การกักเก็บสารสกัดจากใบหม่อน *Morus alba* L. โดยอันตรกิริยาระหว่างพอลิเมอร์

นางสาวเมธาวี เพียรภักดี

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ENCAPSULATION OF EXTRACT FROM MULBERRY *Morus alba* L. LEAVES BY POLYMER-POLYMER INTERACTIONS

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งานวิจัยนี้มีวัตถุประสงค์เพื่อผลิตไมโครแคปซูลจากใบหม่อนด้วยวิธีการเกิดอันตรกิริยาระหว่างพอลิเมอร์ ในขั้นตอนแรกศึกษาการสกัดสารต้านออกซิเดชันจากใบหม่อนโดยแปรความเข้มข้นเอทานอล 4 ระดับ (50, 60, 70 และ 95% v/v) พบว่า สารสกัดที่ใช้เอทานอล 60% (60EM) มีปริมาณสารประกอบฟีนอลิกทั้งหมด 122.21 มิลลิกรัมกรดแกลลิคต่อกรัมใบหม่อนแห้ง และฤทธิ์การต้านออกซิเดชัน (ตรวจวัดด้วยวิธี FRAP และ DPPH) 51.83 และ 10.45 มิลลิโมลโทรลอกซ์ต่อกรัมใบหม่อนแห้ง ซึ่งมีค่าสูงกว่าในสารสกัดอื่นๆ อย่างมีนัยสำคัญทางสถิติ ($p \leq 0.05$) จากการวิเคราะห์ชนิดของสารฟลาโวนอยด์ ด้วย LC-MS พบว่า สารสกัดใบหม่อนประกอบด้วยรูทิน เควอซิทิน ไอโซเควอซิทิน เควอซิทินกลูโคไซด์ เควอซิทริน และแอสทรากาลิน ในกระบวนการเอนแคปซูเลชันโดยใช้หลักการเกิดอันตรกิริยาระหว่างพอลิเมอร์ สารเคลือบคือ โปรตีนถั่วเหลืองสกัด และเพคตินชนิดเมธอคซิลต่ำ และสารแกนกลางคือ สารสกัด 60EM และ95EM ผลการศึกษาพบว่า ไมโครแคปซูลที่บรรจุ 60EM และ 95EM มีค่าร้อยละประสิทธิภาพการกักเก็บ และปริมาณสารประกอบฟีนอลิกทั้งหมดไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p > 0.05$) จึงนำสารสกัดทั้ง 2 ชนิดไปศึกษาค่า pH สุดท้ายของสารผสม (3.5, 4.0 และ 4.5) ที่เหมาะสมในการผลิตไมโครแคปซูล พบว่า ไมโครแคปซูลที่บรรจุ 60EM ที่ผลิตโดยการปรับค่า pH สุดท้ายของสารผสมเป็น 4.0 มีค่าร้อยละผลที่ได้ 50.29 ค่าร้อยละประสิทธิภาพการกักเก็บ 13.55 ปริมาณสารประกอบฟีนอลิกทั้งหมด 57.04 มิลลิกรัมกรดแกลลิกต่อกรัมไมโครแคปซูล ฤทธิ์การต้านออกซิเดชัน 25.20 มิลลิโมลโทรลอกซ์ต่อกรัมไมโครแคปซูล (วิธี FRAP) และ 12.68 มิลลิโมลโทรลอกซ์ต่อกรัมไมโครแคปซูล (วิธี DPPH) ตามลำดับ และปริมาณรูทินและเควอซิทินเท่ากับ 0.49 และ 6.24×10^{-3} มิลลิกรัมต่อกรัมไมโครแคปซูล ตามลำดับ ซึ่งมีค่าสูงกว่าใน ไมโครแคปซูลที่ผลิตโดยใช้ภาวะอื่นๆ อย่างมีนัยสำคัญทางสถิติ ($p \leq 0.05$) เมื่อแปรความเข้มข้นของสารเคลือบ (โปรตีนถั่วเหลืองสกัด และเพคติน) ที่เหมาะสมในการผลิตไมโครแคปซูล 3 ระดับ (2.5, 5.0 และ 7.5% w/v) โดยใช้อัตราส่วนเท่ากับ 1:1 พบว่า ไมโครแคปซูลที่ผลิตโดยใช้โปรตีนถั่วเหลืองสกัด และเพคตินความเข้มข้น 7.5% w/v ให้ค่าร้อยละผลที่ได้ 69.52 ค่าร้อยละประสิทธิภาพการกักเก็บ 15.22 ปริมาณสารประกอบฟีนอลิกทั้งหมด 73.37 มิลลิกรัมกรดแกลลิกต่อกรัมไมโครแคปซูล และฤทธิ์การต้านออกซิเดชัน 27.71 มิลลิโมลโทรลอกซ์ต่อกรัมไมโครแคปซูล (วิธี FRAP) และ 20.13 มิลลิโมลโทรลอกซ์ต่อกรัมไมโครแคปซูล (วิธี DPPH) ซึ่งมีค่าสูงกว่าในไมโครแคปซูลที่ผลิตโดยใช้สารเคลือบความเข้มข้นอื่นๆ อย่างมีนัยสำคัญทางสถิติ ($p \leq 0.05$)

ภาควิชา	เทคโนโลยีทางอาหาร	ลายมือชื่อนิสิต .....
สาขาวิชา	เทคโนโลยีทางอาหาร	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....
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METHAVEE PEANPARKDEE: ENCAPSULATION OF EXTRACT FROM MULBERRY *Morus alba* L. LEAVES BY POLYMER-POLYMER INTERACTIONS. ADVISOR: ASST. PROF. CHALEEDA BOROMPICHAICHARTKUL, Ph.D., CO-ADVISOR: ASST. PROF. KIATTISAK DUANGMAL, Ph.D., 98 pp.

This research was aimed to develop microcapsules from mulberry leaf extract using polymer-polymer interaction method. Firstly, the extraction of antioxidants from mulberry leaves was determined using 4 concentrations of ethanol (50%, 60%, 70% and 95% v/v). It was found that 60% ethanol extract (60EM) had total phenolics content of 122.21 mg GAE/g dried leaves and antioxidant properties (using FRAP and DPPH) of 51.83 and 10.45 mmol trolox/g dried leaves, respectively. These values were significantly higher than those in other extracts. In determination of type of flavonoids using LC-MS, it was found that mulberry leaves extract contained rutin, quercetin, isoquercetin, quercetin glucoside, quercitrin and astragalin. The suitable core material in encapsulation process by polymer-polymer interaction method was then determined. Coating materials were a combination of soy protein isolate (SPI) and low methoxyl pectin and core materials were 60EM and 95EM. The result presented that encapsulation efficiency and total phenolics content of microcapsules from 60EM and 95EM was not significantly different ($p > 0.05$). Therefore, both extracts were used for study the suitable final pH of mixed solution (3.5, 4.0 and 4.5) in polymer-polymer interaction process. It was found that microcapsules from 60EM with pH adjusted to 4.0 showed 50.29% encapsulation yield with 13.55% encapsulation efficiency. Total phenolics content was 57.04 mg GAE/g microcapsules, antioxidant properties was 25.20 mmol trolox/g microcapsules (FRAP) and 12.68 mmol trolox/g microcapsules (DPPH). Amount of rutin and quercetin was 0.49 and 6.24×10^{-3} mg/g microcapsules, respectively. The antioxidant activities of this condition were significantly higher than those in microcapsules produced using other conditions ($p \leq 0.05$). This condition was used for study the suitable concentration of coating materials (SPI and pectin) in microcapsules production. The concentration of SPI and pectin was varied in 3 values (2.5, 5.0 and 7.5% w/v) at a 1:1 ratio. It was found that microcapsules produced by using 7.5% w/v of SPI and 7.5% w/v pectin gave encapsulation yield of 69.52%, encapsulation efficiency of 15.22%, total phenolics content of 73.37 mg GAE/g microcapsules. Antioxidant activities was 27.71 mg trolox/g microcapsules, for FRAP and 20.13 mg trolox/g microcapsules, for DPPH. The antioxidant activities of this condition were significantly higher than those in microcapsules produced using other concentrations of coating materials ($p \leq 0.05$).

Department:	Food Technology	Student's Signature	.....
Field of Study:	Food Technology	Advisor's Signature	.....
Academic Year:	2013	Co-Advisor's Signature	.....

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CHAPTER 1
INTRODUCTION

In recent years, antioxidant has been of interest in the sense of bioactive compounds that have an efficiency to decrease a risk of chronic disease such as coronary heart disease, Alzheimer's disease, cancer and diabetes. Antioxidant compounds are mostly derived from fruits, vegetables, herbs and beverages (Katsube et al., 2006). Furthermore, antioxidant has been widely used in the functional foods, nutraceutical and pharmaceutical industries (Manach et al., 2005).

Mulberry (*Morus alba* L.) leaves is considered as a good source of flavonoids that have different biological activities including antioxidant activity. It has been used as a folk medicine in many Asian countries such as Korea, China, and Japan (Kim and Jang, 2010). Flavonols is the most important kind of flavonoids. Mulberry leaves consist of two type of flavonols; glycosides (flavonoids with sugar in molecules) and aglycones (flavonoids without sugar in molecules) includes rutin, quercetin, isoquercetin (Kim and Jang, 2011; Zhishen et al., 1999). The antioxidant activity of mulberry leaves was reported in a study of Katsube et al. (2006) who studied the suitable concentration of ethanol to extract bioactive compounds in mulberry leaves and found that ethanol extraction of mulberry leaves by 60% v/v ethanol gave highest amount of flavonoids. Nevertheless, extracted flavonoids were easily decomposed by environmental factors such as temperature, light and oxygen.

Microencapsulation process is a beneficial technique to improve the stability of bioactive compounds especially minerals, vitamins, phytosterols, lutein, fatty acids, lycopene and antioxidants (Champagne and Fustier, 2007). The main objective of encapsulation is to protect the core material from environmental conditions, such as light, moisture, oxygen (Shahidi and Han, 1993) and extends shelf life of bioactive compound (Fang and Bhandari, 2010). The polymer-polymer interaction (or coacervation) process is one of microencapsulation. It consists of phase separation by the formation of two coating materials and core material. The coating materials are usually protein and polysaccharide (Silva et al., 2012). Each biopolymer have a different functional properties include solubility, water-holding capacity, viscosity, gelation, coagulation, adhesion, emulsifying, and foaming properties. In addition, functional properties of protein-polysaccharide complexes are generally better than the functional properties of each biopolymer used alone (Schmitt et al., 1998). The

polymer-polymer interaction process is produced at room temperature, therefore this process is suitable for the encapsulation of heat-sensitive ingredients (Gouin, 2004).

The purposes of the present study were to study effects of ethanol concentration on the total phenolics content and antioxidant capacity in mulberry leaves extract, and to encapsulate flavonoids containing mulberry leaves extract by polymer-polymer interaction by using SPI and low methoxyl pectin as coating materials. Microcapsules were evaluated by encapsulation yield, encapsulation efficiency, total phenolics content and antioxidant properties.

The objectives of this research were:

- To determine a suitable concentration of ethanol for extracting flavonoids from mulberry leaves.
- To study the suitable concentration of mulberry extract for using as a core material in encapsulation by polymer-polymer interaction process.
- To study the suitable pH in production of microcapsules by polymer-polymer interaction process
- To study the suitable concentration of coating materials for producing microcapsules.

Scope of the research

This research was separated into 2 part;

Part 1 Study of the effects of concentration of solvent on total phenolics content, antioxidant activities, amount and type of flavonoids in the extract from mulberry leaves

This part is aimed to determine the suitable concentration of solvent to extract flavonoids from mulberry leaves. The mulberry leaves was extracted by various concentration of ethanol: 50, 60, 70 and 95% v/v. Total phenolics content, antioxidant activities (by using FRAP assay and DPPH radical scavenging activity), amount and type of flavonoids (by using High-Performance Liquid Chromatography and Liquid Chromatography-Mass Spectrometry) was determined. The best extract that gave highest total phenolics content and antioxidant activities was selected to be used in the next part of this research.

Part 2 Study of the appropriate conditions to produce microcapsules from mulberry extract by polymer-polymer interaction

The objective of this study was to determine the optimum core material, pH of the process and concentration of coating materials. The microcapsules of mulberry extract were produced by using polymer-polymer interaction method. The freeze-dried microcapsules was determined for terms of encapsulation yield, encapsulation efficiency, solubility, total phenolics content, antioxidant activities, morphology and moisture content.

CHAPTER 2
LITERATURE REVIEW

2.1 Mulberry

2.1.1 Background of mulberry plant

Mulberry or *Morus alba* L. is a plant in the Moraceae family. It is a fast-growing plant and grows worldwide under different conditions such as tropical, subtropical and temperate. In Europe, mulberry fruits are more valuable than their foliage. However, the production of mulberry in most countries in Asia, especially China, India and Thailand is focused on improving the foliage. Mulberry is a well-known economical plant that is a primary source of food for silkworms (*Bombyx mori*). In general, there are three types of mulberry, including white mulberry (*M. alba*), black mulberry (*M. nigra*) and red mulberry (*M. rubra*). The white mulberry (Figure 2.1a) is native to eastern and central China. The red mulberry (Figure 2.1b) is native to eastern United States. The black mulberry (Figure 2.1c) is native to western Asia. It has been grown for its fruits in Europe since before Roman times (Gerasopoulos and Stavroulakis, 1997; Suntornsuk et al., 2003; Reddy et al., 2004; Aramwit et al., 2010)

(a) **(b)** **(c)**

Figure 2.1 Characteristics of (a) white mulberry, (b) red mulberry and (c) black mulberry

Source: John (2011)

In Thailand, white mulberry is widely grown because it is cultivated easily and provides healthy foliage. The major cultivars of white mulberry in Thailand are

separated in eight cultivars; Nakhonratchasima 60, Buriram 60, Chumphon, Wavee, Chaingmai, Pikultong, Kamphaengsaen and Kamnanchul. White mulberry is widely cultivated in north and north-eastern of Thailand (Aramwit et al., 2010; Yamdech et al., 2012).

Mulberry can be used in many ways. In the past, mulberry leaves have mostly been used for silkworm feeding in silk production. Moreover, it has long been used as a medicinal plant in many countries, particularly in eastern countries such as Chinese, Korea and Japan. In Chinese, mulberry leaves are used to treat fever, protect the liver, decrease a risk of diabetes and improve eyesight (Kim et al., 2003a; Zhishen et al., 1999). In Korea and Japan mulberry leaves was used as anti-hyperglycemic nutraceutical foods for diabetic patients because the leaves contain 1-deoxynojirimycin, which is one of the most potent α-glycosidase inhibitors (Kim et al., 2003a). Nowadays, utilization of mulberry leaf tea has been increasing in Japan. It has been interesting because of their benefit for health (Katsube et al., 2006).

2.1.2 Description of mulberry plant

Growth Habit: Mulberry trees can grow well on a good soil as well as gravelly soil or rocky slope. They are propagated by seeding and bud grafting (Ourecky, 1980). Size of three mulberry cultivars is varied sizes. White mulberry can grow up to 80 ft. in drooping or pyramidal shapes. Red mulberry can reach up to 70 ft. in height. The black mulberry is the smallest type of three cultivars. It can grow up to 30 ft. in height. Red mulberry trees can live more than 75 years, while black mulberry can live for hundreds of years (Özgen et al., 2009; Suh et al., 2003).

Foliage: Mulberry leaves are thin, glossy, light green color. Leaves of the red mulberry are larger and thicker. They are rough on their upper surfaces. The black and white mulberry leaves are similar to red mulberry leaves but their leaves are smaller than red mulberry leaves (Özgen et al., 2009; Suh et al., 2003).

Fruit: The color of the fruit does not identify the mulberry species. White mulberry fruits can produce white, lavender or black color. Its fruits are very sweet and low acidity. Red mulberry fruits are usually deep red color, almost black. They are high in dry matter, generally sweet and low acidity. Black mulberry fruits are large and juiciness, with extraordinary color and unique. They have a good balance of sweetness and tartness that makes them the best flavored species of mulberry (Özgen et al., 2009; Suh et al., 2003).

2.2 Phenolic compounds in mulberry leaves

Plants and herbs contain various types of phenolic compounds including flavonoids and phenolic acids. Flavonoids can be separated into 6 subclasses: flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols (catechins and proanthocyanidins). Most of phenolic compounds in mulberry leaves are flavonols (Zhishen et al., 1999). The basic flavonoids structure consists of 15 carbon atoms arranged in three rings (C6–C3–C6), marked as A, B and C (Figure 2.2). Various classes of flavonoids differ in the level of oxidation and saturation of ring C. The differences of structure and substitution will influence the antioxidant properties of flavonoids (Wojdyło et al., 2007). Flavonols (Fig. 2.2) are the highest antioxidant that found in plants and occur as aglycones (such as quercetin) and glycosides, flavonols associated with sugar moiety (such as rutin, isoquercetin) (Manach et al., 2004). The structure of rutin and quercetin are shown in Figure 2.3.

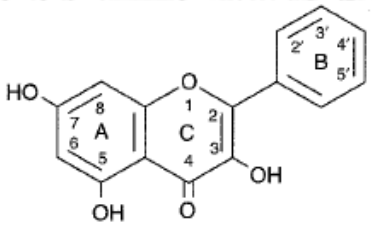
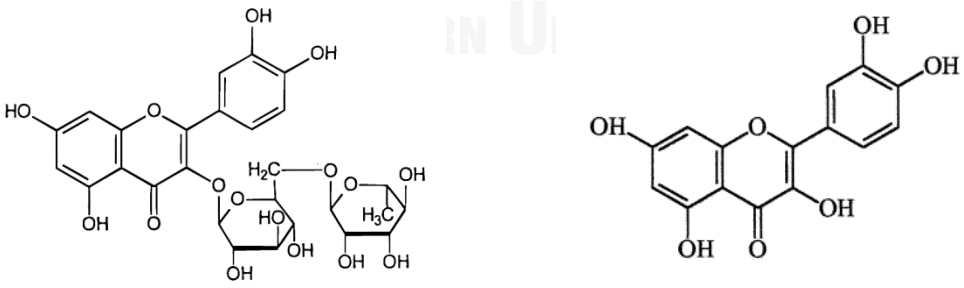


Figure 2.2 Structure of flavonols

Source: Rice-Evans et al. (1997)



(a) (b)

Figure 2.3 Structure of (a) rutin and (b) quercetin

Source: Brewer (2011); Friedman and Jürgens (2000)

Zhishen et al. (1999) studied the separation of flavonoids from mulberry leaves by using high-performance liquid chromatography (HPLC). The mulberry extract was prepared by suspending 1 g of dried mulberry leaves in 0 and 100% v/v of ethanol and extracted in Soxhlet extractor for 1 h. The rutin and quercetin in the extract was determined by using HPLC. They found that mulberry extract contains both of rutin and quercetin and the amount of rutin was higher than quercetin. However, other flavonoids were not identified.

Katsube et al. (2009) determined flavonol glycoside from mulberry leaves by using 100 mg of dried mulberry leaves extracted with 10 mL of 60% v/v ethanol solution and stirred for 2 h at 40 °C in water bath. The mixture was then centrifuged at 13,000 g for 10 min and filtered through a 0.45 μm filter. The extract was analyzed by using HPLC. They found 6 phenolic compounds in mulberry extract: chlorogenic acid (988 mg/100 g of dry weight), rutin (331 mg/100 g of dry weight), isoquercetin (90 mg/100 g of dry weight), quercetin 3-(6-malonylglucoside) (538 mg/100 g of dry weight), astragalin (43 mg/100 g of dry weight) and kaempferol 3-(6-malonylglucoside) (192 mg/100 g of dry weight).

Kim and Jang (2011) analyzed 7 flavonols in mulberry leaves extract by extracting 16 kg of dried leaves with 16 L of distilled water in autoclave at 121 °C for 15 min. The extract was analyzed by using HPLC: kaempferol, quercetin, quercetin-3-**β**-D-glucose, quercetin-3-O-glucose-6”-acetate and rutin was used as standard. The result was shown in Table 2.1, they found that the amount of quercetin-3-**β**-D-glucose was highest followed by quercetin-3-O-glucose-6”-acetate and rutin, respectively. However, kaempferol was not detected.

Table 2.1 Flavonol content of mulberry leaf extract

Flavonols	**Keampferol**	**Quercetin**	**Quercetin-3-β-D-glucose**	**Quercetin-3-O-glucose-6”-acetate**	**Rutin**
Flavonol content (mg/100g dried extract)	ND*	0.03 ± 0.00	1.25 ± 0.02	0.13 ± 0.01	0.09 ± 0.00

Values of flavonol content are presented as mg/100 g extract (dry weight basis).

*Not detectable.

Source: Kim and Jang (2011)

2.3 Extraction conditions of phenolic compounds in mulberry leaves

The effective conditions on the extraction of phenolic compounds in plant materials are type of phenolic compounds in plants, extraction method, solvent, sample amount, particle size, extraction time and temperature (Robbins, 2003). There are many different methods have been used to extract phenolic compounds from plants including liquid-liquid extraction, solid-liquid extraction, Soxhlet extraction, supercritical fluid extraction, pressurized fluid extraction and microwave-assisted extraction. The most widely used procedures to extract phenolic compounds are liquid-liquid and solid-liquid extraction because this method is easy and effective. The type or polarity of solvent has the effect on solubility of phenolic compounds. Alcohols (methanol, ethanol), acetone, diethyl ether and ethyl acetate are mainly used as the solvent. However, phenolic compounds which have high polarity could not be completely extracted with pure organic solvent. Therefore the mixtures of alcohol–water or acetone–water are used (Naczk and Shahidi, 2004; Stalikas, 2007).

Arabshahi-Delouee and Urooj (2007) extracted phenolic compounds by dissolving 15 g of dried mulberry leaves in 100 mL of methanol, acetone and water. The extract was put in a mechanical shaker overnight at room temperature and filtered with Whatman No.1 filter paper. The filtrate extracted with methanol and acetone was evaporated at 40°C in a rotary evaporator then the extract was freeze-dried. Each sample was analyzed in yield and total phenolics content (Table 2.2).

Table 2.2 Extract yield and total phenolics content of different solvent extracts from mulberry leaves

Sample	Yield (g extract/100g dried leaves)	Total phenolics (g gallic acid/100g extract)
Methanol extract	12.35 ± 1.25[a]	9.32 ± 0.10[a]
Acetone extract	8.25 ± 0.95[b]	8.45 ± 0.40[b]
Water extract	6.50 ± 1.50[c]	7.10 ± 0.25[c]

Values in the same column followed by different letters are significantly different ($p \leq 0.05$).

Source: Arabshahi-Delouee and Urooj (2007)

The result showed that methanol extraction gave a highest yield (12.35%) followed by acetone and water extraction, respectively. This result has the same trend as the total phenolics content: when methanol extract > acetone extract > water extract. In this study, they found that methanol was the most effective solvent to extract phenolic compounds from mulberry leaves.

Katsube et al. (2006) studied the extraction of flavonol compounds in mulberry leaves by using various concentrations of ethanol solution. Dried mulberry leaves (2g) were extracted with 20 mL of 0, 20, 40, 60, 70, 80 and 100% v/v ethanol and solution and incubated for 3 h. Each extract was centrifuged at 13,000g for 10 min, the supernatant was then separated. The sediment was resuspended with 20 mL of the same concentration of ethanol and separated. The two supernatant were mixed together and made up to 50 mL. Each sample was determined by HPLC and the result is shown in Figure 2.4. They found that 60% v/v ethanol solution is the most sufficient solvent for extract flavonoids from mulberry leaves.

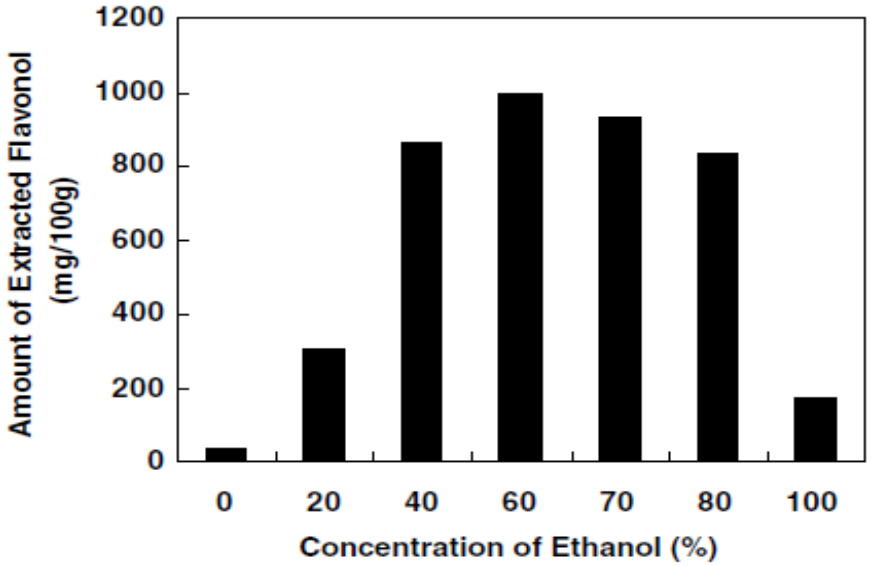


Figure 2.4 Flavonol glycosides in extract which extracted with 0, 20, 40, 60, 70, 80 and 100% (v/v) ethanol solution measured by HPLC

Source: Katsube et al. (2006)

2.4 Determination of total phenolics content and antioxidant activity in plants

A great number of plants contain bioactive compounds including polyphenolic compounds exhibiting antioxidant properties, which resulted in a development of natural additives for food, cosmetic and other applications (Miliauskas et al., 2004).

Crude extracts of fruits, herbs, vegetables, cereals and other plant materials are rich in phenolics. Phenolic substances are a category of phytonutrients that have strong antioxidant properties. The most considerable group of phenolics in food is flavonoids which consist mainly of catechins, proanthocyanins, anthocyanidins and flavones, flavonols and their glycosides. The importance of the antioxidant constituents of plant materials is the efficiency to reduce a risk of coronary heart disease and cancer (Ho, 1992; Kähkönen et al., 1999).

Total phenolics content in plants is generally determined by Folin-Ciocalteu colorimetric method. A major advantage of this procedure is moderately equivalent response to different phenolic substances in biological materials and suitable for measuring accurate mass levels of total phenolics substances (Waterhouse, 2001). Folin-Ciocalteu reagent was used to determine total phenolics content. This reagent is a mixture of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$). During the reaction, a mixture is reduced to blue oxides of tungsten (W_8O_{23}) and molybdenum (Mo_8O_{23}) (OIV, 2009). This reaction occurs under alkaline conditions carried out with sodium carbonate. Blue coloration is followed at 760 nm and the quantity of polyphenols usually expressed as gallic acid equivalent (GAE) (George et al., 2005).

Kaur and Kapoor (2002) studied the total phenolics content of some Asian vegetable and used catechol as standard. The results (Table 2.3) show that total phenolics content of the vegetables varied from 400 mg catechol/100 g fresh weight of mint to 34 mg catechol/100 g fresh weight of round melon.

Table 2.3 Antioxidant activity and total phenolics content of vegetables

Vegetables	Anti-oxidant activity (%)		Total phenolics [mg (100 g)$^{-1}$]
	Ethanol extract	Water extract	
Turmeric fresh (*Curcuma domestica*)	92.45	81.3	175.5 ± 7.2
Kachnar (*Bauhnia variegata*)	91.5	90.3	275.0 ± 10.1
Aonla (*Emblica officinalis*)	86.8	84.3	348.8 ± 13.4
Broccoli (*Brassica oleracea* var. *italica*)	78.4	72.5	87.5 ± 8.1
Mint (*Mentha spicata*)	77.8	73.8	399.8 ± 3.2
Brussel sprouts (*Brassica oleracea* var. *gemmifera*)	73.8	68.5	68.8 ± 1.3
Beet root (*Beta vulgaris*)	73.3	55.0	323.0 ± 11.7
Black carrots (*Daucus carota*)	73.0	61.8	350.5 ± 12.9
Fenugreek (*Trigonella foenum – graceum*)	72.8	62.8	217.5 ± 8.9
Ginger (*Zingiber officinale*)	71.8	65.0	221.3 ± 9.4
Lotus stem (*Nelumbium nelumbo*)	71.8	69.5	85.7 + 1.2
Coriander (*Coriandrum sativum*)	71.8	65.0	82.5 ± 1.9
Yam (*Dioscorea alata*)	71.0	62.8	92.0 ± 2.7
Tomato (*Lycopersicon esculentum*)	70.8	56.3	68.0 ± 1.6

Data expressed as mean ± s.e.m. of three samples analysed separately.

Source: Kaur and Kapoor (2002)

Arabshahi-Delouee and Urooj (2007) estimated the total phonolic content of mulberry leaf extract extracted with various solvent (methanol, acetone and water). The amount of total phenolics expressed as percentage of GAE by weight of dried leaves ranged from 7.10% in water extract to 9.32% in methanol extract

Mostly, antioxidant activity of plants is assembly studied with total phenolics content (Wojdyło et al., 2007). Various methods have been selected to use for determining the antioxidant activity of phenolic compounds in plants. The most popular methods are (1) hydrogen atom transfer (HAT) reactions: including ORAC (oxygen radical absorbance capacity) (Balogh et al., 2010) and (2) single electron transfer (SET): including FRAP assay and TEAC (Trolox equivalent antioxidant capacity). In addition, DPPH (2,2-diphenylpicrylhydrazyl) radical scavenging activity can exhibit both SET and HAT mechanisms.

In HAT mechanism (eq. 1), the antioxidant (ArOH) transfers hydrogen atom to the free radical ($R^{\bullet}$) through homolytic rupture of the O-H bond (Leopoldini et al., 2011):

$$ArOH + R^{\bullet} \longrightarrow ArO^{\bullet} + RH \qquad (1)$$

In SET mechanism (eq. 2), the antioxidant (ArOH) provides an electron to the free radical ($R^{\bullet}$) (Leopoldini et al., 2011):

$$ArOH + R^{\bullet} \longrightarrow ArOH^{+\bullet} + R^{-} \qquad (2)$$

However, each method has a limitation to evaluate. Therefore the appropriate method is depending on the kind of phenolic compounds. For example, DPPH can only dissolve in organic solvent, especially in ethanol. This is an important limitation when determining hydrophilic antioxidants. And the FRAP method is based on the reduction of Fe^{3+} complex of tripyridyltriazine $Fe(TPTZ)^{3+}$ to the blue-color of Fe^{2+} complex $Fe(TPTZ)^{2+}$ by antioxidants in acidic medium to maintain iron solubility (Prior et al., 2005; Wojdyło et al., 2007).

The FRAP assay was originally described by Benzie and Strain (1996) for measuring the ferric reducing ability of plasma. The reaction measures the reduction of ferric 2, 4, 6-tripyridyl-*s*-triazine (Fe(III)-TPTZ) to ferrous form (Fe(II)-TPTZ) (Figure 2.5). Blue coloration is followed at 593 nm.

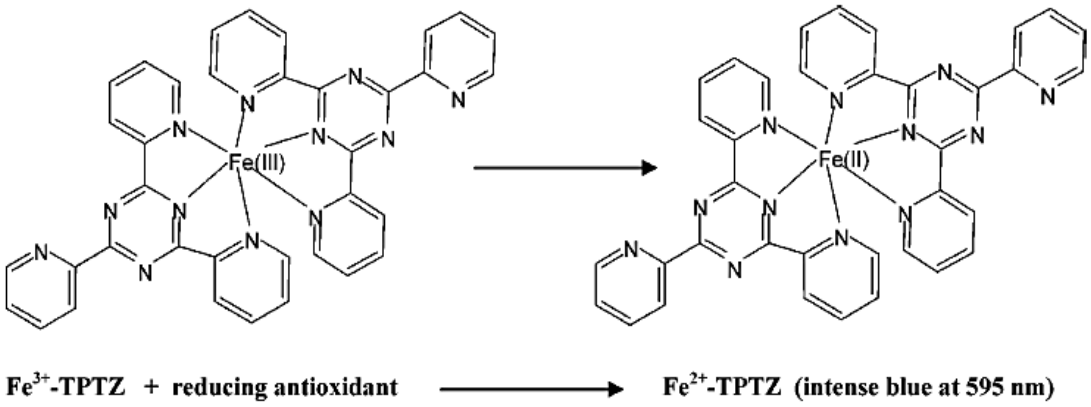


Figure 2.5 The structure of ferric tripyridyltriazine complex and its ferrous form

Source: Prior et al. (2005)

The advantages of the FRAP assay are that it is simple, inexpensive and does not require specialized equipment (Pulido et al., 2000). But the limitation of the FRAP assay is the results can vary tremendously depending on the analysis time. Thus, the endpoint may not represent a completed reaction (Prior et al., 2005).

Another assay which has also commonly used to compare with FRAP assay was DPPH radical scavenging activity. Antioxidant assays are based on measurement of the loss of color of 2, 2-diphenylpicrylhydrazyl (Figure 2.6) at 515 nm after reaction with samples (Bondet et al., 1997).

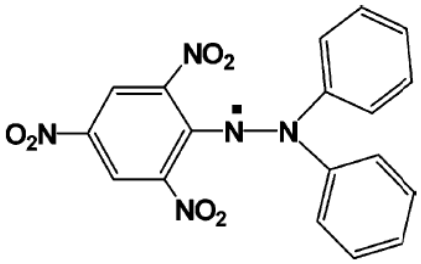


Figure 2.6 The structure of 2, 2-diphenylpicrylhydrazyl

Source: Prior et al. (2005)

The reaction mechanism between the antioxidant and DPPH• depends on the structural conformation of the antioxidant. DPPHH is a product of the reaction between DPPH• and an antioxidant (AH) (Bondet et al., 1997):

$$DPPH\bullet + AH \longleftrightarrow DPPHH + A\bullet$$

The advantage of the DPPH radical scavenging activity is simple and rapid. However, interpretation is complicated when the test compounds have spectra that overlap DPPH at 515 nm (Prior et al., 2005).

2.5 Microencapsulation

Microencapsulation process is the technology that has been used to entrap small particles of liquids, solids, or gases. These particles are called “core material” and it must be insoluble in continuous phase (usually water) that containing the shell, coating material or wall material (Luzzi, 1970). This coating material could be one or two polymers. The purpose of microencapsulation is to protect the core material from environmental factor (such as light, moisture, temperature and oxygen) and extend the shelf life (Gouin, 2004; Shahidi and Han, 1993). Morphology of microcapsules can separate in many types, but the major morphology of microcapsules is combined in 2 types (Figure 2.7). One is microcapsules which have a

single core covered by a wall material called “mononuclear” (Figure 2.7a) and second is microcapsules which have many cores dispersed in a matrix called “aggregate” (Figure 2.7b) (Fang and Bhandari, 2010; Schrooyen et al., 2001).

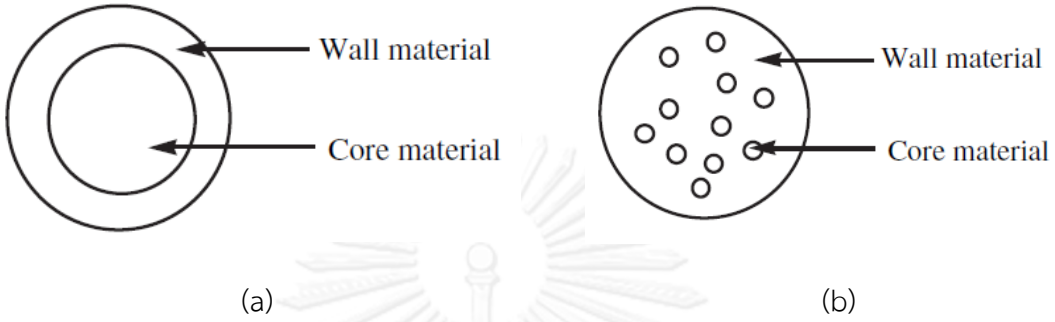
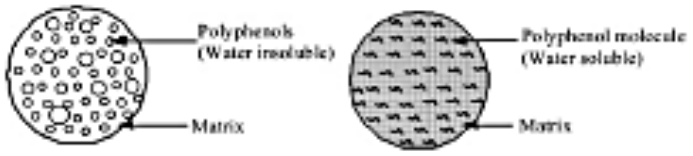
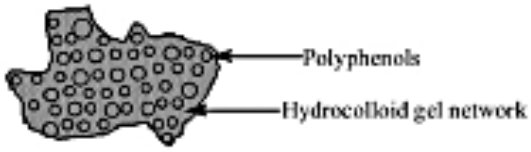
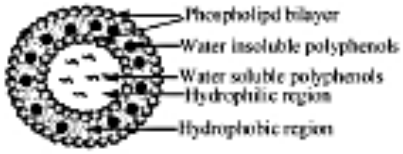
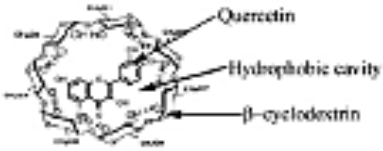
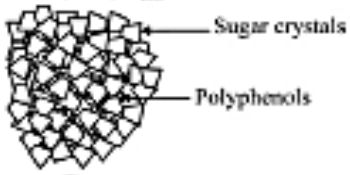
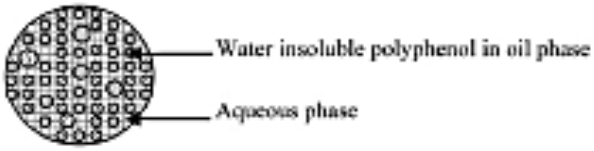
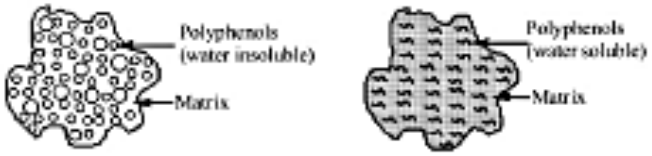
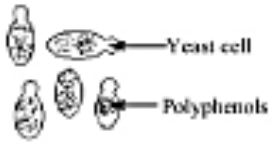
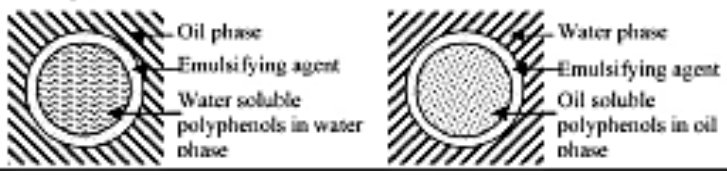


Figure 2.7 Two major forms of encapsulation: (a) mononuclear capsule and (b) aggregate

Source: Fang and Bhandari (2010)

The microencapsulation technique is separated into two classes: chemical and physical methods (Versic et al., 1988). Physical methods such as spray drying, spray cooling/chilling, spinning disk and centrifugal coextrusion, extrusion and fluidized bed. Chemical methods include coacervation, liposomes and inclusion encapsulation (F. Gibbs, 1999; Gouin, 2004). The characteristic of microcapsules produced by each method is different (Table 2.4) (Fang and Bhandari, 2010).

Table 2.4 Characteristics of encapsulated polyphenolic capsules produced by various encapsulation process

Encapsulation technology	Illustration of characteristics
Spray drying	
Coacervation	
Liposomes	
Inclusion	
Cocrystallization	
Nanoparticles	
Freeze drying	
Yeast encapsulation	
Emulsion	

Source: Schmitt et al. (1998)

Coacervation can be divided into “simple” and “complex” coacervation (Wang et al., 1999). This method involves with the phase separation of one or many hydrocolloids from a polymeric solution formed layer around the core material that suspended in the same reaction media (Desai and Jin Park, 2005; Gouin, 2004).

Simple coacervation is the system which containing only one coating material. Pectin is mostly used (Luzzi, 1970). The capability to produce simple coacervation is dependent on the appropriate condition, including pH, ionic strength, temperature, and structure of the macromolecules. For example, when pH of the process is adjusted close to the isoelectric point (IEP) of gelatin at low ionic strength, net charge of gelatin becomes balanced. The molecules unfold and sediment to microcapsules (Schmitt et al., 1998).

Complex coacervation is mostly dependent on pH and concentration of polymer. This process involves the reaction between two oppositely charged polymers (protein and polysaccharides) called “polymer-polymer interaction method”. Polysaccharides shows negatively charged (such as acacia, pectin, alginate and carboxy methyl cellulose) were used to interact with positively charged protein (such as gelatin, SPI and chitosan) (Saravanan and Rao, 2010) by electrostatic interactions. The previous studies have been reported that the mixtures of protein and polysaccharide form an electrostatic complex in a specific pH range rely on IEP of protein and pKa of polysaccharides. At pH below the isoelectric point of the proteins and above the pKa of polysaccharides carboxyl group, they can effectively interact by electrostatic interactions (Giancone et al., 2009).

Nori et al. (2011) studied in the effect of final pH of complex coacervation process and concentration of coating materials on production of microcapsules. Microcapsules produced by entrapping propolis with SPI and pectin. The pH was varied in 4 values: 3.5, 4.0, 4.5 and 5.0. The microcapsules were determined in mass of sediment and found that microcapsules with pH adjusted to 4.0 provided the greatest phase separation. The microencapsulation was then studied in concentration of coating materials by altering in two concentrations: 2.5 and 5.0%. The microcapsules were evaluated in encapsulation efficiency, the result shows that the encapsulation efficiency of microcapsule produced by using 2.5% coating materials was higher than 5.0% coating materials.

Silva et al. (2012) produced microcapsules of lycopene by using gelatin and pectin as coating materials. In coacervation process, they adjusted pH in 4 values: 3.0, 3.5, 4.0 and 4.5. Each microcapsule was analyzed for morphology. The result

(Figure 2.8) shows that the sample with pH adjusted to 3.0 demonstrated a better interaction between gelatin and pectin because microcapsule presents spherical shape with a defined wall and core structure. When pH of the aqueous phase is suitable, the formation of a polymer-rich phase (wall materials) was induced (Jackson and Lee, 1991).

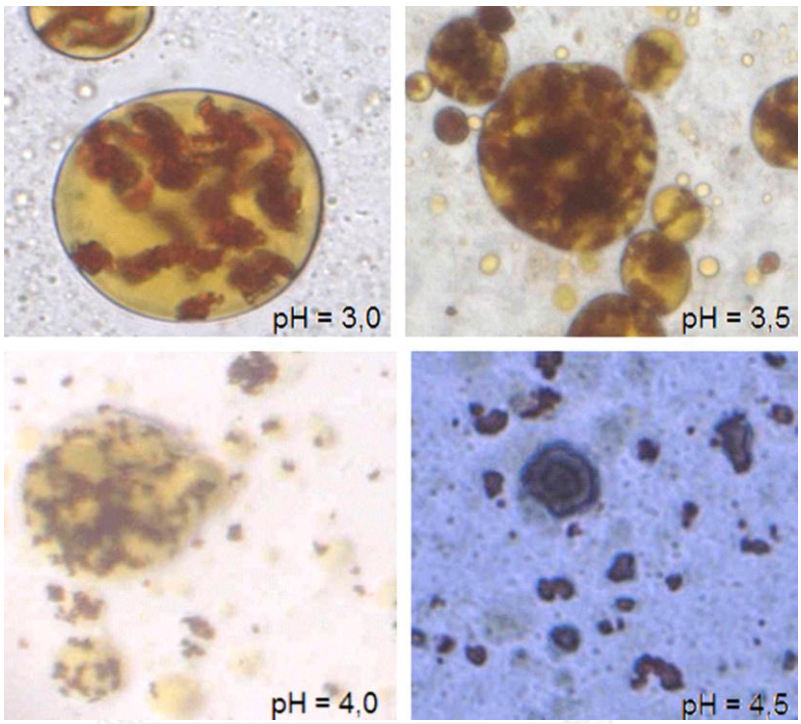


Figure 2.8 Morphology of microcapsules of lycopene with pH adjusted to 3.0, 3.5, 4.0 and 4.5

Source: Silva et al. (2012)

Jun-xia et al. (2011) investigated the effect of pH on coacervation yield of sweet orange oil microcapsules produced by using SPI and gum arabic as coating materials. The pH of polymer-polymer interaction process was varied in 5 values: 2.5, 3.0, 3.5, 4.0 and 4.5. They found that pH 4.0 gave the highest degree of phase separation because the charge densities of the two biopolymers seem to be stoichiometrically balanced at pH 4.0 and pH 4.0 was the electrical equivalence point (EEP) of the SPI–gum arabic system.

2.6 Coating materials

Coating materials in complex coacervation (polymer-polymer interaction) can be used polyelectrolytes-polyelectrolytes or polyelectrolytes-oppositely charged colloids (such as protein) (Kizilay et al., 2011). The two coating materials interact by intermolecular electrostatic interactions (Espinosa-Andrews et al., 2007). The model of the complex between protein and polysaccharide is shown in Figure 2.9.

Figure 2.9 The model of the complex between protein (white ribbon) and polysaccharide (dark spheres)

Source: de Kruif et al. (2004)

Proteins and polysaccharides are present together in many kinds of food systems, and both types of food macromolecules contribute to the structure, texture and stability of food through their thickening or gelling behavior and surface properties (Doublier et al., 2000).

Proteins (such as sodium caseinate, whey protein and SPI) are widely used as coating materials (Kim et al., 1996). Proteins are appropriate for producing microcapsules by polymer-polymer interaction process because of their different chemical groups, amphiphilic properties, high capability to interact with a various types of substances, large molecular weight and molecular chain flexibility (Dalgleish, 1997; Dickinson, 2001; Madene et al., 2006).

Carbohydrates such as starches, corn syrup solids, maltodextrins (DeZarn Thomas, 1995; Kenyon Melanie, 1995) and other polysaccharides have been usually used as encapsulating agents because of the low viscosities at high solids contents and good solubility. Gelling properties of polysaccharides could stabilize emulsions towards flocculation and coalescence (Gharsallaoui et al., 2007).

Protein and polysaccharide can associate by non-covalent interactions (such as electrostatic and hydrophobic interactions, steric exclusion, hydrogen bonding,

etc.). Electrostatic complexes are strongly formed by mixtures of positively charged proteins and negatively charged polysaccharide (at pKa<pH<pI). Reversible complexes tend to be weakly formed when anionic polysaccharides and proteins carrying nearly zero overall charge (pH~pI) or a net negative charge (pH>pI) (Benichou et al., 2007; Dickinson, 2008; McClements, 2006; Rodríguez Patino and Pilosof, 2011; Turgeon et al., 2007). Thus, adjusting the pH of the process during encapsulation is very important.

Jaramillo et al. (2011) studied the effect of pH on the properties of SPI–pectin complexes and reported that the charge on SPI changes when adjusting pH to various values. At pH values below (pH 3) and above (pH 6 and 7) the isoelectric point, protein particles have a strong positive and negative charge. When adjusting pH closer to the isoelectric point (pH 4 and 5), the protein particles have a neutral net charge, while pectin shows negatively charge in all pH value. Therefore, the strongest electrostatic complexes form when the charges between two polymers are opposite.

Rodríguez Patino and Pilosof (2011) also described behavior of protein-polysaccharide mixture (Figure 2.10). In very dilute solutions, the mixture is stable and protein and polysaccharide are co-soluble (Figure 2.10c). When increasing concentration of the polymers, association or segregation phenomena can develop (Figure 2.10a, b and d). Interactions between protein and polysaccharide can be formed to soluble or insoluble complexes (Figure 2.10a and b). The insoluble complexes leads to a phase separation phenomenon called coacervation (Schmitt et al., 1998).

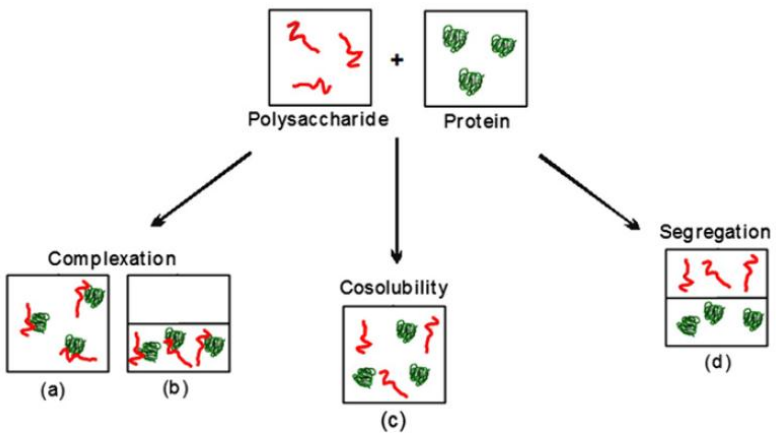


Figure 2.10 Behavior of protein-polysaccharide mixture

Source: Rodríguez Patino and Pilosof (2011)

Phase separation of protein-polysaccharide mixtures occurs above a critical concentration as described quantitatively by phase diagrams (Figure 2.11). The region below the binodal is limited thermodynamic compatibility and the region above the binodal curve is separated into two phases, enriched in protein (A) and enriched in polysaccharide (B). The composition of phase separation is the point of intersection (C) (Rodríguez Patino and Pilosof, 2011).

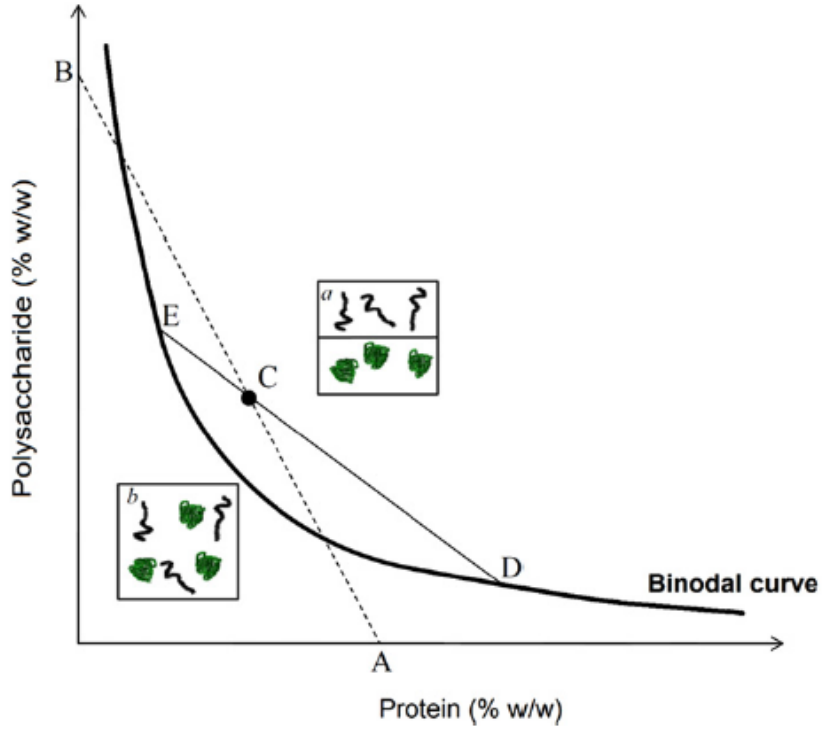


Figure 2.11 Phase diagram of a protein-polysaccharide system Phase diagram of a protein-polysaccharide system

Source: Rodríguez Patino and Pilosof (2011)

2.6.1 Soy protein isolate (SPI)

SPI is a high protein product (consisting of 90% protein) produced from soy flakes. This protein is a large mixture of the two globulin proteins: glycinin (11S, molecular weight about 350,000) and β-conglycinin (7S, molecular weight about 150,000) representing 34% and 27%, respectively in SPI (Iwabuchi and Yamauchi, 1987; Petruccelli and Anon, 1995). β-conglycinin is less heat-stable than glycinin. The denaturation temperature of β-conglycinin is about 77°C and that of glycinin is 92°C

about (German et al., 1982). The isoelectric point (IEP) of SPI is at pH~4.6 (Malhotra and Coupland, 2004).

SPI has been widely used as a source of highly functional proteins to utilize the formulation of food products (Jaramillo et al., 2011), beverage and neutraceutical products (Jiang et al., 2009). Since it can facilitate the formation of emulsion by decreasing interfacial tension between water and oil, helping to stabilize the emulsion by forming a physical barrier at interface (Molina et al., 2001), improving viscosity, forming gel and improving water solubility (Malhotra and Coupland, 2004).

Mendanha et al. (2009) used SPI and pectin as wall materials to produce microcapsules of casein hydrolysate by complex coacervation process. This study aimed to reduce the bitter taste of casein hydrolysate. They found that using SPI and pectin has an efficiency to reduce a bitter taste of casein hydrolysate. In addition, the microcapsules produced in this study may add nutritional value to the product due to properties of the components.

2.6.2 Pectin

Pectin is a class of complex polysaccharides mostly found in the cell walls of higher plants (Thakur et al., 1997). It is also presented in the junction zone between cells with secondary walls including xylem and fiber cells in woody tissue (Mohnen, 2008). Pectin is a naturally occurring biopolymer which has increased applications in pharmaceutical and biotechnology industry. Pectin has been widely used in food and beverage as a thickening agent, gelling agent and colloidal stabilizer. In addition, it can be used as a matrix for the entrapment or delivery drugs, proteins and cells (Sriamornsak, 2003).

Pectin is a heterogeneous anionic polysaccharide present in the cell of most plants such as citrus fruit. It consists mainly of linearly connected α-(1,4)-D-galacturonic acid residues (Figure 2.12), containing carboxyl functional groups (pKa=3.5) (Kim et al., 2003b; Sriamornsak, 2003). Pectins are divided into two major groups on the basis of their degree of esterification (DE): high methoxyl pectin (HMP) and low methoxyl pectin (LMP) (Giancone et al., 2009; Lam et al., 2007). High-methoxyl pectin has more than 50% esterfied carboxylic group. Low methoxyl pectin has less than 50% esterfied carboxylic groups (Girard et al., 2002).

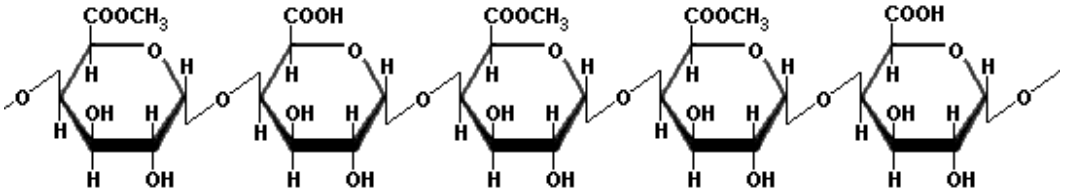


Figure 2.12 Structure of **α**-(1,4)-D-galacturonic acid

Source: Roubroeks et al. (2001)

Girard et al. (2002) studied the complexation between **β**-lactoglobulin and low or high methylated pectin. They found that the carboxyl groups of low methoxyl pectin allow protein complexation more than high methoxyl pectin. This is due to the lower content of carboxylic group in high methoxyl pectin, which limits the possibility of electrostatic interactions with **β**-lactoglobulin. Furthermore low-methoxyl pectin has more H^+ from carboxylic group than high methoxyl pectin. Therefore, low methoxyl pectin can strongly bind with **β**-lactoglobulin.

CHAPTER 3
MATERIAL AND METHODS

3.1 Materials and Equipment

3.1.1 Raw material

The fourth to sixth leaves from the top of mulberry plant (*Morus alba* L.) were collected from Queen Sirikit Sericulture Center (Kanchanaburi, Thailand). Dried leaves powder was prepared as described by Arabshahi-Delouee and Urooj (2007). The leaves were washed and dried in hot air oven at 50°C until moisture content reached approximately 7% dry basis. The dried leaves were ground using blender for 15 sec, passed through a 50-mesh sieve and kept in an air-tight container until use, not more than 3 months. Soy protein isolate (SPI) GS5100 (Gushen Biological Technology Group Co., Ltd, China); Appendix E.1 and the Genu Pectin type LM-104 AS-BG (Food &Cosmetic Systems Co.,Ltd., Thailand); Appendix E.2 and were used as encapsulated agents.

3.1.2 List of chemicals

Table 3.1 Chemical reagents used in this study

Chemical reagents	Supplier name/grade
Ethanol (Absolute)	Macron, Analytical grade
2,2-Diphenyl-1-picrylhydrazyl	Aldrich, Analytical grade
Dry ice pallets (CO_2)	Bangkok dry ice, Thailand
Folin-Ciocalteu's phenol reagent	Merck, Analytical grade
Gallic acid	Ajax Finechem, Analytical grade
Glacial acetic acid	QRëC, Analytical grade
Hydrochloric acid (37%)	QRëC, Analytical grade
Iron (III) chloride hexahydrate	QRëC, Analytical grade
Methanol (Absolute)	QRëC, Analytical grade
Quercetin	Sigma, HPLC grade

Chemical reagents	Supplier name/grade
Rutin Hydrate	Sigma, HPLC grade
Sodium acetate	Ajax Finechem, Analytical grade
Sodium carbonate anhydrous	Ajax Finechem, Reagent grade Aldrich,
Sodium hydroxide	Reagent grade
2,4,6-tri(2-pyridyl])-s-triazine (TPTZ)	Fluka, Analytical grade
Trolox	Aldrich, Analytical grade

3.1.3 List of equipment

Table 3.2 List of equipment used in this study

Equipment	Specification
Blender	HR2061, Philips, Netherlands
Centrifuge	MIKRO 22R, Hettich, Germany
Evaporator and rotavapor	R-200, Buchi, Switzerland
Freeze dryer	FD8, Heto, Denmark
Homogenizer	X10/25, Ystral, Germany
Hot air oven	ED, Binder, Germany
Hot plate stirrer	HL HS-115, Harikul Science, Thailand
HPLC	LC-10, Shimadzu, Japan
LC-MS	amaZon SL, Bruker, USA.
Light microscopes	YS100, Nikon, Japan
Mechanical shaker	SW23, Julabo, Germany
pH meter	Cyberscan 1100, Thermo Fisher Scientific, USA
Scanning electron microscope	JSM-5410LV, Jeol, Japan
Spectrophotometer	Genesys 20, Thermo Scientific, USA.
Magnetic bar	30x6mm, Cowie, England
Magnetic Stirrer	OkWell, Progress Technical, Thailand
Vortex mixer	Vortex-Genie 2, Scientific Industries, USA.

3.2 Experimental procedures

3.2.1 Determination of suitable ethanol concentration for extraction of flavonoids from mulberry leaves

Extraction process

The mulberry leaves was extracted according to the method of Arabshahi-Delouee and Urooj (2007).

Mulberry leaves powder (15 g) was extracted with 100 mL of various concentrations of ethanol (50, 60, 70 and 95% v/v) in Erlenmeyer flask, covered with parafilm and placed in a mechanical shaker at 37 °C for 18 h under dark condition. Each extract was filtered with Whatman No. 1 filter paper. The extract was kept in amber glass bottle at -20 °C until further analysis. The flow chart of the extraction process was shown in Figure 3.1

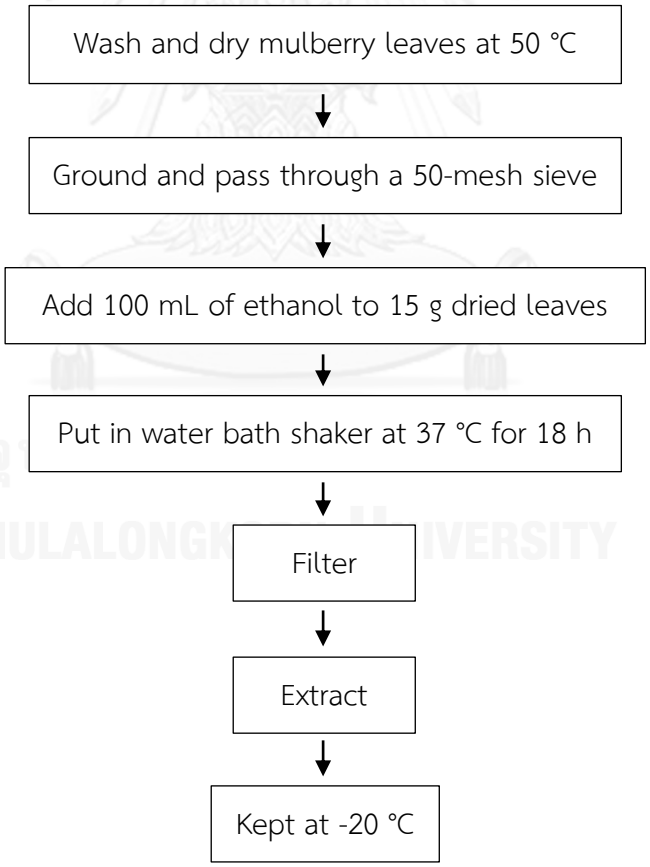


Figure 3.1 Flow chart of the extraction process

Adapted from Arabshahi-Delouee and Urooj (2007)

Chemical properties of mulberry leaves extract were determined as follows:

- Total phenolics content using Folin-Ciocalteu method (Waterhouse, 2001; Appendix A.1)

- The antioxidant activities using Ferric reducing antioxidant power assay (Benzi and Strain, 1996; Appendix A.2) and DPPH radical scavenging activity (Shimada et al. 1992, Li et al. 2010; Appendix A.3)

- Amount of flavonoids compounds in mulberry leaves extract using HPLC (Katsube et al., 2006; Appendix A.4)

- Type of flavonoids compounds in mulberry leaves extract using LC-MS (Appendix A.5)

All analyses were done in triplicates. Analysis of variance (ANOVA) of the experimental data was also performed. Duncan's New Multiple Range Test was used to evaluate the difference between means at the 95% confidence interval.

The most suitable ethanol concentration was the one that gives the highest in total phenolics content and antioxidant activities. The mulberry leaves extract was chosen for using as core material in microencapsulation process by polymer-polymer interaction method. However, the core material in this process should not be soluble in continuous phase. The 95% ethanolic mulberry leaves extract is selected to compare with the best extract for using as core material in polymer-polymer interaction process.

3.2.2 Microencapsulation process

3.2.2.1 Study the suitable ethanolic mulberry leaves extract to be used as core material in microencapsulation process

The microencapsulation process of selected mulberry leaves extract from step 3.2.1 was carried out according to the method of Nori et al. (2011).

To promote phase separation, 2.5 g/100 mL of SPI solution was prepared with pH adjusted to 8.0 (to give highest solubility) with a solution of 0.1 M NaOH and then 2.5 g of ethanol extract was added. The mixture was homogenized at 11,000 rpm for 2 min. Then, the 2.5 g/100 mL of pectin solution was slowly added and pH was adjusted to 4.0 with 1 M HCl. At the end of this process, the material was cooled in a refrigerator at 4 °C for 96 h to promote sedimentation of microcapsules. Then, microcapsules were filtered and frozen using dry ice for 24 h. The sample was

freeze-dried under vacuum (5.2×10^{-3} Mbar) with a chamber temperature of 29 °C and a condenser temperature of –49 °C for 18 h. The freeze-dried sample was stored in Al/PE bag and sealed under vacuum. The encapsulation process was performed in triplicate. The flow chart of microencapsulation process was shown in Figure 3.2.

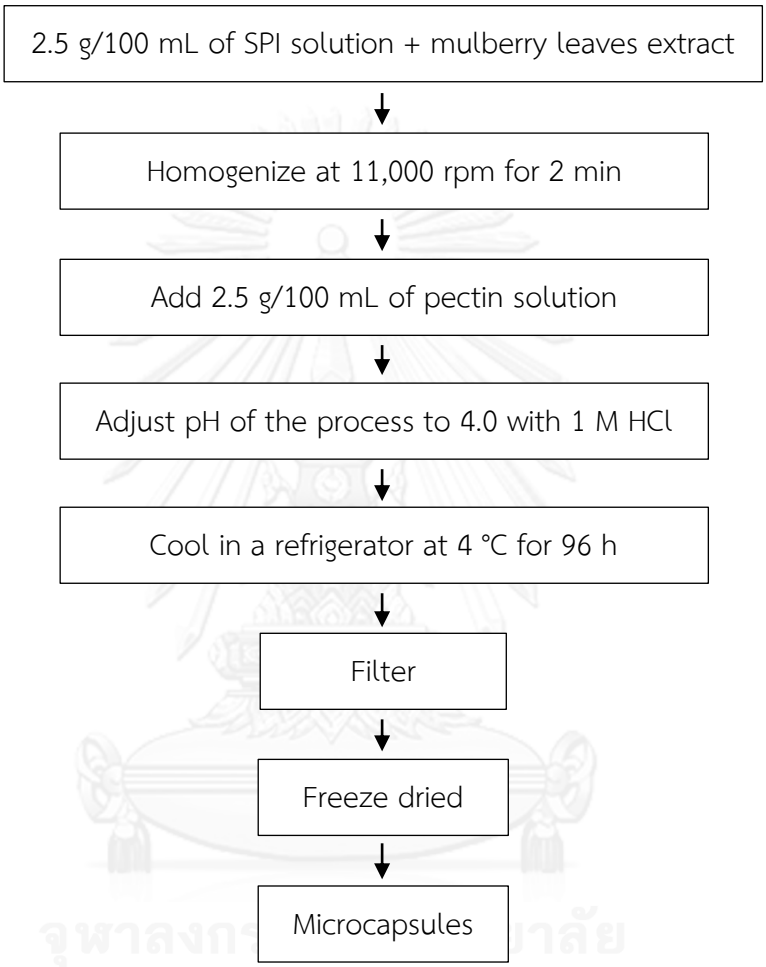


Figure 3.2 Flow chart of microencapsulation process to study the optimum mulberry extract to use as core material

Adapted from: Nori et al. (2011)

Chemical properties, physical properties and efficiency of microcapsules of mulberry leaves extract from 60% and 95% v/v ethanol were determined as follows:

- Solubility of microcapsules (Nori et al., 2011, Cano-Chauca et al., 2005; Appendix A.6)
- Encapsulation yield (Silva et al., 2012; Appendix A.7)

- Encapsulation efficiency (Nori et al. 2011; Appendix A.8)
- Total phenolics content and antioxidant activity (Nori et al. 2011; Appendix A.9)
- Morphology under light microscope (Appendix A.10)

All analyses were done in triplicates. Analysis of variance (ANOVA) of the experimental data was also performed. Duncan's New Multiple Range Test was used to evaluate the different between means at the 95% confidence interval.

The most suitable ethanolic mulberry leaves extract to be used as core material was the one that gives the highest in total phenolics content, antioxidant activities, encapsulation yield, encapsulation efficiency and solubility. The best ethanolic mulberry extract was selected to use as core material for studying the suitable pH of solution in polymer-polymer interaction process in the next part.

3.2.2.2 Study the suitable final pH of mixed solution in polymer-polymer interaction process

The microencapsulation process of ethanolic mulberry leaves extract was carried out by using the same method as 3.2.2.1. The pH of the microencapsulation process was varied into 3 levels: 3.5, 4.0 and 4.5. The microencapsulation process was performed in triplicates. The flow chart of microencapsulation process in this stage was shown in Figure 3.3.

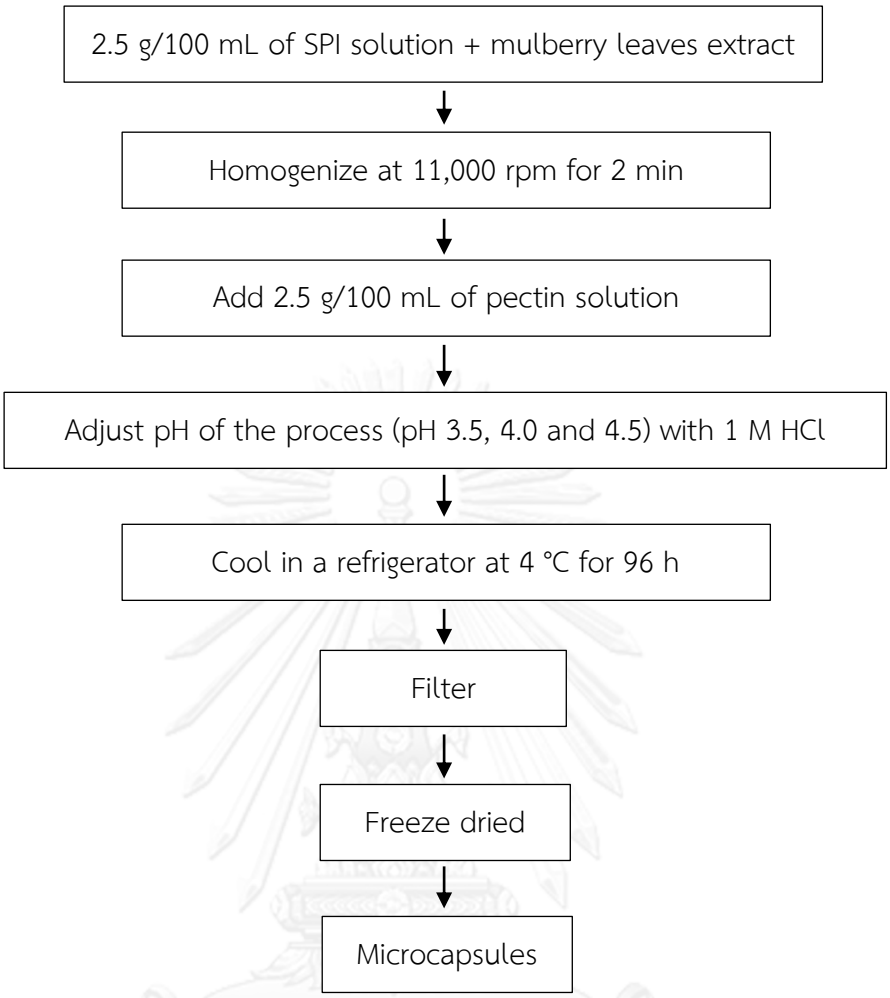


Figure 3.3 Flow chart of microencapsulation process to study the optimum final pH of mixed solution in polymer-polymer interaction process

Adapted from: Nori et al. (2011)

Chemical properties, physical properties and efficiency of microcapsules of mulberry leaves extract from different pH conditions were determined as follows:

- Solubility of microcapsules (Nori et al. 2011, Cano-Chauca et al. 2005; Appendix A.6)
- Encapsulation yield (Silva et al. 2012; Appendix A.7)
- Encapsulation efficiency (Nori et al. 2011; Appendix A.8)
- Total phenolics content and antioxidant activity (Nori et al. 2011; Appendix A.9)

- Amount of flavonoids compounds in mulberry leaves using HPLC (Katsube et al., 2006; Appendix A.4)

- Morphology under light microscope (Appendix A.10)

All analyses were done in triplicates. Analysis of variance (ANOVA) of the experimental data was performed. Duncan's New Multiple Range Test was used to evaluate the difference between means at the 95% confidence interval.

The most suitable pH was the one that gives the highest in total phenolics content, antioxidant activities, encapsulation yield, encapsulation efficiency and solubility. The best final pH of mixed solution was selected for studying the suitable concentration of coating materials in polymer-polymer interaction process in the next part.

3.2.2.3 Study the suitable concentration of coating materials (SPI and pectin) in polymer-polymer interaction process

The microencapsulation process of mulberry leaves extract with pH adjusted to the most suitable value from step 3.2.2.2 was carried out using the same method as 3.2.2.1. Concentration of coating materials in the microencapsulation process was varied to 3 levels: 2.5, 5.0 and 7.5% w/v. The encapsulation process was performed in triplicates. The flow chart of microencapsulation process in this stage was shown in Figure 3.4.

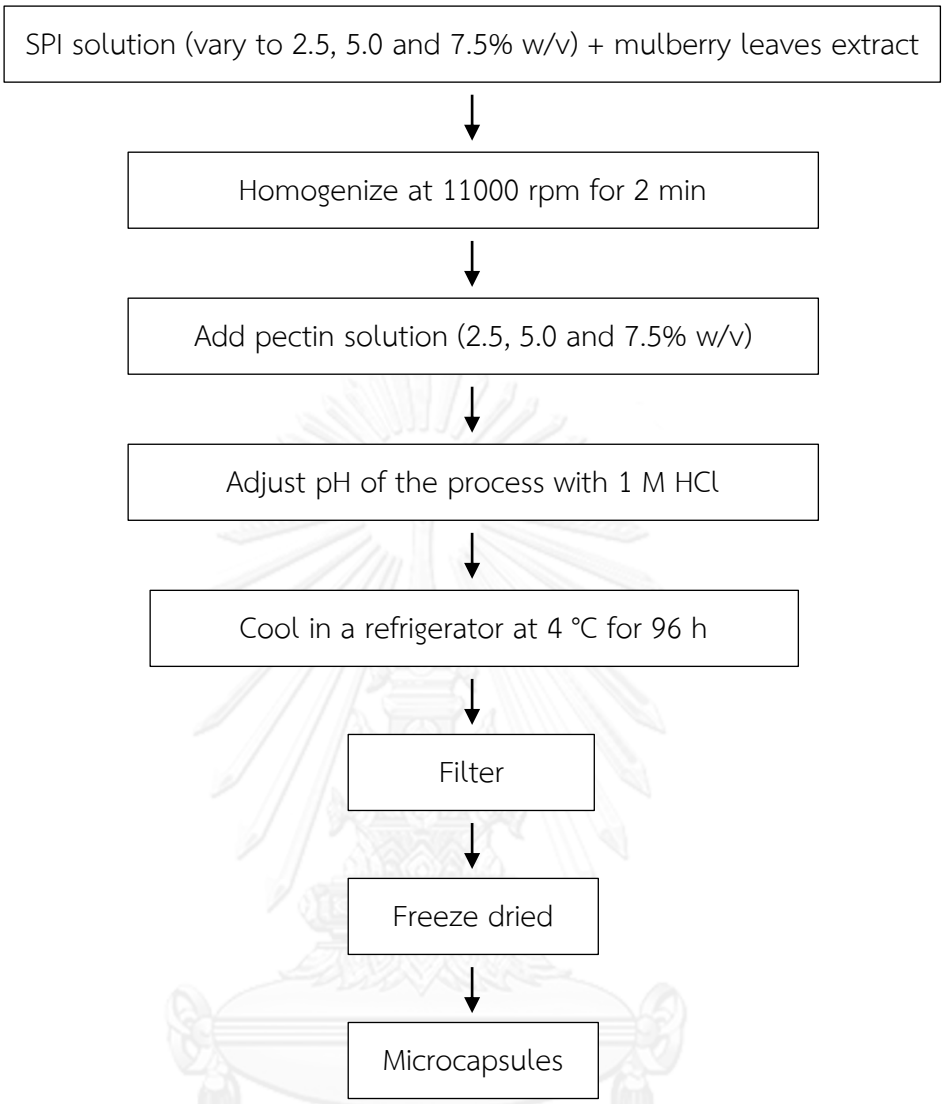


Figure 3.4 Flow chart of microencapsulation process to study the optimum concentration of coating materials

Adapted from the method of Nori et al. (2011)

Chemical properties, physical properties and efficiency of microcapsules of mulberry leaves extract from different concentration of coating materials were determined as follows:

- Solubility of microcapsules (Nori et al. 2011, Cano-Chauca et al., 2005; Appendix A.6)
- Encapsulation yield (Silva et al. 2012; Appendix A.7)
- Encapsulation efficiency (Nori et al. 2011; Appendix A.8)

- Total phenolics content and antioxidant activities (Nori et al. 2011; Appendix A.9)

- Morphology under scanning electron microscope (SEM) (Appendix A.11)

All analyses were done in triplicates. Analysis of variance (ANOVA) of the experimental data was performed. Duncan's New Multiple Range Test was used to evaluate the difference between means at the 95% confidence interval.

The most suitable concentration of coating materials was the one that gives;; the highest in total phenolics content, antioxidant activities, encapsulation yield, encapsulation efficiency and solubility.

CHAPTER 4
RESULTS AND DISCUSSION

This study focused on the suitable ethanol concentration for extraction of flavonoids from mulberry leaves and the suitable conditions (core material, final pH of mixed solution and concentration of coating materials) in microencapsulation process by using polymer-polymer interaction method. The quality attributes of ethanolic mulberry leaves extract are total phenolics content, antioxidant activities, amount and type of flavonoids compounds. Moreover, solubility, encapsulation yield, encapsulation efficiency, total phenolics content, antioxidant activities, type and amount of flavonoids compound and morphology of microcapsules were analyzed in order to select the most appropriate condition for producing microcapsules.

4.1 Determination of suitable ethanol concentration for extraction of flavonoids from mulberry leaves

In this part, the concentration of solvent used to extract antioxidant from mulberry leaves was varied. The flavonoids in mulberry leaves was extracted using ethanol as a solvent because of its capability of extracting flavonoids from plants and safe for use in product. The extracts obtaining from 50, 60, 70 and 95% v/v ethanol (50EM, 60EM, 70EM and 95EM) were then analyzed for total phenolics content, antioxidant activities, amount and type of flavonoids compound.

4.1.1 Total phenolics content

Total phenolics content of mulberry leaves extracts determined using Folin–Ciocalteu method is shown in Table 4.1. It was found that 50EM and 60EM gave the highest amount of total phenolics content, 127.33 and 122.21 mg GAE/g dried leaves, respectively.

Table 4.1 Total phenolics content and antioxidant activities of mulberry leaf extract which extracted by various concentration of ethanol

Extract	Total phenolics content (mg GAE/g dried leaves)	Ferric reducing antioxidant power (mmol Trolox/g dried leaves)	DPPH radical scavenging activity (mmol Trolox/g dried leaves)
50EM	$127.33^{a} \pm 6.26$	$58.42^{a} \pm 4.36$	$7.83^{c} \pm 0.47$
60EM	$122.21^{a} \pm 5.32$	$51.83^{ab} \pm 1.90$	$10.45^{a} \pm 0.16$
70EM	$94.95^{b} \pm 6.49$	$46.67^{b} \pm 0.39$	$9.69^{ab} \pm 0.96$
95EM	$26.23^{c} \pm 1.23$	$18.15^{c} \pm 0.91$	$8.16^{bc} \pm 0.57$

Values followed by different letters are significantly different ($p \leq 0.05$).

Different phenolic compounds in mulberry leaves can be extracted by different concentration of ethanol, due to the polarity of phenolic compounds. The type of flavonoids in mulberry leaves has been reported in many recent studies. Katsube et al. (2009) reported that mulberry leaves contain many types of flavonoids but major flavonoids in mulberry leaves are rutin (573 mg/g sample), isoquercetin (194 mg/g sample) and quercetin-3-(6-malonylglucoside) (900 mg/g sample). In contrast, Zhishen et al. (1999) found two flavonoids in mulberry leaves: rutin and quercetin, the content of rutin was higher than quercetin.

Based on the report of Katsube et al. (2006), the major flavonoids in mulberry leaves are flavonol glycoside (flavonols with sugar in molecules) such as rutin, isoquercetin and quercetin-3-(6-malonylglucoside). The minor flavonoids are flavonols that is quercetin. The flavonols glycoside is a water-soluble flavonoids and water-insoluble quercetin should be dissolved in ethanol. For this reason, the result supported that the low concentration of ethanol has a better ability to extract phenolic from mulberry leaves than high concentration because it can solubilize both flavonols glycoside and aglycones. As the result presented in Table 4.1, 50EM and 60EM gave highest amount of phenolic compounds followed by 70EM and 95EM.

4.1.2 Antioxidant activities

Antioxidant properties of extract were measured using DPPH radical scavenging activity and FRAP assay. The results are shown in Table 4.1.

The result of FRAP assay shows the same tendency as the total phenolics content. It was found that 50EM and 60EM have the highest antioxidant activity (58.42 and 51.83 mmol Trolox/g dried leaves, respectively). This result may be due to the polarity of solvent. The 50 and 60% v/v of ethanol contain water in a high content. Therefore, they have higher efficiency to extract hydrophilic antioxidant than other concentrations.

The result of DPPH radical scavenging activity shows that 60EM has the highest antioxidant activity (10.45 mmol Trolox/g dried leaves). However, 50EM has the lowest antioxidant activity (7.83 mmol Trolox/g dried leaves). This result may be caused by the limitation of DPPH radical scavenging activity method to determine hydrophilic antioxidants (Kaur and Kapoor, 2002). DPPH reagent can only be dissolved in organic solvent (Özyürek et al., 2011) especially in alcohol (Arnao, 2000) (in this study, DPPH reagent was dissolved in 95% ethanol). Therefore, radical scavenging activity of 60EM, 70EM and 95EM was higher than 50EM.

4.1.3 Amount and type of flavonoids compound in mulberry leaves extract

Amount and type of flavonoids in mulberry leaves extract was determined using HPLC and LC-MS (Science Instrument, Rangsit University). Many previous studies reported that mulberry leaves contain high amount of rutin and quercetin aglycone (Suntornsuk et al., 2003; Zhishen et al., 1999). Therefore, rutin and quercetin aglycone were used in this study.

The result is shown in Table 4.2, presence of rutin and quercetin was found in mulberry leaves and rutin presented in a higher amount than quercetin at all of ethanol extract. The amount of quercetin in 50EM, 60EM and 70EM is similar to each other (about 1.10×10^{-3} - 1.15×10^{-3} mg/g dried leaves) but 95EM have a highest amount of quercetin (about 1.52×10^{-3} mg/g dried leaves). For rutin, 70EM gave the highest amount of rutin (0.70 mg/g dried leaves) followed by 60EM, 95EM and 50EM.

Table 4.2 The amount of rutin and quercetin in ethanolic mulberry leaf extracts at various concentration of ethanol

Extract	Rutin (mg/g dried leaves)	Quercetin ($\times 10^{-3}$ mg/g dried leaves)
50EM	$0.26^{c} \pm 0.01$	$1.15^{b} \pm 0.05$
60EM	$0.54^{b} \pm 0.05$	$1.10^{b} \pm 0.01$
70EM	$0.70^{a} \pm 0.06$	$1.10^{b} \pm 0.02$
95EM	$0.38^{c} \pm 0.02$	$1.52^{a} \pm 0.04$

Values followed by different letters are significantly different ($p \leq 0.05$).

From the result, the amount of rutin increased when increasing concentration of ethanol but it decreased at 95% v/v of ethanol. This result may be due to the polarity of each ethanol concentration. The amount of flavonoids in the extracts depends on the polarity of solvents (Stankovic et al., 2010) because each flavonoids has a different polarity (Jayaprakasha et al., 2008). Therefore, ethanol concentration (70% v/v) that has optimum polarity can extract rutin in high amount.

Amount of rutin and quercetin from HPLC are opposed to the total phenolics content and antioxidant activities in Section 4.1.1-4.1.2. This is because mulberry leaves also contain other types of flavonoids. In HPLC chromatogram (Figure 4.1), it was also found that there are other compounds that have high amount in mulberry leaves extract. Moreover, one of compounds (retention time 17.129 min) has higher amount than rutin and quercetin. This type of compound was then determined by using LC-MS (Science Instrument, Rangsit University) and it was identified to be isoquercetin (Figure 4.2), the flavonol glycosides.

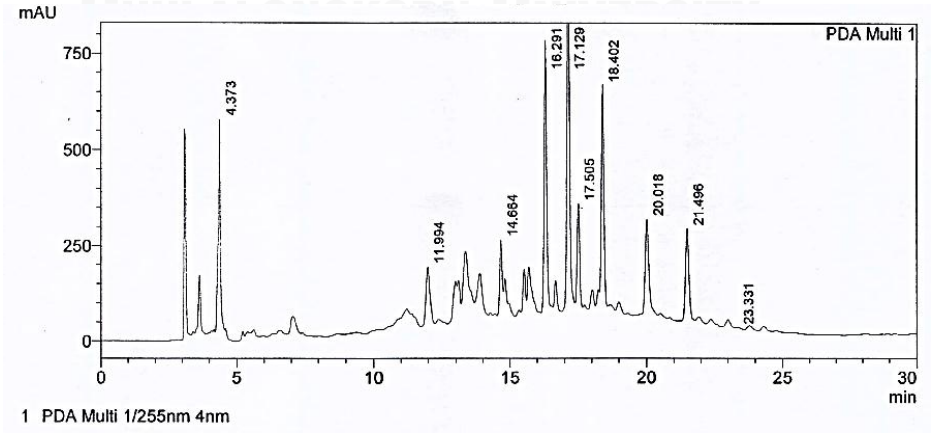


Figure 4.1 The chromatogram of phenolic compounds in 60EM

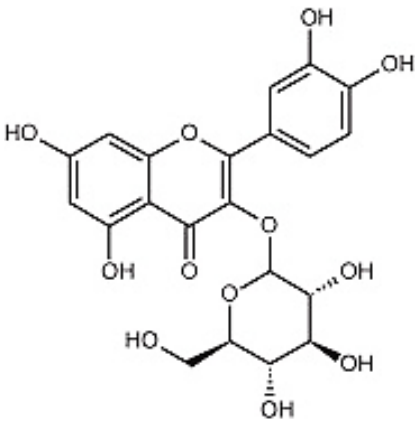


Figure 4.2 Structure of isoquercetin

Source: Cieśla, Kryszeń et al. (2012)

Additionally, other types of phenolic compounds are also determined by using LC-MS and it can classify to quercetin-3-O-(6″-O-malonyl)-**β**-D-glucoside (retention time 18.402 min), quercitrin (retention time 20.018 min) and astragalin (21.496 min). Polarity of flavonoids decreases in the order: rutin > isoquecetin > quercetin-3-O-(6″-O-malonyl)-**β**-D-glucoside > quercitrin > astragalin > quercetin.

Flavonoids in ethanolic mulberry extracts can be separated into 2 groups: flavonol glycosides (hydrophilic antioxidants) and aglycones (hydrophobic antioxidants). Rutin, isoquecetin, quercetin-3-O-(6″-O-malonyl)-**β**-D-glucoside, quercitrin and astragalin are flavonol glycosides. Quercetin is aglycones. Therefore, most of flavonoids in mulberry leaves are flavonol glycosides.

This result supports the high value of total phenolics content and antioxidant activity using FRAP assay of 50EM and 60EM (Table 4.1). A 50 and 60% v/v of ethanol contain high polarity, thus they have a higher capability to extract flavonol glycosides than other concentration of ethanol.

Kim et al. (1999) also found rutin, quercetin, isoquercetin, quercetin-3-O-(6″-O-malonyl)-**β**-D-glucoside and astragalin in mulberry leaves. They studied the antioxidant activity of each flavonoid by using DPPH radical scavenging activity. The radical scavenging activity of flavonoids decreases in the order: quercetin > quercetin 3-O-(6″-O-malonyl)-**β**-D-glucoside > rutin > isoquercetin. Nevertheless, radical scavenging activity of astragalin can not detect in this study. Katsube et al. (2006) also found three flavonol glycosides which have the strongest antioxidant activities: rutin, isoquercetin and quercetin-3-O-(6″-O-malonyl)-**β**-D-glucoside. This method is

suitable for determining antioxidant activity of hydrophobic antioxidant. However, radical scavenging activity of quercetin was highest due to its hydrophobicity.

In Table 4.1, 50EM shows the lowest radical scavenging activity because 50% v/v of ethanol has a lower amount of ethanol than other concentrations. Thus, it has a low capability to extract aglycones from mulberry leaves.

Therefore 60EM was selected for using as core material in encapsulation process by polymer-polymer interaction method. However, the core material of this process should be water-insoluble extract; hence 95EM was also selected to compare with 60EM.

4.2 Microencapsulation process

To produce microcapsules with high efficiency, the suitable conditions (core material, final pH of mixed solution and concentration of coating materials) in microencapsulation process by using polymer-polymer interaction method were studied in this part.

4.2.1 Study the suitable ethanolic mulberry leaves extract to be used as core material in microencapsulation process

The selected ethanolic mulberry leaves extracts from step 4.1 were used as core material in polymer-polymer interaction process. Two ethanolic mulberry leaves extracts (with different hydrophilicity and hydrophobicity) were compared in this part in order to select the most suitable extract to use as core material. The properties of microcapsules were analyzed in term of solubility, encapsulation yield, encapsulation efficiency, total phenolics content, antioxidant activities and morphology.

4.2.1.1 Solubility of microcapsules

The solubility of microcapsules was analyzed by using the same method as dissolving microcapsules for determining total phenolics content and antioxidant activities. Microcapsules produced by polymer-polymer interaction process are generally water-insoluble (Dong et al., 2008). Therefore the method used to analyzed solubility of microcapsules was adjusted. Solubility of microcapsules was determined by first dissolving flavonoids from the surface of microcapsules by using ethanol and

separating a supernatant. Then, distilled water (with pH adjusted to 8.0) at 50 °C and ethanol (60 or 95% v/v) was added to the filtrate for dissolving flavonoids in the inner of microcapsules. All supernatant was dried in hot air oven. The solubility (%) was calculated by weight difference (Appendix A.5) as shown in Table 4.3.

Table 4.3 Solubility of microcapsules from 60EM and 95EM

Microcapsules	Solubility (%), [NS]
60EM	47.21 ± 1.70
95EM	46.33 ± 4.28

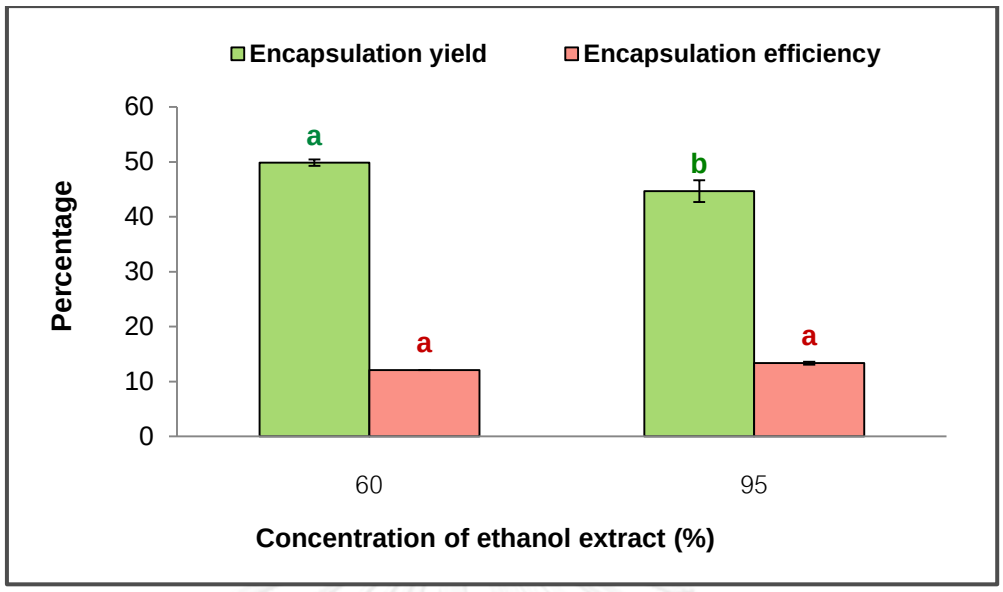
NS = Not significant ($p > 0.05$).

The solubility of microcapsules from 60EM and 95EM was 47.21 and 46.33%, respectively. There is no significant difference ($p > 0.05$) among different treatments because both of microcapsules were produced by same coating materials (SPI and pectin). Moreover, ethanol (60 or 95% v/v) has an efficiency to dissolve antioxidant from each sample. Therefore, the solubility of microcapsules from 60EM and 95EM was not significantly different ($p > 0.05$).

The solubility of microcapsules was lower than 50% because each particle of microcapsules has a limitation of solubility. Microcapsules include SPI, pectin and flavonoids which can be dissolved in different conditions: SPI dissolves in distilled water (with pH adjusted to 8) at 90 °C, pectin dissolves in distilled water at 50°C and flavonoids dissolves in ethanol. While microcapsule was dissolved in solvent, some particle still insoluble and precipitate. Furthermore, the solvent used for solubility of microcapsules cannot dissolve all particles in microcapsules. Hence, solubility of microcapsules from 60EM and 95EM was lower than 50%.

4.2.1.2 Encapsulation yield and encapsulation efficiency

The effects of different core material on the encapsulation yield and encapsulation efficiency were presented in Figure 4.3.



Values followed by different letters are significantly different ($p \leq 0.05$)

Figure 4.3 Encapsulation yield and encapsulation efficiency of microcapsules from 60EM and 95EM

The encapsulation process used 60EM as core material provided greater phase separation than 95EM (49.86 and 44.68%, respectively). However, there is no significant difference in encapsulation efficiency of 60EM and 95EM (12.10 and 13.35%, respectively).

The difference in encapsulation yield between microcapsules of 60EM and 95EM may be caused by the polarity of flavonoids in each extract. A 95EM contains higher amount of low-polarity flavonoids (a poorly water soluble compounds) than 60EM. This type of flavonoids can be dissolved in high ethanol concentration (Rice-evans et al., 1995). The continuous phase in polymer-polymer interactions process is water, when 95EM was added into SPI solution and homogenized, some of flavonoids in the extract can be sedimented. The sedimented flavonoids can not interact with SPI and pectin and form to microcapsules. Therefore, encapsulation yield of microcapsules from 95EM was lower than 60EM.

4.2.1.3 Total phenolics content and antioxidant properties of microcapsules

To determine total phenolics content and antioxidant properties, microcapsules were dissolved at the same condition used in section 4.2.1.1.

Microcapsules were analyzed in total phenolics content and antioxidant properties by FRAP assay and DPPH radical scavenging activity as shown in Table 4.4.

Table 4.4 Total phenolics content and antioxidant activities of microcapsules from 60EM and 95EM

Microcapsules	Total phenolics content (mg GAE/g microcapsules)	Ferric reducing antioxidant power (mmol Trolox/g microcapsules)	DPPH radical scavenging activity (mmol Trolox/g microcapsules)
60EM	$57.04^{ns} \pm 4.94$	$25.20^{a} \pm 2.40$	$12.68^{a} \pm 0.68$
95EM	$52.05^{ns} \pm 2.78$	$15.59^{b} \pm 1.12$	$9.37^{b} \pm 0.35$

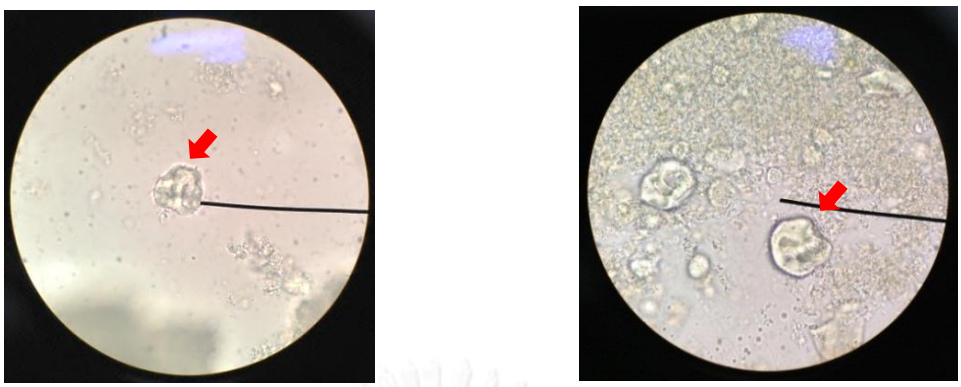
Values followed by different letters are significantly different ($p \leq 0.05$).

Total phenolics content of microcapsules of 60EM and 95EM was not significantly different ($p > 0.05$). Microcapsules of 60EM gave higher antioxidant activities in both methods (FRAP assay and DPPH radical scavenging activity) compared to 95EM.

The amount of flavonoids in core material (ethanolic mulberry leaves extract) affects antioxidant activities of microcapsules. From the result of the amount of rutin and quercetin in 60EM and 95EM from Table 4.2, it was found that 60EM has a greater amount of rutin than 95EM (0.54 and 0.38 mg/g dried leaves, respectively). However, 60EM has a little lower amount of quercetin than 95EM (1.10×10^{-3} and 1.52×10^{-3} mg/g dried leaves, respectively). Therefore, antioxidant activities of microcapsules from 60EM were higher than microcapsules from 95EM because rutin is dominant antioxidant which also exhibits higher activity than quercetin.

4.2.1.4 Morphology of microcapsules

The morphology of microcapsules from 60EM and 95EM was presented in Figure 4.4. It was found that microcapsules have an irregular shape with rough surface because of the process used to dehydrate microcapsules.



(a) (b)

Figure 4.4 Morphology of microcapsules produced by using different core material: (a) 60EM and (b) 95EM obtained under light microscopes (100x)

The lyophilization process promoted the breakage of unstable electrostatic interactions of microcapsules and the loss of the initial structure or round form due to the loss of water content during the freeze-drying process. Therefore, the freeze-drying microcapsules had irregular shape and wrinkled surface (Kwok et al., 1991; Silva et al., 2012).

The weakened of electrostatic interactions between SPI and pectin may result in the low value of encapsulation efficiency of microcapsules from ethanolic mulberry leaves extract. The interaction between SPI and pectin was broken, microcapsules can not entrap flavonoids. Therefore, the encapsulation efficiency of microcapsules was lower than 20% (Figure 4.2).

Both 60EM and 90EM was chosen to be used as core material in microencapsulation process by polymer-polymer interaction method in order to study the suitable final pH of mixed solution in polymer-polymer interaction process. Since, the encapsulation efficiency and total phenolics content of both microcapsules are not significant difference ($p>0.05$) but the encapsulation yield and antioxidant activities analyzed in both methods (FRAP assay and DPPH radical scavenging activity) of microcapsules produced from 60EM was higher than 95EM. Therefore, adjusting the final pH of mixed solution in polymer-polymer interaction process may increase entrapment efficiency of microcapsules from 95EM. Therefore to confirm that 60EM or 95EM can be used as the suitable core material, 95EM was also used in comparison with 60EM in the next part.

4.2.2 Study the suitable final pH of mixed solution in polymer-polymer interaction process

In this part, two ethanolic mulberry leaf extracts (with different polarity) and three final pH of mixed solution (3.5, 4.0 and 4.5) were varied in order to study the most suitable extract and final pH of mixed solution to produce microcapsules using polymer-polymer interaction process. The efficiency of microcapsules was analyzed in term of solubility, encapsulation yield, encapsulation efficiency, total phenolics content, antioxidant activities, type of flavonoids compounds and morphology.

4.2.2.1 Solubility of microcapsules

The solubility of microcapsules is shown in Table 4.5. There is no significant difference ($p > 0.05$) between microcapsules of 60EM and 95EM at all pH values (3.5, 4.0 and 4.5).

Table 4.5 Solubility of microcapsules from 60EM and 95EM with pH adjusted to 3.5, 4.0 and 4.5

Microcapsules	pH	Solubility (%), [NS]
60EM	3.5	50.75 ± 2.26
	4.0	49.06 ± 0.58
	4.5	48.97 ± 0.81
95EM	3.5	45.98 ± 3.51
	4.0	46.34 ± 2.64
	4.5	46.39 ± 2.60

NS = Not significant ($p > 0.05$).

The low solubility of microcapsules (<50%) may be related to a limitation of solubility of each particle in microcapsules: SPI, pectin and flavonoids. SPI dissolves in distilled water (with pH adjusted to 8) at 90°C, pectin dissolves in distilled water at 50°C and flavonoids dissolves in ethanol. Furthermore, the solvent used to analyze solubility of microcapsules can not dissolve all particles in microcapsules, some

particle still insoluble and precipitate. Hence, solubility of microcapsules from 60EM and 95EM was lower than 50%.

4.2.2.2 Encapsulation yield and encapsulation efficiency

The effect of pH on the encapsulation yield and encapsulation efficiency of microcapsules from 60EM and 95EM is presented in Table 4.6. The encapsulation process using 60EM as core material with pH adjusted to 3.5, 4.0 and 4.5 provided greater phase separation than 95EM but the encapsulation efficiency of microcapsules of 60EM and 95EM with pH adjusted was not significantly different ($p > 0.05$).

Table 4.6 Encapsulation yield and encapsulation efficiency of microcapsules from 60EM and 95EM ethanol extract with pH adjusted to 3.5, 4.0 and 4.5

Microcapsules	pH	Encapsulation yield (%)	Encapsulation efficiency (%)
60EM	3.5	$51.12^{a} \pm 3.02$	$13.58^{a} \pm 0.50$
	4.0	$50.29^{a} \pm 0.34$	$13.55^{a} \pm 0.86$
	4.5	$51.25^{a} \pm 1.46$	$11.19^{ab} \pm 0.67$
95EM	3.5	$18.12^{d} \pm 1.29$	$10.40^{c} \pm 0.46$
	4.0	$41.22^{b} \pm 1.20$	$11.49^{ab} \pm 1.10$
	4.5	$34.91^{c} \pm 1.75$	$12.73^{bc} \pm 0.69$

Values followed by different letters are significantly different ($p \leq 0.05$).

The difference in encapsulation yield between microcapsules of 60EM and 95EM may cause by the polarity of flavonoids in each extract as described in section 4.2.1.2.

In case of final pH of mixed solution in polymer-polymer interaction process, Giancone et al. (2009) reported that the mixtures of protein and polysaccharide form an electrostatic complex in a specific pH range. This phenomenon relies on isoelectric point (IEP) of protein and pKa of polysaccharides. At pH below the isoelectric point of the proteins and above the pKa of polysaccharides carboxyl

group, protein and polysaccharide can effectively interact by electrostatic interactions. In this study, final pH of mixed solution in polymer-polymer interaction process was varied in value above pKa of pectin (3.5) and below the isoelectric point of SPI (4.6). From the result, final pH of mixed solution in polymer-polymer interaction process has an effect on encapsulation yield of microcapsules from 95EM (at pH 4.0 > 4.5 > 3.5). On the other hand, final pH of mixed solution in polymer-polymer interaction process has no effect on encapsulation yield of microcapsules from 60EM because pH has an effect on solubility of flavonoids (Lauro et al., 2002). Adjusting pH of the mixture can improve the solubility of sedimented flavonoid of 95EM, flavonoids can interact with SPI and pectin, and form to microcapsules. For this reason, encapsulation yield of microcapsules from 95EM was increased when final pH of mixed solution in polymer-polymer interaction process was adjusted to a suitable value.

4.2.2.3 Total phenolics content and antioxidant properties of microcapsules

The result of total phenolics content and antioxidant properties by FRAP assay and DPPH radical scavenging assay are shown in Table 4.7.

Table 4.7 Total phenolics content and antioxidant properties of microcapsules from 60EM and 95EM with pH adjusted to 3.5, 4.0 and 4.5

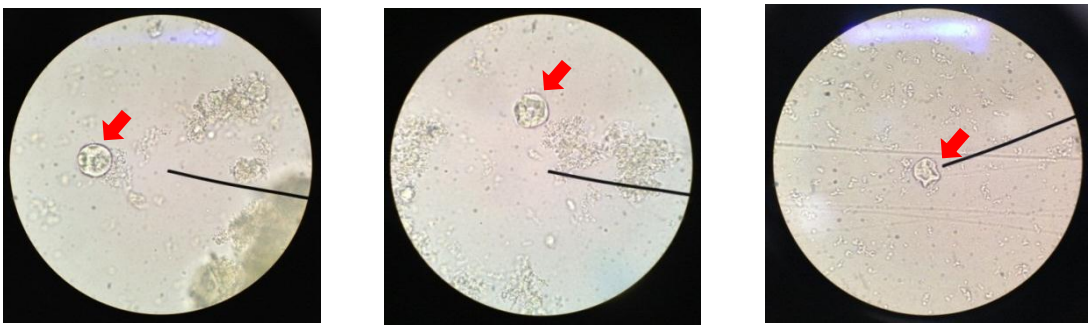
Microcapsules	pH	Total phenolics content (mg GAE/g microcapsules)	Ferric reducing antioxidant power (mmol Trolox/g microcapsules)	DPPH radical scavenging activity (mmol Trolox/g microcapsules)
60EM	3.5	$61.87^{ab} \pm 5.64$	$20.21^{a} \pm 0.45$	$14.02^{b} \pm 0.45$
	4.0	$66.41^{a} \pm 0.50$	$20.76^{a} \pm 0.25$	$16.18^{a} \pm 0.50$
	4.5	$69.13^{a} \pm 0.49$	$20.33^{a} \pm 0.22$	$16.71^{a} \pm 0.18$
95EM	3.5	$53.60^{c} \pm 1.07$	$15.02^{b} \pm 0.99$	$9.88^{c} \pm 0.09$
	4.0	$57.64^{bc} \pm 4.48$	$15.70^{b} \pm 0.11$	$9.72^{c} \pm 0.34$
	4.5	$54.47^{bc} \pm 1.11$	$14.54^{b} \pm 0.46$	$9.48^{c} \pm 0.51$

Values followed by different letters are significantly different ($p \leq 0.05$).

Microcapsules from 60EM gave higher amount of total phenolics content and antioxidant activities in both methods (FRAP assay and DPPH radical scavenging activity) than microcapsules from 95EM. This result is depending on encapsulation efficiency of microcapsules. Microcapsules from 60EM have a higher of capability to entrap flavonoids than microcapsules from 95EM. Therefore, total phenolics content and antioxidant activities of microcapsules from 60EM were higher than microcapsules from 95EM.

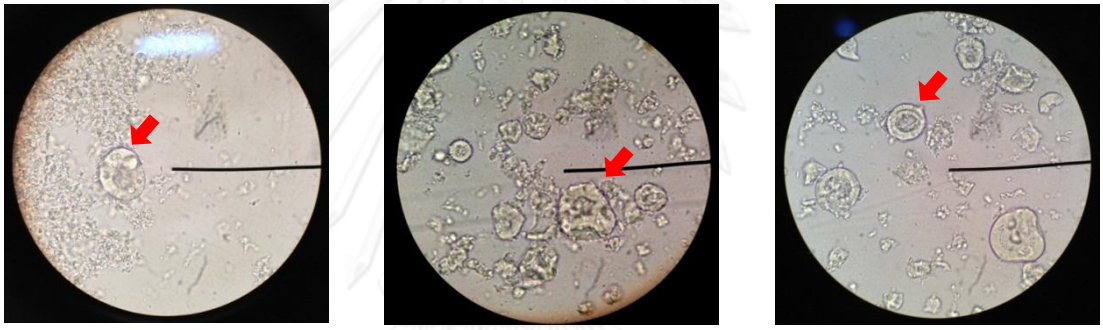
4.2.2.4 Morphology of microcapsules

The morphology of microcapsules from 60EM and 95EM at various pH (3.5, 4.0, 4.5) are shown in Figure 4.5 and 4.6.



(a) (b) (c)

Figure 4.5 Morphology of microcapsules from 60EM at various pH: (a) pH 3.5, (b) pH 4.0 and (c) pH 4.5 under light microscopes (100x)



(a) (b) (c)

Figure 4.6 Morphology of microcapsules from 95EM at various pH: (a) pH 3.5, (b) pH 4.0 and (c) pH 4.5 under light microscopes (100x)

Microcapsules of both core materials at all pH values have the irregular shape but microcapsules of 60EM tend to be more round shape compared to microcapsules of 95EM. This irregular shape of microcapsules may relate to the method used to produce and dehydrate microcapsules as described in section 4.1.1.1

4.2.2.5 Amount of flavonoids compound in microcapsules

The amount of rutin and quercetin of microcapsules produced by 60EM and 95EM with pH adjusted to 3.5, 4.0 and 4.5 was analyzed by using HPLC as shows in Table 4.8.

Table 4.8 The amount of rutin and quercetin in microcapsules produced by 60EM and 95EM with pH adjusted to 3.5, 4.0 and 4.5

Microcapsules	pH	Rutin (mg/g microcapsules)	Quercetin ($\times 10^{-3}$ mg/g microcapsules)
60% ethanol extract	3.5	$0.46^{a} \pm 0.01$	$5.52^{a} \pm 0.42$
	4.0	$0.49^{a} \pm 0.01$	$6.24^{a} \pm 0.60$
	4.5	$0.49^{a} \pm 0.02$	$5.99^{a} \pm 0.45$
95% ethanol extract	3.5	$0.21^{c} \pm 0.01$	ND*
	4.0	$0.32^{b} \pm 0.06$	ND*
	4.5	$0.20^{c} \pm 0.01$	ND*

Values followed by different letters are significantly different ($p \leq 0.05$)

*Not detectable.

The entrapment efficiency of microcapsules from 60EM with pH adjusted to 3.5, 4.0 and 4.5 was higher than microcapsules from 95EM. Microcapsules from 60EM can entrap rutin (about 0.46-0.49 mg/g microcapsules) and quercetin (about 5.52×10^{-3} - 6.24×10^{-3} mg/g microcapsules). Moreover rutin was also found in microcapsules from 95EM in all pH value (about 0.20-0.32 mg/g microcapsules) but quercetin was not found in microcapsules.

The amount of rutin in microcapsules from 60EM was higher than 95EM due to the amount of rutin in each extract. From step 4.1.3, It was found that 60EM gave a higher amount of rutin than 95EM (0.54 and 1.10×10^{-3} mg/g microcapsules, respectively). The result of losing quercetin in microcapsules from 95EM may be caused by the polarity of the extract as described in section 4.2.1.2.

From the result in this part, there is no significant difference ($p \leq 0.05$) in chemical properties, physical properties and efficiency of microcapsules between 60EM at pH 4.0 and 4.5. Therefore, the suitable condition was selected by encapsulation efficiency of microcapsules because it related to the stability of flavonoids in microcapsules. Entrapped flavonoids can be separated in 2 groups: flavonoids at the inner of microcapsules and flavonoids at the surface of microcapsules. Encapsulation efficiency is the factor that indicated the amount of

flavonoids at the inner microcapsules. They were protected from environmental conditions (light, moisture, oxygen and temperature) by coating materials (SPI and pectin). Moreover, flavonoids at the surface of microcapsules may have low stability and easily decompose by environmental factor after storage. The result in Table 4.6 shows that encapsulation efficiency of microcapsules from 60EM at pH 4.0 was slightly higher than at pH 4.5 (13.55 and 11.19%, respectively). Although the total phenolics content and DPPH radical scavenging activity of microcapsules of 60EM at pH 4.5 were slightly higher than at pH 4.0 (Table 4.7), they are the result that analyzed from all microcapsules (inner and surface).

Thus, 60EM was selected to use as core material and adjusting final pH of mixed solution to 4.0 was chosen as optimum condition to produce microcapsules for using in the next step because of high encapsulation yield, encapsulation efficiency, total phenolics content and antioxidant activities.

4.2.3 Study the suitable concentration of coating materials (SPI and pectin) in polymer-polymer interaction process

The selected ethanolic mulberry leaves extracts and final pH of mixed solution from step 4.2.2 was used to produce microcapsules in polymer-polymer interaction process. In this part, three concentrations (2.5, 5.0 and 7.5% w/v) of coating materials (SPI and pectin) were compared in order to select the most suitable concentration of coating materials for using in polymer-polymer interaction process. The efficiency of microcapsules was analyzed in solubility, encapsulation yield, encapsulation efficiency, total phenolics content, antioxidant activities and morphology.

4.2.3.1 Solubility of microcapsules

The solubility of microcapsules was shown in Table 4.9. There is not significantly different ($p > 0.05$) between microcapsules from all concentration (2.5, 5.0 and 7.5% w/v) of coating materials (SPI and pectin).

Table 4.9 Solubility of microcapsules from 60EM at various concentration of coating materials (SPI and pectin)

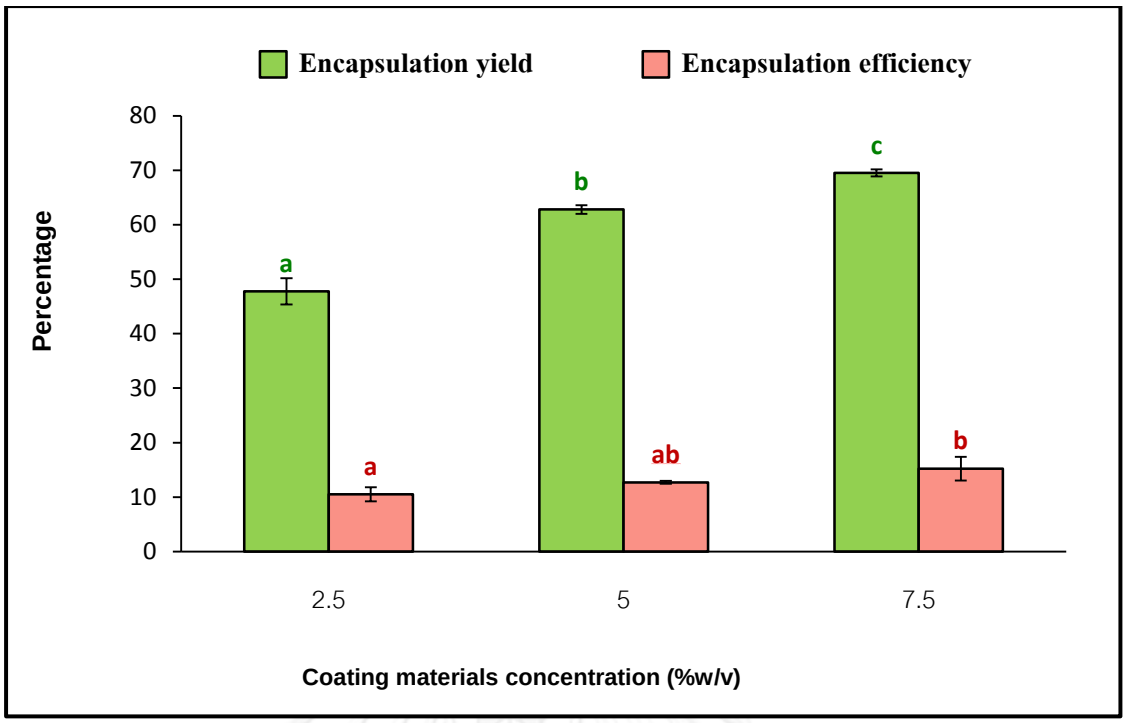
Concentration of coating materials (% w/v)	**Solubility (%),** [NS]
2.5	48.93 ± 0.81
5.0	49.53 ± 0.31
7.5	50.13 ± 1.79

NS = Not significant ($p > 0.05$).

Due to the limitation of solubility of each particle in microcapsules, the solubility of microcapsules was lower than 50%. SPI, pectin and flavonoids which are components in microcapsules can be dissolved in different conditions. SPI dissolves in distilled water (with pH adjusted to 8) at 90 °C, pectin dissolves in distilled water at 50 °C and flavonoids dissolves in ethanol. Therefore, the solvent can not dissolve all particles in microcapsules.

4.2.3.2 Encapsulation yield and encapsulation efficiency

The effects of concentration of coating materials on the encapsulation yield and encapsulation efficiency of microcapsules were presented in Figure 4.7. Concentration of 7.5% coating materials (SPI and pectin) provided the greatest phase separation and encapsulation efficiency during encapsulation process (69.52 and 15.22%, respectively).



Values followed by different letters are significantly different ($p \leq 0.05$).

Figure 4.7 Encapsulation yield and encapsulation efficiency of microcapsules from 60EM at various concentration of coating materials (SPI and pectin)

Extending of electrostatic interactions between protein and polysaccharide is depending on the amount of each molecule available to interact (Vies et al., 1967). At high concentration of coating materials (SPI and pectin), they have more efficiency to interact with electrostatic interactions, entrap flavonoids from mulberry leaves and sediment to microcapsules. Therefore, encapsulation yield and encapsulation efficiency of microcapsules increase when produced by using high concentration of coating materials.

On the other hand, using concentration of coating materials higher than 7.5% w/v is non-practical because of high viscosity of pectin solution. Furthermore, pectin can not mix with the mixture of SPI-ethanolic mulberry leaf extract in polymer-polymer interaction process.

4.2.3.3 Total phenolics content and antioxidant properties of microcapsules

The result of total phenolics content and antioxidant properties by FRAP Assay and DPPH radical scavenging activity were shown in Table 4.10. Microcapsules produced by using 7.5% concentration of coating materials gave higher total phenolics content and antioxidant activities in both methods FRAP assay and DPPH radical scavenging activity) than other concentration.

Table 4.10 Total phenolics content and antioxidant activities of microcapsules from 60EM at various concentration of coating materials (SPI and pectin)

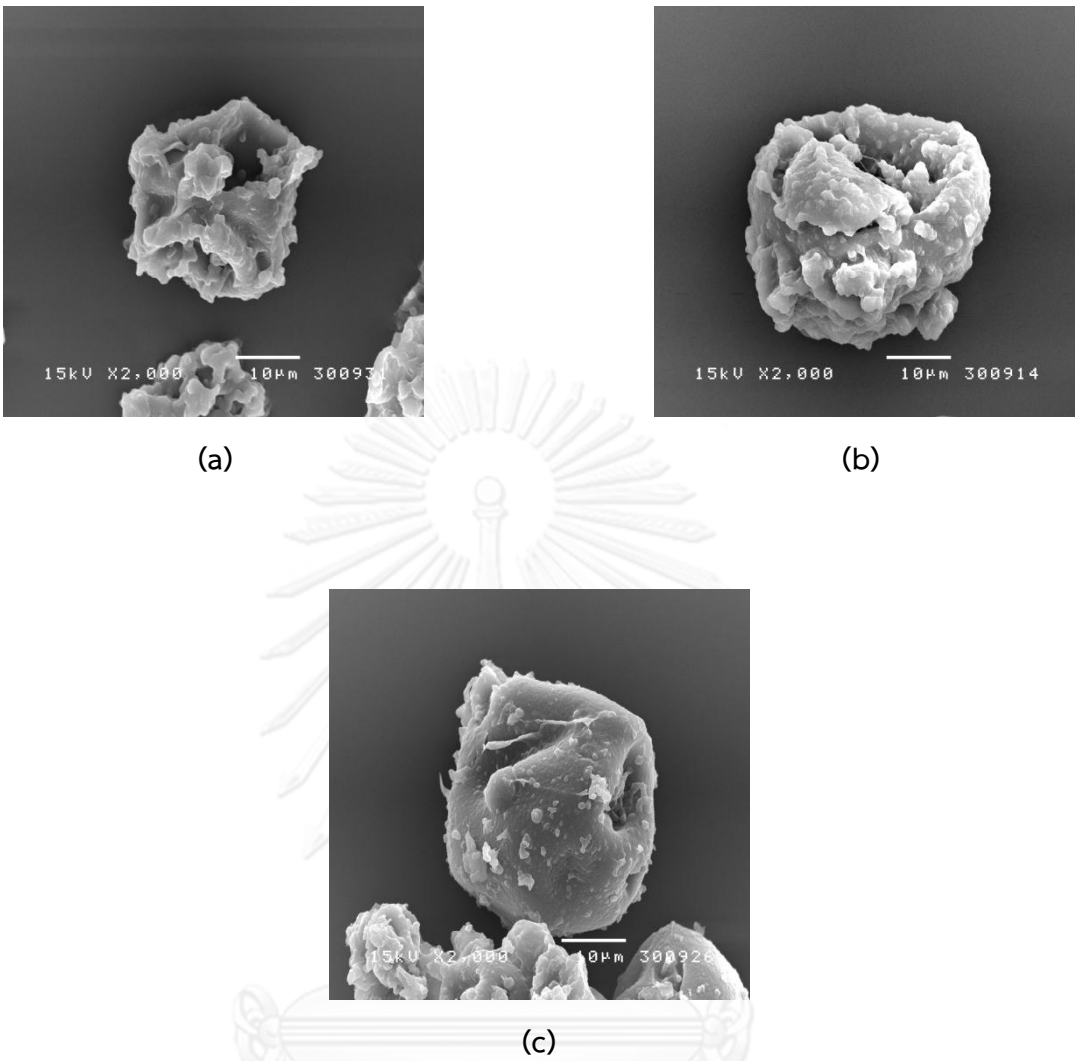
Concentration of coating materials (% w/v)	Total phenolics content (mg GAE/g microcapsules)	Ferric reducing antioxidant power (mmol Trolox/g microcapsules)	DPPH radical scavenging activity (mmol Trolox/g microcapsules)
2.5	$61.17^{b} \pm 0.59$	$22.29^{b} \pm 0.48$	$13.98^{b} \pm 0.45$
5.0	$71.01^{a} \pm 0.87$	$24.16^{b} \pm 0.71$	$20.42^{a} \pm 0.08$
7.5	$73.37^{a} \pm 3.54$	$27.71^{a} \pm 1.20$	$20.13^{a} \pm 0.43$

Values followed by different letters are significantly different ($p \leq 0.05$).

Total phenolics content and antioxidant activities of microcapsules from 60EM at 2.5, 5.0 and 7.5% w/v of coating materials are depending on encapsulation efficiency of microcapsules. Microcapsules produced at 7.5% w/v of coating materials have the highest of capability to entrap flavonoids than microcapsules produced at 2.5 and 5.0% w/v. Therefore, total phenolics content and antioxidant activity of microcapsules produced at 7.5% w/v was higher than other concentration.

4.2.3.4 Morphology of microcapsules

The morphology of microcapsules from 60EM at various concentration of coating material is presented in Figure 4.8.



(a) (b)

(c)

Figure 4.8 Morphology of microcapsules from 60EM at various concentration of coating materials (SPI and pectin): (a) 2.5% (w/v), (b) 5.0% (w/v) and (c) 7.5% (w/v) obtained under scanning electron microscopes: SEM (2,000x)

It was found that microcapsules produced in all concentration of coating material have irregular shape with rough surface. This result may be due to the process of producing and drying microcapsules: polymer-polymer interaction process and freeze-drying process as described in section 4.1.1.1.

Thus, using 7.5% w/v concentration of coating materials (SPI and pectin) was chosen as a suitable condition to produce microcapsules because of a high encapsulation yield, total phenolics content and antioxidant activity.

CHAPTER 5
CONCLUSIONS

The suitable concentration of ethanol to extract flavonoids from mulberry leaves is 60% v/v. A 60EM contains higher total phenolics content and antioxidant activities (DPPH radical scavenging activity and FRAP assay) than other extracts. Therefore 60EM was chosen to use as core material in microencapsulation process by polymer-polymer interaction method compared with 95EM.

Statistical analysis showed that solubility, encapsulation efficiency and total phenolics content between microcapsules from 60EM and 95EM is not significantly different ($p>0.05$). Accordingly, the suitable core material was not selected in this section.

In the study of a suitable final pH of mixed solution (3.5, 4.0 and 4.5) in polymer-polymer interaction process, it can be identified that using 60EM as core material are better than 95EM. However, the statistical analysis of physical properties, chemical properties and efficiency of microcapsules from 60EM at pH 4.0 and 4.5 is not significantly different. The most suitable pH value was selected by using encapsulation efficiency due to the stability of flavonoids in microcapsules. Therefore, pH 4.0 was chosen.

The highest concentration of SPI and pectin (7.5% w/v) can produce high ability microcapsules. They have higher encapsulation yield, encapsulation efficiency, total phenolics content and antioxidant activities than other conditions.

The best condition to encapsulate flavonoids from mulberry leaves by using polymer-polymer interaction process is using 60EM (as core materials), 7.5% of coatings material and adjusted final pH of mixed solution to 4.0. It has high efficiency to let the coating materials: soy protein isolate and low methoxyl pectin interacted by electrostatic interactions and entrapped flavonoids from the extract. The efficiency of microcapsules indicated through encapsulation yield, encapsulation efficiency, total phenolics content and antioxidant activities.

Recommendations

1. Mulberry leaves consist of many types of phenolic compounds. The standard solutions used in this study are rutin and quercetin. However, the chromatogram from HPLC also shows high peaks of other phenolic compounds. Using rutin and quercetin as a standard is not enough to clarify types and concentration of phenolic compounds in mulberry leaves. Therefore, determination of other phenolic compounds should be considered in further research.

2. Applications and stability of microcapsules of flavonoids from mulberry leaves should be studied in further research.

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APPENDICES

APPENDIX A

Analytical methods

Appendix A.1: Determination of total phenolics content by Folin-Ciocalteu assay (Waterhouse, 2001)

1. Preparation of gallic acid standard solution

1.1 To prepare a standard solution, 0.5 g of gallic acid was dissolved in 10 mL of ethanol (Macron, Malaysia) and adjusted volume to 100 mL with distilled water in a volumetric flask.

1.2 0, 1, 2, 5, 7.5, 10, 15 mL of gallic acid stock solution was diluted with 100 mL distilled water in a volumetric flask. The final concentration of gallic acid standard solutions was 0, 50, 100, 250, 375, 500, 750 mg/L.

2. Preparation of Sodium carbonate solution

Dissolved 200g of sodium carbonate in 800 mL of distilled water and heated. After the solution cooled, it was filtered with Whatman No.1 filter paper. The volume of solution was made up to 100 mL in a volumetric flask.

3. Folin-Ciocalteu assays

To determine the total phenolics content, 0.1 mL of sample or standard solution or distilled water was added to test tube, 7.9 mL of distilled water was then added. 0.5 mL of Folin-Ciocalteu reagent was added and the mixture was then swirled by vortex mixer and incubated 5 min at room temperature. 1.5 mL of sodium carbonate solution was subsequently added and mixed by vortex mixer. The solution was incubated in the dark at room temperature for 30 min. The absorbance was measured by a spectrophotometer at 765 nm.

The standard curve was shown in Figure A.1

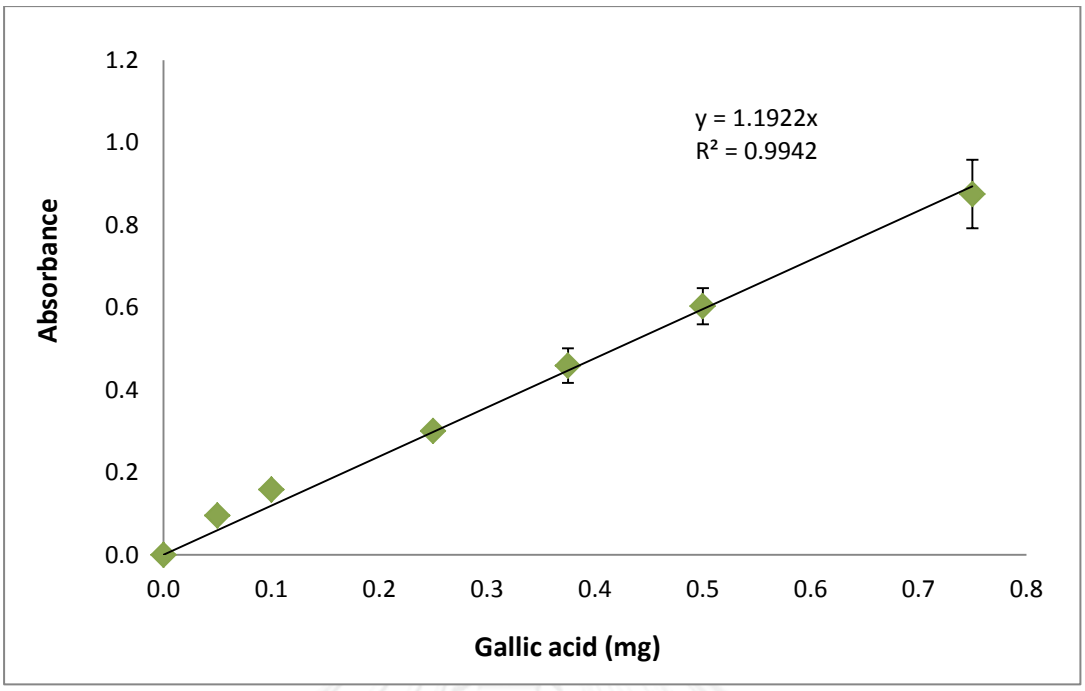


Figure A.1 Gallic acid standard curve for total phenolics content determination

4. **Calculation of total phenolics content**

The amount of total phenolics in crude extract was calculated using gallic acid standard curve. The value was expressed as mg gallic acid equivalent (GAE)/g dried sample.

Appendix A.2: Determination of antioxidant activity by ferric reducing antioxidant power (FRAP) assay (Benzie and Strain, 1996)

1. **Preparation of Trolox® standard curve**

To prepare various concentration of trolox standard solution, 500 µM trolox solution was prepared by dissolving 0.0062 g of trolox with 50 mL of absolute methanol. The 500 µM trolox was then diluted to 100 µM, 10 mL of 500µM trolox was added in volumetric flask and made up to 50 mL with distilled water. The dilution of trolox standard solution was prepared as listed in Table A.1.

Table A.1 Preparation of standard Trolox® solution for FRAP assay

100μM Trolox® volume (mL)	Distilled water volume (mL)	Final concentration (μM)
10	0	100
8	2	80
6	4	60
4	6	40
2	8	20
0	10	0

2. Preparation of reagents

2.1 300 mM Acetate buffer, pH 3.6: 3.1 g of sodium acetate: $CH_3COONa•_3H2O$ was dissolved in 1.6 mL of glacial acetic acid and adjusted volume to 1000 mL with distilled water in a volumetric flask.

2.2 40mM HCl: 1.46 mL of 37% HCl was diluted and made up to 1000 mL with distilled water.

2.3 10mM 2,4,6-tri(2-pyridyl])-s-triazine (TPTZ) solution: dissolve 0.031 g of TPTZ in 10 mL of 40 mM HCl at 50°C.

2.4 20mM Ferric chloride ($FeCl_3$) solution: dissolve 0.054 g of Iron (III) chloride hexahydrate in 10 mL of distilled water.

2.5 FRAP reagent: 300 mM Acetate buffer, 20 mM Ferric chloride solution and 10 mM TPTZ solution were mixed in the ratio of 4:1:1.

3. FRAP assay

FRAP reagent was warmed at 37°C in water bath for 10 min before used. Antioxidant activity was estimated by adding 7.5 mL of FRAP solution to 0.5 mL of trolox solutions or samples or distilled water in test tube and mixing by vortex mixer.

The mixer was held in the dark at room temperature for 30 min. The absorbace of the mixture was measured at 593 nm.

The antioxidant activity was calculated using trolox standard curve as shown in Figure A.2. The value was expressed as mM trolox/g dried sample.

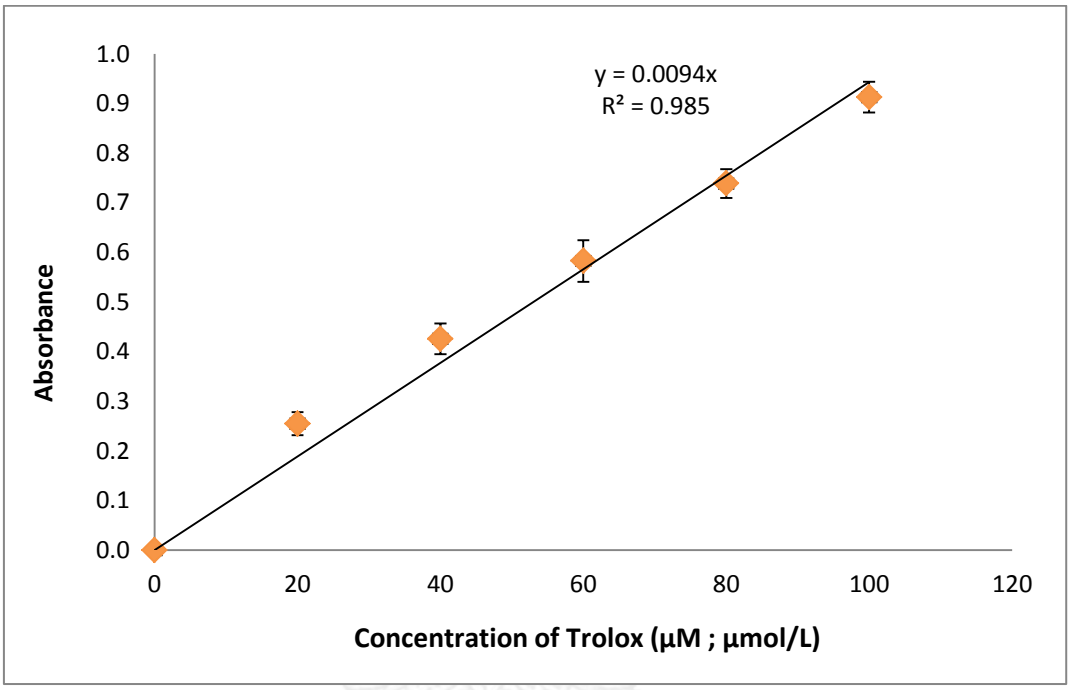


Figure A.2 Trolox® standard curve for FRAP assay

Appendix A.3: Determination of antioxidant activity by DPPH radical scavenging assay (Brand-Williams et al., 1995)

1. **Preparation of Trolox® standard curve**

To prepare various concentration of Trolox® standard solution, 500 µM trolox solution was prepared by dissolving 0.0062 g of Trolox® with 50 mL of absolute methanol. The dilution of Trolox® standard solution was prepared as listed in table A.2.

Table A.2 Preparation of standard Trolox® solution for DPPH assay

500μM Trolox® volume (mL)	Distilled water volume (mL)	Final concentration (μM)
2.8	7.2	140
2.4	7.6	120
2.0	8.0	100
1.6	8.4	80
1.2	8.8	60
0.8	9.2	40
0.4	9.6	20
0	0	0

2. Preparation of DPPH solution

0.1 mM DPPH solution was prepared by dissolving 0.005 g of 2,2-Diphenyl-1-picrylhydrazyl in 100 mL of 95% ethanol.

3. DPPH assay

5 mL of DPPH solution was added to 0.5 mL of Trolox® solution or samples or 95% ethanol (as blank) in a test tube and mixed by vortex mixer. The mixtures were incubated in the dark at room temperature for 30 min. The absorbance was measured at 515 nm.

The result was calculated in % inactivation by:

$$\% \text{ inactivation} = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

Where: A_{blank} = absorbance of blank

A_{sample} = absorbance of sample

The antioxidant activity of sample was calculated using Trolox® standard curve as shown in Figure A.3. The value was expressed as mM trolox/g dried sample.

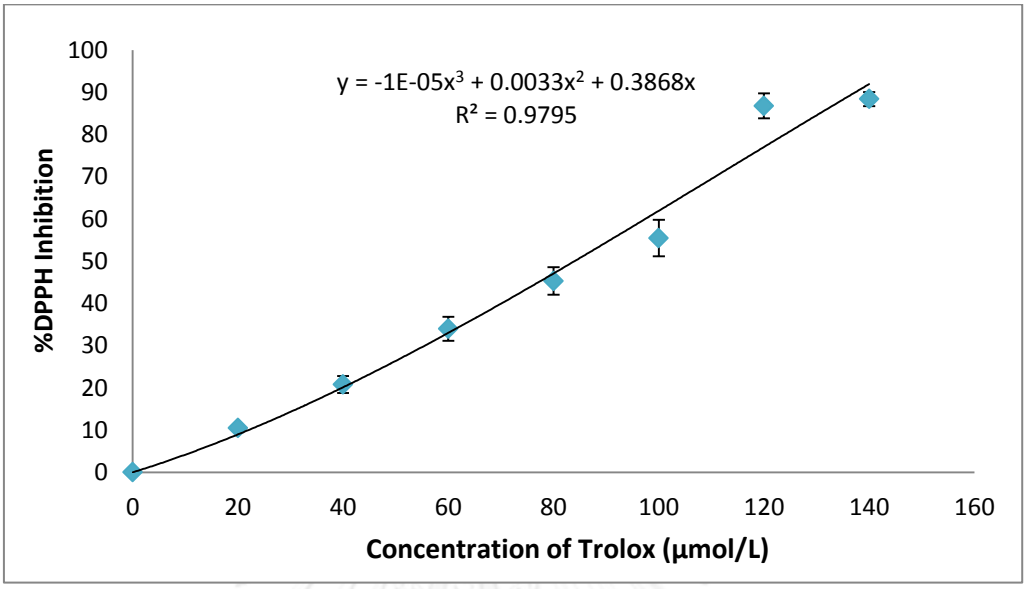


Figure A.3 Trolox® standard curve for DPPH assay

Appendix A.4: Quantitative determination of antioxidant compounds by High Performance Liquid Chromatography: HPLC (analyzed by Science Instrument, Rangsit University)

Each of samples was filtered for HPLC analysis, using a 0.45 µm filter. Flavonol compounds of the extract were analyzed by a quantitative HPLC with Diode Array Detector, using a Inertsil-ODS-3 column (250 x 4.6 mm: 5 µm): solvent, 0 min acetonitrile/1% acetic acid (10:90), 15–20 min acetonitrile/1% acetic acid (40:60), 30 min acetonitrile/1% acetic acid (60:40), 45–55 min acetonitrile/1% acetic acid (80:60), 60-70 min acetonitrile/1% acetic acid (100:0), 75-90 min acetonitrile/1% acetic acid (10:90); flow rate 0.8 mL/min. Rutin and quercetin were used as a standard

Appendix A.5: Determination of antioxidant compounds by Liquid Chromatography and Mass Spectrometry: LC-MS (analyzed by Science Instrument, Rangsit University)

LC-MS Conditions:

Mobile phase : A = 1% acetic acid, B = Acetonitrile

		gradient = 0 min 10%B, 15-20 min 40%B, 30 min 60%B, 45-55 80%B, 60-70 min 100%B, 75-90 min 10%B
Flow rate	:	0.3 mL/min
Oven	:	35 ℃
Column	:	Luna C 18 (2.1 x 150 mm: 3 mm)
Injection	:	10 mL

Appendix A.6: Determination of solubility of microcapsules (Nori et al.; Cano-Chauca et al., 2005)

The solubility of microcapsules was determined by adding 1.0 mL of ethanol to 0.1 g of each microcapsule and vortex for 2 min. The mixture was then centrifuged at 4000 g for 20 min and the supernatant was separated (A). A 2.5 mL of distilled water (pH 8.0) at 50 ℃ was added to the filtrate and mixed for 2 min, 2.5 mL of ethanol was then added and mixed for 2 min and centrifuged at 4000 g for 20 min. The supernatant was separated and mix with (A). The mixture was immediately oven-dried at 105 ℃ until the weight was constant. The solubility (%) was calculated by weight difference by using the following equation:

The encapsulation yield was calculated using the following equation:

$$\text{Solubility of microcapsules (\%)} = \frac{\mathbf{Mx}}{\mathbf{My}} \times 100$$

Where M_x is the total mass of supernatant after drying

M_y is the total mass of microcapsules used to determine the solubility

Appendix A.7: Determination of encapsulation yield (Silva et al., 2012)

The encapsulation yield was calculated using the following equation:

$$\text{Encapsulation yield (\%)} = \frac{\mathbf{Ma}}{\mathbf{Mb}} \times 100$$

Where M_a is the total mass of microcapsules after freeze dried.

M_b is the total mass of solids (mulberry leaves extract, SPI and pectin) that feed in encapsulation process.

Appendix A.8: Determination of encapsulation efficiency (modified from Nori et al., 2010)

To determine the amount of flavonoids at the inner of microcapsules, flavonoids at the surface of microcapsules was removed by add 1.0 mL of ethanol to 0.1 g of each sample and vortex the mixture for 2 min. Then centrifuged at 4000 g for 20 min and the supernatant were separated. To break the interaction between SPI and pectin, 2.5 mL of distilled water (pH 8.0) at 50 ℃ was added to the filtrate and mixed on a vortex mixer for 2 min. Then, 5.0 mL of ethanol was added and mixed for 2 min for dissolving flavonoids at the inner of microcapsules. The mixture was centrifuged at 4000 g for 20 min. The total phenolics content of supernatant (W_1) was determined using Folin–Ciocalteu method as show above.

The encapsulation efficiency was calculated using the following equation:

$$\text{Encapsulation efficiency (\%)} = \frac{W1}{W2} \times 100$$

Where W_1 is the amount of total phenolics at the inner of microcapsules.

W_2 is the amount of total phenolics added to the preparation of microcapsules.

The total phenolics content was determined using Folin–Ciocalteu method as show above.

Appendix A.9: Determination of total phenolics content (TPC) and antioxidant properties of microcapsules

The analysis of total phenolics content (TPC) and antioxidant properties of microcapsules was modified from the method of Nori et al. (2010). The microcapsules (0.2 g) were dissolved in 5.0 mL of distilled water (pH 8.0) at 50 ℃ and mixed on a vortex mixer for 2 min. Then, 5.0 mL of ethanol was added and mixed for 2 min. The mixture was centrifuged at 4000 g for 20 min.

The supernatant was analyzed for the total phenolics content and antioxidant activities (Ferric Reducing Antioxidant Power Assay and DPPH radical-scavenging activity).

Appendix A.10: Morphology of microcapsules under light microscopes

Sample was prepared according to the method of (Zuanon et al., 2013). To analyze the morphology, dried microcapsules were spreaded over a glass slide and added a drop of water for microcapsule rehydration. The sample was then covered with a coverslip and analyzed under light microscopes with magnification power 100x.

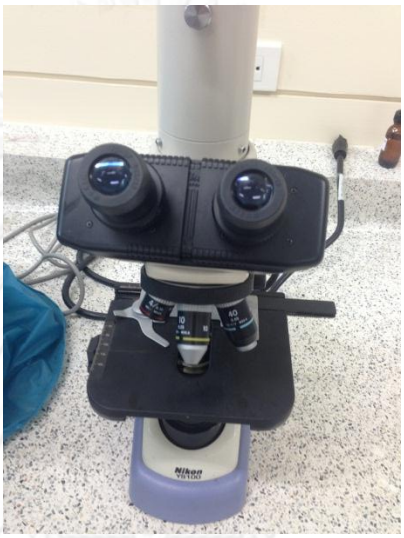


Figure A.4 Light microscopes apparatus

Appendix A.11: Morphology of microcapsules by scanning electron microscope (SEM)

Microcapsules were prepared by mounting on circular aluminium stubs with double-sided sticky tape, coating with gold (15 mm) through Sputer Coating Attachment of Balzers in vacuumed evaporators. Samples were then observed and photographed in a scanning electron microscope at a voltage of 10 kV with 750 and 2,000 of magnification.

APPENDIX B

Chromatograms of phenolic compounds from HPLC

Appendix B.1: The chromatogram of phenolic standard

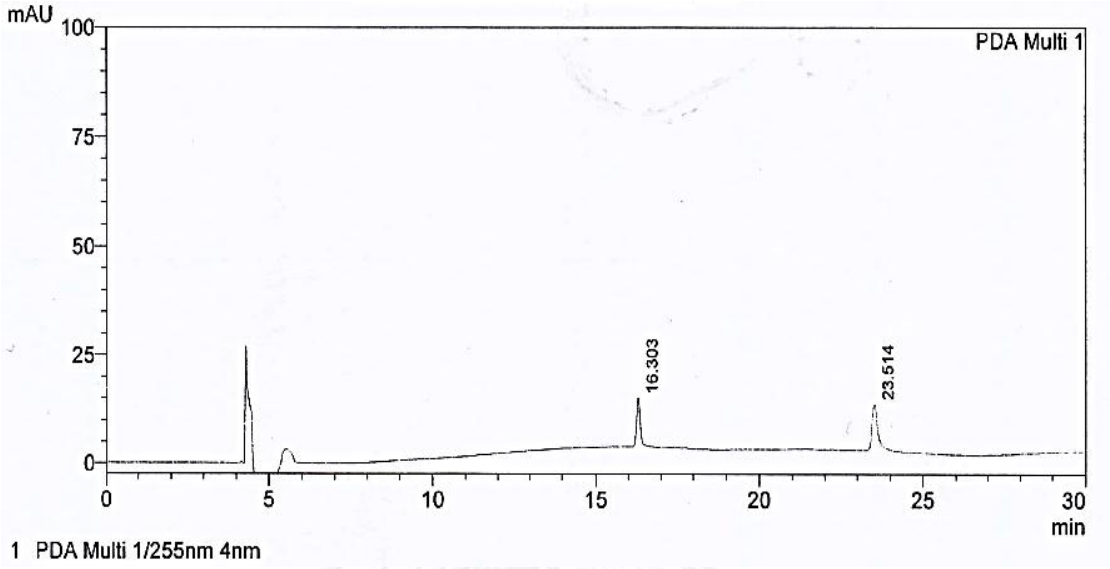


Figure B.1 The chromatogram of rutin and quercetin

Appendix B.2: The chromatogram of phenolic compounds in ethanolic mulberry extract

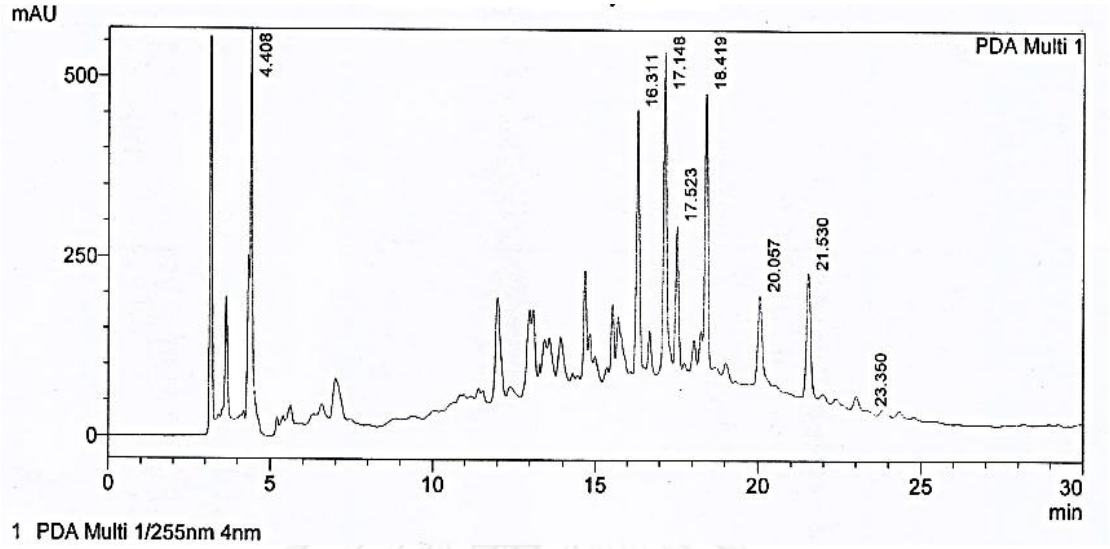


Figure B.2 The chromatogram of phenolic compounds in 50EM

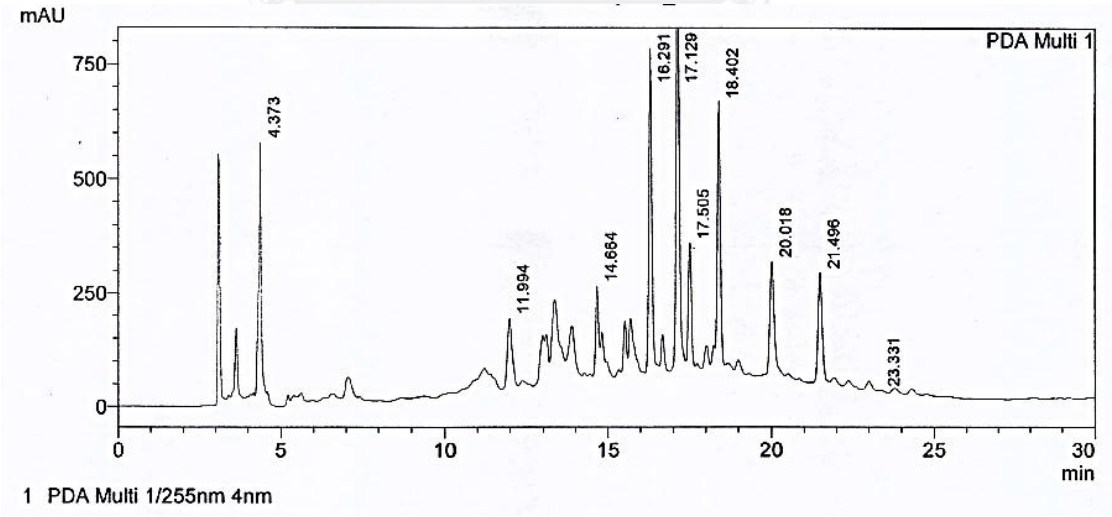


Figure B.3 The chromatogram of phenolic compounds in 60EM

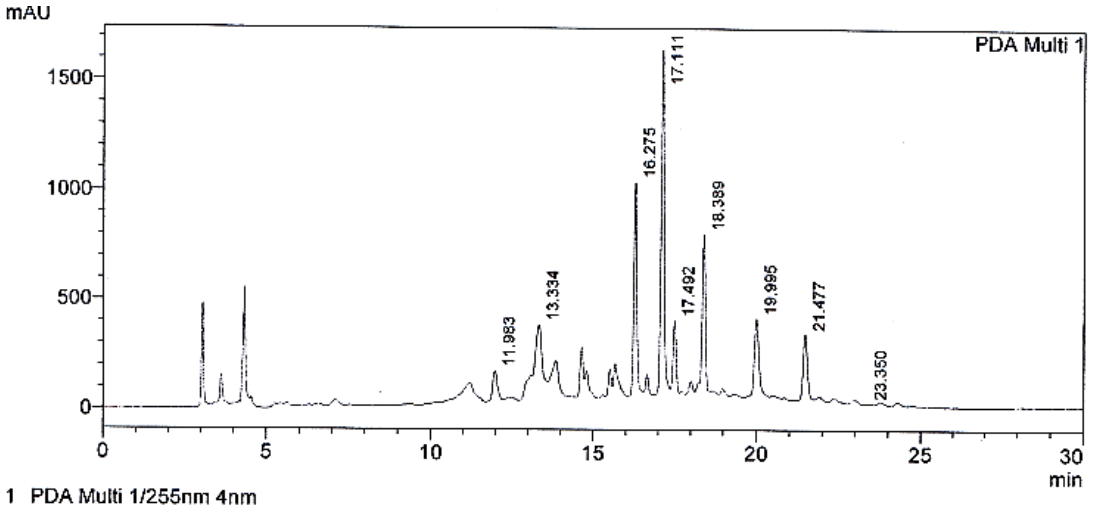


Figure B.4 The chromatogram of phenolic compounds in 70EM

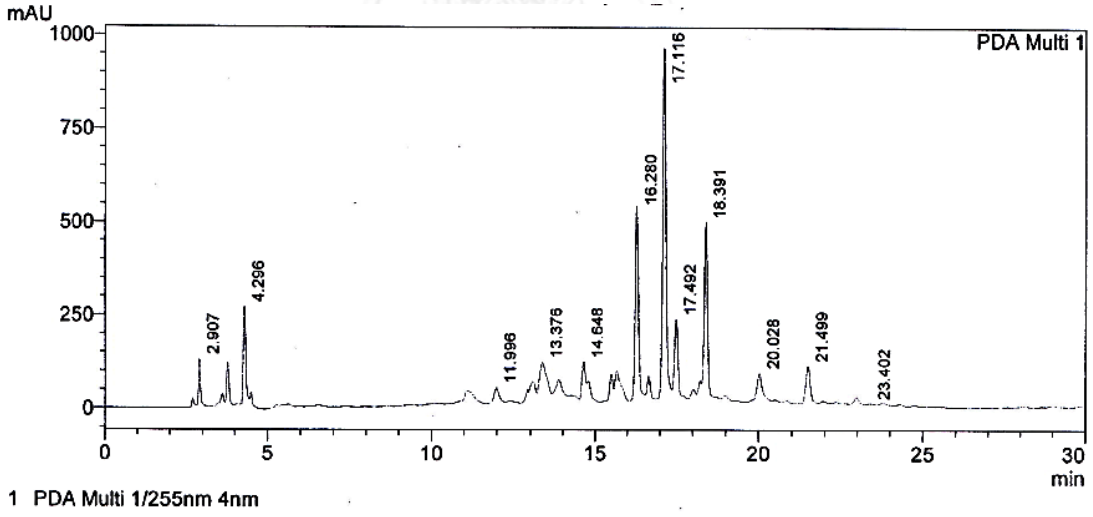


Figure B.5 The chromatogram of phenolic compounds in 95EM

Appendix B.2: The chromatogram of phenolic compounds in microcapsules from 60EM at various pH

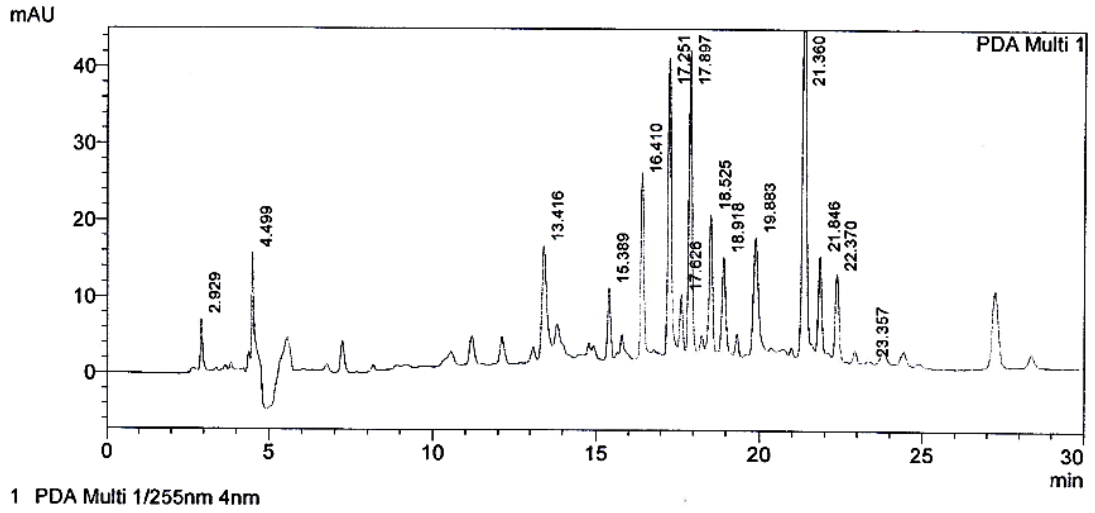


Figure B.6 The chromatogram of phenolic compounds in microcapsules from 60EM with pH adjusted to 3.5

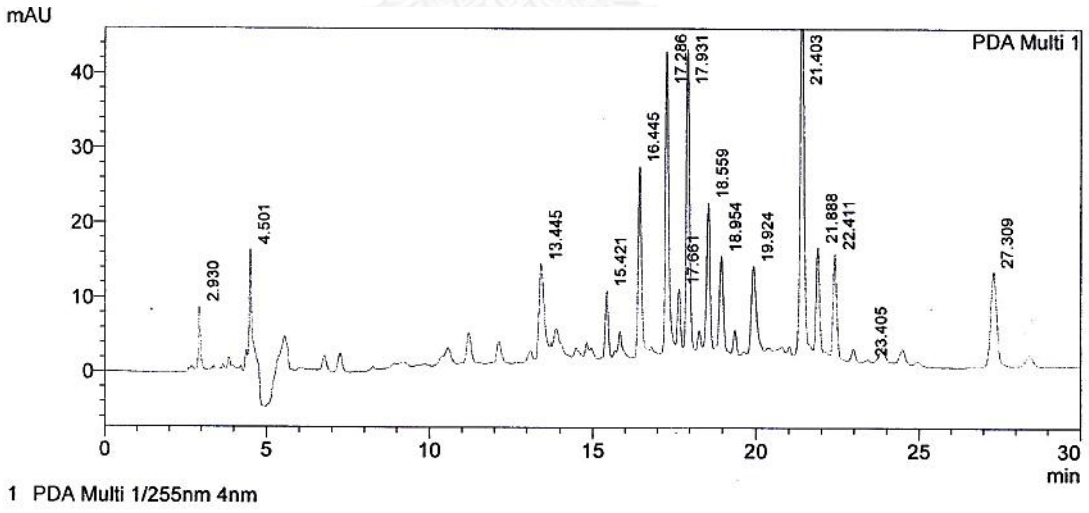


Figure B.7 The chromatogram of phenolic compounds in microcapsules from 60EM with pH adjusted to 4.0

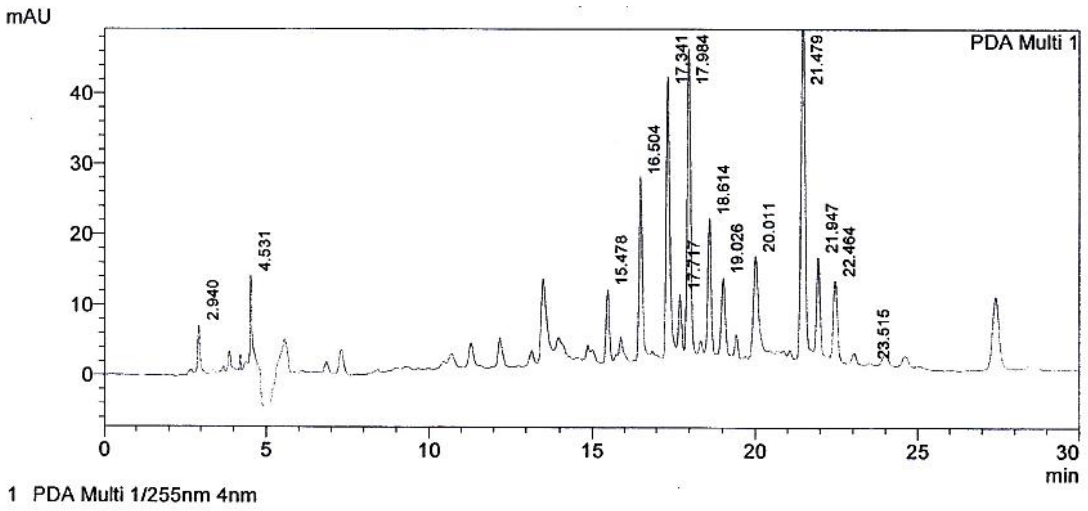


Figure B.8 The chromatogram of phenolic compounds in microcapsules from 60EM with pH adjusted to 4.5

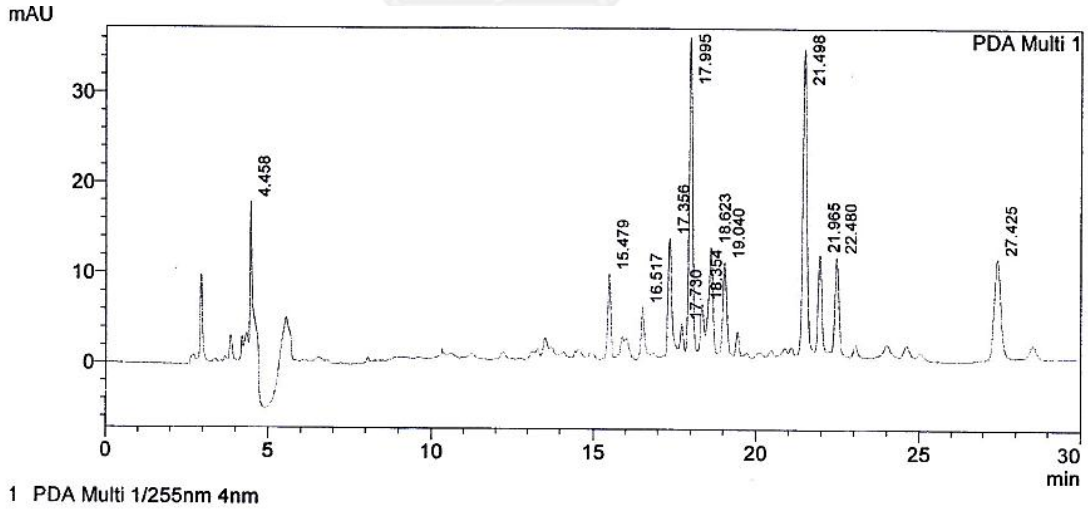


Figure B.9 The chromatogram of phenolic compounds in microcapsules from 95EM with pH adjusted to 3.5

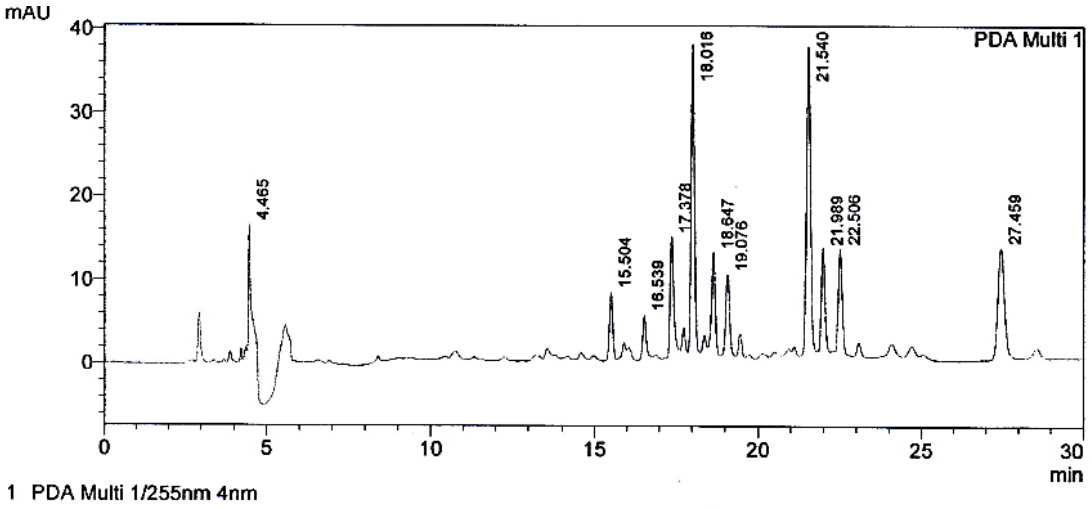


Figure B.10 The chromatogram of phenolic compounds in microcapsules from 95EM with pH adjusted to 4.0

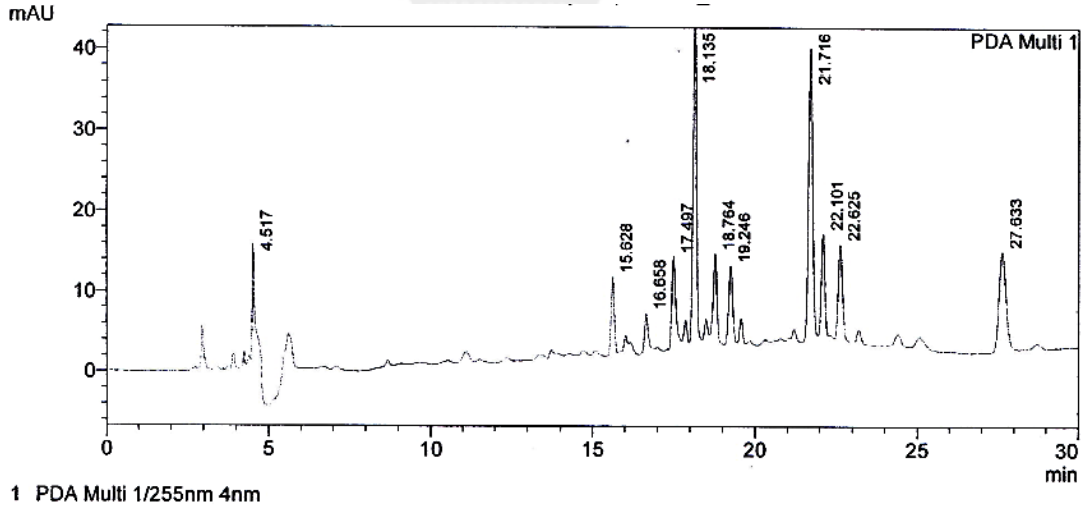


Figure B.11 The chromatogram of phenolic compounds in microcapsules from 95EM with pH adjusted to 4.5

APPENDIX C

Mass spectra of phenolic compounds

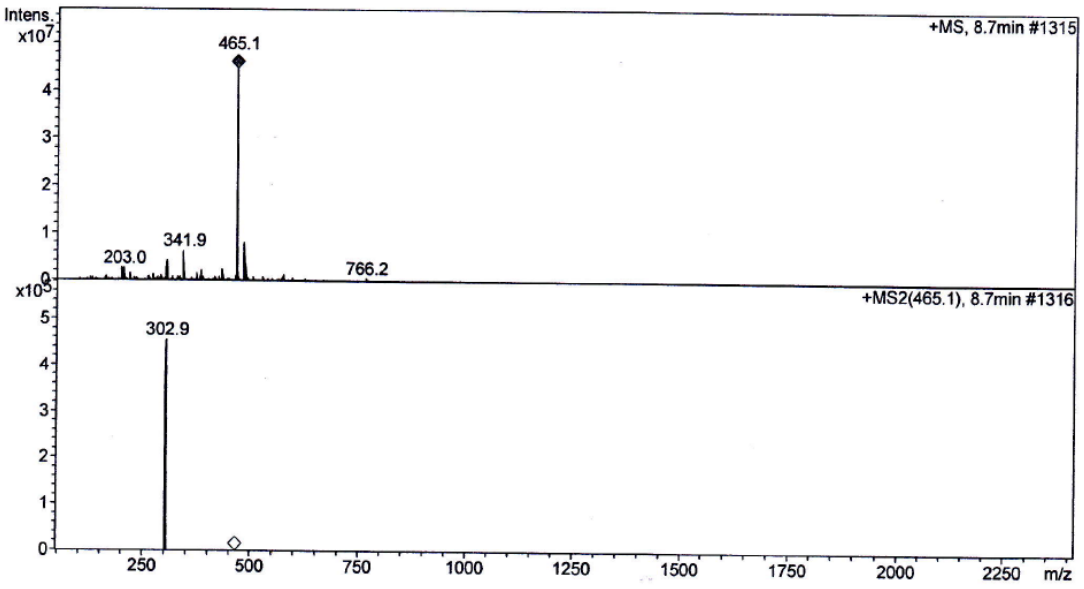


Figure C.1 Mass spectrum of isoquercetin

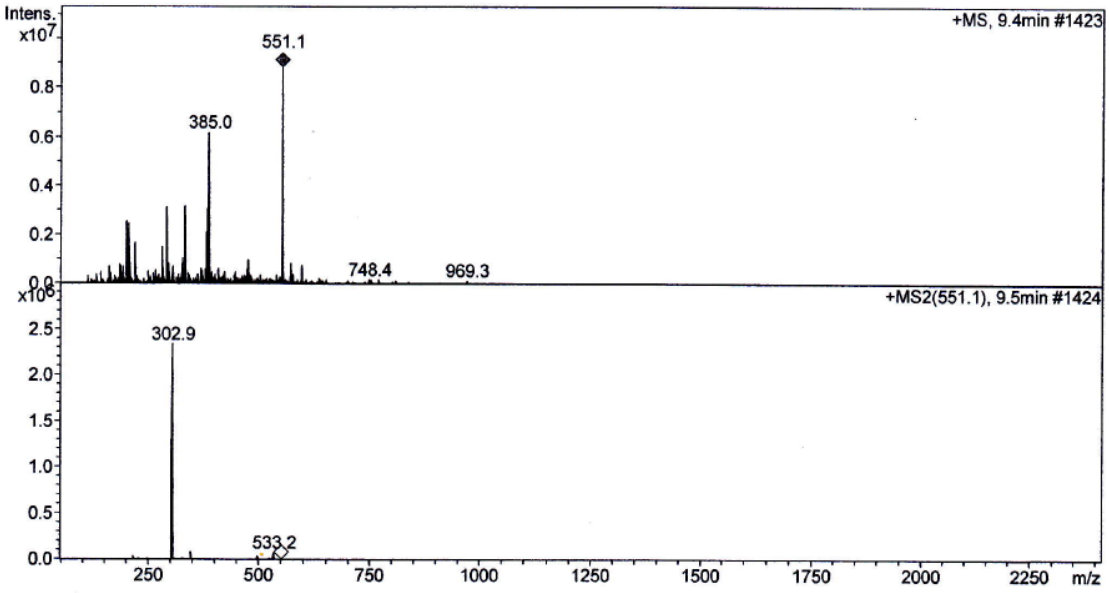


Figure C.2 Mass spectrum of quercetin 3-O-(6″-O-malonyl)-β-D-glucoside

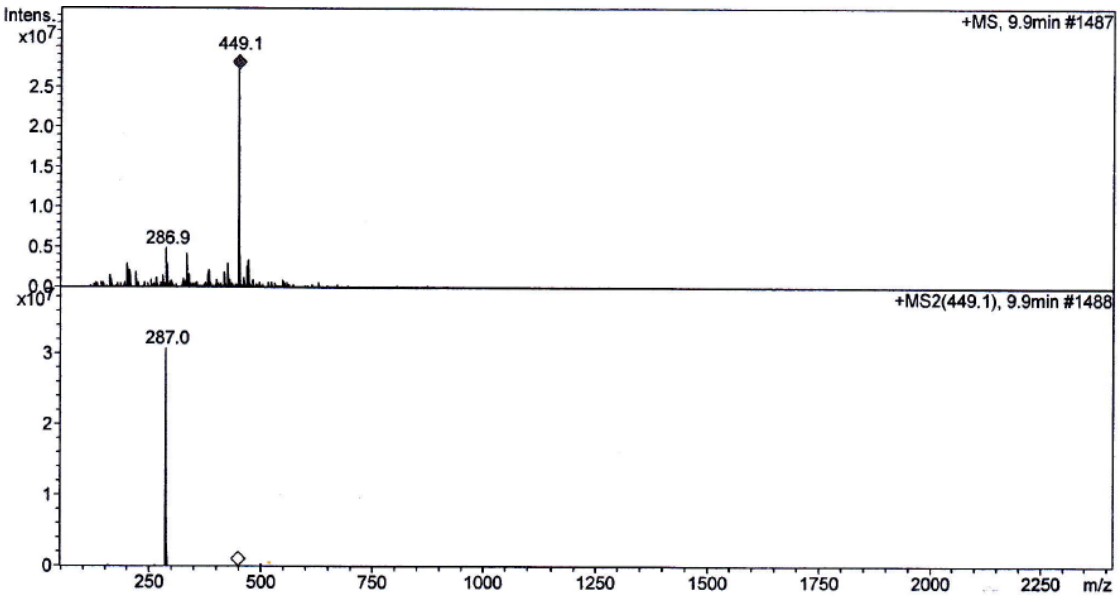


Figure C.3 Mass spectrum of quercitrin

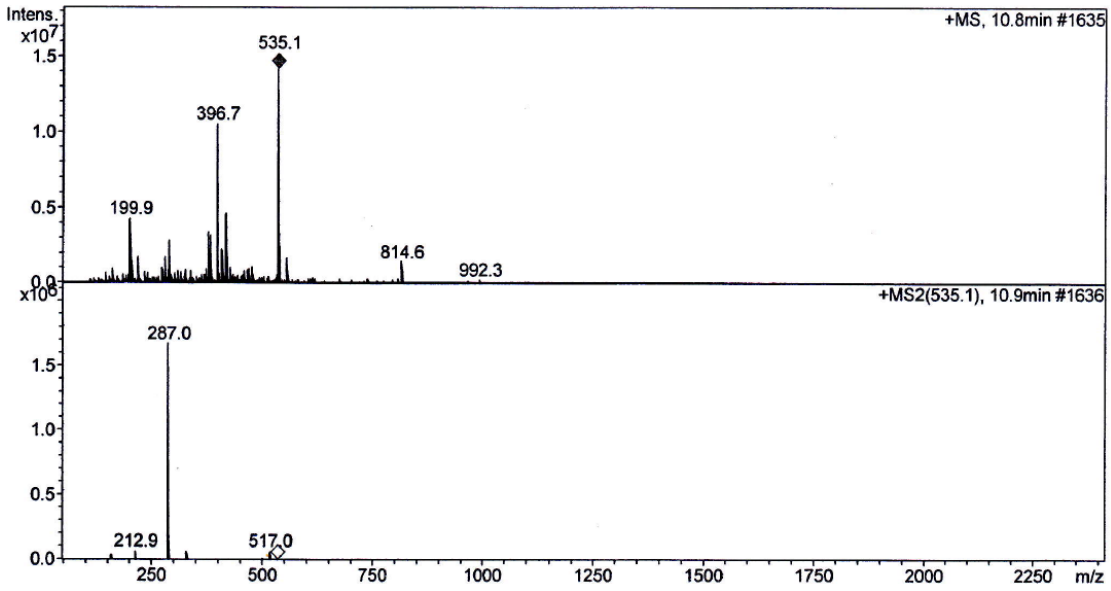
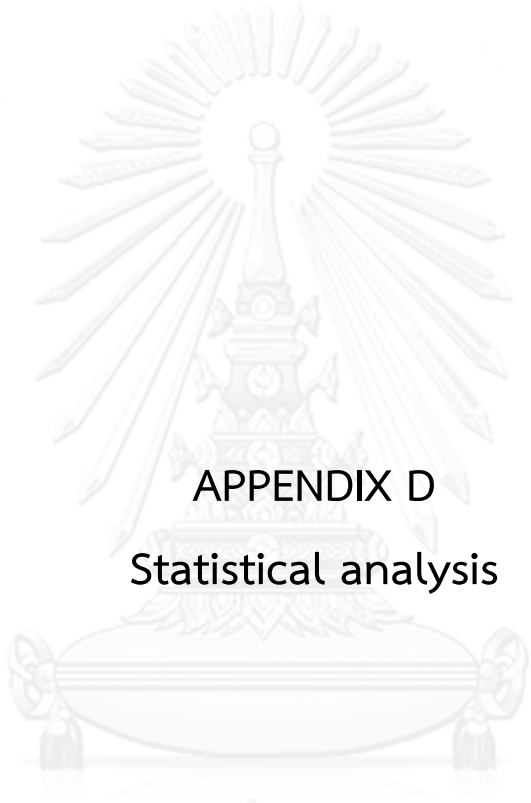


Figure C.4 Mass spectrum of astragalin



APPENDIX D

Statistical analysis

Table D.1 The ANOVA table showing the effect of concentration of ethanol used to extract antioxidant from mulberry leaves on total phenolics content, DPPH radical scavenging activity and FRAP assay at the 95% confidence interval

		Sum of Squares	df	Mean Square	F	Sig.
TPC	Between Groups	12987.039	3	4329.013	155.878	.000
	Within Groups	111.087	4	27.772		
	Total	13098.126	7			
DPPH	Between Groups	9.322	3	3.107	8.322	.034
	Within Groups	1.493	4	.373		
	Total	10.815	7			
FRAP	Between Groups	1888.944	3	629.648	106.597	.000
	Within Groups	23.627	4	5.907		
	Total	1912.571	7			

Table D.2 The ANOVA table showing the effect of concentration of ethanol used to extract antioxidant from mulberry leaves on the amount of rutin and quercetin at the 95% confidence interval

		Sum of Squares	df	Mean Square	F	Sig.
Rutin	Between Groups	.216	3	.072	40.249	.002
	Within Groups	.007	4	.002		
	Total	.223	7			
Quercetin	Between Groups	.000	3	.000	6.627	.050
	Within Groups	.000	4	.000		
	Total	.000	7			

Table D.3 The ANOVA table showing the effect of different core material (60EM and 95EM) on the solubility, encapsulation yield and encapsulation efficiency **of** microcapsules at the 95% confidence interval

		Sum of Squares	df	Mean Square	F	Sig.
Solubility	Between Groups	1.162	1	1.162	.110	.757
	Within Groups	42.359	4	10.590		
	Total	43.521	5			
Yield	Between Groups	40.300	1	40.300	19.203	.012
	Within Groups	8.395	4	2.099		
	Total	48.695	5			
Efficiency	Between Groups	2.369	1	2.369	72.367	.001
	Within Groups	.131	4	.033		
	Total	2.500	5			

Table D.4 The ANOVA table showing the effect of different core material (60EM and 95EM) on total phenolics content, antioxidant activities (by using DPPH radical scavenging activity and FRAP assay) of microcapsules at the 95% confidence interval

		Sum of Squares	df	Mean Square	F	Sig.
TPC	Between Groups	37.350	1	37.350	2.321	.202
	Within Groups	64.365	4	16.091		
	Total	101.715	5			
DPPH	Between Groups	16.467	1	16.467	57.165	.002
	Within Groups	1.152	4	.288		
	Total	17.620	5			
FRAP	Between Groups	138.432	1	138.432	39.444	.003
	Within Groups	14.038	4	3.510		
	Total	152.471	5			

Table D.5 The ANOVA table showing the effect of pH of mixed solution in polymer-polymer interaction process on the solubility, encapsulation yield and encapsulation efficiency of microcapsules at the 95% confidence interval

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Solubility	34.767[a]	2	17.383	4.417	.046
	Yield	1280.191[b]	2	640.096	12.932	.002
	Efficiency	4.553[c]	2	2.276	1.256	.330
Intercept	Solubility	347.114	1	347.114	88.195	.000
	Yield	261.578	1	261.578	5.285	.047
	Efficiency	23.122	1	23.122	12.760	.006
Core	Solubility	33.835	1	33.835	8.597	.017
	Yield	1137.048	1	1137.048	22.971	.001
	Efficiency	4.551	1	4.551	2.512	.147
pH	Solubility	.932	1	.932	.237	.638
	Yield	143.143	1	143.143	2.892	.123
	Efficiency	.002	1	.002	.001	.978
Error	Solubility	35.422	9	3.936		
	Yield	445.487	9	49.499		
	Efficiency	16.309	9	1.812		
Total	Solubility	27617.481	12			
	Yield	22044.726	12			
	Efficiency	1793.060	12			
Corrected Total	Solubility	70.189	11			
	Yield	1725.679	11			
	Efficiency	20.861	11			

a. R Squared = .495 (Adjusted R Squared = .383)

b. R Squared = .742 (Adjusted R Squared = .684)

c. R Squared = .218 (Adjusted R Squared = .045)

Table D.6 The ANOVA table showing the effect of pH of mixed solution in polymer-polymer interaction process on total phenolics content, antioxidant activities (by using DPPH radical scavenging activity and FRAP assay) of microcapsules at the 95% confidence interval

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	TPC	367.126[a]	2	183.563	17.651	.001
	DPPH	108.555[b]	2	54.278	74.879	.000
	FRAP	86.045[c]	2	43.022	95.016	.000
Intercept	TPC	470.898	1	470.898	45.281	.000
	DPPH	46.333	1	46.333	63.919	.000
	FRAP	94.885	1	94.885	209.556	.000
Core	TPC	334.119	1	334.119	32.129	.000
	DPPH	105.910	1	105.910	146.110	.000
	FRAP	85.975	1	85.975	189.877	.000
pH	TPC	33.008	1	33.008	3.174	.109
	DPPH	2.645	1	2.645	3.649	.088
	FRAP	.070	1	.070	.155	.703
Error	TPC	93.594	9	10.399		
	DPPH	6.524	9	.725		
	FRAP	4.075	9	.453		
Total	TPC	44424.870	12			
	DPPH	2039.146	12			
	FRAP	3875.842	12			
Corrected Total	TPC	460.721	11			
	DPPH	115.079	11			
	FRAP	90.120	11			

a. R Squared = .797 (Adjusted R Squared = .752)

b. R Squared = .943 (Adjusted R Squared = .931)

c. R Squared = .955 (Adjusted R Squared = .945)

Table D.7 The ANOVA table showing the effect of pH of mixed solution in polymer-polymer interaction process on the amount of rutin and quercetin in microcapsules at the 95% confidence interval

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	Core	3542.110[a]	2	1771.055	119.945	.000
	pH	.002[b]	2	.001	.006	.994
Intercept	Core	3980.504	1	3980.504	269.581	.000
	pH	6.214	1	6.214	27.998	.000
Rutin	Core	22.979	1	22.979	1.556	.244
	pH	.000	1	.000	.002	.965
Quercetin	Core	291.268	1	291.268	19.726	.002
	pH	.001	1	.001	.006	.941
Error	Core	132.890	9	14.766		
	pH	1.998	9	.222		
Total	Core	75750.000	12			
	pH	194.000	12			
Corrected Total	Core	3675.000	11			
	pH	2.000	11			

a. R Squared = .964 (Adjusted R Squared = .956)

b. R Squared = .001 (Adjusted R Squared = -.221)

Table D.8 The ANOVA table showing the effect of concentration of coating materials (SPI and pectin) in polymer-polymer interaction process on the solubility, encapsulation yield and encapsulation efficiency of microcapsules at the 95% confidence interval

		Sum of Squares	df	Mean Square	F	Sig.
Solubility	Between Groups	7.966	2	3.983	13.906	.030
	Within Groups	.859	3	.286		
	Total	8.826	5			
Yield	Between Groups	496.952	2	248.476	115.353	.001
	Within Groups	6.462	3	2.154		
	Total	503.414	5			
Efficiency	Between Groups	22.074	2	11.037	15.417	.026
	Within Groups	2.148	3	.716		
	Total	24.222	5			

Table D.9 The ANOVA table showing the effect of concentration of coating materials (SPI and pectin) in polymer-polymer interaction process on total phenolics content, antioxidant activities (by using DPPH radical scavenging activity and FRAP assay) of microcapsule at the 95% confidence interval

		Sum of Squares	df	Mean Square	F	Sig.
TPC	Between Groups	167.637	2	83.819	18.471	.021
	Within Groups	13.613	3	4.538		
	Total	181.250	5			
DPPH	Between Groups	52.998	2	26.499	203.553	.001
	Within Groups	.391	3	.130		
	Total	53.389	5			
FRAP	Between Groups	30.257	2	15.129	21.019	.017
	Within Groups	2.159	3	.720		
	Total	32.417	5			

APPENDIX E

Specification of coating materials

Appendix E.1: The specification of SPI

Soy protein isolate (SPI) GS 5100

Technical data:

Appearance	:	Light-yellow powder
Moisture	:	≤ 7%
Crude protein	:	≥ 90%
NSI	:	≥ 90%
pH value	:	7.0 + 1
Ash	:	≤ 6.0%
Fat	:	≤ 1%
Particle (through 100 mesh)	:	min 97%

Biological data:

Total bacteria	:	≤ 20000/g
Coliforms	:	Negative
E. coli	:	Negative
Salmonella	:	Negative
Yeast and mold	:	100/g

Typical minerals (mg/100g product)

Sodium	:	800-1400
Potassium	:	200-500
Calcium	:	50-150
Phosphorus	:	700-1100
Iron	:	10-15
Magnesium	:	50-110

Typical amino acids (g/100g product)

Aspatic acid	:	9.49
Threonine	:	3.39
Serine	:	3.94
Glutamic acid	:	14.11
Glycine	:	3.36
Alanine	:	3.37
Valine	:	3.58
Methionine	:	1.08
Isoleucine	:	3.53
Leucine	:	6.74
Tyrosine	:	3.07
Phynylalanine	:	4.37
Lysine	:	5.12
Histidine	:	1.83
Arginine	:	6.04
Proline	:	4.77
Tryptophan	:	0.75
Cystine	:	1.66

Heavy metals (mg/kg product)

Lead	:	< 0.01
Cadmium	:	< 0.01
Mercury	:	< 0.005
Arsenic	:	< 0.01
Manganese	:	< 20
Selenium	:	< 0.3
Zinc	:	20-50
Copper	:	< 20

Vitamins (mg/100g product)

Vitamin A	:	10 IU/100g
Vitamin B1	:	0.09
Vitamin B2	:	0.02
Vitamin C	:	0

Appendix E.2: The specification of pectin

The Genu Pectin type LM-104 AS-BG

Description

- Low methoxyl pectin (DE 28%)
- White to light brown/tan powder.
- Gelling agent, thickening agent and stabilizer in food

Chemical parameters

- Loss on drying % : 5.9
- pH (1% solution) : 4.4
- Ca-test 80 ppm, 25%, g TA-XT2 : 21.7
- Ca-test 160 ppm, 25%, g TA-XT2 : 30.8

Microbiological parameters

- Total plate count : < 500cfu/g
- Yeast & moulds : < 10 cfu/g
- Enterobacteriacae : absent (1 g)
- Salmonella : absent (25 g)

VITA

Miss Methavee Peanparkdee was born on May 24, 1989 in Bangkok, Thailand. She obtained a Bachelor of Food science and Technology, Faculty of Agro-Industry Kasetsart University in academic year 2010. In 2011, she enrolled the master degree program at Department of Food Technology, Faculty of Science, Chulalongkorn University.

She presented her research in a poster at The International Symposium on Agri-Foods for Health and Wealth (AFHW2013) in the topic “Encapsulation of extract from mulberry Morus alba L. leaves by polymer-polymer interactions” at Golden Tulip Sovereign Hotel, Bangkok, Thailand during August 5-6 2013. On 4 December 2013, she presented as oral presenter at The 2nd Southeast Asia Symposium on Quality Management in Postharvest Systems (SEAsia2013), Vientiane, Laos in the topic “Effects of pH and concentration of coating materials on antioxidant activity of mulberry leaf extract microcapsules”