Valorization of the Sea Buckthorn (*Hippophae rhamnoides)* Pomace for Potential Food Application

December 2019

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A Dissertation Submitted to

the School of the Integrative and Global Majors,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy in Food Innovation

(Doctoral Program in Life Science Innovation)

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Abstract

In this study, we investigated oil-in-water (O/W) emulsion-assisted extraction of β-sitosterol and carotenoids from sea buckthorn pomace. We compared this new green extraction method with conventional extraction using organic solvents and oils. The objective of this study was to evaluate the efficiency of these different extraction systems on the yields of bioactive compounds from plant-based materials, and to determine the optimum extraction conditions for maximum extraction yield. Our results indicated that O/W emulsions, prepared without emulsifier using a high-pressure homogenizer had the highest extraction capability for β-sitosterol and carotenoids, as compared with the other extraction systems. The optimum conditions were achieved at 65 °C for 1 h extraction, using emulsifier-free soybean O/W emulsions. Under these conditions, the extracted amounts were up to 32.0 mg/g dw (dry weight) for β-sitosterol and 1.44 mg/g dw for total carotenoids. The obtained compounds were relatively stable at 5 °C and 25 °C for up to 28 days of storage. This emulsion-based extraction method is promising for the extraction of β-sitosterol and carotenoids that can be further applied into dietary nutritional of supplements and fortified food

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List of Abbreviations

MCT	Medium-chain triglyceride oil
O/W	Oil-in-water
W/O	Water-in-oil
DW	Dry based weight
LDL	Low density lipoprotein
RSH	Rotary-stator homogenization
HPH	High-pressure homogenization
ANOVA	Analysis of variance
UV	Ultraviolet-visible
v/v	Volume/Volume
w/w	Weight/Weight

Chapter 1

General introduction

1.1 Background

Food processing industries produce large amounts of by-products including pomace which contains rich valuable compounds. By-products and wastes are partly used for animal feed or fertilizer but are mainly discarded. Besides that, these by-products can be directly and indirectly used in development of new food applications (Badjona *et al.*, 2019).

Recently, researchers are interested in utilizing fruits and vegetables waste for functional food and pharmaceutical applications due to their nutrients composition. Many studies reported some valuable compounds extracted from fruit and vegetable wastes: for instance, carotenoids, dietary fiber and protein found in banana peels (Subagio, Morita, & Sawada, 1996). Also, Ajila *et al.* (2010) reported that mango peels are rich in nutrient compounds including vitamins, fibers and phenolic compounds.

Sea buckthorn (*Hippophae rhamnoides*) fruits has attracted growing interest in recent years, because of their rich nutrient compounds. The berries are rich in lipophilic compounds such as β-carotene, lycopene, tocopherol and phytosterols (Beveridge *et al.*, 1999). These compounds have numerous biological properties, including antioxidant and antidiabetic activities, and the ability to reduce the risks of cardiovascular diseases (Stobdan *et al.*, 2013).

Processing of sea buckthorn berries generates high amount of pomace that are not efficiently valorized. The pomace obtained after their juice extraction is considered as by-product and is primarily used as animal feed. This pomace by-product is rich in various bioactive compounds, such as phytosterols and carotenoids, that have great potential to be used for the development of natural nutritional foods and supplements.

In addition, people may consider diet related health problems, in which they prefer to use natural ingredients than synthetic compounds (Schieber *et al.*, 2001).

1.2 Sea buckthorn fruits

The sea buckthorn is one of the precious fruits having good health benefit. It is used in the food, pharmaceutical and cosmetics industries.

Sea buckthorn fruits are distributed in Asia and Europe such as China, Mongolia, India, Pakistan and Romania. In Asia, sea buckthorn berries are growing wildly in total 740,000 ha and cultivating area is 300,000 ha. Mongolia is at the 5th place by its natural and cultivated sea buckthorn resource in the world and 2nd place in Asia. It is wild growing of 13,000 ha and cultivating area of 6,000 ha in Mongolia. There are cultivating 10 varieties of sea buckthorn fruit cultivated in 6 provinces including Selenge, Bulgan, Zavkhan, Govi-Altai, Khovd and Uvs (Gonchigsumlaa, 2016; Nawaz *et al.*, 2019).

Sea buckthorn fruits are medium sized belonging Elaeagnaceae family. This fruit has 6 species and 12 subspecies. Sea buckthorn fruit has 0.5–6 m height shrub and leaves are 3–8 cm long. Their berries are yellow to orange color. Sea buckthorn fruits start shoots from seeds around 4–5 years and plant life is 7–8 years (Ozturk *et al.*, 2018). The whole berries contain many different types of nutrients and bioactive compounds which are vitamins, minerals, carotenoids, flavonoids, phenolic compounds and essential oils. It also contains high antioxidant compounds such as tocopherol and flavonoids (Stobdan *et al.*, 2013).

1.2.1 Sea buckthorn fruits historic applications

Sea buckthorn, which is one of the most famous tradition fruits, was used by the ancient Greek people as a fodder for their horses to promote weight gain and enhance

shiny pretty coat. It is believed that the latin name of sea buckthorn fruit is "*Hippophae*" which literally could be translated into "shiny horse" due to it ability to improve the brightness and lumonisty of horse's coat. Also, sea buckthorn fruit has been known for widely distributed and being used for centuries in Europe and Asian regions due to its effective pharmaceutical properties. It was reported that sea buckthorn fruits showed an efficient therapeutic effect through lowering fever, reducing inflamation, counteracting the toxicity, healing abscesses, cleaning lungs, and treating cold and digestive system (Chaurasia & Ahmed, 2005). Furthermore, some Chinese and ancient Tibetan medical literatures documented using sea buckthorn fruit for treating hepatic diseases, circulatroy disorders, ischematic heart diseases, and metabolic disorders and gaecological diseases (Ballabh & Chaurasia, 2007). While in Russia, the extracted oil from sea buckthorn fruits was used to treat dermatological problems such as dermatitis, chronic dermatoses, eczema and lupus disease. In Mongolia this fruit has been used in many traditional treatment mostly for gastric and cold.

1.2.2 Chemical composition of seabuckthorn fruit

Sea buckthorn fruits rich in nutrients contain different types of vitamins, minerals (24 types), phenolic compounds, flavonoids, phytosterols, carotenoids, antioxidant compounds (flavonoids, phenolic acids and tocopherols), 18 kinds of amino acids, essential oils, protein, carbohydrates and volatile compounds.

All parts of sea buckthorn contain valuable compounds, but most of leaves and berries are more widely use for therapeutic medicine ingredients. The leaves contain many nutrients including sterols, carotenoids, flavonoids (0.31–2.1 mg/100 g dw), tannin and protein (20.6 g/100 g dw leaves) (Stobdan *et al.*, 2013).

Sea buckthorn fruit is rich in vitamin C (53-3,909 mg/100g dw fruit) which is almost 12 times higher than oranges, and in vitamin A 3 times higher than carrots. Furthermore, the fruit contains some other vitamins including vitamin E (160 mg/100g dw fruit), B_6, B_2, B_3 and B_5 (Tables 1-1,1-2, and 1-3). The sea buckthorn fruit contains 20–87% of moisture, 1.8% of ash and 5.6–22.7% of soluble sugar (Bal *et al.*, 2011). The fruits have some special aroma and sour taste reason that they contain some esters and organic acid (major malic and quinic). Sea buckthorn seed oil contains higher phytosterol (23 g/kg seed oil) than soybean, sunflower and olive oil. However, the amount of nutrients are different, depening on the sea buckthorn fruits variety, origin and harvesting time.

1.2.3 Phytosterols

Phytosterols are natural plant sterols and they have same structure as a cholesterol. Major phytosterols are β-sitosterol, stigmasterol, campesterol and brassicasterol. There are around 250 phytosterols discovered in the world. The main sources of sterols are vegetable oils, cereals, margarine and grains. Sterols contain in vegetable oils around 1-5 g/kg such as sesame oil, corn oil and rice bran oils. The sterols contain 350-1200 mg/kg fresh weight in the cereals, 116–228 mg/kg in the fruits, 60–279 mg/kg in the berries. The sterols are important for control of cholesterol serum levels and their dietary useful for treatment of hypercholesterolemia (Menedez-Carreño *et al.*, 2008).

Recently, researchers interest increases to use β-sitosterol for dietary. Studies reported that around 1.5-3 g phytosterol consumption can decrease the LDL cholesterol by 10-15 % (Hendriks *et al*., 1999). Decreasing cholesterol causes to prevent heart and cardiovascular diseases. Many countries using phytosterols for milk products, soy based drinks , salad dressing, rye bread and oils.

The sea buckthorn fruit contains quiet high compared to soybean oil. The phytosterols contain in oil 330–355 mg/kg, seed oil 23 mg/g oil, fresh pulp and peel 29 mg/g of oil (Table 1-4). Sistosterol is the main sterol of sea buckthorn, contain around 61–83% of seed and soft-tissue sterols (Stobdan *et al.*, 2013). Sterols are easy to be oxidized by lights, air and enzyme reaction because of their double-bond structure.

1.2.4 Carotenoids

Carotenoids are natural red, orange, yellow pigments and they can present foods color. They are well documented for their beneficial biological activities, such as strengthening the immune system, and reducing the risk of cancer and heart diseases. Researchers discovered total 700 carotenoids and many studies approved their benefit for the health. Around 50 types of carotenoids can be pro-vitamin A.

Carotenoids are divided into 2 groups which are xantophylls (lutein and zeaxanthin) having oxygen as functional group, and carotenes (lycopene, α-carotene and β-carotene) having hydrocarbon chain. Many fruits and vegetables contain different types of carotenoids that can be good effect for the human health. The carotenoids have good antioxidant acivity that can be support immune system.

Carotenoids are one of the major compound in sea buckthorn fruits. Berries color orange can be explained, being rich in carotenoids. The sea buckthorn oil contains high amount of carotenoids about 3.14–21.3 mg/g oil, pulp and fruit oils 10–90 mg/g oil (Bal *et al.*, 2011) as shown in Table 1-4. The main carotenoids in the sea buckthorn berries are zeaxanthin, β-carotene and lutein. Zeaxanthin role in the prevention of age-related macular degeneration the leading cause of blindness. Zeaxanthin is mostly used for feed additive and colorant in food (Sajilata *et al.*, 2008).

1.3 Sea buckthorn fruits production and valorization of pomace

Demands for sea buckthorn fruits products is increasing year by year on the international market. According to the report, China is the biggest sea buckthorn berries producing and exporting country in the Asia. They harvest 8.5 million sea buckthorn berries, exporting 1.4 billion US$ of products (Vilariño *et al.*, 2017). Mongolia is the second sea buckthorn berries harvesting country in Asia. There are total 37 sea buckthorn processing factories which have the capacity to process over 5,200 tons of sea buckthorn annually. A total of 1.08 million US$ worth of sea buckthorn oil, fruit juice and dried leaves were exported to Russia, Japan and Singapore in 2015 from Mongolia. Also, they are using sea buckthorn berries for cosmetic products and exporting abroad. Sea buckthorn extracts used in pharmaceutical and cosmetic industries such as hair oil, shampoo and cream. Furthermore many industries producing jam, jelly, wine, dietary supplements and food additives. Sea buckthorn fruit has peels, pulp, seed and leaves. Average weight of 1 berry about 0.7–0.8 g dw that contains 23% of seed , 68% of pulp and 9% of peels (Beveridge *et al.*, 1999).

In addition, seed contains 7–8% oil and pulp contains 29–48% of oil (Stobdan *et al.*, 2013). Main sea buckthorn berries products are juice and oil. During juice processing technology 55% was seperated to liquid phase, called as sea buckthorn berries juice. The 45% solid part was eliminated from berries, called as pomace. The pomace contains uncracked seeds that will be separated for extraction of seed oil. Around 1 ton berries are used to produce 600 L juice, 26–50 kg of oil and 350–374 kg pomace (Figure 1-1).

Particularly, this juice pomace is rich in nutrients and some studies reported that it contains flavonoids, phenolic compounds, antioxidant compounds, vitamins, phytosterols and carotenoids. Hungary researchers extracted sterols, carotenoids, ursolic acid and tocopherol from sea buckthorn pomace using supercritical carbon dioxide extraction. They recovered β-sitosterol 2.50–4.25 mg/(g dry pomace), 0.20–1.60 ursolic acid mg/(g dry pomace), 0.04–0.18 carotenoid mg/(g dry pomace), 0.35–0.42 mg/(g dry pomace) total tocopherol from sea buckthorn pomace (Cossuta *et al.*, 2007).

Furthermore, another study reported that extraction of β-carotene from sea buckthorn by-products using solvents and vegetable oils. They obtained that 0.5 mg/(g dry pomace) β-carotene from hexane extraction, 0.4 mg/(g dry pomace) and 0.3 mg/(g dry pomace) β-carotene from soybean oil and rapeseed oil extraction (Li *et al.*, 2013). Besides that, some studies analyzed total phenolic content (303 mg/(g extract)) and palmitoleic acid (Li *et al.*, 2013; Klaas & Meurer, 2004). All these resuls shows sea buckthorn pomace contain valuable compounds.

1.4 Extraction methods for bioactive compounds

Extraction is one of the methods to separate bioactive compounds of plant and animal tissue from the inactive components by using solvents in standard extraction procedures. Food industries use different types of extraction methods to extract valuable compounds from by-products and wastes. Many researchers are innovating new extraction techniques, especially to extract valuable compounds. However, more consideration to improve extraction efficiency with less loss of bioactive compounds, seems to be needed. Some studies reported extraction of valuable compounds using

different methods such as soxhlet extraction, microwave assisted extraction, ultrahigh pressure-assisted, ultrasound extraction, pulsed electric field extraction, subcritical carbon dioxide extraction, subcritical water extraction, subcritical fluid extraction, hot water extraction, high thermal liquid extraction, enzyme-assisted extraction (Zhang *et al.*, 2018).

One of the common extraction methods is solvent extraction. When solid material and solvent interact with each other, the components dissolve to the solvent from solid material. The main principle is mass transfer process between the sample and solvent. There is equilibrium point in which mass transfer stopped from dissolving component to the solvent; however, mass transfer of the dissolving components related to their solubility in the solvent. The mass transfer normally is increasing with the solvent heating temperature. During solvent extraction, one of the considerations is that solvent should be used more than the sample amount (Warkoyo & Saati, 2012). There is a simple method for using solvent extraction, such as shaking and stirring. In this method, the speed and temperature were need to be set up in the extraction techniques. Many industries using organic solvent for extraction; however, trend is changing to use green extraction method due to organic solvents toxicity and environmental hazards (Yara-Varón *et al.*, 2017). Nowadays researchers interested to use “green extraction” technology (Figure 1-2) which is safety, less toxicity, eco-friendly and less cost extraction. Recently researchers using vegetable oils and emulsions to extract some valuable compounds instead of organic solvents (Roohinejad *et al.*, 2014).

1.5 Emulsions

The emulsions include many different foods such as beverages, cream, infant formula ice cream and mayonnaise. In addition, emulsions are part of medicine and functional food ingredients because of improving delievery of bioactive compounds.

An emulsion consists of at dispersed phase, a continuous phase and emulsifier, in a homogeneous mixture prepared with high and low energy. The dispersed phase is the substances which is involving make droplets in the emulsion, the continuous phase which is around the dispersed phase. Generally, emulsions divided 2 type which is O/W and W/O emulsion (Figure 1-3). In each O/W emulsion, a continuous phase is water and a dispersed phase is oil (e.g., soybean oil and rapeseed oil). Emulsion stability and droplet sizes can be affected by storage period and visual appearance of some food products.

Oil and water are not soluble each other, in which emulsifiers are important component in emulsion stability. The emulsifiers have molecules which can be attached hydrophobic and hydrophilic parts in the emulsions. The industries produce food-grade emulsifiers from some raw materials such as fats, sugar, oils and organic acids. Also there is some synthetic emulsifiers with different physicochemical properties. Different types of emulsifier, such as water soluble, oil soluble and intermediate, are available. Depending on the different emulsion types industries need to choose right emulsifiers to produce their products.

Many industries (mostly food industries) use mechanical techniques which is high speed and pressure blender and homogenizer to making emulsions. They mostly use high shear mixer, colloid mill, and high pressure homogenizer. High pressure

homogenizer commonly used in food industry makes fine emulsions, because it is more useful for not high viscosity products which need small droplet size.

1.6 Research objectives

Valorization of fruits and vegetables by-products is increasing economic value for the food industry along with addressing food and nutrition insecurity. Sea buckthorn fruit is one of the rich nutrients fruit and processing of sea buckthorn into juice, which results in generation of 45% of sea buckthorn pomace. This pomace good source of valuable bioactive compounds such as carotenoids and phytosterols. The aim of this thesis is to develop the valuable products that could be generated from sea buckthorn pomace by utilizing bioactive compounds including carotenoids and sterols which can be good functional ingredient for dietary and supplement drink.

The objectives of this study are as follows:

- To investigate the efficiency of extraction systems using various organic solvents and oils, O/W emulsions on the recovery of β-sitosterol and carotenoids from sea buckthorn pomace,
- To analyze the effect of extraction conditions on the obtained yields of β-sitosterol and carotenoids,
- To evaluate the long-term chemical stability of β-sitosterol and carotenoids at various temperatures.

Table 1-1: Chemical composition of sea buckthorn fruits (Bal *et al*., 2011)

Chemical composition	**Sea buckthorn berries**		
	Berries/Juice	**Seed and oil**	**Pulp from berries**
Moisture (%)	52.4	22.4	84.9-97.6
Ash (%)	1.8	-	-
TSS (°Brix)	10.7-13.2	-	26.2-27.9
Oil content (%)	-	9.69-20.2	-
Flavonoids (mg/100g)	1000	-	-
Vitamin C	509	-	223.2
Vitamin E	216	64-93	481
Vitamin K	110-230	-	109.8-230
Fe	4-15	0.36-0.647	0.703-1.127
Mg	150-240	1.8-3.4	0.62-1.92
Cu	-	0.023-0.097	0.09-1.33
Zn	-	0.497-2.83	0.817-2.5
As	-	0.063-0.145	0.06-0.2
Na	6.9	0.05-0.49	0.47-0.63
K	62.2	9.33-13.42	10.12-14.84
P	-	0.61-0.69	-
Ca	67.1	-	-

Table 1-2: Phenolic compounds content of sea buckthorn fruits (Stobdan *et al*., 2013)

	Phenolic compounds (µg/g of dry plant material)			
	Seed	**Leaves**	**Pulp**	**Whole fruit**
Rutin	-	313.8	300.2	136
Quercetin-3-*O*-galactoside	23.9	280.8	194.7	108.2
Quercetin	22.9	-	-	-
Myricetin	1.26	136.8	50.1	17.3
Kaemferol	-	45.8	12.6	3.7
Isorhamnetin	2.22	120.0	61.5	41.2

Table 1-3: Fatty acids content of sea buckthorn fruits (Molar %) (Stobdan *et al*., 2013)

Fatty acid	Berry		Seed	
	ssp.*sinensis*	**ssp.*mongolica***	**ssp.*sinensis***	**ssp.*mongolica***
16:00	16.4	21.1	14.1	17.0
16:1n-7	15.9	21.5	<0.5	<0.5
18:00	1.6	2.4	3.3	6.0
18:1n-9	18.5	4.3	22.2	10.0
18:1n-7	9.2	8.5	4.7	4.3
18:2n-6	22.2	32.1	42.7	47.7
18:3n-3	16.2	10.1	13.0	14.8
Total	100	100	100	100

Table 1-4: Bioactive compounds content of sea buckthorn fruits (Stobdan *et al*., 2013; Pop *et al*., 2013)

Bioactive compounds	**Sea buckthorn berries *(Hipophae rhamnoides)***		
	Fresh berry	**Seed**	**Fresh pulp/peel**
Total sterol (mg/kg)	385	1369	278.8
Campesterol (mg/kg)	7	40	3.8
Stigmastadienol (mg/kg)	-	-	3.3
Sitosterol (mg/kg)	256	931	198
Stigmastanol (mg/kg)	2	50	1.3
Other sterols (mg/kg)	120	-	-
Total carotenoids (mg/100g dw)	96.7	22.2	527.8
Zeaxanthin (mg/100g dw)	42.4	-	-
Lutein (mg/100g dw)	30	-	-
β–carotene (mg/100g dw)	10.9	-	-
Lycopene (mg/100g dw)	3	-	-
Other carotenoids (mg/100g dw)	10.4	-	-

Figure 1-1: Sea buckthorn fruits juice and oil processing sheme

(Beveridge, 1999)

Figure 1-2: Green extraction technology

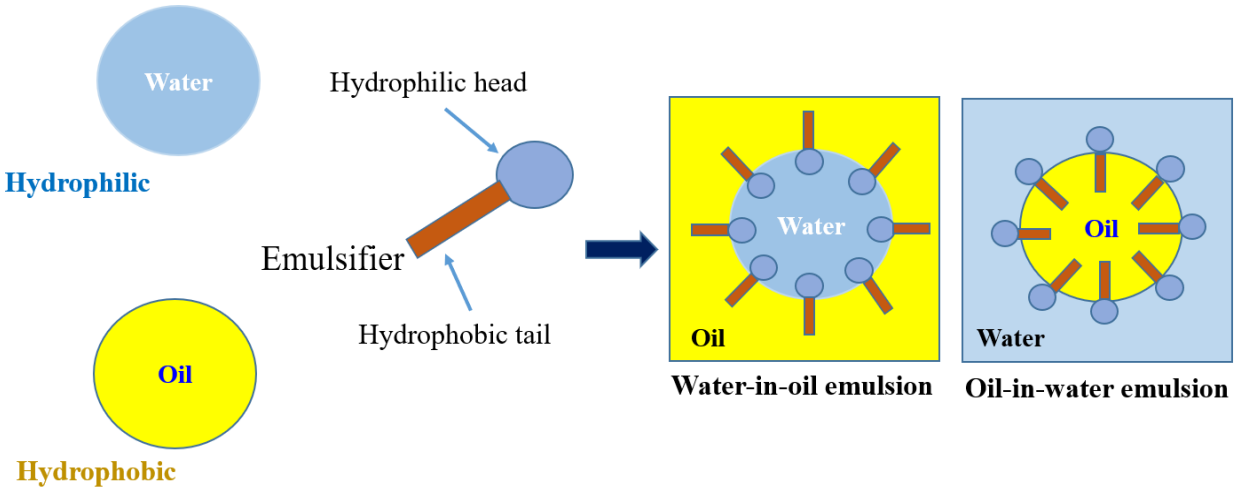


Figure 1-3: Different types of emulsions

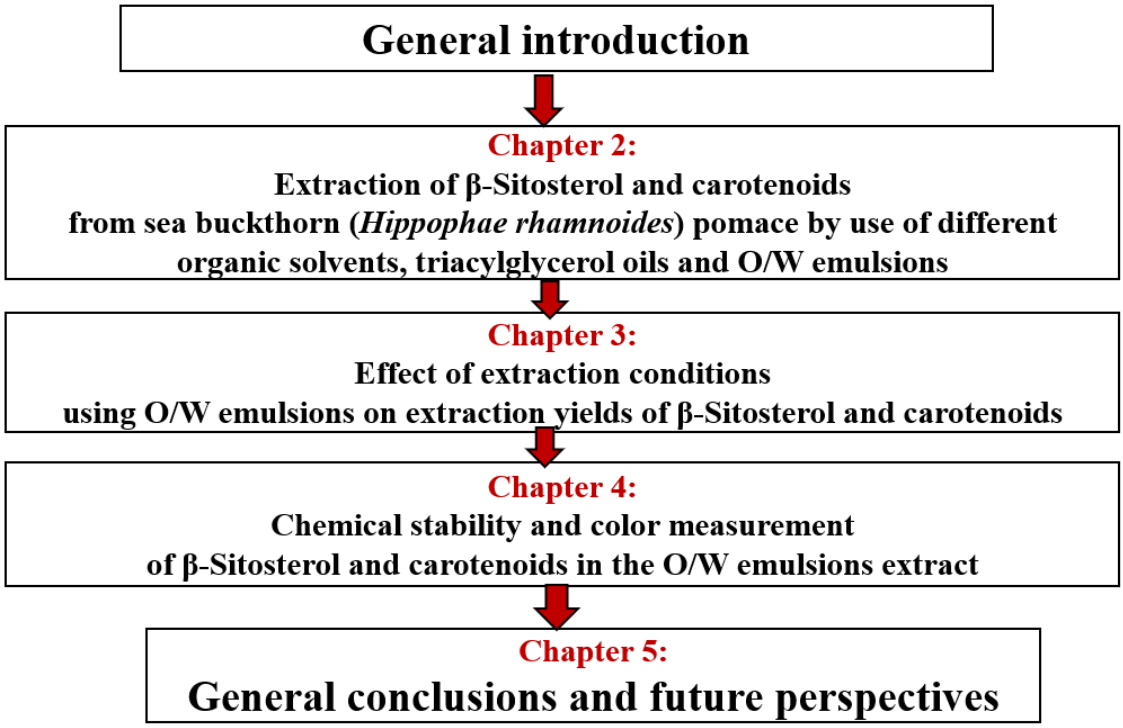


Figure 1-4: Research outline of this PhD thesis

Chapter 2

Extraction of β-sitosterol and carotenoids from sea buckthorn (*Hippophae rhamnoides*) pomace by use of different organic solvents, triacylglycerol oils and O/W emulsions

2.1 Introduction

Processing of sea buckthorn berries generates large amounts of by-products that are not efficiently valorized. The pomace obtained after juice extraction is considered as a waste product and is primarily used as animal feed. This pomace is rich in multiple bioactive compounds, such as phytosterols and carotenoids.

Recently, phytosterols have attracted much attention because of their multiple bioactive properties (Hicks, 2001; Jones & Vanstone, 1997). Previous studies have reported the extraction of β-sitosterol from sea buckthorn pomace using vegetable oils or supercritical carbon dioxide (Chemat, Périno-Issartier, Loucif, Elmaataoui, & Mason, 2012; Cossuta, Simandi, Hohmann, Doleschall, & Keve, 2007). Others studies have also evaluated the efficiency of organic solvents in the extraction of tocopherols, carotenoids, and sitosterol from this plant-based material (Klaas & Meurer, 2004). The extraction in all these studies was performed using the conventional methods for extract of bioactive compounds (Sabir *et al.*, 2005). However, despite their efficiency in some cases, the organic solvent extraction methods require large quantities of the solvents, are costly, environmentally hazardous, and require expensive disposal procedures (Mustafa *et al.*, 2012). On the other hand, oil-assisted extraction is generally accepted, but can have limited efficiency because of the low diffusion properties of vegetable oils, even at high temperatures (Goula *et al.*, 2016).

Emulsions are homogeneous mixtures of two or more immiscible liquids, with one of the liquids being dispersed in the other liquid(s) in the form of small spherical droplets (Gadhave & Waghmare, 2014). Previous studies reported the application of microemulsion for the extraction of bioactive compounds from solid plant-based materials such as carrot and tomato pomace (Roohinejad, Oey, Everett, & Niven,

2014; Amiri-Rigi & Abbasi, 2019; Amiri-Rigi, Abbasi, & Scanlon, 2016). Roohinejad *et al.* (2014) reported that the extraction of β-carotene using microemulsions was much more efficient than extraction using hexane or glycerol monocaprylocaprate oil. In all these studies, the authors used high concentrations of synthetic emulsifiers to increase the stability of prepared emulsions. However, in recent years, there have been increasing concerns about the use of synthetic emulsifiers for food and cosmetic applications (Singh *et al.*, 2005). Therefore, it is essential to reduce their use in food and pharmaceutical products, for increased acceptability by general consumers.

The objective of this chapter was to investigate the efficiency of extraction systems using various organic solvents and oils on the yields of β-sitosterol and total carotenoids from sea buckthorn pomace. O/W emulsion-assisted extraction was investigated as a green extraction method and compared with solvent extraction systems.

2.2 Materials and methods

2.2.1. Organic solvent-assisted extraction

A 2 g sample of dried pomace was mixed with 20 mL of a given solvent and stirred at 25 °C for 24 h. The suspensions were sonicated for 1 h, and then centrifuged at 9,100 x *g* for 1 h to remove the undissolved particles. The supernatant was finally filtered (PTFE-0.45 μm), and the solvent was evaporated using a rotary evaporator (Eyela EVP-1100, Tokyo Rikakikai Co., Ltd., Tokyo) at 35 °C.

2.2.2 Oil-assisted extraction

For oil-assisted extraction, the solid-to-liquid ratio and extraction parameters were similar to those described in chapter 2.2.1. The supernatants were directly used for analysis without evaporation, because of their composition.

2.2.3 O/W emulsion-assisted extraction

O/W emulsions were prepared by homogenizing 20 mL of the oil phase (refined soybean or rapeseed oil) with 80 mL of an aqueous phase, containing 1% (w/w) Tween 20, unless stated otherwise. Coarse emulsions were initially prepared using a rotor-stator homogenizer (Polytron, PT-3000 Kinematica-AG, Littace, Switzerland) at 7,000 rpm for 5 min. Fine emulsions were subsequently prepared by high-pressure homogenization of coarse emulsions (NanoVater, NV200, Yoshida Kikai Co., Ltd., Nagoya, Japan) at 100 MPa for 4 passes. Prepared emulsions (100 mL) were then mixed with 2 g of dried pomace and stirred at 750 rpm at different temperatures (25, 50, 65, 75, and 80 °C), and for different extraction time (1, 2, and 5 h). The suspensions were sonicated for 1 h at 25 °C, and then centrifuged at 9,100 x *g* for 1 h to remove the undissolved particles.

2.2.4 β-Sitosterol and carotenoids analysis

The β-sitosterol content was determined following the method of (Daksha, Jaywant, Bhagyashree, & Subodh, 2010). Briefly, the organic solvent extracts were re-dissolved in 10 mL of chloroform, following which, 3 mL of the solution was mixed with 2 mL of Liberman-Burchard reagent (0.5 mL of sulfuric acid dissolved in 10 mL of acetic anhydride) and incubated in the dark for 15 min. The absorbance was then measured at 640 nm using a UV spectrophotometer (V-530, Jasco Corporation, Tokyo,

Japan). In the case of oil and O/W emulsion extraction, 1 g of the supernatant was mixed with 10 mL of chloroform and analyzed using the same method as described before.

Carotenoids were determined directly at 450 nm using a UV spectrophotometer. In the case of oil and O/W emulsion extract, 200 μg of the supernatant was mixed with 5 mL of chloroform, and then measured at the same wavelength as mentioned before.

2.2.5 Calculation of β-sitosterol and carotenoid extraction yields, contents and total yields

The content (%) and extraction yields (mg/g dw), total yields (%) of β-sitosterol and carotenoid from dried sea buckthorn pomace, were determined using equations 2-1, 2-2 and 2-3 respectively, as follows:

❑ Extraction yield of bioactive compound

$$\text{Extraction yield, mg/g dw} = \frac{\text{β-sitosterol or carotenoids weight in the extract (mg)}}{\text{Dried pomace weight (g)}} \quad (2\text{-}1)$$

❑ The percent of a specified compound in an impure sample

$$\text{Compound content in extract (\%)} = \frac{\text{Targeted compound weight (g)}}{\text{Recovered extract weight (g)}} \times 100 \quad (2\text{-}2)$$

❑ The solvent efficiency to extract specific components from the original material

$$\text{Total yield (\%)} = \frac{\text{Recovered extract weight (g)}}{\text{Dried pomace weight (g)}} \times 100 \quad (2\text{-}3)$$

2.2.6 Measurement of droplet size and size distribution

The droplet size and size distribution of the prepared emulsions were measured using a laser diffraction particle size analyzer (LS 13 320, Beckman Coulter, Inc.,

Fullerton, USA). The refractive index used for water was 1.33, and that for soybean and rapeseed oils was 1.47.

The average droplet diameter values were reported as volume mean diameter ($d_{4,3}$):

$$d_{4,3} = \sum n_i d_i^4 / \sum n_i d_i^3 \quad (2\text{-}4)$$

where d_i is the diameter and n_i is the number of droplets having diameter d_i.

2.2.7 Statistical analysis

All experiments were repeated at least triplicate for per sample. The analysis of variance (ANOVA) was used to compare the extraction yield under different treatment conditions at a 95% confidence level (p <0.05) using Statistix 8.1 software (Tallahassee, USA).

2.3 Results and Discussion

2.3.1 Organic solvent-assisted extraction

The β-sitosterol yield and content in extracts, obtained using various organic solvents with different dielectric constants, are shown in Figure 2-1a. Overall, 50% (v/v) aqueous-ethanol resulted in the lowest extraction yield, whereas extraction with other organic solvents resulted in comparatively similar yields. The yield and content of β-sitosterol were in the range of 5.45–8.27 mg/g dw and 3.02%–6.03% (w/w), respectively. The highest yield of β-sitosterol was obtained by hexane. The carotenoids yield and content for organic solvent extracts are shown in Figure 2-1b. The yield and content of carotenoids were in the range of 0.69–1.12 mg/g dw and 0.46%–0.75% (w/w), respectively.

The highest yield of carotenoids was obtained for acetone and ethyl acetate extraction, whereas chloroform was a less efficient solvent. The lowest yield of carotenoids was obtained for 50% (v/v) aqueous-ethanolic extract. Figure 2-2 shows total yield of extract. There was no difference between organic solvents extract except hexane extract. Hexane extraction obtained lower total yield and other. The ethanol 50 % organic solvents extract was shown yellow as a color and other organic solvents extract were in the same orange color (Figure 2-3).

This study findings are similar to those of a previous study on corn silk waste, wherein hexane was more efficient for β-sitosterol extraction than acetone and ethyl acetate (Zhang *et al.*, 2017). In addition, another study reported that the sterol yield sugarcane was increased by reducing the solvent polarity, which is in good agreement with this study findings (Luo *et al.*, 2014). Wei, Wang, Liu, & Wang (2010) found that the solubility of werity was in the following order: n-hexane < ethanol < acetone < ethyl acetate. Therefore, the differences observed in β-sitosterol yields across the different organic solvents used in this study could be due to the difference in solvent polarity, which plays an important role in the extraction of sterols. Ethanol is a polar protic solvent with a dielectric constant of 24, whereas acetone, ethyl acetate, chloroform, and hexane have lower dielectric constants (about 21, 6, 4.81, and 1.88, respectively). Therefore, the use of solvents with low dielectric constant is recommended for the extraction of β-sitosterol from sea buckthorn pomace.

The results of carotenoid extraction from sea buckthorn pomace by organic solvents in this study accordance with those of Sachindra, Bhaskar, & Mahendrakar (2006). The authors found that the efficiency of carotenoid recovery from shrimp waste was in the following order: acetone > ethyl acetate > ethanol > hexane. Strati &

Oreopolou (2011) also reported that acetone and ethyl acetate were more efficient than other solvents for carotenoid extraction from tomato waste. Accordingly, Warkoyo & Saati (2012) observed that acetone was more suitable than ethanol for carotenoid extraction from *Eucheuma cottonii* seaweed. In the present study, it was found out that acetone and ethyl acetate were more efficient for carotenoids extraction than all the other organic solvents used in this study. Cossuta *et al.* (2007) reported that higher yields of β-sitosterol and carotenoids can be obtained by organic solvents extraction than with supercritical fluid extraction. Their extraction yields were about 2.0–4.25 mg/g dw for β-sitosterol and 0.04–0.18 mg/g dw for carotenoids, while those in this study were 5.45–8.27 mg/g dw for β-sitosterol and 0.69–1.12 mg/g dw for carotenoids.

In general, acetone and ethyl acetate extraction exhibited the highest carotenoids yield among all the organic solvents. This could be due to their polar and water-miscible properties, which may allow for a better extraction efficiency of bioactive compounds from wet plant-based materials. Therefore, in the extraction of wet samples, the use of non-polar solvents may not be advisable, as their penetration through the hydrophobic matrix surrounding the pigment is limited. The pomace used in this study was obtained in hydrated form and also contains mainly polar carotenoids such as zeaxanthin and lutein which may have resulted in better extraction yields with acetone and ethyl acetate.

2.3.2 Oil-assisted extraction

For many years, food and cosmetic industries have used oils to extract natural compounds from plant-based materials, as a green and environmentally friendly option for organic solvent extraction. Oils also have a good dissolving power for hydrophobic bioactive compounds and can prevent their oxidation during extraction, which provides

additional advantages for food and pharmaceutical industries. In this section was used different oils to extract β-sitosterol and carotenoids from sea buckthorn pomace, and compared their efficiency with the other extraction systems.

As shown in Figure 2-4, the yield of β-sitosterol extracted using different oils was in the range of 5.19–12.5 mg/g dw, while that of carotenoids was in the range of 1.03–1.15 mg/g dw. Soybean and rapeseed oils resulted in higher yields of β-sitosterol than other oils and caprylic acid. However, they all provided a similar extraction yield for carotenoids. Oils extract color was orange and there was no color difference (Figure 2-5).

Sachindra & Mahendrakar (2005) reported that soybean oil and MCT oil result in similar extraction yields of carotenoids from shrimp waste. The better extraction yields of β-sitosterol using soybean and rapeseed oils than that of MCT oil can be explained by their long-chain fatty acids composition, which provides increased hydrophobic properties and oxidative stability compared with the medium chain fatty acids found in MCT (Crozier, 1988; Odle, 1997).

The results showed that oils were able to extract a slightly higher amount of β-sitosterol from sea buckthorn pomace than organic solvents, but the yield of carotenoids was almost similar to the previously obtained results. This is in accordance with the previously reported findings, where a good extraction efficiency of carotenoids from pumpkin was observed, using both ethyl acetate and virgin coconut oil (Norshazila *et al.*, 2017). Extraction using oils may be more acceptable than organic solvent extraction, and is recommended due to their environment-friendly and ability to prevent oxidation.

2.3.3 O/W emulsion-assisted extraction

Emulsions are homogeneous mixtures of two or more immiscible liquids, which can provide particular properties during extraction. In this section, based on oils extraction results the soybean and rapeseed oils were selected for O/W emulsion-assisted extraction. As shown in Figure 2-6, the highest yield of β-sitosterol (41.9 mg/g dw) was obtained using soybean O/W emulsions, whereas rapeseed oil-based emulsions provided the highest yield of carotenoids (1.73 mg/g dw). Interestingly, the O/W emulsions extraction was obtained 3 times higher β-sitosterol compared to organic solvents and oils extraction. Furthermore, for both bioactive compounds, the addition of emulsifier resulted in a lower extraction efficiency by emulsions. The yield of β-sitosterol, for example, decreased from 41.9 mg/g dw to 22.7 mg/g dw, following the addition of Tween 20 during emulsion preparation. The addition of emulsifiers during emulsion formulation may suppress the partitioning of bioactive compounds from the plant matrix to the oil system during extraction, by creating a barrier around the oil droplets. This is the first systematic evaluation of the effect of emulsifier addition on the extraction efficiency of O/W emulsions. Therefore, the addition of emulsifier during emulsion preparation should be further investigated in the future. O/W emulsions extract had orange color (Figure 2-7).

Next, the effect of emulsion preparation method was evaluated on the extraction yields of β-sitosterol and carotenoid from sea buckthorn pomace, to analyze the effect of emulsion properties on extraction efficiency (Figure 2-8). The emulsions prepared by high-pressure homogenization (HPH) had a smaller droplet size ($d_{4,3}$: 2.77 μm) than those prepared by rotary-stator homogenization (RSH) ($d_{4,3}$: 61.9 μm). The difference in droplet size can be explained by the high energy density input of HPH compared with RSH, which resulted in smaller particle size and more stable emulsions. The HPH

emulsions, which have smaller droplet sizes, provided better yields of β-sitosterol and carotenoid than RSH emulsions (Figure 2-9).

Overall, the results presented in this study revealed that O/W emulsions were more efficient than conventional solvent extraction systems for the extraction of bioactive compounds from plant-based materials. Emulsions with smaller droplet size were found to be better than those with large droplets. Roohinejad *et al.* (2014) reported that more carotenoids were obtained using microemulsions than those with hexane or glycerol monocaprylocaprate oil. In their study, the authors used 20% Tween 80 which was high concentration of emulsifier, resulting in expansion of the cells due to high osmotic pressure and improvement in the overall extraction efficiency. However, emulsions with 1% Tween 20 of lower emulsifier concentration could extract high amount of bioactive compounds. Besides this, emulsifier-free O/W emulsion was found to give better extraction yields than stable emulsions with emulsifier.

The mechanism of the emulsion-assisted extraction may be considered as follows: a) the presence of a high volume of water enhances the swelling of plant cell in the pomace b) diffusion of oil droplets through the cell membrane, and c) dissolution/ solubilization of the bioactive in the oil. Probably the less interaction between bioactive compounds and oil droplets covered by emulsifiers may occur due to their barrier, compared with emulsifier-free emulsion extraction system (Figure 2-11). In present study new finding is that emulsifier-free O/W emulsion system was so suitable for the extraction of bioactive, such as carotenoids and β-sitosterol.

Hildebrand solubility theory is known theory for solubilization or extraction systems (Hansen, 2000). The Hildebrand solubility parameter can be used to determine the energy needed to generate space in the molecule to fit other molecules.

The solubility parameter "distance" Ra, between β-sitosterol and carotenoid against organic solvents and oils were calculated based on their respective partial solubility parameter components following equation (Hansen, 2000) :

$$(Ra)^2 = 4(\delta D_2 - \delta D_1)^2 + (\delta P_2 - \delta P_1)^2 + (\delta H_2 - \delta H_1)^2 \qquad (2\text{-}5)$$

$\delta_{D1,}$ δ_{D2}- Dispersion solubility parameter of material 1 and 2

δ_{P1}, δ_{P2}- Polar solubility parameter of material 1 and 2

$\delta_{H1,}$ δ_{H2}- Hydrogen bonding solubility parameter of material 1 and 2

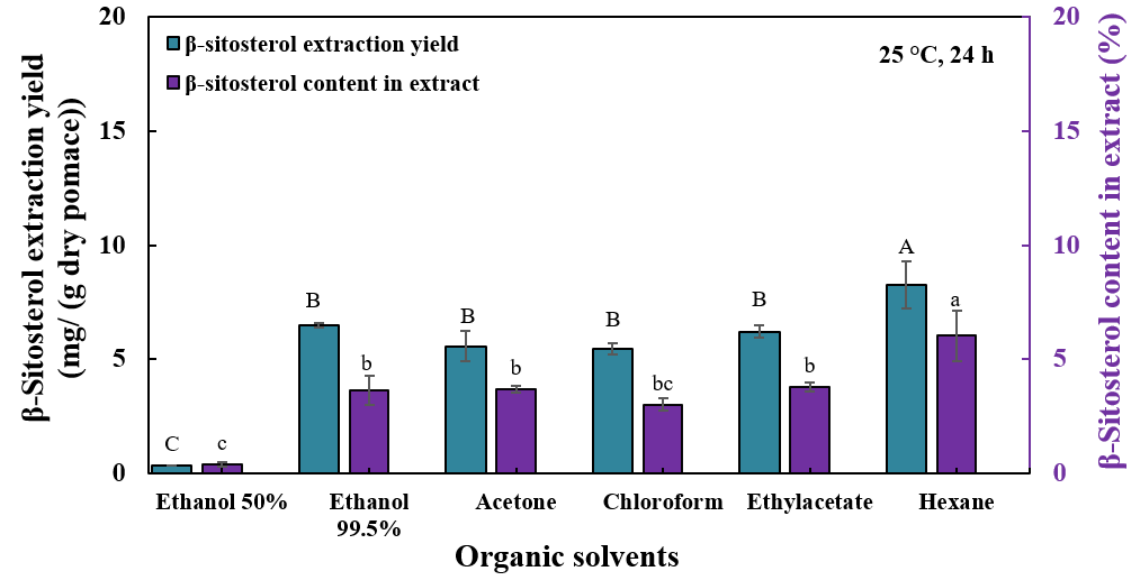
Table 2-1 shows that Hildebrand solubility and Hansen parametres of organic solvents, oils and cholesterol and β-carotene. For the calculation, cholesterol and β-carotene was used instead of β-sitosterol and carotenoid. Figure 2-10 shows yield of β-sitosterol and carotenoids were plotted against Ra, showing that negative correlation was observed (correlation coefficients are around 0.3-0.6). The extraction yields of bioactive compounds increase, if Ra values increase. Compared with the one-phase solvent extraction systems, two-phase O/W emulsion systems showed significantly higher values, which may suggest that dynamics through the cell walls are also important factor, such as diffusion and dissolution equilibrium.

2.4. Conclusions

In this chapter, new green extraction technology which is O/W emulsion-assisted extraction was proposed and investigated. The O/W emulsions were more efficient than conventional solvent extraction and oils systems for the extraction of β-sitosterol and carotenoids. The addition of emulsifier effected to decrease of extraction yields. Emulsifier-free O/W emulsions extraction obtained higher yields of bio active compounds. The emulsions prepared by high-pressure homogenization (HPH) had a

smaller droplet size ($d_{4,3}$: 2.77 μm) than those prepared by rotary-stator homogenization (RSH) ($d_{4,3}$: 61.9 μm). The next chapter effect of extraction conditions experiments was carried out using emulsifier-free soybean O/W emulsions.

(a)



(b)

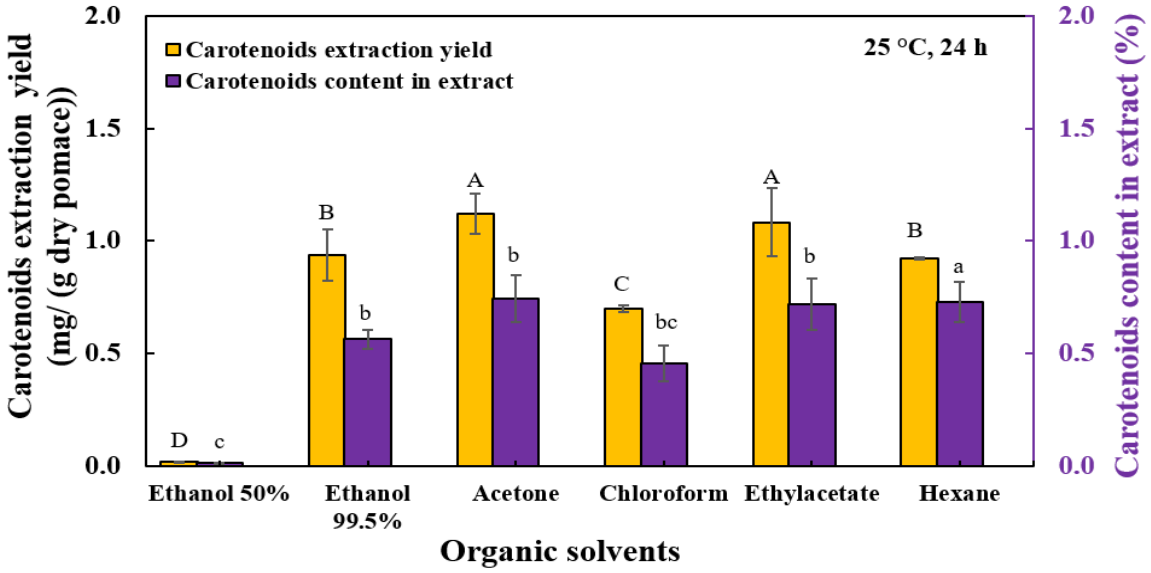


Figure 2-1: The extraction yields and extract content of (a) β–sitosterol (b) carotenoids from sea buckthorn pomace in different organic solvent.

*Different letters on the graphics indicate significant differences (ANOVA test $p<0.05$).

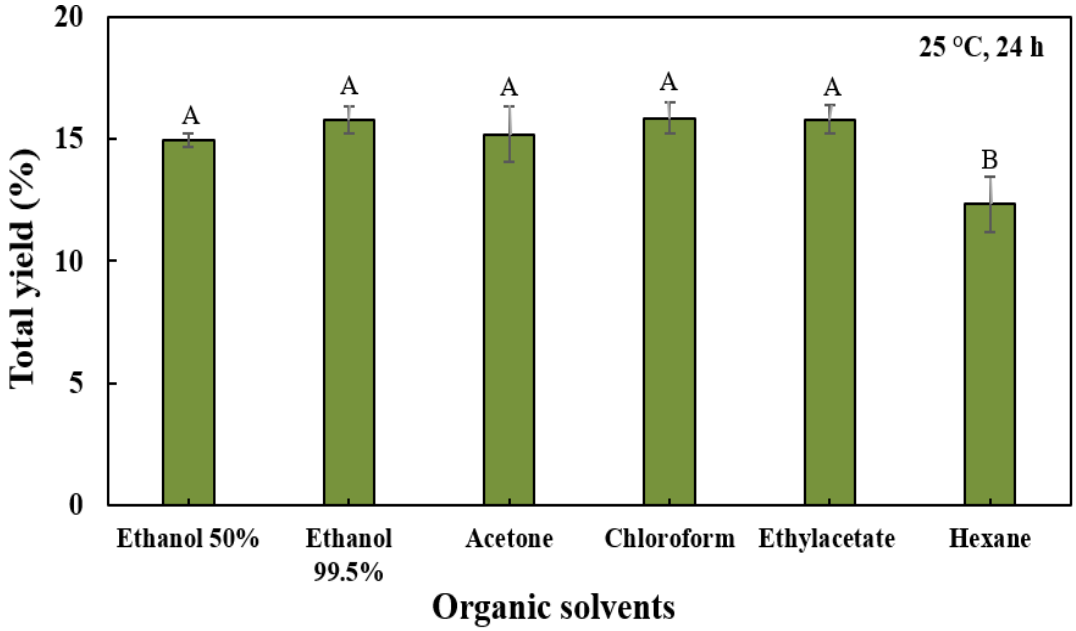


Figure 2-2: Total yields of recovered weight using different organic solvents from sea buckthorn pomace.

*Different letters on the graphics indicate significant differences (ANOVA test $p<0.05$).

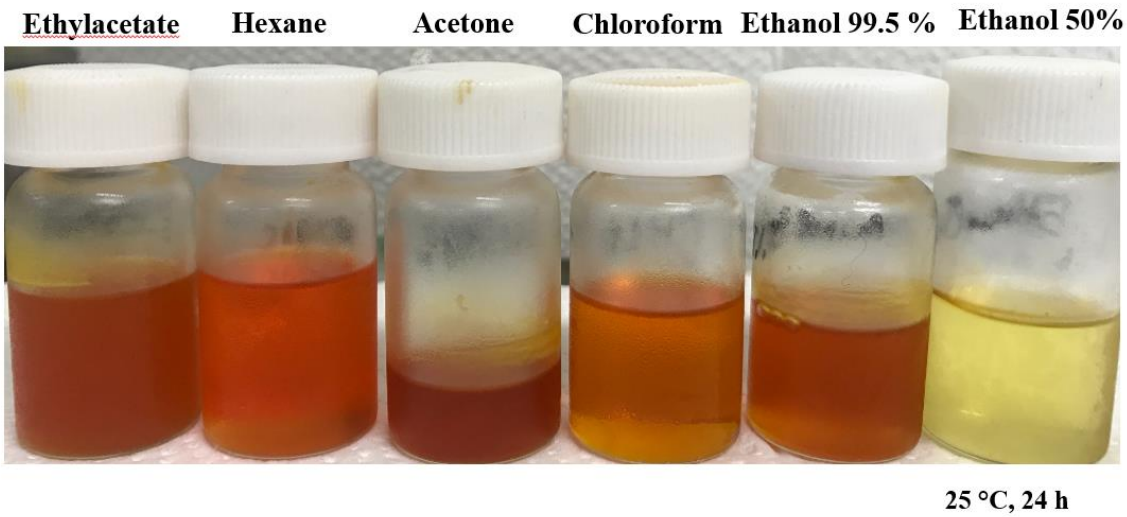
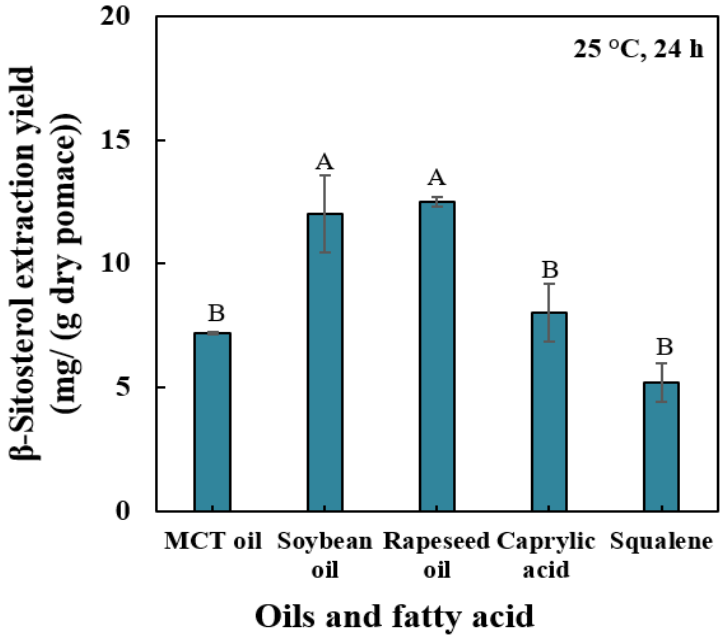


Figure 2-3: Appearance of sea buckthorn pomace different organic solvents extract

(a)



(b)

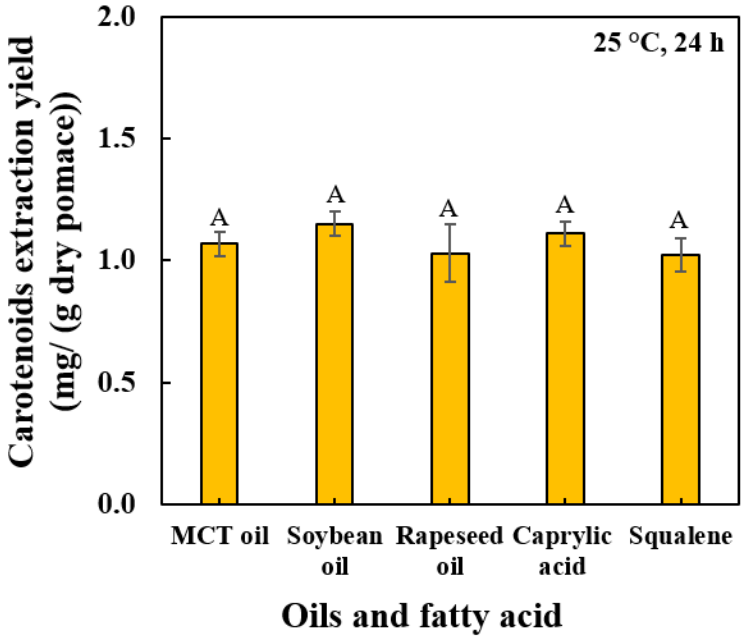


Figure 2-4: Extraction yield of (a) β–sitosterol and (b) carotenoids in sea buckthorn pomace extracts, prepared using different oils and fatty acids

*Different letters on the graphics indicate significant differences (ANOVA test $p<0.05$).

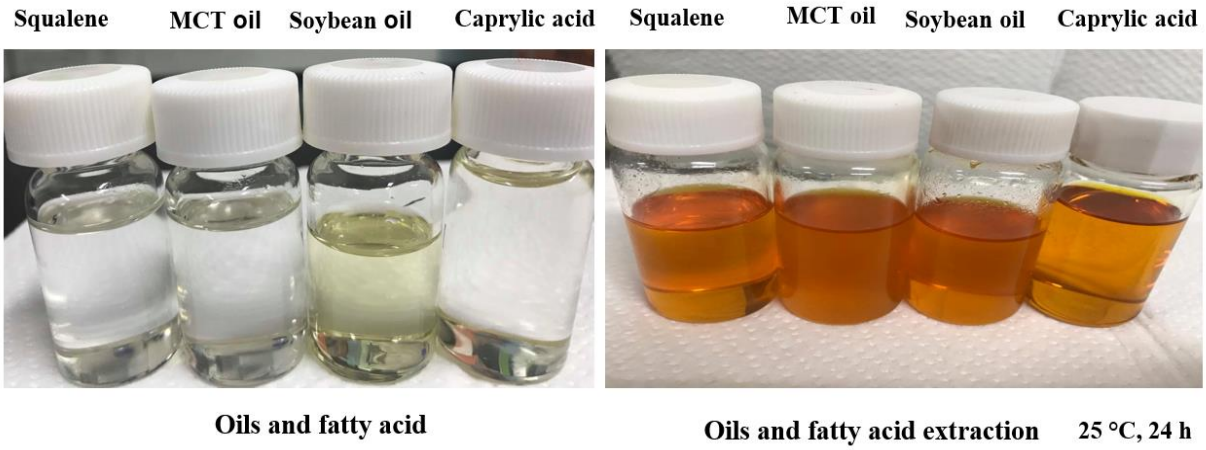
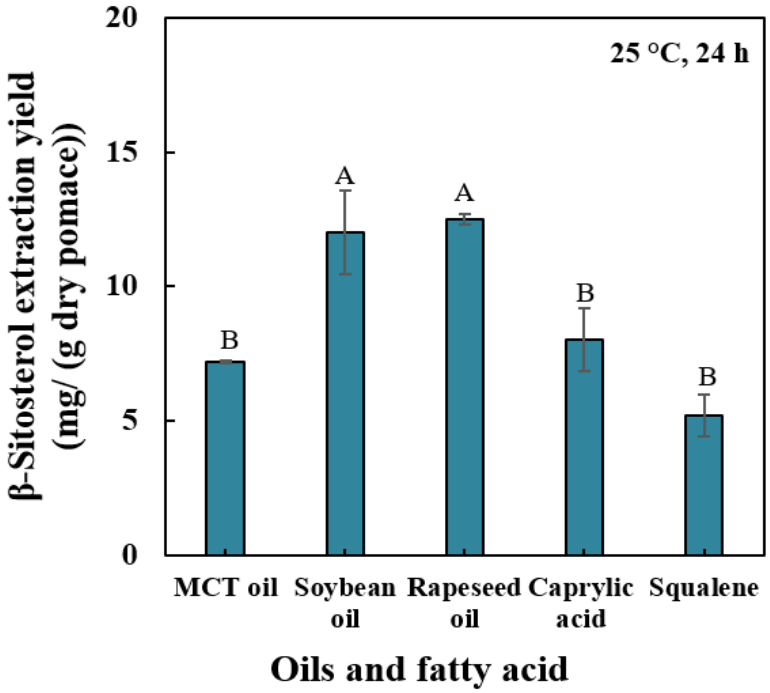


Figure 2-5: Appereance of sea buckthorn pomace different oils extract

(a)



(b)

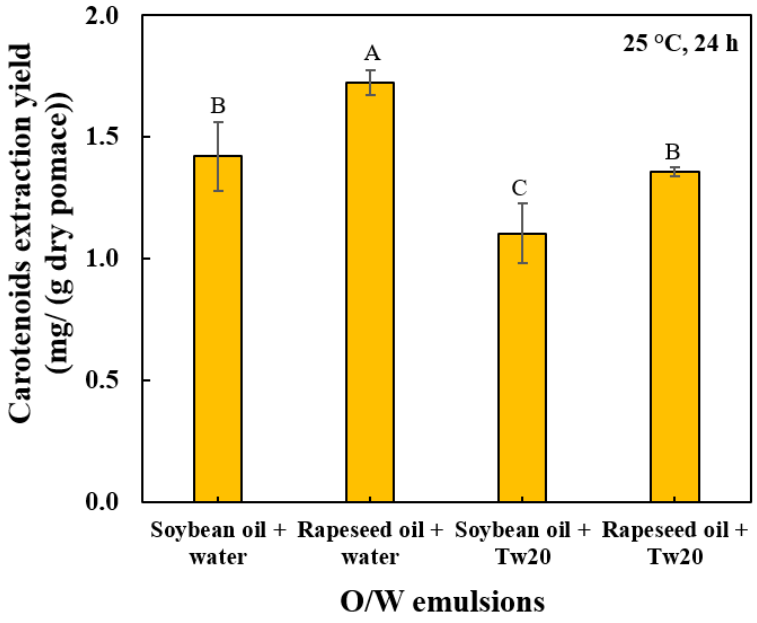


Figure 2-6: Extraction yield of (a) β–sitosterol and (b) carotenoids in sea buckthorn pomace extracts, prepared using O/W emulsions

*Different letters on the graphics indicate significant differences (ANOVA test $p<0.05$).

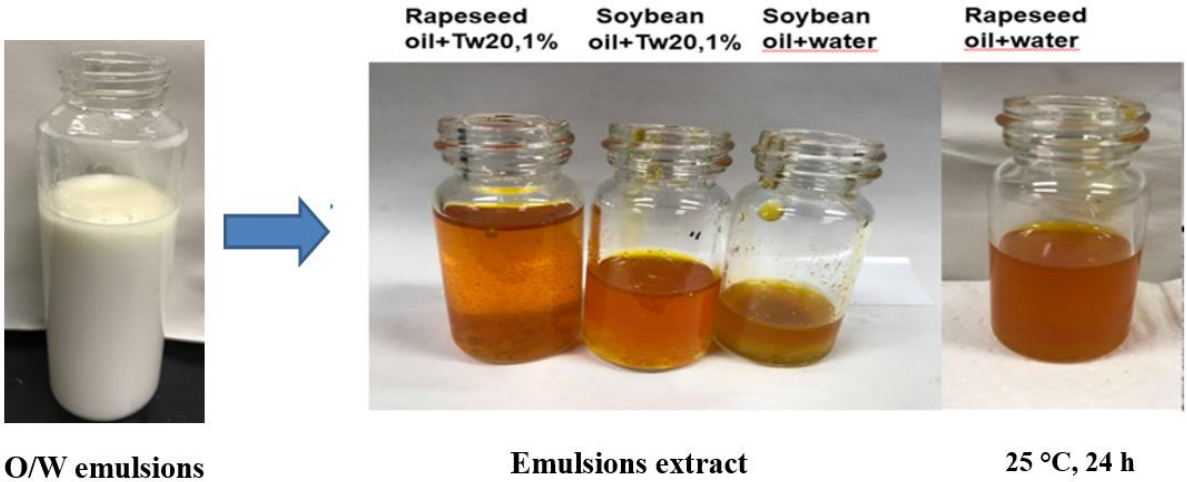


Figure 2-7: Appereance of O/W emulsions-assisted extract

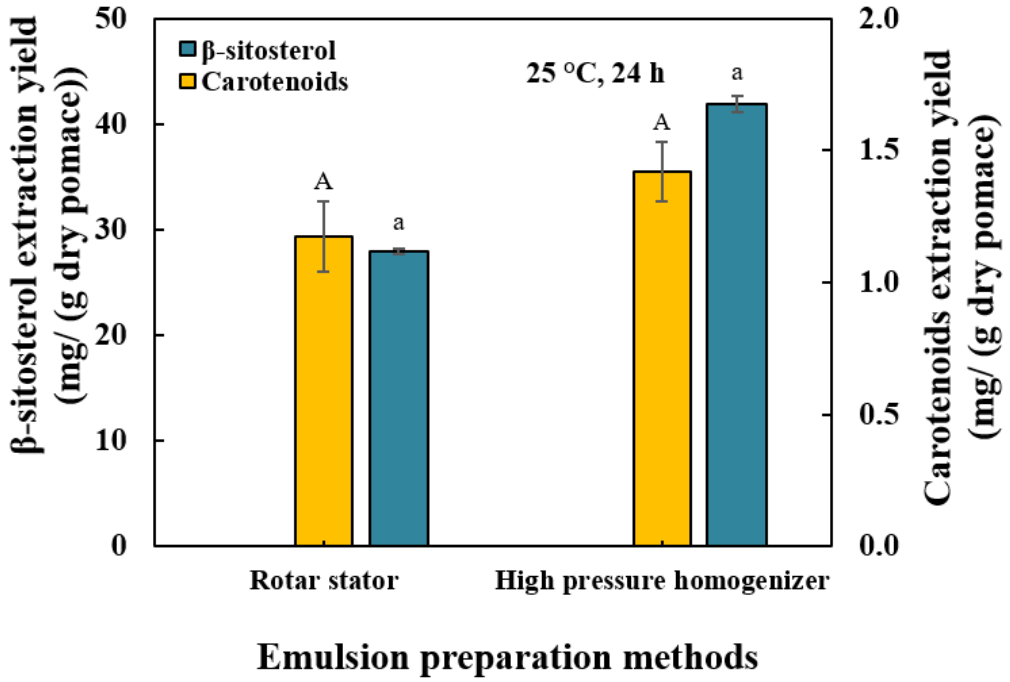


Figure 2-8: Effect of emulsion preparation method on β-sitosterol and carotenoids yield in sea buckthorn pomace extracts, prepared using O/W emulsions

*Different letters on the graphics indicate significant differences (ANOVA test $p<0.05$).

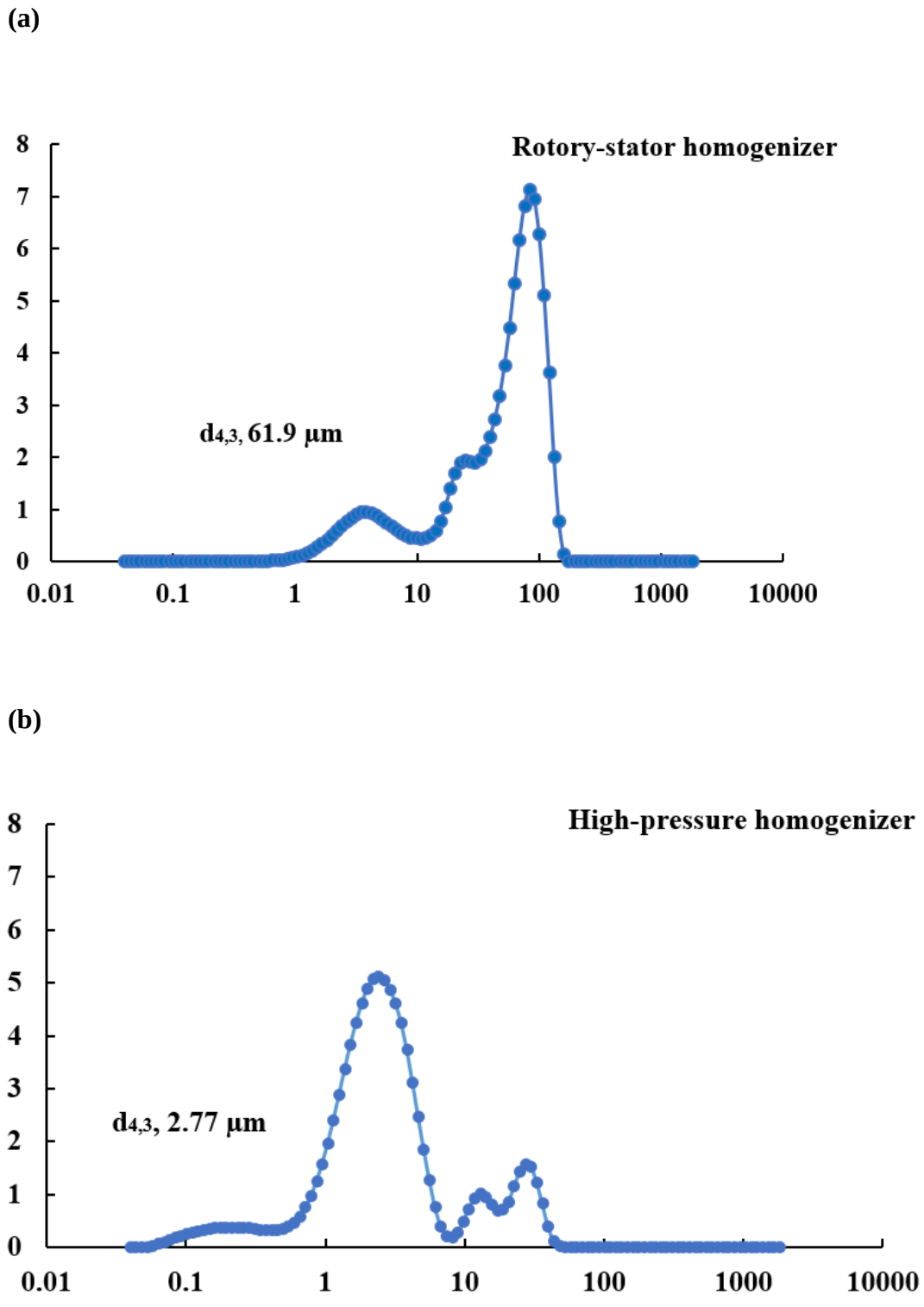
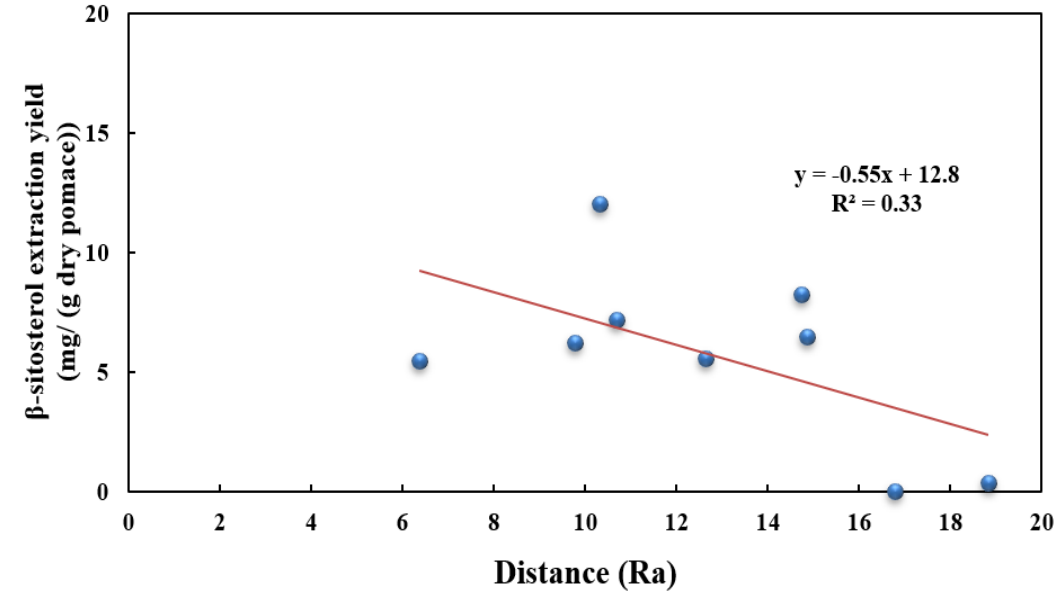


Figure 2-9: Droplet size distribution of emulsifier-free O/W emulsions prepared using (a) high-pressure homogenization or (b) rotory-stator homogenization

Table 2-1: Hildebrand solubilty of different organic solvents, water and oils (Hansen, 2000)

Organic solvents and oils	**Hildebrand solubility**	**Hansen parameter**		
		Dispersion	Dipolar	H-bond
	σ	σd	σp	σh
	[MPA $^{1/2}$]	[MPA $^{1/2}$]	[MPA $^{1/2}$]	[MPA $^{1/2}$]
Ethanol 50%	28.79	15.45	16.36	17.96
Ethanol 99.5%	26.52	15.8	8.8	19.4
Acetone	19.94	15.5	10.4	7
Chloroform	18.9	17.8	3.1	5.7
Ethylacetate	19.9	15.8	5.3	7.2
Hexane	14.9	14.9	0	0
Coconut oil	16.62	16.2	2.5	2.8
Soybean oil	16.84	16.5	2	2.7
Water	24.86	18.1	17.1	16.9
β-Carotene	17.14	16.18	2.24	5.21
Cholestserol	22.64	20.4	2.8	9.4

(a)



(b)

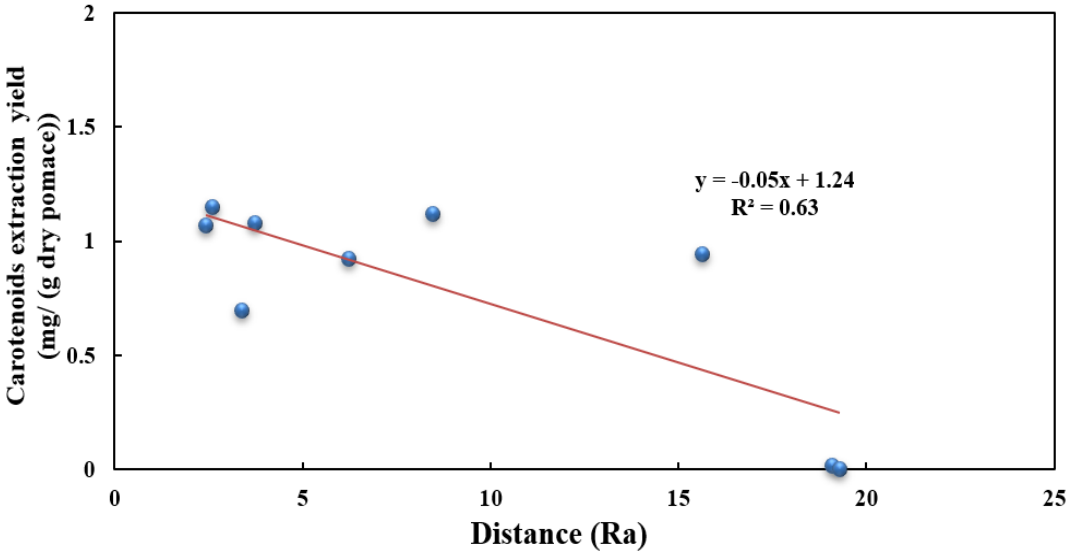


Figure 2-10: The solubility parameter distance, between β–sitosterol and carotenoid against organic solvents and oils

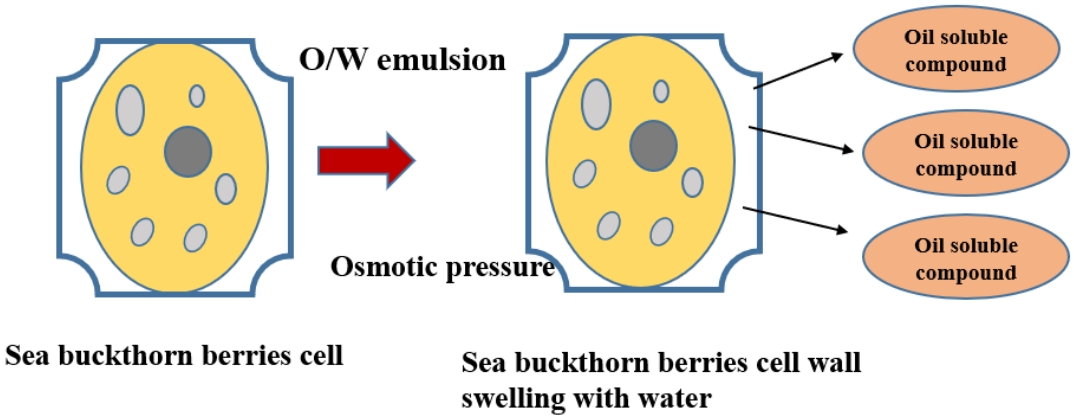


Figure 2-11: Possible mechanism of O/W emulsions extraction

Chapter 3

Effect of extraction conditions using O/W emulsions on extraction yields of β-sitosterol and carotenoids

3.1 Introduction

The extraction methods need to choose extraction conditions such as temperature, time, solvents, the size of the samples, the solvent-to-solid ratio considering targeted compounds. Previous studies evaluated effect of extraction temperature, time and extraction steps on the extraction yields of carotenoids and sterols (Menedez-Carreño *et al.*, 2008; Strati & Oreopoulou, 2011). They found that increasing of extraction temperature results increase of extraction yields. The extraction temperature increasing was effect to increase the solubility and diffusion, and decrea se of solvent viscosity and surface tension.

The extraction efficiency increases with the increase in extraction time range, While some studies reported that carotenoids degredated at high temperature with long extraction time. The sterols yields increased with high temperature and long extraction time. Sachindra (2005) reported that carotenoid yields reduced considerably at a temperature above 70°C, while there was a marginal decrease in carotenoid yield above 150 min of heating (Sachindra & Mahendrakar, 2005). Cossuta *et al.* (2007) found that 60 °C is optimal temperature for carotenoids and β-sitosterol using supercritical carbon dioxide from sea buckthorn pomace. In addition, Roohinejad *et al.* (2014) evaluated effect of different temperature (30-70 °C) and time (10-110 min) on the yields of β-carotene from carrot using microemulsion. They found that 49.4 min 52.2 °C was optimal conditions for extract β-carotene from carrot.

The objective of this chapter was to evaluate effect of extraction temperature and time on the yields of β-sitosterol and carotenoids using emulsifier free soybean O/W emulsions.

3.2 Materials and methods

Dried sea buckthorn pomace consists of peels and seeds (<0.5 mm) was supplied by Eco-Erdene LLC (Ulaanbaatar, Mongolia). Refined soybean oil purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

O/W emulsions preparation and extraction method used in this chapter is based on chapter 2.3.3. Briefly, O/W emulsions were prepared by homogenizing 20 mL of the oil phase (refined soybean or rapeseed oil) with 80 mL of an aqueous phase. Coarse emulsions were initially prepared using a rotor-stator homogenizer at 7,000 rpm for 5 min. Fine emulsions were subsequently prepared by high-pressure homogenization of coarse emulsions at 100 MPa for 4 passes. Prepared emulsions (100 mL) were then mixed with 2 g of dried pomace and stirred at 750 rpm at different temperatures (25, 50, 65, 75, and 80 °C), and for different extraction time (1, 2, and 5 h). The suspensions were sonicated for 1 h at 25 °C, and then centrifuged at 9,100 x *g* for 1 h to remove the undissolved particles. After centrifugation the oil part was collected and used for analysis β-sitosterol and carotenoids. The β-sitosterol and carotenoids were analyzed using same methods as like chapters 2.2.4 and 2.2.5.

3.3 Results and discussion

3.3.1 Effect of extraction conditions using O/W emulsions on the yield of β-sitosterol and carotenoids

The effect of extraction conditions on yields of β-sitosterol and carotenoids from sea buckthorn pomace was evaluated using emulsifier-free soybean O/W emulsions as a model emulsion-based extraction system. As shown in Figure 3-1a, the yields of β-sitosterol increased slightly upon increasing the extraction temperature from 25 °C to

80 °C, while the yield of carotenoids increased by increasing the temperature from 25 °C to 65 °C. However, the yield of carotenoids decreased after 75 °C (Figure 3-1b), with a short extraction time of 1 h. The highest yield of β-sitosterol (36.5 mg/g dw) was obtained at 80 °C, and that of carotenoid (1.44 mg/g dw) was obtained at 65 °C, for an extraction time of 1 h. However, any further increase in the extraction temperature or time reduced the yield of carotenoids. These findings agree with those from previous studies, which reported that temperatures higher than 65 °C resulted in increased β-carotene degradation (Baysal, Ersus, & Starmans, 2000).

In addition, previous studies suggested that phytosterol recovery can significantly improve by increasing the extraction temperature (Zhang, Cao, Liu & Shang, 2017; Feng, Luo, Zhong, Jiang & Tang, 2014). This can be explained by the breakdown of pomace cell walls at high temperatures, which favors the extraction of bioactive compounds as well as the reduction of solvent viscosity; thus, improving the cell penetration.

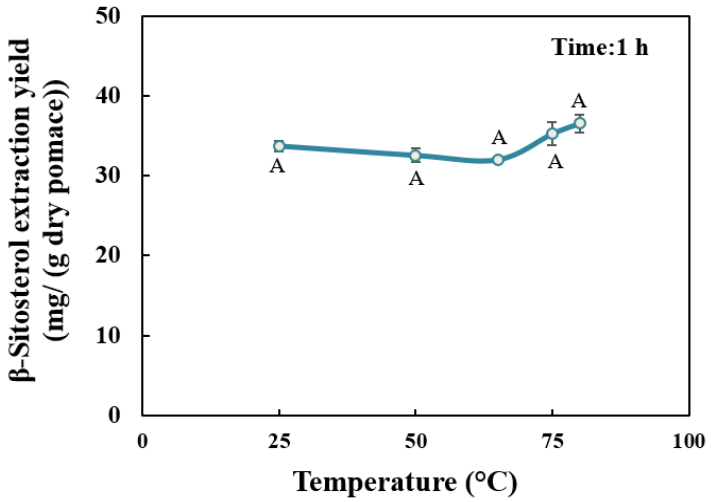
The effect of extraction time on β-sitosterol and carotenoids recovery at 65 °C was evaluated. As shown in Figure 3-2, the yields of β-sitosterol and carotenoid changed slightly after 1 and 2 h of continuous extraction, showing the high efficiency of emulsion extraction systems on the recovery of bioactive compounds from plant-based materials. However, extending the extraction time up to 5 h resulted in a decrease in the concentration of bioactive compounds, suggesting a chemical degradation of extracted compounds at elevated temperatures (65 °C). A previous study reported that the extraction time of 1 h as favorable for maximal extraction of β-carotene (Roohinejad *et al.*, 2014). Figure 3-3 shows effect of different temperature and time on

β-sitosterol and carotenoids yield. The on β-sitosterol yield increased with high temperature, but carotenoids yields decreased.

3.4 Conclusion

The yields of β-sitosterol increased by increasing temperature, while the yield of carotenoids increased from 25 °C to 65 °C but decreased at higher tempetures. Long extraction time effected yields of β-sitosterol and carotenoids. The suitable extraction conditions were obtained using emulsifier-free soybean O/W emulsions at 65 °C and for 1 h. In this study extract contain 3170 µg/mL β-sitosterol and 142.94 µg/mL carotenoids. The extracts obtained using soybean O/W emulsions at 65°C for 1 h were selected to evaluate the long-term chemical stability of β-sitosterol and carotenoids at various temperatures, and the following experiment was carried out under these conditions.

(a)



(b)

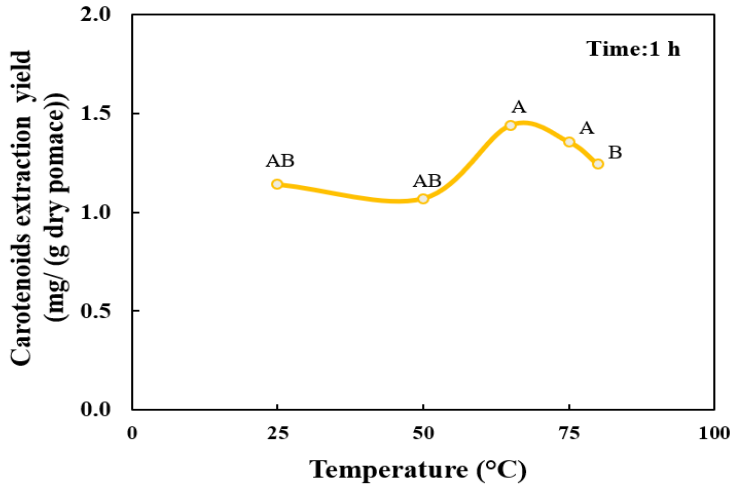
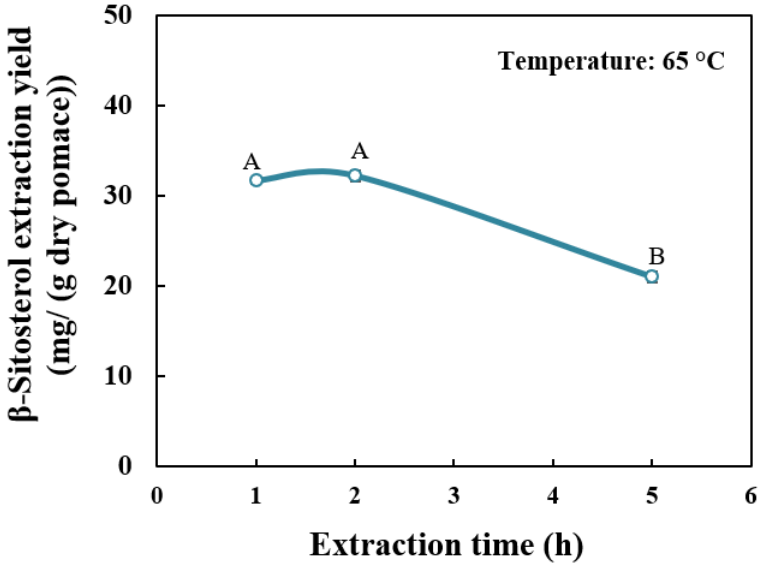


Figure 3-1: Effect of extraction temperature on (a) β-sitosterol and (b) carotenoids extraction yield

*Different letters on the graphics indicate significant differences (ANOVA test $p<0.05$).

(a)



(b)

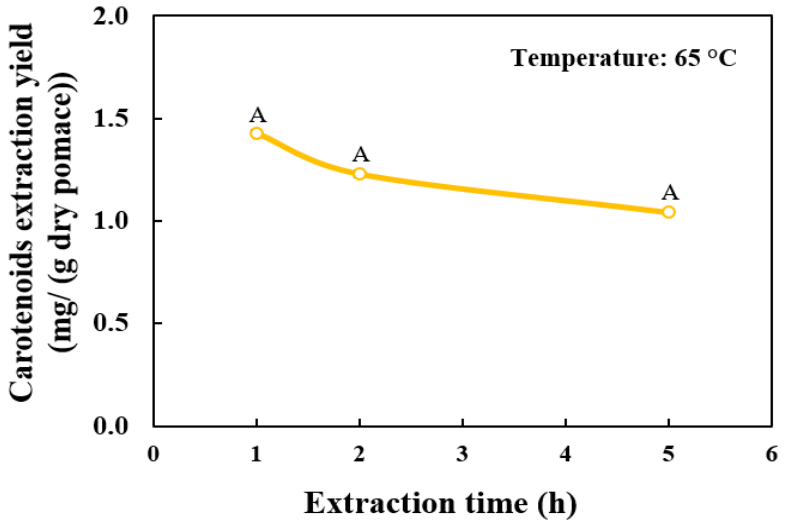
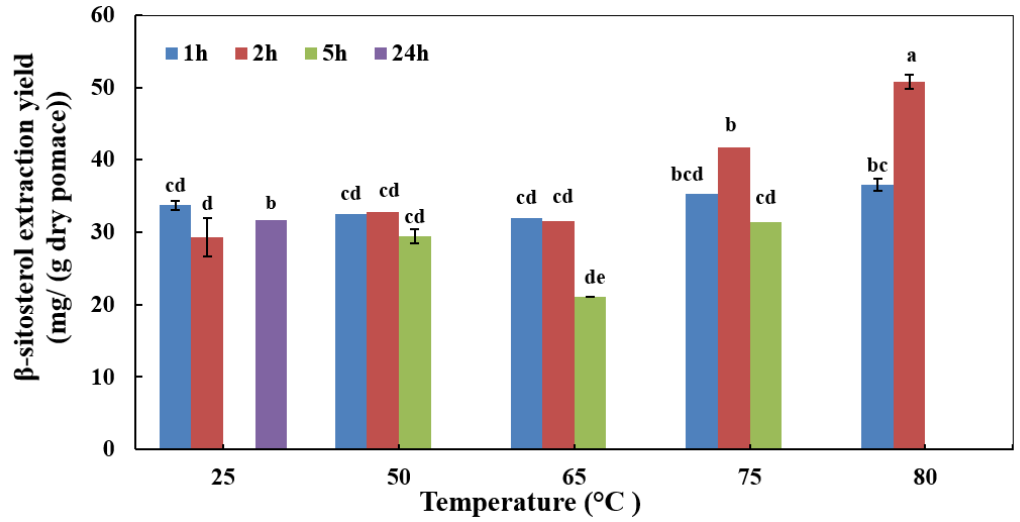


Figure 3-2: Effect of extraction time on (a) β-sitosterol and (b) carotenoids extraction yield

*Different letters on the graphics indicate significant differences (ANOVA test $p<0.05$).

(a)



(b)

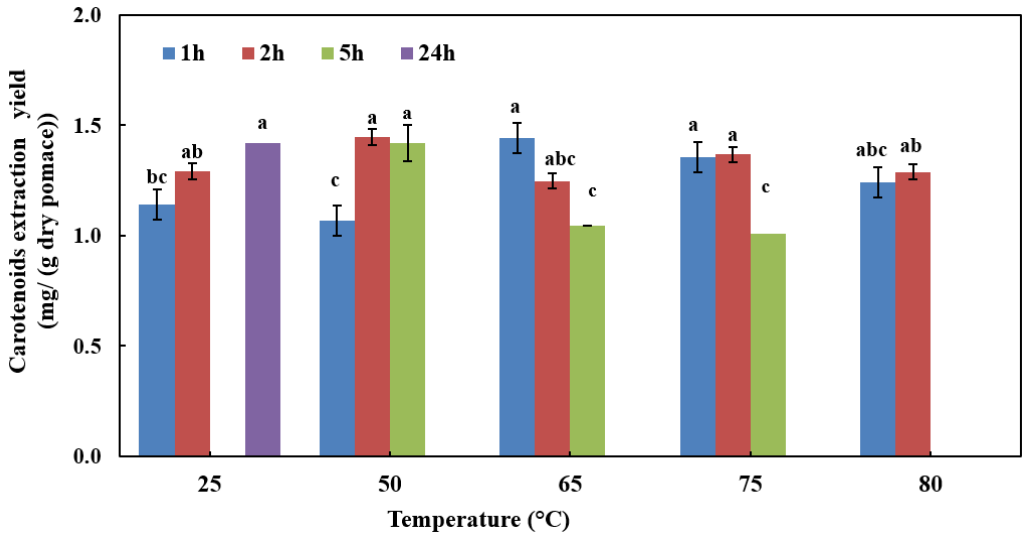


Figure 3-3: Effect of different extraction condition (temperature and time) on β-sitosterol and carotenoids extraction yield

*Different letters on the graphics indicate significant differences (ANOVA test $p<0.05$).

Chapter 4

Chemical stability and color measurement of β-sitosterol and carotenoids in the O/W emulsions extract

4.1 Introduction

In this chapter, the extracts obtained using soybean O/W emulsions at 65 °C continuously for 1h were selected to evaluate the long-term chemical stability of β-sitosterol and carotenoids at various temperatures, and the following experiment was carried out under these conditions. The β-sitosterol and carotenoids are easy to oxidize and unstable with some conditions. Heat, light, singlet oxygen, acid, iron, iodine, and free radical promote this degradation (Dutta *et al.*, 2005). Piorkowski & McClements (2014) revealed that one of the major factors limiting the incorporation of carotenoids into many food and beverage products is their high susceptibility to chemical degradation. Degradation of carotenoids in foods is complex in nature as various factors such as nature and composition of foods, processing treatments, packaging and storage conditions, activity of lipooxygenase and other enzymes, and coupled oxidation with lipids are considered to play a vital role. Boon *et al.* (2010) explained that the conjugated polyene chain which is characteristic of carotenoids makes these compounds susceptible to degradation. At the high temperature oxidation of phytosterols occurring in foods encompasses a sequence of reactions resulting in primary (hydroperoxides), secondary (polar: ketones, alcohols, epoxides; unpolar: steradienes, steratrienes) and tertiary oxidation products (dimers, oligomers, polymers).

The objectives of this chapter were as follows:

- To evaluate the long-term chemical stability of β-sitosterol and carotenoids at various temperatures in the emulsifier-free soybean O/W emulsions extract,
- To measure color change of O/W emulsions extract at different temperatures during storage.

4.2 Materials and methods

4.2.1 Chemical stability of β-sitosterol and carotenoids during storage

For chemical stability and color measurement test, soybean O/W emulsions without emulsifier were chosen to extract prepared with 65 ℃ for 1 h. Then the supernatant part was collected and stored different temperatures (5, 25, and 50 ℃) for 28 days. The β-sitosterol and carotenoids were analyzed by methods described in chapters 2.2.4 and 2.2.5.

4.2.2 Kinetic data analysis

According to previous study (Song *et al.*, 2018), kinetic degredation of β-sitosterol and carotenoids calculated effect of different storage temperatures at 5, 25 and 50 ℃, first-order kinetics Equation (4-1) were hypothesized to determine the reaction order of individual carotenoid;

$$\ln C = \ln C_0 - kt \qquad (4\text{-}1)$$

where C is the concentration (%) of the individual β-sitosterol and carotenoids at time ; C_0 is the concentration (%) of the individual β-sitosterol and carotenoids at time zero; k is the reaction rate constant (day^{-1}); t is the reaction time (day)

The relationship between the reaction rate and storage temperature was quantified by the Arrhenius Equation (4-2);

$$k = k_0 \exp(-E_a/RT) \qquad (4\text{-}2)$$

where E_a is the activation energy of the reaction (kJ/mol), and k_0 is the pre-exponential constant; R is the gas constant (8.314 J/(mol K); T is the absolute temperature of the storage temperature (K).

4.2.3 Color measurement

Color measurement was performed by similar previous study. O/W emulsion extract color was assessed using Spectrophotometer CM-5 machine and reported as L*, a* and b* values. The L* value represents lightness, ranging from 0 (black) to 100 (white), and indicates how dark/light the sample is; the a* value exhibits redness (positive) and greenness (negative); and the b* value exhibits yellowness (positive) and blueness (negative). The total colour difference (ΔE) is used to characterize the overall change in colour during the drying process. The ΔE value is calculated by the following equation:

$$\Delta E = \sqrt{\left(L_0^* - L^*\right)^2 + \left(a_0^* - a^*\right)^2 + \left(b_0^* - b^*\right)^2} \tag{4-3}$$

where L*,a*,b* are the measured colour coordinates of the extract at storage time and L_o*,a_o*,b_o*are the initial color coordinates of the extract.

4.3 Results and discussions

4.3.1 Chemical stability of β-sitosterol and carotenoids in O/W emulsion-based extracts

As shown in Figure 4-2, the concentrations of β-sitosterol and carotenoids were relatively stable at 5 °C and 25 °C over the entire period of 28 days of storage. However, they dramatically decreased at 50 °C, resulting in an appreciable change of color. β-sitosterol was more unstable to degradation at high temperatures than carotenoids. In this study results agree with those from previous studies that reported a rapid

degradation of phytosterol at high temperatures. For example, Carreño, Ansorena, & Astiasaran (2008) found that sterols degrade more rapidly at high temperatures (90 °C, 15 min) than at low temperatures (65 °C, 24 h) in sterol-enriched milk. Rudzińska, Przybylski, & Wąsowicz (2014) also found that the degradation of sterols in enriched margarine was 1.5-times more rapid at 20 °C than at 4 °C. Tian *et al.* (2017) reported that the concentration of β-carotene in sunflower oil decreased gradually from 100% to 8%, after 27 days of storage at 37 °C. Qian *et al.* (2012) also reported a similar finding on β-carotene degradation in O/W emulsions.

4.3.2 Color measurement

The influence of storage temperature on the total color difference is shown in Figure 4-2. The rapid acceleration in colour fading was clearly highlighted when the storage temperature increased from 5 to 50°C. The extract total color faded to orange to yellow during 28 days storage at 50 °C (Figure 4-3). However, total color did not change at 5 °C and 25 °C. At 50 °C *L (lightness) value increased and *a (redness),*b (yellowness) values decreased during storage. At 25 °C and 5°C these values (*L,*a,*b) did not change much.

Previous studies have also found a relatively rapid loss of β-carotene in nanoemulsions stored at elevated temperatures (Mao *et al*., 2009; 2010; Ribeiro, Chu, Ichikawa, & Nakajima, 2008). These results highlight the importance of preparing, transporting and storing β-sitosterol and carotenoids-enriched extract under relatively cool conditions to avoid color fading and potential loss of bioactivity. The transmittance of extract color was analyzed during storage time (Figure 4-4). Transmitance data shows there was no change at 5 ºC and 25 ºC but at 50 ºC after 21 days increased. The extract orange color faded to yellow with transmittance increasing. The reason that

degredation of carotenoids C=C double bond decreasing effected to the extract color change following : red <vermeil <orange <yellow.

4.3.3 Degradation kinetics of β-sitosterol and carotenoids

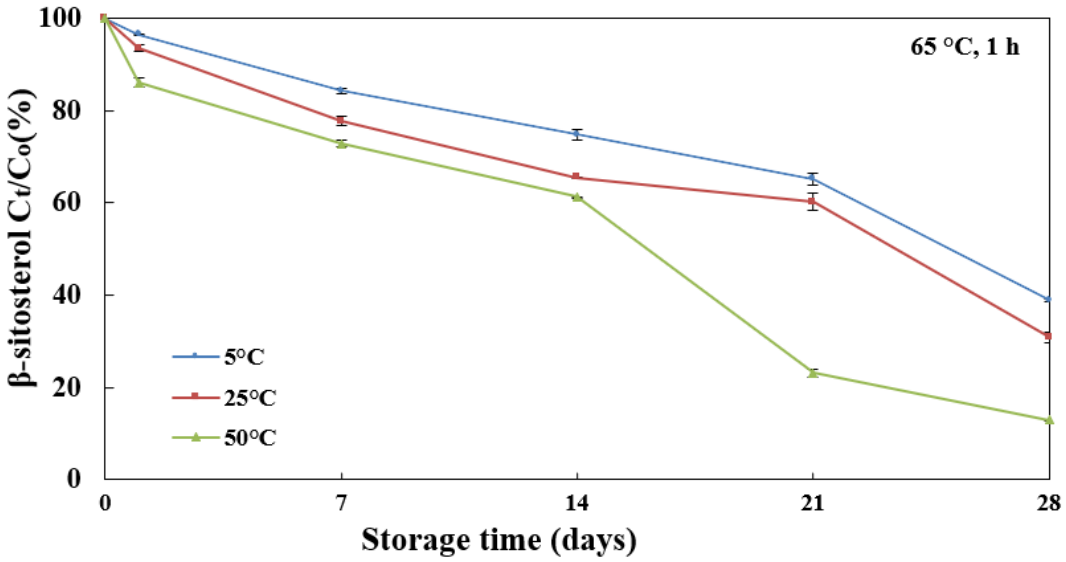
The β-sitosterol and carotenoids relative concentrations dramatically decreased from 100% reached β-sitosterol 12.9% and carotenoids 22.8% after 28 days storage at 50°C.

The reaction rate constants for β-sitosterol and carotenoids loss were significantly influenced by storage temperature (Figure 4-5). The reaction rate was β-sitosterol 0.029-0.07 and carotenoids 0.01-0.045. When storage time increased, the β-sitosterol and carotenoids degredation rate also increased. The carotenoids degredation rate was lower than β-sitosterol degredation rate. The activation energy for β-sitosterol and carotenoids thermal degredation was about 14.51 kJ/mol and 24.3 kJ/mol (Figure 4-6). The carotenoids activation energy was higher than β-sitosterol. This result agreed with previous studies that β-carotene degredation kinetic increased with increasing temperature.

4.4 Conclusion

The β-sitosterol and carotenoids were relatively stable at 5 ℃ and 25℃; however, they dramatically decreased at 50 ℃ during storage. The extract total color faded to orange to yellow during 28 days storage at 50 ℃. However, total color did not change at 5 ℃ and 25 ℃. Degradation kinetics are analyzed in the long term experiments and reaction rate constants of 5, 25, 50 ℃ and activation energy estimated.

(a)



(b)

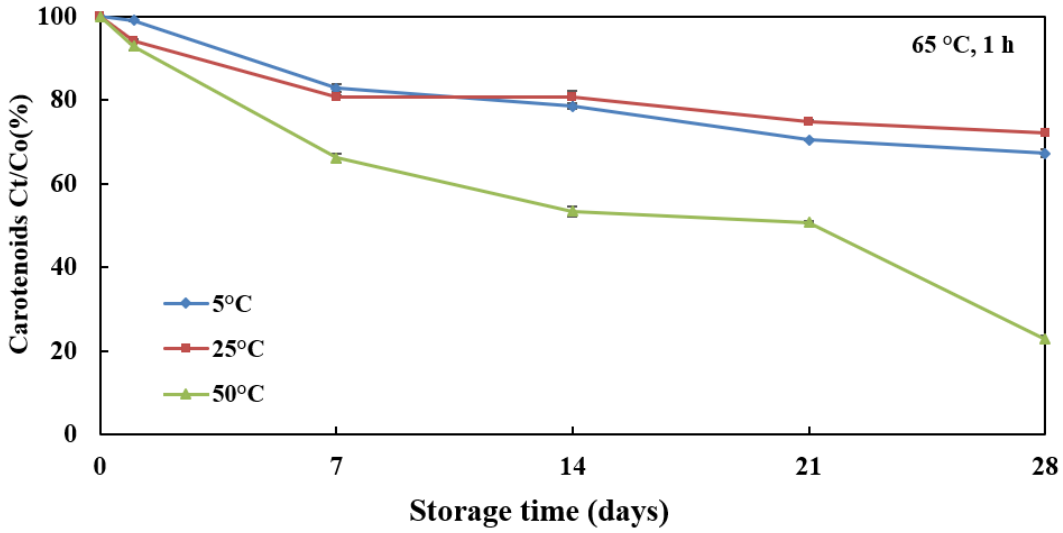


Figure 4-1: Chemical stability of (a) β-sitosterol and (b) carotenoids in O/W emulsion extract during storage

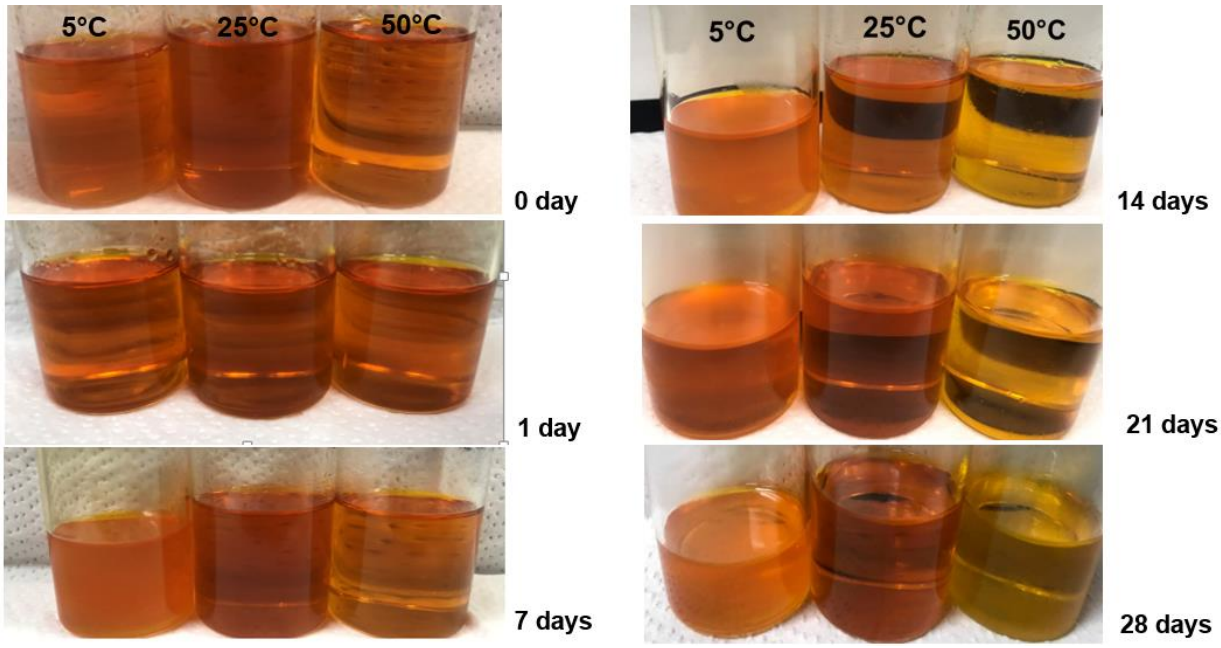


Figure 4-2: Appearance of β-sitosterol and carotenoids loaded emulsifier-free soybean O/W emulsions extract during storage with different temperatures

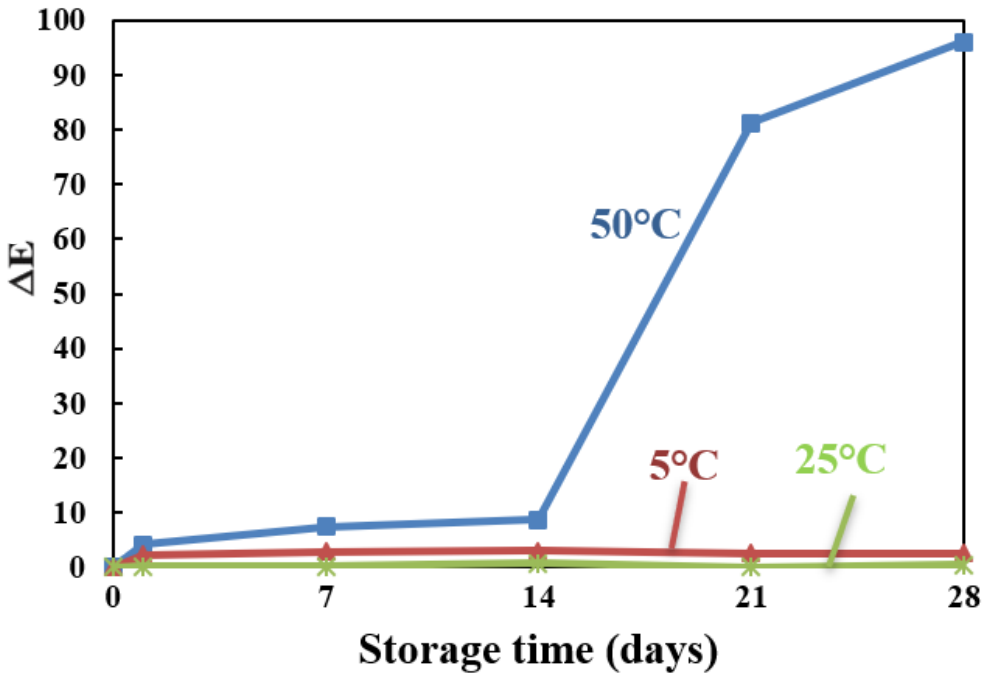
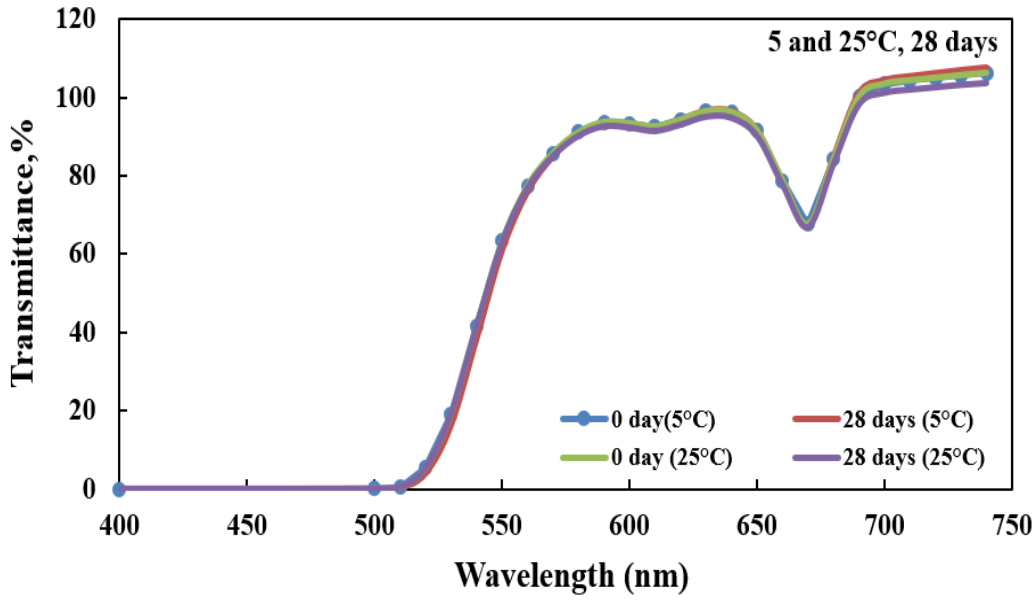


Figure 4-3: Total color difference (ΔE) of emulsifier-free soybean O/W emulsions extract during storage

(a)



(b)

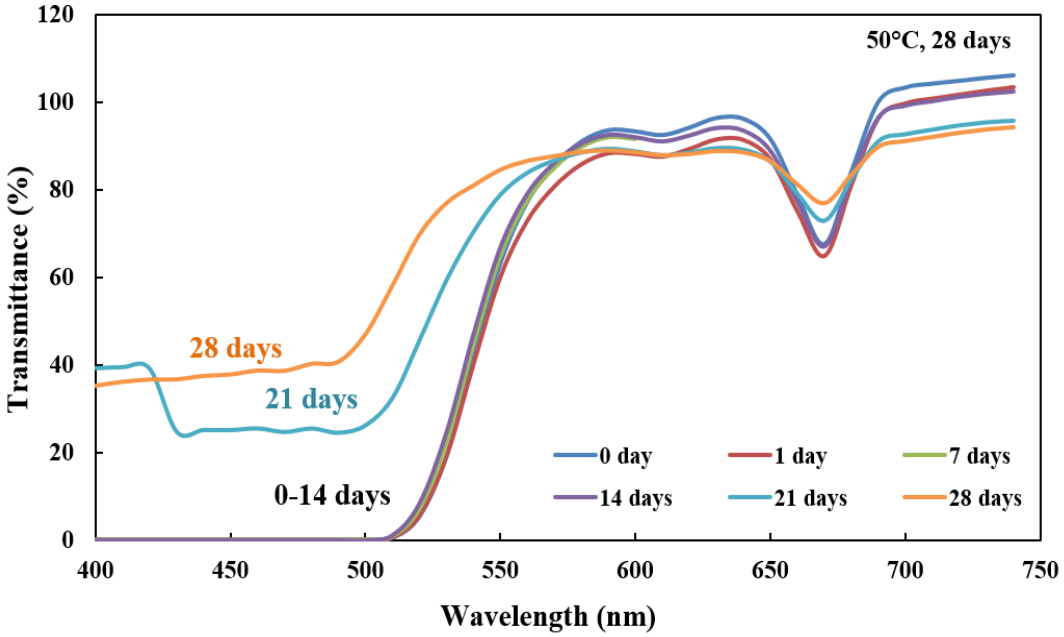
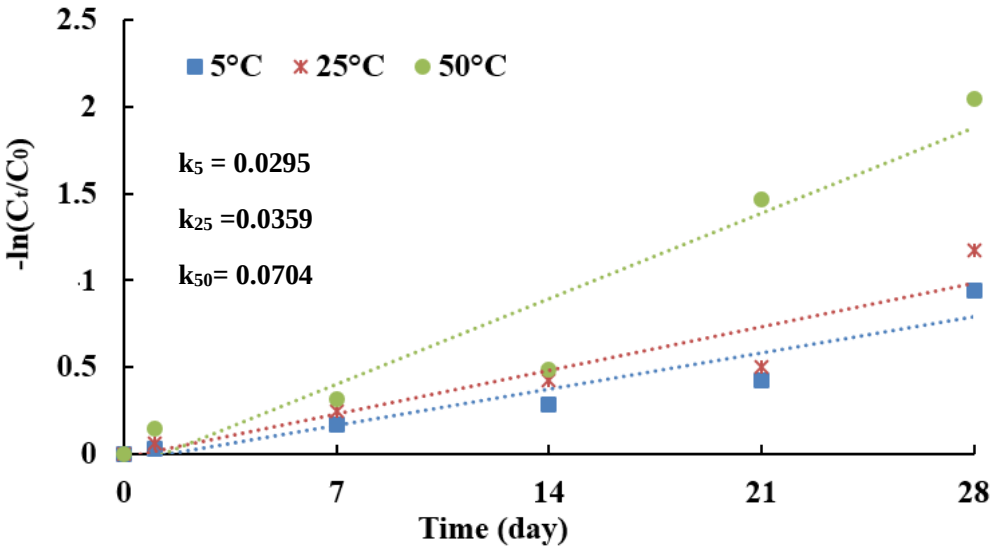


Figure 4-4: Transmittance of (a) β-sitosterol and (b) carotenoids emulsifier-free soybean O/W emulsions extract during storage

(a)



(b)

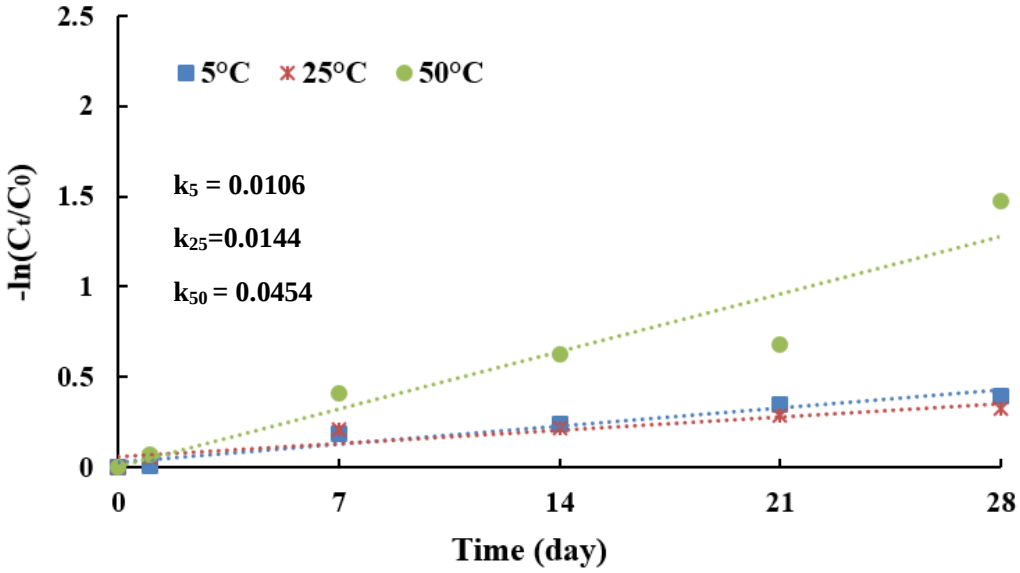
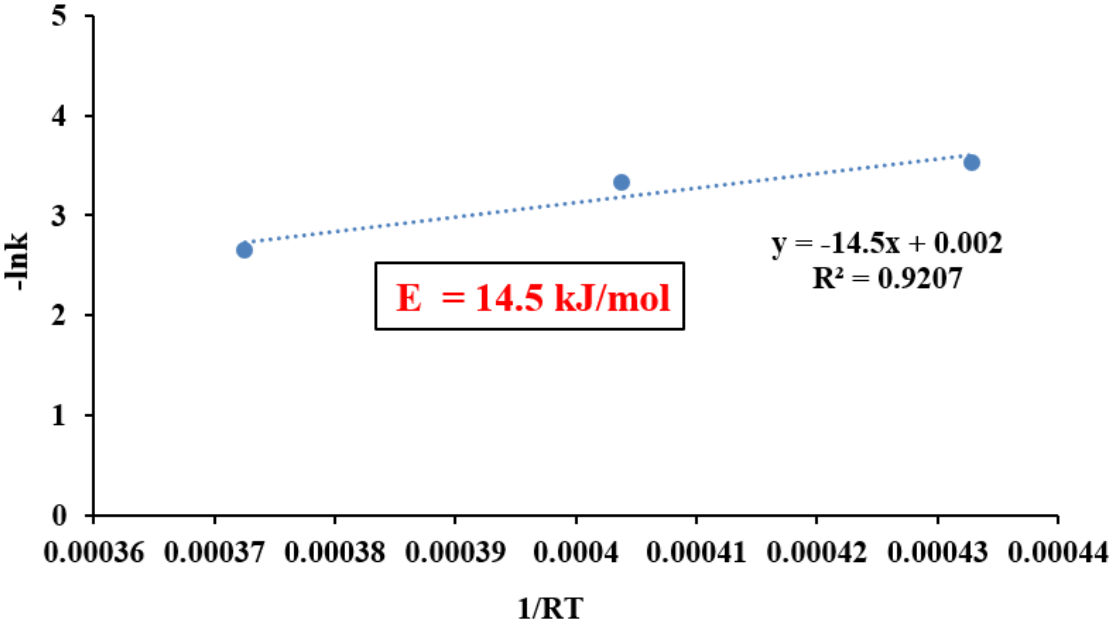


Figure 4-5: Degredation kinetics of (a) β-sitosterol and (b) carotenoids in emulsifier-free soybean O/W emulsion extract during storage

(a)



(b)

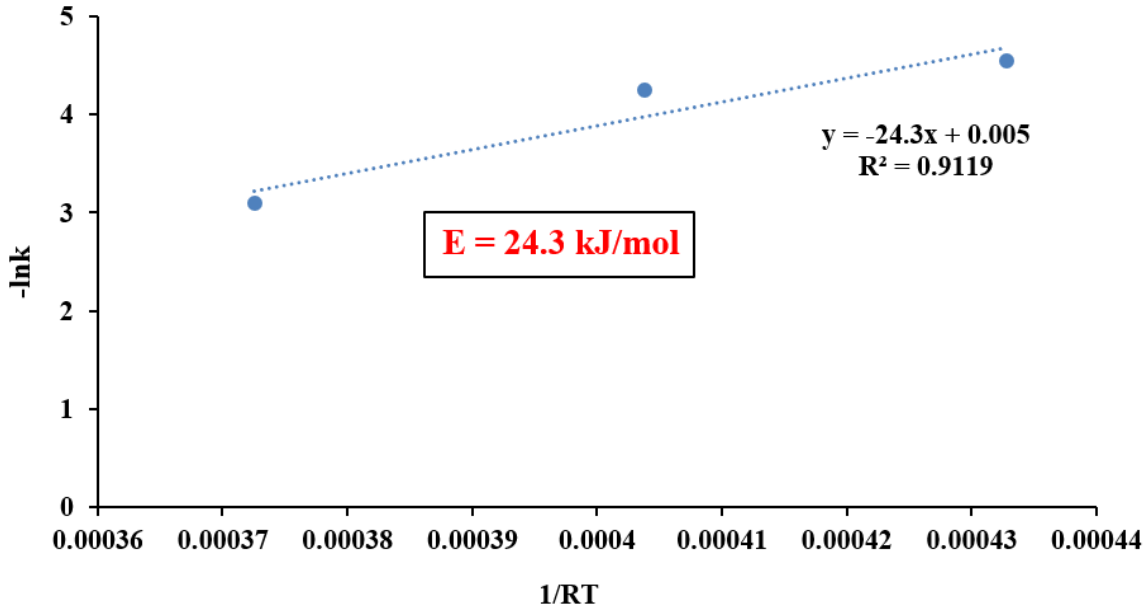


Figure 4-6: Activation energy for degredation kinetis of (a) β-sitosterol and (b) carotenoids in the emulsifier-free soybean O/W emulsion extract

Chapter 5

General conclusions and future perspectives

5.1 Summary of chapters

Chapter 1: In this chapter, sea buckthorn fruits and their distribution, chemical, physical properties and major applications were briefly overviewed. In addition, the valorization of sea buckthorn pomace was explained with some previous studies report. Also, extraction methods and emulsions system were explained in this chapter.

Chapter 2: In this chapter, extraction efficiency of different solvent system on the yields of β-sitosterol and carotenoids was evaluated using sea buckthorn pomace. The organic solvents, oils and emulsions system were chosen for extracting β-sitosterol and carotenoids. The aim of this chapter was to investigate new and efficient green extraction method for bioactive compounds.

Chapter 3: This chapter aimed to evaluate the effect of extraction temperature and time using O/W emulsions extarction on the yields of β-sitosterol and carotenoids.

Chapter 4: In this chapter, the chemical stability and color measurement of β-sitosterol and carotenoids was evaluated in the O/W emulsions extract. The chemical stability of β-sitosterol and carotenoids were analyzed during 28 days storage with different temperatures. This chapter aimed to find suitable storing temperature for O/W emulsions extract with less degredation of these valuable compounds. The extract color was also measured by using color measurement machine.

5.2 General conclusions

In this study, the efficiency of different extraction systems was evaluated on the extraction of β-sitosterol and carotenoids from sea buckthorn pomace. O/W emulsions were found to have much better extraction efficiencies than organic solvents and oils. However, the O/W emulsion structure had an adverse effect on the extraction yields of

bioactive compounds from the pomace. This could be addition of emulsifiers may suppress the partitioning of bioactive compounds from the plant-based material to the oil system. It was found that HPH treatment and smaller droplet sizes affected an increase in extraction yields, compared with RSH. Among these emulsifier-free systems with HPH treatment, the suitable extraction condition was obtained using soybean O/W emulsions at 65 °C with 1 h of continuous extraction. Under these conditions the β-sitosterol and carotenoids were obtained up to 32.0 mg/g dw and 1.44 mg/g dw of respectively. The extracted compounds were unstable to a high temperature (50 °C), but was relatively stable at lower temperatures (5 and 25 °C), indicating a potential application for the formulation of green-label food and beverages. During storage at 50 °C, the color of the extracts changed orange to yellow but at 5 and 25 °C their color hardly changed.

5.3 Future perspectives

In the present work, it was found that O/W emulsions extraction is efficient and green extraction system. This study revealed that O/W emulsions extraction method has a higher capability to extract β-sitosterol and carotenoids compared with other solvent extraction systems. The O/W emulsions extraction method can be considered a green technology, with a low cost application for extracting β-sitosterol and carotenoids that can be used in food and pharmaceutical industries.

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List of publication

Tsogtoo, B., Taarji, N., Melanie, H., Khalid, N., Tsolmon, S., Kobayashi, I., Neves, M.A., Nakajima, M., (2019). Emulsion-based extraction of β-sitosterol and carotenoids from sea buckthorn (*Hippophae rhamnoides*) pomace, *International Food Research Journal,* in press

Acknowledgements

First of all, I would like to express my sincere gratitude to my supervisors, Prof. Mitsutoshi Nakajima and Prof. Isao Kobayashi for their guidance, encouragement and valuable advice during my PhD journey in University of Tsukuba, Japan.

I thank Prof. Marcos A. Neves for his all valuable comment and suggestion, Prof. Sosaku Ichikawa and Prof. Kunihiko Uemura for their all advice and comments, Mrs. Mitsuko Kawamoto for her kind support and help during my study in University of Tsukuba.

I gratefully acknowledge Mongolian-Japan Higher Engineering Education Development project (MJEED 1000 engineers) for the financial support granted during my study.

I also thank to Bio-Engineering for Human Sustainable Development team of MJEED project giving me an opportunity to continue my study in Japan, specially my deepest grateful to Dr. Soninkhishig Tsolmon for her valuable encouragement, support and advice.

I would like to thank for all my collagues in Food Resources Engineering Laboratory for giving me warm and friendly environment also their kind support. I thank for Mr. Aymen Halleb, Dr. Noamane Taarji, Mr. Lenin Kandasamy, Ms. Samar Yahia, Mr. Sekove Vodo, Dr. Hakiki Melanie, Ms. Meryem Bouhoute and other my labmates their all friendship during my study in University of Tsukuba.

Finally, I deeply thank for my family and friends who has supported and motivated me during my study time. My deepest thank to my mother Oyunbileg Badrakh, sister Tserendolgor Tsogtoo and brother-in-law Baasanbayar Tudev for their encouragement, inspiration and supporting. This journey would not have been possible without the dedicated support of my family.

December, 2019

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