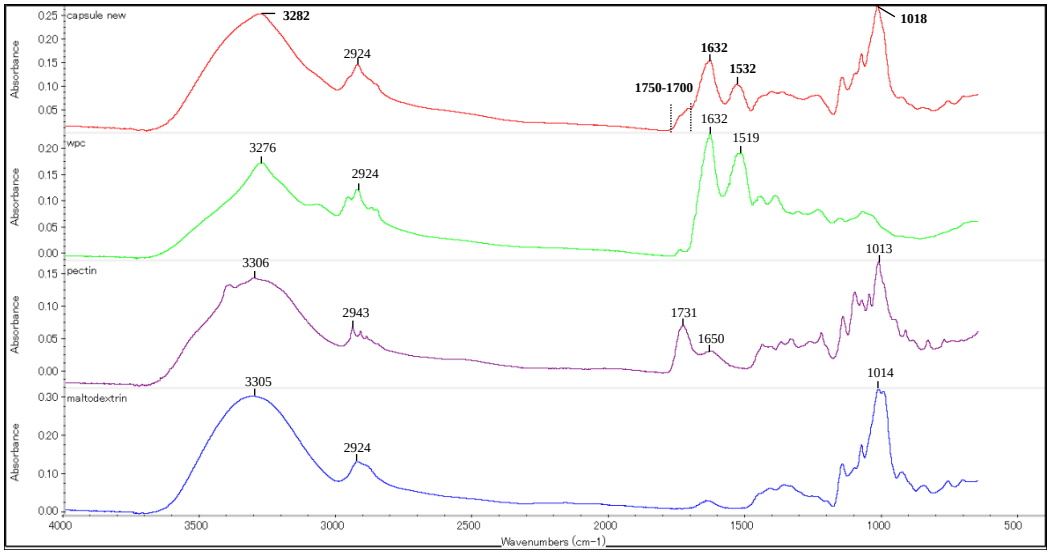
report (Tisserand and Young, 2014). γ-Terpinene ranks third also showed a percentage increased after encapsulation, from 8.43% in LEO increased to 9.26 % in LEONCs. Different results were shown on β-pinene and α-pinene which decreased in percentage after encapsulation. Thus, encapsulation LEO in whey protein-complex changed the volatile compositions.

3.3.3 Molecular analysis of NCs, LEO and LEONCs

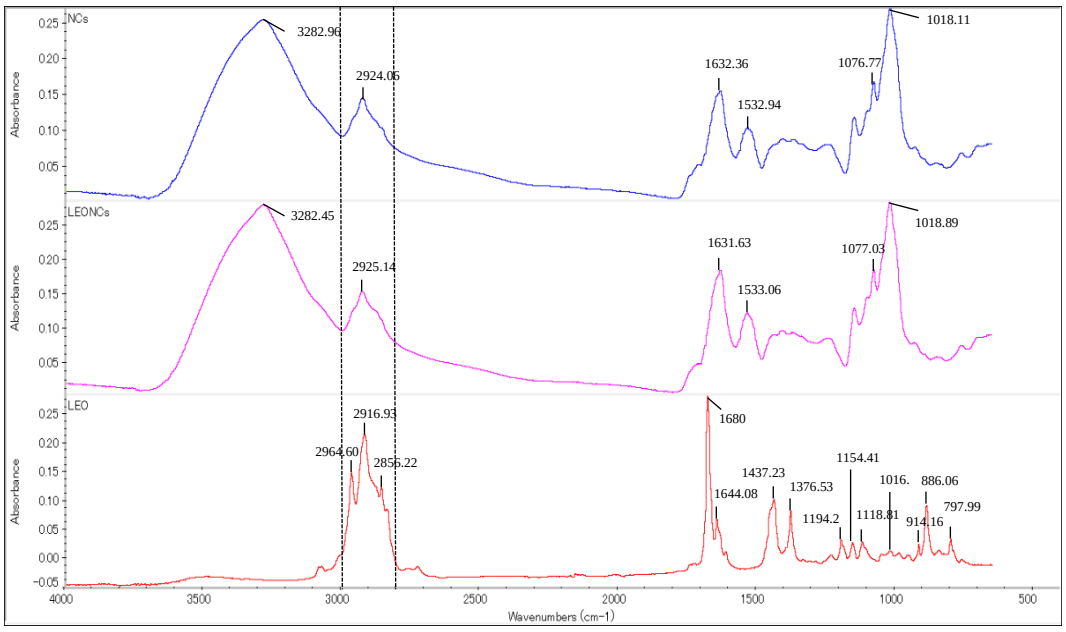
In the spectral range studied of 4000 to 400 cm^{-1} there are numerous peaks which corresponds to the different molecular bonds of the sample components interacting with IR radiation. At shown in **Fig. 23a**, pectin showed its characteristic absorption at 1731 and 1650 cm^{-1} which were assigned to stretching of COOR (esterified carboxyl group), and COO^- (free carboxyl group), respectively (Gnanasambandan and Proctor, 2000). The absorbance at 1731 cm^{-1} is higher than 1650 cm^{-1} indicated high methoxyl pectin. In the spectrum of WPC showed characteristic absorption at 1632, 1519, 1446, 1392, 1236 and 1072 cm^{-1}, were attributed to the stretching vibration amide I (C=O, C-N), amide II (N-H, C-N), C-H, COO^-/COOH (fat related), and C-N/CO bonds, respectively (Assadpour et al., 2017, and Ghasemi et al, 2017). Maltodextrin present featuring the band

assigned to C-O at 1014 cm^{-1}. During the complex coacervation, carboxyl groups in pectin interact with amino groups in WPC. The NCs showed a broad band at 3282 cm^{-1}, shifted to another position from 3276 and 3306 cm^{-1} in WPC and pectin respectively, attributed to NH2 and OH groups vibration (Espinosa-Andrews et al. 2010).

After formation of NCs, the intermolecular interaction between WPC-pectin were explained by several major changes in FT-IR spectra. First, at peek 3000 to 3500 cm^{-1} , related to hydrogen bonding in WPC (3276 cm^{-1}) and amino groups in pectin (3306 cm^{-1}), was shifted to another position in 3282 cm^{-1} in the NCs which attributed to NH_2 and OH groups vibration (Espinosa-Andrews et al. 2010). These shifts clearly indicated the enhancements of hydrogen bonding is also involved in the interactions between polymers and the consequent formation of complex coacervates. The new region formed around 1750-1700 cm^{-1} which was connected to the band 1632 cm^{-1}, which could be the carboxyl groups of pectin between the amide groups of WPC. Furthermore, peak of amide II at 1519 cm^{-1} shifted to 1532 cm^{-1} in NCs indicated the interaction of amide groups and carboxyl groups. Addition, the peak at 1013 and 1014 cm^{-1} of pectin and maltodextrin respectively shifted to 1018 cm^{-1} indicated the interaction between polymers.



(a)



(b)

Fig. 23. FTIR spectra of (a) NCs and the compositions (maltodextrin, pectin, and WPC) (b) LEONCs and the compositions (LEO and NCs)

Identifical spectral features of LEO can be seen in **Fig. 23b.** Main peaks were observed at 2964, 2916, 2856, 1644, 1437, 1376, 1194, 1154, 1118, 1016,

914, 886, and 797 cm^{-1}.The vibrational band around ~2900, ~1700, and ~1100 cm^{-1} may include spectral features a rising from C-H, C=O, and C-O respectively, stretching vibrations of terpenoids components (Hasani et al., 2018). The peak at 2964 cm^{-1} corresponds to the $-CH_3$ asymmetric and symmetric stretching vibrations (Berechet et al., 2015). The peaks at 2916, 1680, 1644, 1437, 1376, 1154, 886, and 797 cm^{-1} corresponds to C-H stretching vibrations of alkanes, C=O stretching vibrations, C=C stretching vibrations of alkanes, C-H bending vibrations of alkanes, C-H stretching vibrations of aromatics, and C=C bending vibrations of alkanes, respectively (Berechet, et al., 2015; Benoudjit et al., 2017). Several major changes in FTIR spectra identified after loaded LEO in NCs in LEONCs formation, including the shifted peaks from 3282.96 to 3282.45; from 2924.06 to 2925.14; from 1632.36 shifted to 1631.63; from 1532.94 to 1533.06; from 1076.77 to 1077.03; and from 1018.11 to 1018.89. It indicated the interaction between LEO and NCs in the formation of LEONCs.

3.3.4 Morphology, topography, and particle size analysis

The morphology and topography of LEONCs was analysed by SEM and AFM. SEM photographs (**Fig. 24**) showed that LEONCs have a non-uniform spherical shape.

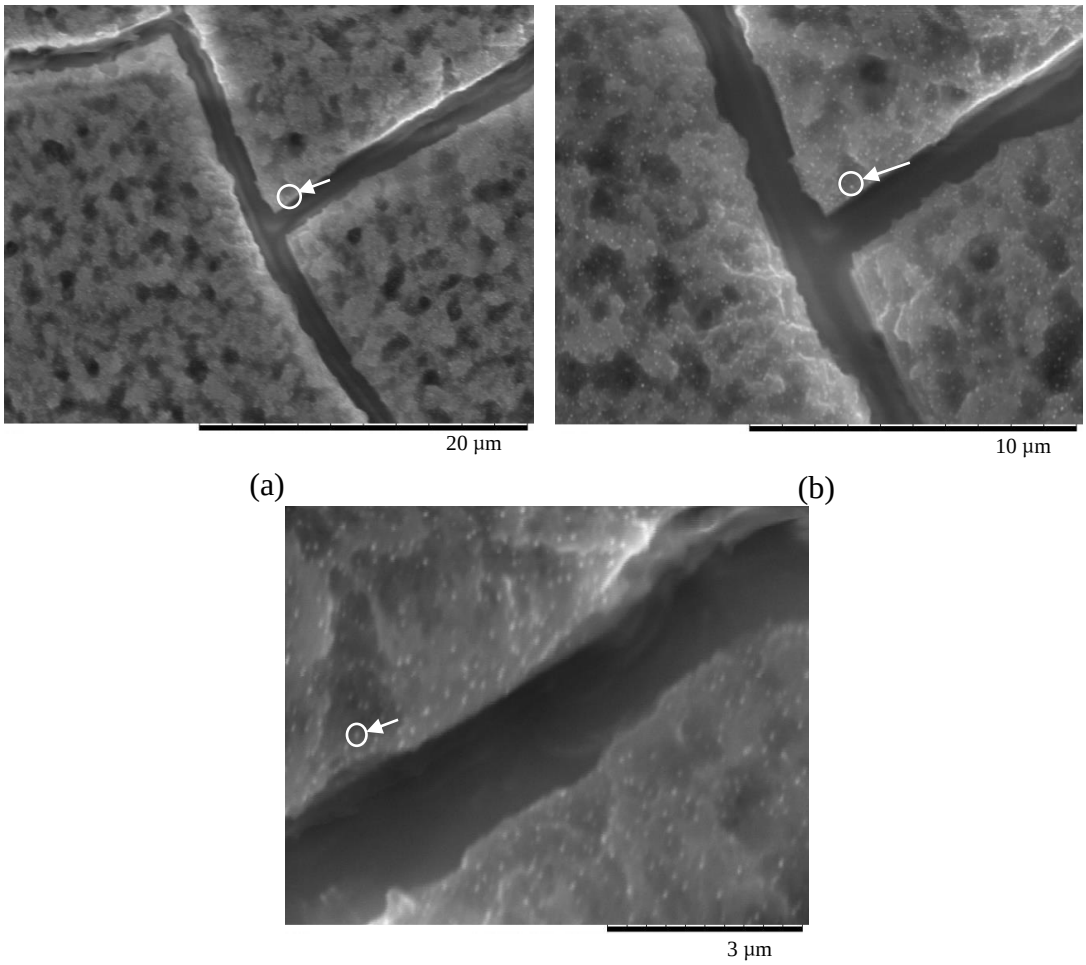
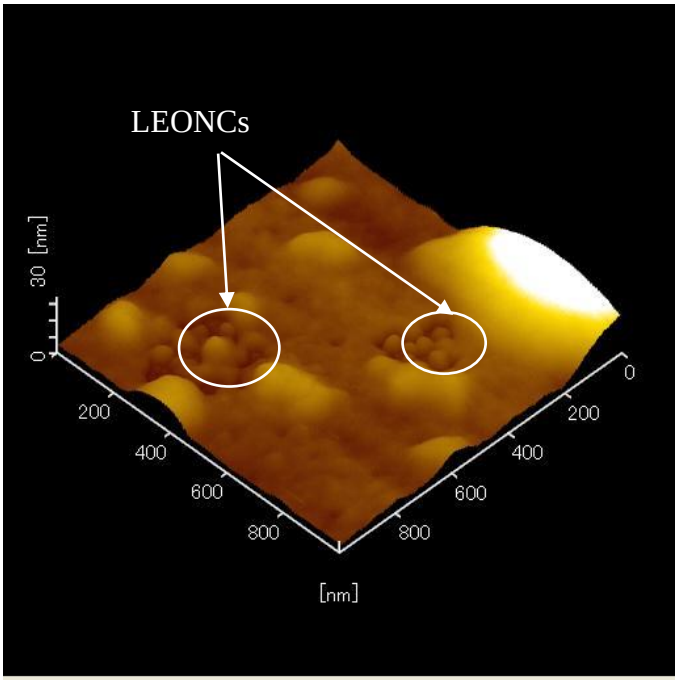
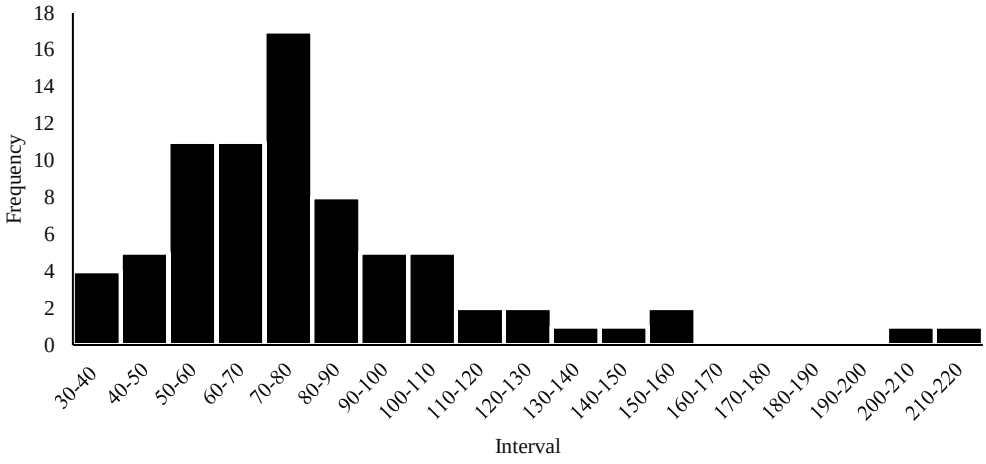


Fig. 24. SEM photographs of LEONCs at (a) 5,000, (b) 10,000, and (c) 20,000 of magnifications



(a)



(b)

Fig. 25. AFM analysis: (a) the images of LEONCs, and (b) particle size distribution of LEONCs

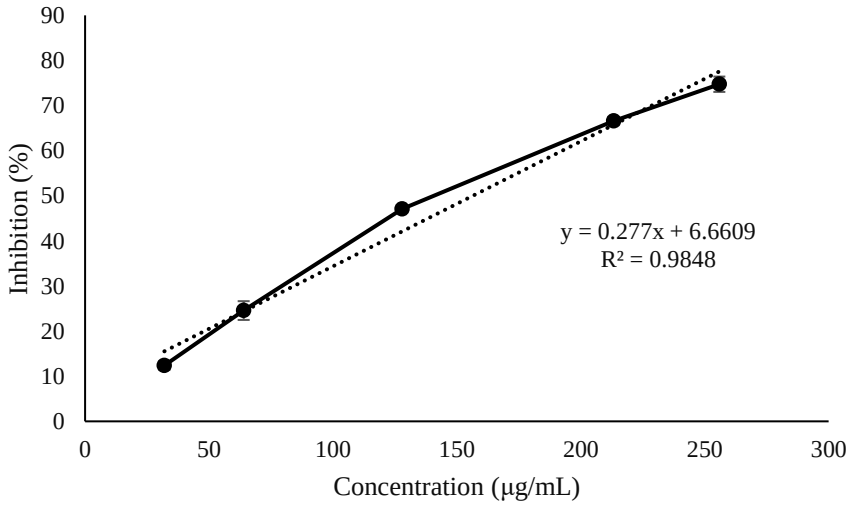
The particle size of LEONCs was between 30.79 nm – 210.83 nm and average size of 80.91 nm.

3.3.5 Antioxidant activity

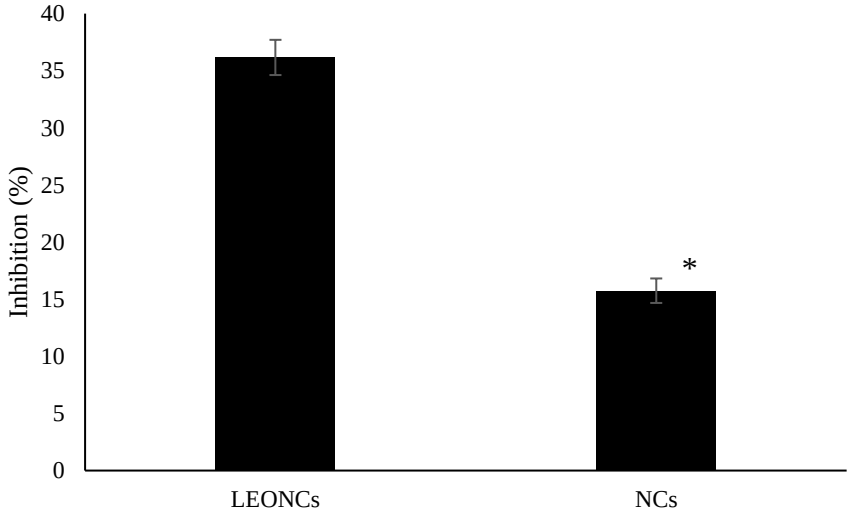
LEO was able to reduce the stable, purple-colored radical DPPH into yellow-colored DPPH-H with IC_{50} of 156.4 ± 2.3 µg/mL. Frassinetti et al. (2011), demonstrated the scavenging abilities of Citrus spp. essential oil was ranging from 20 to 70%. Another research by Moosavy et al. (2017) reported that antioxidant activity of essential oil from lemon was 55.09%. The antioxidant activity is related to the chemical composition of LEO that rich in monoterpenes (D-limonene and α-pinene) (Wei and Shibamoto, 2007). D-Limonene, α-pinene, and ß-pinene individually tested do not have significant antioxidant activity compared to the same constituents when tested together (Ruberto and Baratta, 2000). LEONCs showed higher antioxidant activity than NCs. This indicated the LEO loaded in LEONCs.

Capsule-forming polymer also contributes to the antioxidant activity of LEONCs, as shown in the result of NCs. NCs was composed of WPC and pectin. Whey protein is well known to exhibit antioxidant activity (Corrochano et al., 2018). The whey protein antioxidant activity was shown to be dose-dependent (20 - 100 mg/mL) by the DPPH assay which measures the ability of a compound to scavenge the DPPH radical (Gad et al., 2011). Pectin (from apple pomace and

citrus) at the concentration higher than 4.5 mg/mL was reported have DPPH scavenging activity more than 60 % (Wang et al., 2014).



(a)



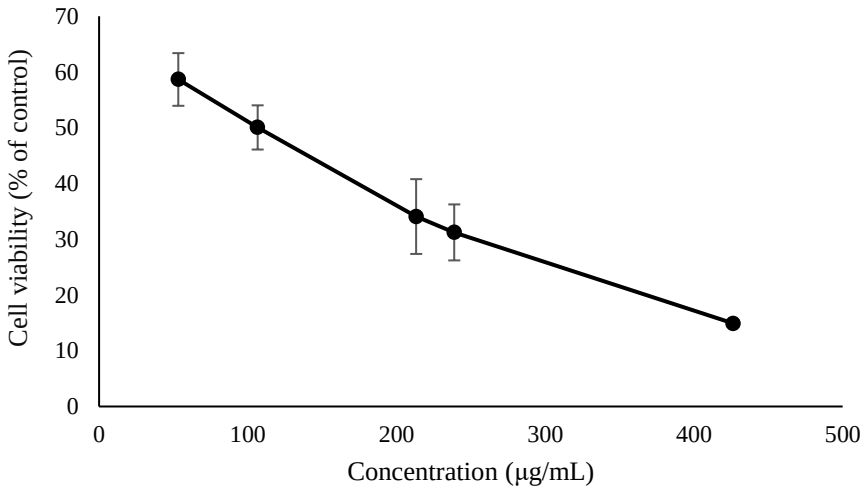
(b)

Fig. 26. Antioxidant activity by DPPH assay; (a) LEO, and (b) LEONCs and NCs at the same concentration (15 mg/mL). Data represented the mean ± SD. *Significant differences by *the least significant difference* at $p < 0.01$

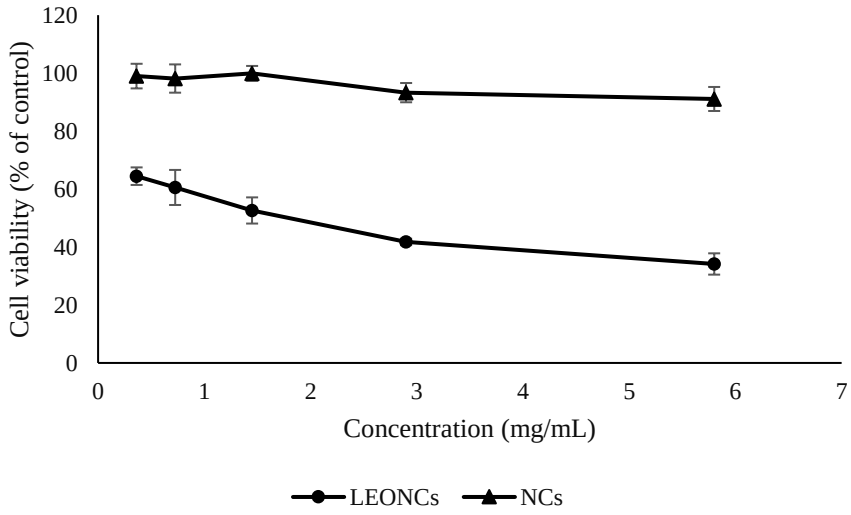
3.3.5 Anticancer activity

The in vitro assessment of anticancer activity of LEO and LEONCs showed have a significant cytotoxic effect against colon-26 cells, which IC_{50} value was 105.88 ± 15.83 µg/mL and 2.26 mg/mL, respectively. Previously study reported that LEO inhibited 50% of cells viability of PC-3, A549, and MCF-7 cells lines at the concentrations of 0.083; 0.061; and 0.143 (% v/v), respectively (Zu et al., 2010).

The antiproliferation effect might be related to D-limonene in LEO, which reported can induce apoptosis by up-regulating of pro-apoptotic factors and down-regulating anti-apoptotic factors (Mukhtar et al., 2018). Other components of LEO, α-pinene, has been also reported involved in antiproliferative activity by stimulating apoptosis, proved by initial disruption of mitochondrial function, reactive oxygen species formation, improved caspase-3 properties, heterochromatin aggregation, DNA disintegration, and exposure of phosphatidylserine on the cell surface (Matsuo et al., (2011).



(a)



(b)

Fig. 27. Cell viability of colon-26 cells by CCK-8 assay after treatments with (a) LEO, and (b) LEONCs and NCs. Data represented the mean ± SD (n=3)

At the same concentration of LEONCs, NCs consisting of WPC and pectin also showed antiproliferative activity although not significant. Decreasing of cell

viability in NCs might be correlated with WPC and pectin components. Whey protein which is composed of β-lactoglobulin (β-Lg) and α-lactalbumin (α-LA) reported have anticancer activity. α-Lactalbumin possesses anti proliferative effects on colon adenocarcinoma cell line (CaCo2 or HT-29 mono layers) (Sternhagen and Allen, 2001). α-LA can also be a potent calcium concentration-elevating and apoptosis-inducing agent. Multimeric form of α-LA was shown to promote apoptosis in transformed and immature cells while sparing mature epithelial cells. During this process calcium levels are elevated, allowing a connection between calcium levels and apoptosis (Hakansson et al., 1995). The mechanism of anticancer activity of β-Lg may be related to its sulphur aminoacid content. This suggests a possible role in protecting DNA in methylated form. Indeed, the aminoacid composition of β-Lg plays an important role in preventing oxidative damage. Particularly, β-Lg influences tissue levels of the thiol-glutathione, a multifunctional tripeptide, that binds and eliminates endogenous and exogenous mutagens and carcinogens (Pepe et al., 2013).

Enzymatically extracted apple pectin reported reduced the viability of HCT 116 and Caco-2 colorectal cancer cells, induced apoptosis, and increased intracellular reactive oxygen species production (Palko-Labuz et al., 2021).

3.3.7 Volatile compounds stability

LEO are a mixture of volatile compounds and consist mainly of monoterpene hydrocarbons which poses high levels of unsaturation and are generally unstable due to many factors such as light, heat, oxidation, and hydration. To know the effect of encapsulation on the volatile compound stability, LEO and LEONCs was stored at different temperatures (4 ^{o}C and 40 ^{o}C) for 90 days. Volatile compound composition during storage period was analyzed and the results was shown at **Table 9.**

There was no significant differences in D-limonene content in LEONCs after storage at 4 ^{o}C and 40 ^{o}C, while in LEO there was significant differences. It is indicated that encapsulation LEO in WPC-pectin complex could protect the D-limonene content. The biggest changes was in γ-terpinene, where in LEO after storage at 40 ^{o}C decreased more than 99 % and at 4 ^{o}C decreased more than 20 %. γ-terpinene in LEONCs was no significant differences after storage at 4 ^{o}C, but at 40 ^{o}C, there was decreasing about 33 %. There was no significant difference of α-pinene in LEO and LEONCs after storage at 4 ^{o}C, but both showed significant differences at 40 ^{o}C. ß-Pinene content in LEO was significant difference after storage at 40 ^{o}C, while at 4 ^{o}C, there was no significant difference. ß-Pinene

content of LEONCs was significant difference after storage at 4 ^{o}C, and at 40 ^{o}C there was no significant difference.

Table 9. Volatile compounds composition during storage for 90 days at 4 ^{o}C and 40 ^{o}C

Samples	Area (%)		
	Initial	4 ^{o}C	40 ^{o}C
	D-limonene		
LEO	59.8±1.35^{a}	66.20±0.90^{c}	56.33±0.56^{b}
LEONCs	74.01±0.84	72.06±1.82	70.56 ± 0.32
	ß-pinene		
LEO	15.31±0.15^{a}	15.03±0.54^{a}	16.74 ±0.29^{b}
LEONCs	9.61±0.14^{a}	8.51±0.43^{b}	10.11±0.04^{a}
	γ-terpinene		
LEO	8.43±0.20^{a}	6.68±0.27^{b}	0.01±0.00^{c}
LEONCs	9.26±0.10^{a}	9.30±0.13^{a}	6.19±0.26^{b}
	α-pinene		
LEO	2.97±0.27^{a}	2.89±0.31^{a}	3.71±0.03^{b}
LEONCs	0.91±0.01^{a}	0.93±0.04^{a}	1.15±0.12^{b}

Data presents as mean±SD (n=3). The different letters in one row represented the significant differences in one row by *the least significant differences* at $p < 0.01$.

As terpenoids (D-limonene, ß-Pinene, γ-terpinene, α-pinene) and tend to be both volatile and thermolabile and may be easily oxidized or hydrolyzed depending on their respective structure (Scott, 2005). It is well accepted that the chemical composition of essential oils is moreover dependent on the conditions during processing and storage of the plant material, upon distillation as well as in

the course of subsequent handling of the oil itself (Turek and Stinzing, 2013). The results indicated that encapsulation in WPC-pectin complex increased the stability of volatile compounds composition during storage period for 30 days at 4 ^{o}C and 40 ^{o}C.

3.4 Conclusion

The main component of LEO was dominated by 4 volatile compounds consisting of D-limonene, β-pinene, γ-terpinene, and α-pinene. The encapsulation efficiency of LEO in the WPC-pectin complex was 77.37 %, which means that was efficient absorption of LEO in the complex. LJNCs have a spherical shape with an average size of 80.91 nm. The encapsulation significantly changed the composition of LEO by increasing the D-limonene, and γ-terpinene and decreasing β-pinene, and α-pinene. From molecular analysis by ATR-FTIR, it was found that there were shifts in the peak of the functional groups after the encapsulation process which indicated the success of the loading of LEO in NCs.

The in vitro assessment of anticancer activity of LEO and LEONCs showed have a significant cytotoxic effect against colon-26 cells, which IC_{50} value was 105.88 ± 15.83 µg/mL and 2.26 mg/mL, respectively. They also showed

antioxidant activity against DPPH radicals. This proved that encapsulation did not remove the biological activities of LEO. Encapsulation in WPC-pectin complex increased the stability of volatile compounds composition of LEO during storage period at different temperatures (4 ^{o}C and 40 ^{o}C). It becomes interesting for the further development of LEONCs in food product applications.

References

Asikin Y, Taira I, Inafuku S, Sumi H, Sawamura M, Takara K, Wada K. Volatile aroma components and antioxidant activities of the flavedo peel extract of unripe Shiikuwasha (*Citrus depressa Hayata*). *Journal of Food Science*. 2012, 77 (4), C649-C675.

Assadpour E, Jafari SM, Maghsoudlou Y. Evaluation of folic acid release from spray dried powder particles of pectin-whey protein nano-capsules. *International Journal of Biological Macromolecules*. 2017, 95, 238-247.

Bae EK, Lee SJ. Microencapsulation of avocado oil by spray drying using whey protein and maltodextrin. *Journal of Microencapsulation*. 2008, 25(8), 549-560.

Bagamboula CF, Uyttendaele M, Debevere J. Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool, and p-cymene toeards Shigella sonmei and S. Flexneri. *Food Microbiology*. 2004, 21, 33-42.

Benoudjit F, Maameri L, Ouared K, HistoryA. Evaluation of the quality and composition of lemon (*Citrus limon*) peel essential oil from an Algerian fruit juice industry. *Alger. J. Environ. Sci. Technol*. 2020, 6, 1575–1581.

Berechet MD, Stelescu MD, Manaila E, Craciun GD. Chemical composition of the essential oil of *Artemisia absinthium* from Romania. *REV.CHIM*. 2015, 66 (11), 1814-1818.

Bourgou A, Rahai F, Ourghemmi I, Tounsi MS. Changes in peel essential oil composition of four Tunisian citrus during fruit maturation. *The Scientific World Journal*. 2012, 4, 528-593.

ChunYan H, Hong P, ZhenYu Z, Jing, S. Evaluation of antioxidant and antitumour activities of lemon essential oil. *Journal of Medicinal Plants Research*. 2010, 4 (18), 1910-1915.

Corrochano AR, Buckin V, Kelly PM, Giblin L. Invited review: whey proteins as antioxidants and promoters of cellular antioxidant pathways. *Journal of Dairy Science*. 2018, 101 (6), 4747-4761.

Cortez-Camargo S, Cruz-Olivarez, Barragan-Huerta B, Dublan-Garcia O, Roman-Guerrero A, Perez-Alonso C. Microencapsulation by spray drying of lemon essential oil: Evaluation of mixtures of mesquite gum-nopal mucilage as new wall materials. *Journal of Microencapsulation*. 2017, 34 (4), 395-407.

Dugo P, Mondello L. 2011.*Citrus Oils: Composition, Advanced Analytical Techniques, Contaminants, and Biological Activity*. Vol. 49. Boca Raton, FL: CRC Press.

Espinosa-Andrews H,Sandoval-Castila O, Vazquez-Torez H, Vernon-Carter EJ, Lobato-Calleros C. Determination of the gum-Arabic-chitosan interactions by Fourier Transform Infrared Spectroscopy and characterization of the microstructure and rheological features of their coacervates. *Carbohydrate Polymers.* 2010, 79, 541-546.

Fathi M, Mozafari MR, Mohebbi M. nanoencapsulation of food ingredients using lipid-based delivery systems. *Trends in Food Science & Technology.* 2012, 23 (1), 13-27.

Frassinetti S, Caltavuturo L, Cini M, Croce CMD, Maserti B. Antibacterial and antioxidant activity of essential oils from Citrus spp. *Journal of Essential Oil Research*. 2011, 23 (1), 27-31.

GLOBOCAN (Global Cancer Observatory) Data Base. 2000. In Sung H, Ferlay J, Siegel RL. Laversanme M, Soerjomataram, I, Jemal A, Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca Cancer J Clin*. 2021, 71, 209-249.

Gad AS, Khadrawy YA, El-Nekeety AA, Mohamed SR, Hassan NS, Abdel-Wahhab MA. Antioxidant activity and hepatoprotective effects of whey protein and Spirulina in rats. *Nutrition*. 2011, 27 (5), 582-589.

Ghasemi S, Jafari SM, Assadpour E, Khomeiri M. Production of pectin-whey protein nano-complexes as carriers of orange peel oil. *Carbohydrate Polymers*. 2017, 177 (9), 369-377.

Ghasemi S, Jafari SM, Assadpour E, Khomeiri. Nanoencapsulation of D-limonene within nanocarriers produced by pectin-whey protein complexes. *Food Hydrocolloids*. 2018, 77, 152-162.

Gnanasambandam R, Proctor A. Determination of pectin degree of esterification by diffuse reflfectance fourier transform infrared spectroscopy. *Food Chemistry*. 2000, 68 (3), 327-332.

Hakansson A, Zhivotovsky B, Orrenius S, Sabharwal H, Svanborget C. Apoptosis induced by a human milk protein. *Proceedings of the National Academy of Sciences of the United States of America.* 1995, 92 (17), 8064–8068.

Hasani S, Ojagh SM, Ghorbani M. Nanoencapsulation of lemon essential oil in Chitosan-Hicap system. Part 1: study on its physical and structural sharacteristics. *Int J Biol Macromol*. 2018, 115, 143-151.

Jafari SM, Assadpoor A, He Y, Bhandari B. Encapsulation efficiency of food flavours and oils during spray drying. *Drying Technology*. 2008, 26 (7), 816-835.

Jiang T, Wang C, Liu W, Li Y, Luan Y, Liu P. Optimization and characteriation of lemon essential oil entrapped from chitosan/cellulose nanocrystals microcapsules. *Journal of Applied Polymer Sciences.* 2021, 138 (43), 51625.

Kausadikar S, Gadhave A, Waghmare J. Microencapsulation of lemon il by spray drying and its application in flavor tea. *Advances in Appplied Science Research*. 2015, 6 (4), 69-78.

Matsuo, AL, Figueiredo, CR, Arruda DC, Pereira FV, Scutti JAB, Massaoka MH, Travassos LR, Sartorelli P and Lago JHG, 2011: α-pinene isolated from *Schinus terebinthifolius Raddi* (*Anacardiaceae*) induces apoptosis and confers antimetastatic protection in a melanoma model. *Biochem Biophys Res Commun*. 2018, 411 (2), 449-454.

Moosavy MH, Hassanzadeh P, Mohammadzadeh E, Mahmoudi R, Khatibi A, Mardani K. Antioxidant and antimicrobial activities of essential oil of lemon (*Citrus limon*) peel in vitro and in a food model. *Journal of Food Quality and Hazard control*. 2017, 42-48.

Mukhtar YM, Adu-Frimpong M, XU X, Yu J. Biochemical significance of limonene and its metabolites: Future prospects for designing and developing highly potent anticancer drugs. *Biosci Rep*. 2018, 38 (6), BSR20181253.

Palko-Labuz A. Maksymowicz J, Sobieszczanska B, Wikiera A, Skonieczna M, Wesolowska O, Sroda-Pomianek K. Newly obtained apple pectin as an adjunct to irinotecan therapy of colorectal cancer reducing *E. coli* adherence and β-glucuronidase activity. *Cancers*. 2021, 13, 2952, 1-21.

Pepe G, Tenore GC, Mastrocinque R, Stusio P, Campiglia P. Review article: potential anticarcinogenic peptides from bovine milk. *Journal of Amino Acids*. 2013, ID 939804, 1-7.

Priftis D, Tirrell M. Phase behaviour and complex coacervation of aqueous polypeptide solutions. *Soft Matter*. 2012, 8, 9396-9405.

Ruberto G, Baratta MT. Antioxidant activity of selected essential oil components in two lipid model systems. *Food Chem*. 2000, 69, 167-174.

Scott RPW. 2005. *Essential oils.* In: Worsfold P, Townshend A, Poole C, editors. Encyclopedia of analytical science. 2nd ed. Amsterdam, London, New York: Elsevier. p 554–61.

Sternhagen LG, Allen JC. Growth rates of a human colon adenocarcinoma cell line are regulated by the milk protein alpha-lactalbumin. *Advances in Experimental Medicine and Biology.* 2001, 501, 115–120.

Timilsena YP, Akanbi TO, Khalid N, Adhikari B, Barrow CJ. Complex coacervation: principles, mechanisms and applications in microencapsulation. *Int J Biol Macromol.* 2019, 121, 1276-1286.

Tisserand, R., Young, R. *Essential Oil Safety*, 2nd ed.; Elsevier: New York, NY, USA, 2014.

Turek C, Stizing FC. Stability of Essential Oils: A. review. *Comprehensive Reviews in Food Science and Food Safety*. 2013, 12, 40-53.

Wang X, Chen Q, and Lu X. Pectin extracted from apple pomace and citrus peel by subcritical water. *Food Hydrocolloids*. 2014, 38, 129-137.

Wei A, Shibamoto T. Antioxidant activities and volatile constituents of essential oils. *J. Agric. Food Chem.* 2017, 54, 1737-1742.

Zu Y, Yu H, Fu Y, Efferth T, Liu X, WU N. Activities of ten essential oil towards *Propionibacterium acnes* and PC-3, A-549, and MCF-7 cancer cells. Molecules. 2010, 15 (5), 3200-3210.

CHAPTER 4. CONCLUSION AND RECOMMENDATION

In this study, two bioactive compounds from lemon fruits, namely LJ and LEO were encapsulated in the WPC-pectin complex. To get the optimum condition on encapsulation of LJ, response surface methodology was used by maximizing the response of the antioxidant activity and D-limonene content. The optimum encapsulation condition of LJ was found at 6 % WPC, 3 % pectin, and at pH 3.1. At this condition obtained the LJNCs with optimum antioxidant activity and D-limonene content. LJNCs have a spherical shape with an average size of 22.3 nm, and an encapsulation efficiency of 66.07 %. Molecular analysis showed that after the complex coacervation, there were found several shifts and changes in the peak of the functional groups associated with the synthesis of NCs and LJNCs. Interaction of carboxyl groups of pectin and amino groups of WPC was clearly demonstrated by FTIR spectra. Loaded LJ in the WPC-pectin complex as indicated by the formation of a new peak at 1230 cm^{-1}, the sharper region between 1750-1700 cm^{-1}, and the shifted of peak 1632 to 1630 cm^{-1}. The encapsulation efficiency and FTIR analysis proved the success of encapsulation of LJ in the WPC-pectin complex (formation of LJNCs).

Encapsulation in the WPC-pectin complex showed the protection function of bioactive compounds in LJ. The encapsulation increased the retention of ascorbic acid after storage for 30 days at 4 ^{o}C and 40 ^{o}C (about 29.5 % and 27.8%, respectively). Thus, will increase the shelf life of LJ. LJ and LJNCs were reported to have the anticancer activity against colon-26 cells with IC_{50} at 1.12 mL/100 mL and 13.13 mg/mL, respectively. They also showed antioxidant activity against DPPH radicals. The results indicated that the nano-system of the WPC-pectin complex increased the stability of LJ during storage without removing the biological activities. Thus, it's potential to develop as a functional food in the future.

LEO was encapsulated by oil-in-water emulsification followed by complex coacervation. The main component of LEO was dominated by 4 volatile compounds consisting of D-limonene, β-pinene, γ-terpinene, and α-pinene. The encapsulation efficiency of LEO in the WPC-pectin complex was 77.37 %, which means that was efficient absorption of LEO in the complex. LJNCs have a spherical shape with an average size of 80.91 nm. The encapsulation significantly changed the composition of LEO by increasing the D-limonene, and γ-terpinene and decreasing β-pinene, and α-pinene. From molecular analysis by ATR-FTIR, it was

found that there were shifts in the peak of the functional groups after the encapsulation process which indicated the success of the loading of LEO in NCs.

The in vitro assessment of anticancer activity of LEO and LEONCs showed have a significant cytotoxic effect against colon-26 cells, which IC_{50} value was 105.88 µg/mL and 2.26 mg/mL, respectively. They also showed antioxidant activity against DPPH radicals. This proved that encapsulation did not remove the biological activities of LEO. Encapsulation in WPC-pectin complex increased the stability of volatile compounds composition of LEO during storage period at different temperatures (4 ^{o}C and 40 ^{o}C). It becomes interesting for the further development of LEONCs in food product applications.