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# ENCAPSULATION OF ASTAXANTHIN-ENRICHED CAMELINA SEED OIL OBTAINED BY ETHANOL-MODIFIED SUPERCRITICAL CARBON DIOXIDE EXTRACTION

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# ENCAPSULATION OF ASTAXANTHIN-ENRICHED CAMELINA SEED OIL OBTAINED BY ETHANOL-MODIFIED SUPERCRITICAL CARBON DIOXIDE EXTRACTION

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Astaxanthin is a high-value carotenoid widely used in the food, feed, nutraceutical, and pharmaceutical industries. Natural sources of astaxanthin are preferable to synthetic sources. However, current sources of natural astaxanthin are limited and fall short of global demand. In addition, effective extraction and stabilization strategies are needed before the utilization of natural astaxanthin in food applications. This work investigated 1) the feasibility of ethanol-modified supercritical carbon dioxide (SC-$CO_2$) extraction of astaxanthin from engineered camelina seed, a potential source of natural astaxanthin, and 2) the potential of an emulsion-based system to protect astaxanthin after extraction. The physical properties of the emulsion were characterized, and the protection of astaxanthin by emulsion was evaluated.

The results from the extraction project indicated that ethanol-modified SC-$CO_2$ was a feasible green extraction technique to extract astaxanthin from a high oil, low water content material. In comparison to other techniques, ethanol-modified SC-$CO_2$ was found to be more effective than accelerated ethanol extraction or accelerated hexane extraction,

and as effective as hexane at extracting astaxanthin from engineered camelina seed. As opposed to hexane-based extractions, ethanol-modified SC-$CO_2$ can generate products that are compliant with clean labeling. An emulsion system made by egg albumin (EA) and gum arabic (GA) with and without tannic acid cross-linking were used to inhibit the effects of UV light and heat on astaxanthin. Compared to oil from non-engineered camelina seed oil, engineered camelina seed oil had higher antioxidant activity, which can be further improved by tannic acid crosslinking. The EA/GA- stabilized emulsion with tannic acid crosslinking was able to better protect astaxanthin against UV light and heat, and therefore further broaden the food applications of astaxanthin-enriched camelina oil.

Optimized ethanol-modified SC-$CO_2$ can be used for efficient recovery of astaxanthin from the engineered camelina seed. The EA/GA- stabilized astaxanthin-enriched emulsion product may be used as a food ingredient in various food applications.

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## Organization

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References can be found at the end of each chapter.

# Chapter 1. Introduction and Thesis Objectives

## 1.1 Introduction

There has been a growing awareness of the health benefits of foods enriched in bioactive compounds (Pereira & Meireles, 2010). Bioactive compounds are extranutritional constituents that typically occur in small quantities in foods (Kris-Etherton et al., 2002). Many of these bioactive compounds are lipophilic. Lipophilic bioactive compounds, such as several vitamins, carotenoids, essential fatty acids, and phytosterols, have poor solubility in water, which limits their food applications (Raikos & Ranawana, 2017). These bioactive compounds belong to different classes of chemicals; thus, they can be obtained by several extraction techniques (Pereira & Meireles, 2010). The lipophilic character of these compounds requires suitable solvents to extract them from food matrices. These compounds are commonly extracted using hazardous organic solvents. Although some lipophilic compounds are relatively stable in the matrix, once extracted they may be sensitive to light, heat or oxygen (Zaghdoudi et al., 2015). Thus, it is necessary to develop a technology that considers the stability and the safety concern of the solvent for the extraction of food grade bioactive compounds. The molecular affinity between solvent and solute, mass transfer, and financial feasibility should also be considered in solvent selection for bioactive compound extraction (Azmir et al., 2013).

Supercritical fluid extraction (SFE) is an important extraction method in the food, pharmaceutical, and cosmetic industries because it is possible to generate products without toxic products, with no degradation of active principle, and with purity (Pereira & Meireles, 2010). Carbon dioxide is considered as an ideal solvent for SFE. Studies on

the extraction of various lipid, fat, and non-polar substances have been reported, such as essential oils, carotenoids, and tocopherols (Pereira & Meireles, 2010).

Lipophilic bioactives are chemically sensitive; thus, the degradation of those compounds could occur during processing and storage due to their high sensitivity to environmental factors resulting in the loss of bioactive properties. Incorporating lipophilic compounds into emulsion could be an effective method to protect them, improve their bioavailability, and broaden their food applications (Khalid, Shu, Kobayashi, Nakajima, & Barrow, 2017).

This thesis reports that astaxanthin was recovered by ethanol-modified SC-$CO_2$ extraction from engineered camelina seed. The effects of SC-$CO_2$ extraction parameters, such as pressure, temperature, and ethanol concentration on astaxanthin concentration were determined. Different extraction methods were compared in terms of total oil yield, astaxanthin concentration, tocopherol content, and antioxidant activity. A natural biopolymer-based emulsion was developed to encapsulate astaxanthin-enriched camelina oil. The properties of the emulsion system were characterized. Furthermore, the effect of crosslinking by tannic acid was evaluated.

### 1.2 Hypothesis

It was hypothesized that ethanol-modified SC-$CO_2$ can extract astaxanthin from a high oil content material. It was hypothesized egg albumin (EA)/gum arabic (GA)-stabilized emulsion with tannic acid crosslinking can protect astaxanthin against environmental factors effectively.

## 1.3 Thesis objectives

The goal of this study was to use a natural biopolymer based emulsion system to encapsulate astaxanthin-enriched camelina seed oil extracted by ethanol-modified SC-$CO_2$. The first objective is to extract astaxanthin from engineered camelina seed using ethanol-modified SC-$CO_2$ extraction. The purpose of the first objective is to investigate the feasibility of ethanol-modified SC-$CO_2$ extraction of astaxanthin from a potential astaxanthin source. The second objective is to encapsulate astaxanthin-enriched camelina seed oil into EA/GA- stabilized emulsion with and without crosslinking by tannic acid. The purpose of the second objective is to improve astaxanthin water solubility and protect astaxanthin during processing and storage.

The specific objectives are to:

1) Study the effect of ethanol as co-solvent on the astaxanthin concentration in camelina oils;

2) Optimize the ethanol-modified SC-$CO_2$ extraction conditions;

3) Compare optimized ethanol-modified SC-$CO_2$ extraction with hexane extraction and accelerated solvent extraction;

4) Characterize EA/GA- stabilized astaxanthin-enriched emulsion; and

5) Investigate the effect of tannic acid crosslinking on emulsion structure and functionality.

## 1.4 References

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carotenoids from: Tunisian Kaki (*Diospyros kaki* L.), peach (*Prunus persica* L.) and apricot (*Prunus armeniaca* L.). *Food Chemistry, 184*, 131-139. doi:http://dx.doi.org/10.1016/j.foodchem.2015.03.072

# Chapter 2. Literature Review

## 2.1 Astaxanthin

The research in this thesis focuses on astaxanthin which belongs to the carotenoid family. Over 500 members consist of the great family of carotenoids, which can be naturally found in vegetables (Maria, Graziano, & Nicolantonio, 2015). According to their chemical structures, carotenoids can be divided into two groups: carotene and xanthophylls. Beta-carotene and lycopene are two carotenes, and xanthophylls include lutein, canthaxanthin, zeaxanthin, and astaxanthin. Astaxanthin (3, 3'-dihydroxy-β, β-carotene-4, 4'-dione) can be naturally found in various microorganisms and aquatic animals like shrimps, crabs, and salmon. *Haematococcus pluvialis* has the highest concentration of astaxanthin in nature, at up to 1.5~3.0% (w/w) on a dry weight basis (Lemoine & Schoefs, 2010).

### 2.1.1 Astaxanthin structure and properties

Astaxanthin consists of one conjugated polyene system, and two terminal rings with a molecular formula of $C_{40}H_{52}O_4$ and molecular weight of 596.84 g/mol (Lorenz & Cysewski, 2000). As shown in Figure 2.1, the two-asymmetric carbons located at the 3, 3' positions of the benzenoid ring contain hydroxyl groups and at 4, 4' positions have keto groups. Astaxanthin exists in three configurational isomers, two enantiomers (3S, 3'S and 3R, 3'R) and one meso form (3R, 3'S) (Khalid & Barrow, 2018). *Haematococcus pluvialis* produces the (3S, 3'S)-isomer, which is most abundant in nature. The yeast *Xanthophyllomyces dendrorhous* biosynthesizes the (3R, 3'R)-isomer.

Depending upon the source and origin, astaxanthin is often esterified at the hydroxyl group with different fatty acids. If hydroxyl groups are reacted with a fatty acid, it will form monoester, whereas when both hydroxyl groups are reacted with fatty acids, the diester is formed (Ambati, Phang, Ravi, & Aswathanarayana, 2014). In nature, astaxanthin primarily exists as a protein-conjugated form such as in exoskeleton of crustaceans or a fatty acid-esterified form such monoester or diester (Yang, Kim, & Lee, 2013).

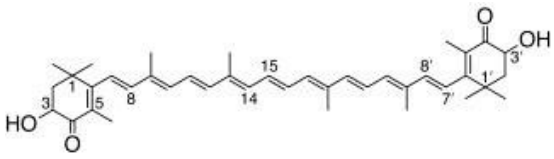


**Figure 2.1** Structure of astaxanthin (Guerin, Huntley, & Olaizola, 2003).

In general, carotenoids are not soluble in water, but soluble in solvents such as acetone, ethanol, chloroform, and dichloromethane (Mezzomo & Ferreira, 2016). Conjugated double bonds, hydroxyl and keto groups all exist in astaxanthin, which makes astaxanthin have both lipophilic and hydrophilic properties (Ambati et al., 2014). The conjugated double bonds at the center of the compound give the red color. This type of conjugated double bonds acts as a strong antioxidant by donating electrons and reacting with free radicals to convert them to be a more stable product and stop free radical chain reaction (Guerin et al., 2003).

### 2.1.2 Source of astaxanthin

Natural astaxanthin can be found in algae, yeast, salmon, trout, krill, shrimp, and crayfish (Ambati et al., 2014). The commercial astaxanthin is mainly from green algae *Haematococcus pluvialis*, red yeast *Phaffia rhodozyma* and through chemical synthesis. Commercially grown *Haematococcus pluvialis* contains between 1.5 and 3.0% (w/w) astaxanthin, which consists approximately of 70% monoesters, 25% diesters, and 5% free form (Machmudah, Shotipruk, Goto, Sasaki, & Hirose, 2006). Recovery of astaxanthin from crustacean byproducts is another source of natural astaxanthin. The carotenoid content in shrimp and crab byproducts varies between 119 and 148 μg/g, and astaxanthin is mainly found free or esterified with fatty acids (Higuera-Ciapara, Félix-Valenzuela, & Goycoolea, 2006). For different sources, the composition and profile of astaxanthin are different (Yuan, Peng, Yin, & Wang, 2011).

Biosynthesis of astaxanthin in plants through metabolic engineering is a promising method. Jayaraj, Devlin, & Punja (2008) demonstrated astaxanthin production in the storage root of a high β-carotene containing crop plant, and the potential of using engineered carrots to produce astaxanthin. Huang, Zhong, Liu, Sandmann, & Chen (2013) illustrated another approach of production of astaxanthin in engineered tomato.

### 2.1.3 Astaxanthin and human health

Free radicals (e.g. hydroxyl and peroxyl radicals) and highly reactive forms of oxygen (e.g. singlet oxygen) are produced in the body during normal metabolic reactions and processes (Guerin et al., 2003). External sources such as exposure to X-rays, ozone, cigarette smoking, and air pollutants can enhance the production of such agents (Lobo,

Patil, Phatak, & Chandra, 2010). DNA, proteins and lipid membranes can be damaged by free radicals.

The health benefits of astaxanthin have been associated with its antioxidant activity. It has been reported that astaxanthin showed higher scavenging capacity against peroxyl radicals and hypochlorous acid than that of α-tocopherol, lutein, lycopene, and β-carotene (Yang et al., 2013). Due to the antioxidant effects of astaxanthin, it may have preventive activities in the pathogenesis of multiple diseases mediated by oxidative stress in the human body (Yang et al., 2013). Additionally, the powerful antioxidant activity of astaxanthin may play a key role in conditions that are triggered by oxidative damage such as UV-light damage, cancer, inflammation, and ulcerous infections (Olaizola, 2007).

## 2.2 Supercritical fluid (SCF) technology

Supercritical fluid technology uses a fluid at a temperature and pressure above its critical point (Fig. 2.2). The supercritical fluid (SCF) exists in a phase between liquid and gas phases in the supercritical region. In this region, the SCF possess unique physical properties. The density of a SCF is similar to a liquid, its viscosity is similar to a gas, and its diffusivity is between that of the two states (Herrero, Cifuentes, & Ibañez, 2006). The "gas-like" behavior of SCFs allows them to have superior penetration properties into the target material, compared to liquid solvents (Sapkale, Patil, Surwase, & Bhatbhage, 2010).

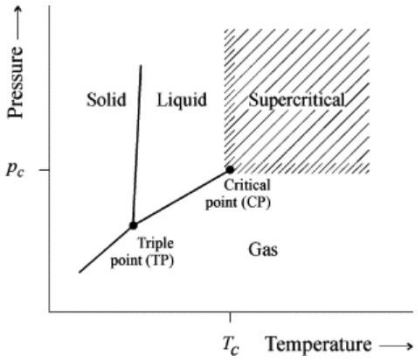


**Figure 2.2** A phase diagram of supercritical fluids (Brunner, 2005).

When processing with SCF, the difference from the equilibrium state determined the driving potential for heat and mass transfer (Brunner, 2005). The information about the capacity of a supercritical solvent, the amount of solvent and the selectivity of a solvent can be provided by the equilibrium state (Brunner, 2005). If capacity and selectivity are known, a good guess can be made about whether a separation problem can be solved with SCFs (Brunner, 2005). The selection of the fluid used as the supercritical fluid depends on the purpose, cost, properties of the fluid, and applicability (Sapkale et al., 2010).

Besides the type of solvent used in the process, other factors could affect the ability to remove a solute from the solid matrix such as the solubility of the solute in the SCF, interactions of the solute-solid matrix, localization of solute in the matrix, and the porosity of the extractor bed (Pereira & Meireles, 2010).

### 2.2.1 Supercritical carbon dioxide (SC-$CO_2$) technology

$CO_2$, ethylene, ethane, and ammonia are commonly used supercritical solvents (Meziani, Pathak, & Sun, 2009). Among those solvents, SC-$CO_2$ is the most commonly and widely used in pharmaceutical, chemical, and food industries. SC-$CO_2$ has a near-ambient critical temperature (31.1°C) and relatively low critical pressure (7.38 MPa). Degradation of heat liable compounds can be avoided during the processing because of the low critical temperature of $CO_2$. In addition, unlike other commonly used solvents, $CO_2$ is nontoxic, nonflammable, abundant, and generally regarded as safe (GRAS) (Ciftci, Calderon, & Temelli, 2012).

The solvent power (capacity) of SC-$CO_2$ can be summarized by a few rules: (i) it dissolves non-polar or slightly polar compounds; (ii) the solvent power for compounds with low molecular weight is high and decreases with increasing molecular weight; (iii) SC-$CO_2$ has high affinity with oxygenated organic compounds of medium molecular weight; (vi) SC-$CO_2$ is capable of separating compounds that are less volatile (Brunner, 2005).

SC-$CO_2$ technology has been widely in various applications, such as polymer modification, lipid extraction, aerogel drying, and particle formation (Belayneh, Wehling, Cahoon, & Ciftci, 2015; Nalawade, Picchioni, & Janssen, 2006; Ubeyitogullari & Ciftci, 2016; Yang & Ciftci, 2016). Moreover, SC-$CO_2$ has been used for the extraction of high-value compounds, such as essential oils, phytosterols and carotenoids (Belayneh, Wehling, Reddy, Cahoon, & Ciftci, 2017; Jaime et al., 2015; Sánchez-Camargo, Meireles, Ferreira, Saito, & Cabral, 2012). Additionally, optimization of SC-$CO_2$ extraction of carotenoids based on response surface design has been reported from different materials (de Andrade Lima, Charalampopoulos, & Chatzifragkou, 2018;

Hosseini, Tavakoli, & Sarrafzadeh, 2017; Shazaha, Masturah, Badlishah, Rashidi, & Russly, 2016).

SC-$CO_2$ extraction basically occurs in two steps: the solubilization of the chemical compounds present on the solid matrix and its separation into the SC-$CO_2$ (da Silva, Rocha-Santos, & Duarte, 2016). During the extraction, SC-$CO_2$ flows through the packed bed, solubilizing the target compounds present in the matrix. Subsequently, the SC-$CO_2$ exits the extractor carrying the solubilized compounds, and as the SC-$CO_2$ depressurizes, becomes gaseous, and dissipates resulting in the extract being solvent free.

### 2.2.2 SC-$CO_2$ extraction of astaxanthin

As an efficient alternative for the extraction of natural compounds, SC-$CO_2$ has been used for the extraction of astaxanthin from various sources for over a decade. There are some studies reporting astaxanthin extraction by SC-$CO_2$ from different materials, such as *Haematococcus pluvialis*, *Agrobacterium aurantiacum*, Red spotted shrimp waste and tiger shrimp (Chougle, Bankar, Chavan, Patravale, & Singhal, 2016; Machmudah et al., 2006; Radzali, Baharin, Othman, Markom, & Rahman, 2014; Sánchez-Camargo, Martinez-Correa, Paviani, & Cabral, 2011).

Pressure is one of the principal parameters studied in astaxanthin extraction using SC-$CO_2$. Generally, the optimization of the pressure for maximum extraction of astaxanthin was done by varying the pressure from 20 MPa to 50 MPa (Ali-Nehari, Kim, Lee, Lee, & Chun, 2012; Chougle et al., 2016; Machmudah et al., 2006; Nobre et al., 2006; Wang, Yang, Yan, & Yao, 2012). The optimum pressure in astaxanthin extraction

depends not only on the material but also on extraction setting. The astaxanthin recovery increased to a certain value along with the increasing pressure and decreased thereafter (Chougle et al., 2016; Machmudah et al., 2006). Higher pressure increases fluid density and increases solute solubility (Wang et al., 2012). However, recovery of astaxanthin decreases as pressure is increased further due to increased compression of $CO_2$ resulting in stronger solvophobic interactions between solute and solvent (Chougle et al., 2016).

Another important parameter in astaxanthin extraction is temperature. Because astaxanthin is a thermolabile molecule, the selected temperature range for extraction is very important in order to avoid degradation. Additionally, at a fixed temperature, an increase in temperature reduces the density of SC-$CO_2$, thus reducing the solvent power of the SC-$CO_2$; however, it increases the vapor pressure of astaxanthin. In general, astaxanthin extractions have been performed over a temperature range of 35 °C to 65 °C (Chougle et al., 2016; Nobre et al., 2006; Wang et al., 2012).

Pure SC-$CO_2$ is non-polar; thus it is unsuitable for the extraction of slightly polar solutes due to the low solute solubility. The polarity of pure SC-$CO_2$ can be modified by polar co-solvents. Water, methanol, and ethanol are typical candidates for the polar co-solvent. Since astaxanthin was more soluble in organic solvents, which are less polar than water, the use of methanol and ethanol as the co-solvents gave better recovery of astaxanthin than that obtained using water (Radzali et al., 2014). Besides, the use of methanol could cause potential health issues; thus, food-grade ethanol further makes it favorable for food applications. Edible vegetable oils, such as soybean oil, canola oil, and olive oil, have also been proposed to enhance SC-$CO_2$ extraction (Krichnavaruk, Shotipruk, Goto, & Pavasant, 2008; Sun & Temelli, 2006). In general, the additional co-

solvent could improve the extraction recovery of astaxanthin (Krichnavaruk et al., 2008; Machmudah et al., 2006; Radzali et al., 2014; Sánchez-Camargo et al., 2011).

The solubility of astaxanthin in SC-$CO_2$ depends not only on physical and chemical properties but also on the operating conditions such as pressure, temperature, and solvent flow rate in the supercritical region (Chougle et al., 2016). The presence of co-solvent could further increase the astaxanthin extraction yield by modifying the polarity of pure SC-$CO_2$.

## 2.3 Emulsion-based encapsulation technology

Encapsulation technology can be very effective for enhancing the solubility, stability, and bioavailability of astaxanthin (Khalid & Barrow, 2018). Astaxanthin has been successfully incorporated into emulsions, liposomes, and solid lipid nanoparticles (Kamezaki et al., 2016; Khalid et al., 2017; Li, Zahi, Yuan, Tian, & Liang, 2016). Comparing with other systems, emulsion-based systems are very good encapsulation candidates for lipophilic compounds and have been widely used for many decades on protection and delivery of flavors, colors and other bioactive compounds (Ozturk, Argin, Ozilgen, & McClements, 2015).

In general, an emulsion system contains two immiscible liquid phases (usually oil and water). A system which consists of oil droplets dispersed in an aqueous phase is called an oil-in-water (O/W) emulsion, whereas a system that consists of water droplets dispersed in an oil phase is called a water-in-oil (W/O) emulsion. In a conventional O/W emulsion, oil droplets are surrounded by a thin interfacial layer consisting of emulsifier

molecules (Raikos & Ranawana, 2017). Lipophilic bioactive compounds, such as beta-carotene, lycopene, and astaxanthin are dissolved in the oil phase and constitute the dispersed phase, where this dispersed phase can be produced by various emulsification processes, like high-pressure homogenization, microfluidization, ultrasonication and solvent diffusion (Tadros, Izquierdo, Esquena, & Solans, 2004).

### 2.3.1 Encapsulation of carotenoid using emulsion systems

Carotenoids, such as β-carotene, lycopene, lutein, and astaxanthin, are the red, orange and yellow pigments found in plants, algae, and microorganisms. The conjugated polyene chain in those compounds makes them susceptible to degradation induced by chemical, mechanical, and thermal stresses (Boon, McClements, Weiss, & Decker, 2010). Besides, since carotenoids are primarily lipid-soluble, the low water solubility of these compounds further limits their food applications.

Emulsions have been found to be an effective way of protecting carotenoids. Qian, Decker, Xiao, & McClements (2012) dispersed β-carotene in orange oil and studied the stability of β-carotene-enriched nanoemulsions. Their results showed that β-carotene could be effectively protected with food-grade nanoemulsions stabilized by β-lactoglobulin or Tween 20. In the O/W emulsion system, β-carotene was in the core of the oil droplets surrounded by emulsifier molecules; thus, the oxidation of β-carotene could be effectively inhibited in the emulsion system. A whey protein isolate-pectin stabilized microemulsion gave lycopene protection against environmental stresses, such as pH, NaCl concentration and temperature (Shi et al., 2015). Liu, McClements, Cao, &

Xiao (2016) successfully fabricated astaxanthin-enriched emulsion stabilized by sodium caseinate and reported that the chemical stability of astaxanthin against pH and light exposure was improved in the emulsion system.

The fatty acid composition and minor components in oil could influence the carotenoid degradation in the emulsion as well. For instance, carotenoids dispersed in unsaturated oils have lower oxidative stability than those dispersed in saturated oils due to the increased potential for the production of radical species that could, in turn, react with carotenoids (Boon et al., 2010). Minor components in the oil, like tocopherols and flavonoids, may act as natural antioxidants, which in turn improve the oxidative stability of carotenoids. Boon et al. (2008) prepared lycopene-enriched O/W emulsions with corn oil, corn oil stripped of its minor components, and hexadecane. They found that lycopene degradation was faster in the stripped corn oil compared with the other two treatments. In this study, astaxanthin was extracted along with camelina oil which has high omega-3 fatty acids content. On the other hand, tocopherols, important minor lipid components in camelina seed, were also extracted. Thus, the high omega-3 fatty acids content of camelina oil and tocopherols may play important roles in the stability of astaxanthin.

### 2.3.2 Emulsifier

Emulsifiers, usually are amphiphilic molecules, exist at the interface between two immiscible liquids such as oil and water, allowing them to be blended into stable emulsions. The traditional food emulsifiers including synthetic surfactants such as Tween 20, Tween 80, and carboxymethyl cellulose.

Food industries have been showing a growing interest in using natural emulsifiers, such as proteins, polysaccharides, and phospholipids (Ozturk et al., 2015). The amphiphilic nature of protein makes it a good food-grade emulsifier. At the interface, flexible proteins, like casein and gelatin, re-align themselves to position their surface hydrophobic amino acids within the oil phase and hydrophilic amino acids within the aqueous phase (Lam & Nickerson, 2013). Globular proteins, such as whey, egg and soy proteins, may partially unfold after adsorption and form cohesive viscoelastic layers (Ozturk & McClements, 2016). Whey protein isolate, casein, egg albumin, and soy protein are commonly used as emulsifiers by the food industry (Lam & Nickerson, 2013). Some natural polysaccharides have good emulsifying properties because they have non-polar groups or proteins as their side chains (Ozturk & McClements, 2016). Gum arabic, pectin and galactomannans are the most common examples of this type of polysaccharide (Ozturk & McClements, 2016). Lecithin, a mixture of phospholipids, is used in a variety of products, including bakery, ice cream, salad dressing, and mayonnaise. The hydrophilic head and hydrophobic tail of lecithin allow it to interact with both oil and water simultaneously.

Egg albumin (EA), a common commercial albumin product, is the major protein present in egg white (~ 65%). It is a monomeric phosphoglycoprotein of 42-47 kDa molecular weight and composed by 385 amino acids (Feng, Cai, Wang, Li, & Liu, 2018). The isoelectric point of EA is around 4.8 and, thus, the protein carries negative charges at neutral pH. EA is an important food ingredient with good emulsifying, foaming and gelling properties (Sponton, Perez, Carrara, & Santiago, 2015). However, protein-stabilized emulsions are unstable to aggregation under certain conditions such as near the

isoelectric point of the proteins and during heat exposure. EA-anionic polysaccharide complexes showed a better emulsifying ability compared with EA alone (Niu et al., 2015). Also, there is a growing interest in using polysaccharide-protein complexes as the emulsifier to stabilize the emulsion (Evans, Ratcliffe, & Williams, 2013).

Gum arabic (GA), a complex and highly branched polysaccharide, has been widely used in food industries as a stabilizer, thickening agent, and an emulsifier (Ali, Ziada, & Blunden, 2009). GA is a tree gum exudate from the stems and branches of *Acacia Senegal* and *Acacia Senegal* (Evans et al., 2013). It consists of a core of β-1,3 linked galactose residues with branches consisting of galactose, arabinose, rhamnose, and glucuronic acid. A small amount of protein also exists in its structure (Evans et al., 2013). The hydrophobic protein fraction that is covalently linked to hydrophilic polysaccharide structures contributes to the excellent surface activity, and therefore make GA as an effective emulsifier at the oil-water interface (Ozturk et al., 2015). The protein/GA complexes, including whey protein isolate/GA, egg albumin/GA, and fish gelatin/GA, were reported for emulsion preparation (Klein, Aserin, Svitov, & Garti, 2010; Niu et al., 2015; Piacentini, Giorno, Dragosavac, Vladisavljević, & Holdich, 2013).

EA/GA- stabilized emulsions can be formed by different preparation methods (Niu et al., 2017). However, the dissociation between EA and GA could occur due to environmental stresses. Crosslinking of biopolymers in food emulsions by physical, chemical and enzymatic treatments, has been reported to enhance their stability and functionality (Zeeb, Gibis, Fischer, & Weiss, 2012). For instance, crosslinking by chemical agents, like formaldehyde and glutaraldehyde, can be applied to stabilize and modify the microstructure and functional properties of the complexes (Anvari & Chung,

2016). Chen, Li, Ding, & Suo (2012) reported that the network of whey protein isolate/beet pectin complex was restructured after the crosslinking as it was evidence in particle size distribution, rheological properties, and microstructure observation. However, the safety concern about those aldehyde agents limits their applications in food and pharmaceutical industries. Tannic acid, a commercial form of tannin, contains abundant hydroxyl groups to form complexes with macromolecules such as polysaccharides, proteins (Anvari & Chung, 2016; Xie, Wehling, Ciftci, & Zhang, 2017). Anvari & Chung (2016) reported that tannic acid crosslinking improved gelling ability and mechanical properties of the fish gelatin/GA gels. Another study by Zhang, Pan, & Chung (2011) found that the gelatin/GA coacervates crosslinked with tannic acid were capable of sustaining the release of allyl isothiocyanate under simulated gastrointestinal conditions. Therefore, the effect of tannic acid crosslinking on the EA/GA- stabilized emulsion structure and functionality are evaluated in this study.

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# Chapter 3. Extraction of astaxanthin from engineered *Camelina sativa* seed using ethanol-modified supercritical carbon dioxide*

## 3.1 Abstract

Natural astaxanthin, a high-value carotenoid that is currently extracted mainly from marine organisms, was extracted from engineered camelina seed using ethanol-modified supercritical carbon dioxide (SC-$CO_2$) for the first time, and compared with hexane and accelerated solvent extraction using hexane and ethanol. Response surface methodology (RSM) based on the central composite rotatable design was employed to investigate the effect of pressure (30-45 MPa), temperature (40-60 °C), and ethanol concentration (10-35%, wt.). RSM-optimized conditions (41.6 MPa, 36.6 °C and 42.0% ethanol concentration) predicted the astaxanthin concentration was 437 μg/g oil, whereas the actual concentration was 421 ± 14 μg/g oil. Astaxanthin concentration in accelerated solvent extracted oil was significantly lower than that in ethanol-modified SC-$CO_2$- and hexane-extracted oils ($P < 0.05$). Oils extracted with ethanol-modified SC-$CO_2$ had the highest antioxidant activity. Results indicated that ethanol-modified SC-$CO_2$ extraction method can be successfully used as a green method to extract astaxanthin from high oil feedstocks.

**Keywords:** Astaxanthin; supercritical carbon dioxide; extraction; camelina seed.

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## 3.2 Introduction

Astaxanthin (3,3ʾ-dihydroxy-β-carotene-4,4ʾ-dione) is a red color fat-soluble pigment which belongs to the keto-carotenoid family. It has been reported that astaxanthin's antioxidant activity is ten times higher than other carotenoids such as beta-carotene and lutein, and over 500 hundred times higher than tocopherol (Dong, Huang, Zhang, Wang, & Liu, 2014). Growing evidence also shows that astaxanthin provides beneficial effects on human health, including the enhancement of general well-being and immune system, protection against lipid membrane peroxidation and DNA damage, gastrointestinal cancers, degenerative ailments such as Parkinson's and Alzheimer's diseases; chronic inflammatory diseases; metabolic disorders such as diabetes; and cardiovascular diseases (Sánchez-Camargo et al., 2012; Yuan, Peng, Yin, & Wang, 2011).

Astaxanthin is the highest value carotenoid that is currently used in the food, feed, nutraceutical and pharmaceutical industries. Currently, more than 95% of the astaxanthin used in aquaculture is synthesized artificially (Sánchez-Camargo et al., 2012). There is an increasing demand for natural astaxanthin due to the growing demand for natural ingredients. Currently, the most popular source of natural astaxanthin is microalgae *Haematococcus pluvialis*. Various microorganisms such as green algae *Haematococcus pluvialis* and *Chlorella zofingiensis*, red yeast *Phaffia rhodozyma* and marine bacterium *Agrobacterium aurantiacum* can produce astaxanthin (Kittikaiwan, Powthongsook, Pavasant, & Shotipruk, 2007). Other natural sources of astaxanthin include wild Pacific sockeye salmon, lobster, arctic shrimp, crab, crawfish, red trout, algae, and krill. However, the potential of current sources is limited and is not sufficient to supply the global astaxanthin market (Breitenbach et al., 2016).

In recent years, there have been some attempts to synthesize astaxanthin in carrot, tomato, and corn through metabolic engineering (Breitenbach et al., 2016). However, the engineered plants make the development of new extraction methods crucial as current methods are based on marine organisms, which are high water content materials. Camelina seed contains a high amount of oil (~40%) ,and its water content is much lower than that of current natural astaxanthin sources, which in turn affect the selection of the solvent and extraction method and conditions for efficient recovery of astaxanthin from the source.

Astaxanthin is sensitive to light and oxygen; therefore, may degrade during extraction. Moreover, there is a growing demand for clean extraction methods by consumers and manufacturers for labeling and marketing purposes. Supercritical carbon dioxide (SC-$CO_2$) has been used as an alternative clean extraction method for various oilseeds such as flaxseed, sunflower seed, watermelon seed, and various lipid compounds such as lycopene from tomato seeds and lutein from spinach (Jaime et al., 2015). Pure SC-$CO_2$ is nonpolar; therefore, it extracts nonpolar compounds. Because astaxanthin is slightly polar, the polarity of SC-$CO_2$ was improved by modifying ethanol, which is a food grade solvent, to extract astaxanthin from *Haematococcus pluvialis*, Brazilian redspotted shrimp waste and tiger shrimp (Sánchez-Camargo et al., 2011).

There is no reported study on the extraction of astaxanthin from an oilseed using SC-$CO_2$ or ethanol-modified SC-$CO_2$. In this study, engineered camelina seed was used for the first time as an alternative non-marine astaxanthin source. The main objective of this study was to investigate the feasibility of ethanol-modified SC-$CO_2$ extraction of astaxanthin from engineered camelina seed. The specific objectives were: (i) to study the effects of ethanol modified-SC-$CO_2$ extraction parameters, namely, pressure, temperature

and ethanol concentration on the extraction of astaxanthin using response surface methodology (RSM); (ii) to optimize the ethanol-modified SC-$CO_2$ extraction conditions using RSM; (iii) to compare the SC-$CO_{2,\,opt}$ extraction with conventional hexane extraction and accelerated ethanol and hexane extractions in terms of oil yield and astaxanthin concentrations of the extracted oils; and iv) to evaluate the effect of the extraction method on the antioxidant activity of the astaxanthin-rich camelina seed oils.

## 3.3 Materials and methods

### 3.3.1 Materials

Astaxanthin-enriched camelina seeds were provided by the Plant Innovation Center at the University of Nebraska-Lincoln. Seeds were ground using an analytical mill (A11 basic, IKA Works, Inc., Wilmington, NC, USA) and sieved to obtain the particles smaller than 0.3 mm. $CO_2$ (99.99% purity) was purchased from Matheson (Lincoln, NE, USA). Astaxanthin from *Haematococcus pluvialis* (≥ 97% purity) and trans-β-apo-8'-carotenal (≥ 96% purity) standard were purchased from Sigma Aldrich (St Louis, MO, USA). Rac-5,7-Dimethyltocol was purchased from Matreya LLC. (State College, PA, USA). All other reagents and solvents were of the analytical or chromatographic grade.

### 3.3.2 Ethanol-modified SC-$CO_2$ extraction

Ethanol-modified SC-$CO_2$ extractions were carried out in a laboratory scale SC-$CO_2$ extraction system (SFT 110, Supercritical Fluids, Inc., Newark, DE, USA). Schematic

diagram of the system was reported previously (Belayneh et al., 2017). For each experiment, the extraction vessel was loaded with 10 g of ground camelina seed. The air in the vessel was flushed out by opening the $CO_2$ cylinder before each run for 2 min. Then, the shut-off valve was closed, and the extraction vessel heated to the extraction temperature in the oven of the system. After reaching the set extraction temperature, $CO_2$ was pumped into the system using the high-pressure $CO_2$ pump, and ethanol was pumped into the system at an inlet point before the extraction vessel using the co-solvent pump at the predetermined flow rates to attain the set ethanol concentrations. Extraction pressure was monitored and maintained constant using the $CO_2$ pump. A static extraction time of 20 min was established by keeping the shut-off valve closed. Then, the shut-off valve was opened, and the extracted oil was collected continuously in an amber glass sample collection vial held in a cold trap at -10 °C. The $CO_2$ flow rate was maintained at 1 L/min (measured at ambient conditions) with a heated micrometering valve, and measured by the gas flow meter placed after the sample collection vial. The ethanol in the extracted oils was evaporated under nitrogen flow at 40 °C. The amount of oil extracted was determined gravimetrically, and the oil yield (%, w/w) was obtained by dividing the mass of oil extracted by the mass of camelina seed used for extraction. The headspace of the vials containing the extracted oil were filled with nitrogen and stored at -20 °C until analyzed for astaxanthin concentration.

### 3.3.3 Experimental design

RSM based on a central composition rotatable design (CCRD) with three variables at three levels were used to investigate the effects of extraction variables (pressure, temperature, and ethanol concentration) on the astaxanthin concentration of the

extracted oils. Extraction time was limited to 180 min. The three different levels of the three variables were represented in codes as -1, 0, and +1. Two extreme levels were coded as -1.68 and +1.68. The actual levels of the coded and uncoded variables generated by the Design Expert software (Stat-Ease Inc., Minneapolis, MN, USA) were shown in Table 3.1. The total number of experiments was 20 ($2^k$+2k+6), where k is the number of independent variables. In order to determine the pure error, five replications were performed at the center point. The levels of the variables were determined based on the capabilities of the SC-$CO_2$ extraction system and the preliminary study.

**Table 3.1.** Independent variables and levels used for central composite rotatable design (CCRD).

| **Variable** | **Symbol coded** | **Levels** | | | | |
|---|---|---|---|---|---|---|
| | | -1.68 | -1 | 0 | +1 | +1.68 |
| **Pressure (MPa)** | $X_1$ | 24.9 | 30.0 | 37.5 | 45.0 | 50.1 |
| **Temperature (°C)** | $X_2$ | 33.2 | 40.0 | 50.0 | 60.0 | 66.8 |
| **Ethanol concentration (%)** | $X_3$ | 1.5 | 10.0 | 22.5 | 35.0 | 43.5 |

### 3.3.4 Hexane (Soxhlet) extraction

Ground camelina seeds (12 g) were extracted with hexane (250 mL) in a Soxhlet apparatus for 6 hrs in the dark to prevent photooxidation of astaxanthin. Hexane was separated from the oil using a rotary vacuum evaporator (Buchi Labortechnik AG, model B-490, Flawil, Switzerland) at 22 °C after each extraction. The resulting oil was weighed,

and the total oil yield was reported as (weight of oil/weight of ground seed used for extraction) x 100. The oil extracts were flushed with nitrogen and stored at -20 °C until analyzed for astaxanthin concentration.

### 3.3.5 Accelerated solvent extraction

Accelerated solvent extractions (ASE) using hexane and ethanol were performed using an accelerated solvent extractor (Dionex ASE 350, Sunnyvale, CA, USA) according to Zaghdoudi et al. (2015) with minor modifications. Ground camelina seed (3 g) was mixed with diatomaceous earth (1:3) to reduce the dead volume and then loaded into the 34 mL-extraction cell. Extractions were performed at 1500 psi (10.3 MPa) and 40 °C for 5 static cycles of 5 min. The cell was rinsed with the extraction solvent, and the solvent was purged from the cell with nitrogen for 60s. The solvent in the extract was evaporated under the nitrogen, and the oils were stored at -20 °C until analyzed for astaxanthin concentration.

### 3.3.6 Analysis of astaxanthin

Astaxanthin concentration of the oil samples was determined by a reversed phase high performance liquid chromatography (RP-HPLC) according to Du et al. (2016) with minor modifications. The samples were dissolved in acetone (70 mg/mL), and 15 µL of trans-β-Apo-8'-carotenal (internal standard) solution (1 mg/mL in acetone) was added onto each sample. An aliquot (10 µL) was injected into an HPLC (Agilent 1100, Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector (DAD). Samples were separated on a C18 column (150 × 4.6 mm; 5 µm particle size; Phenomenex

Inc., Torrance, CA, USA) using a mobile phase of methanol: acetonitrile (3:97, v/v) at a flow rate of 1 mL/min. The column temperature was set at 30 °C, and the elution was detected at 474 nm.

### 3.3.7 Tocopherol analysis

Tocopherols were analyzed according to the method of Belayneh et al. (2015) with minor modification. Ten mg of each extract was dissolved in 1 mL methanol: dichloromethane (9:1, v/v) solvent mixture and 20 µL of rac-5,7-Dimethyltocol (internal standard) solution (0.05 mg/mL) was added onto each sample. Then the samples were analyzed by a high performance liquid chromatography (HPLC) (1200 Series, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a fluorescence detector set at an excitation wavelength of 292 nm and an emission wavelength of 330 nm. An aliquot (70 µL) of prepared solution was separated on a reversed-phase Eclipse XDB-C18 column (150 × 4.6 mm; 5 µm particle size; Agilent Technologies, Inc., Santa Clara, CA, USA) using a mobile phase of methanol: water (95:5, v/v) at a flow rate of 1.5 mL/min. Retention time of tocopherol standards was used for identification. Tocopherol content was expressed as the sum of all tocopherols in mg tocopherol per kg oil.

### 3.3.8 Measurement of antioxidant activity

The antioxidant capacity of the oils obtained by different extraction methods was measured by $ABTS^{\bullet+}$ radical cation. The $ABTS^{\bullet+}$ radical cation was generated by reacting 7 mmol/L ABTS and 2.45 mmol/L potassium persulfate after incubation at room temperature for 16 h in the dark. The $ABTS^{\bullet+}$ radical solution was diluted with ethanol to

an absorbance of 0.700 ± 0.02 at 734 nm. 6 mg of each extract was added to react with 2 mL of ABTS solution. The mixture was stored in the dark for 6 min, and the absorbance at 734 nm was recorded using an Evolution 201 UV-Visible Spectrophotometer (ThermoFisher, Waltham, MA). Ethanol was used as the control. Each measurement was conducted in duplicate. The scavenging of free radical was calculated according to the Eq. (1):

$$ABTS\ Scavenging\ activity\ (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \quad (1)$$

### 3.3.9 Statistical analysis

Design Expert software 10.0.6 was used for regression and graphical analysis of the data. A quartic polynomial equation that correlates the response (astaxanthin concentration, µg/g oil) as a function of the independent variables and their interaction was developed. Analysis of variance (ANOVA) was used to determine the significance of the model through regression and mean square of residue error. The coefficient of determination ($R^2$) was used to assess the quality of the developed model. Analysis of the data to determine the statistical differences was performed by ANOVA and least-squares difference (LSD) using the SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) at 95% confidence interval.

## 3.4 Results and discussions

### 3.4.1 Model fitting

Preliminary studies were performed to observe the effect of ethanol concentration on the astaxanthin extraction and to determine the range of ethanol concentrations to be used in the RSM design, because ethanol-modified SC-$CO_2$ extraction of astaxanthin was not performed on an oilseed before. Total oil yield increased with increasing ethanol content in the SC-$CO_2$ (Fig. 3.1). The highest oil yield of 25.6% was obtained at 15% ethanol concentration, whereas oil yield was only 18.1% with pure SC-$CO_2$. Moreover, ethanol addition into SC-$CO_2$ increased the rate of the extraction, which is observed from the slope of the extraction lines in the first 120 min of the extraction (linear region) (Ciftci et al., 2012). Astaxanthin content of the seed was 200 μg/g seed, including both free and esterified astaxanthin. Preliminary studies revealed that the astaxanthin content of the oils increased with increasing ethanol concentration (Fig. 3.2). Astaxanthin content of the oil extracted with pure SC-$CO_2$ was 190 μg/g oil, whereas it was 304 μg/g oil for the oil extracted with 15% ethanol in SC-$CO_2$. In a study where astaxanthin was extracted from redspotted shrimp waste using SC-$CO_2$, the highest yield (2.3% dry wt.) was obtained at 30 MPa and 50 °C (Sánchez-Camargo et al., 2011). Sánchez-Camargo et al. (2012) reported that the astaxanthin concentration increased from 26 to 35 μg/g dry residue when SC-$CO_2$ was modified with 15% ethanol at 30 MPa and 50 °C. SC-$CO_2$ is nonpolar; therefore, it cannot extract polar compounds and has limited capacity to extract slightly polar compounds. Ethanol increases the polarity of SC-$CO_2$; therefore, increases the solubility of the astaxanthin in the ethanol-modified SC-$CO_2$ due to the slightly polar structure of astaxanthin. In addition, the SC-$CO_2$-expanded ethanol helps to swell the pores of the seeds, which in turn improves the interaction of the solvent with the inner parts of the seed matrix. In a study by López, Arce, Garrido, Ríos, & Valcárcel (2004), maximum

astaxanthin yield was obtained with 15% ethanol, whereas, any further increase in the ethanol amount in the SC-$CO_2$ caused a considerable decrease in the astaxanthin yield. Effect of ethanol content of the SC-$CO_2$ depends on the matrix, polarity of the target compound, extraction pressure and temperature; therefore, optimization of the extraction conditions is required for each case.

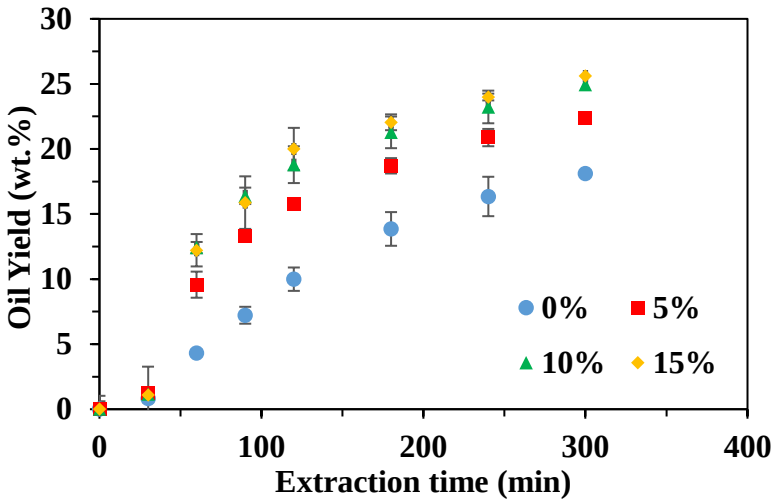



**Figure 3.1.** Ethanol-modified SC-$CO_2$ extraction curves of camelina seed oil at 30 MPa and 50 °C at varying ethanol concentrations.

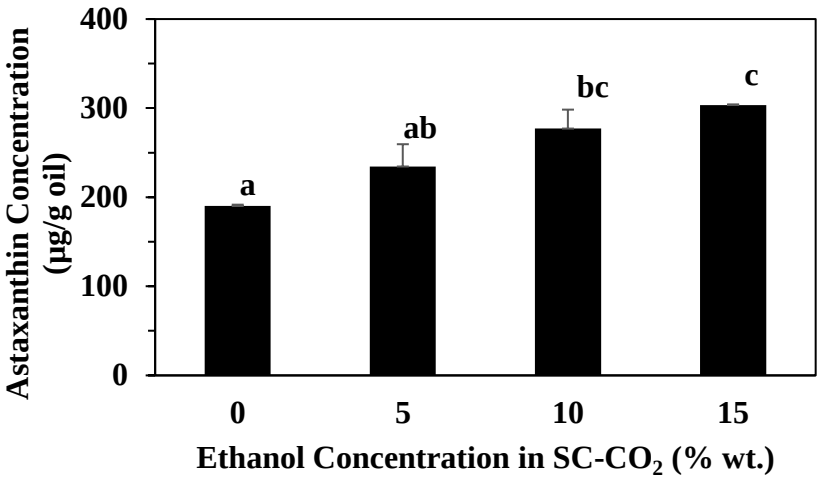



**Figure 3.2.** Effect of the ethanol concentration in the SC-$CO_2$ on the astaxanthin concentration of the extracted oils obtained at 30 MPa and 50 °C. Different lowercase letters are significantly different for each extraction conditions ($p < 0.05$).

Multiple regression was used to determine the effect of pressure, temperature and ethanol concentration on the SC-$CO_2$ extraction of astaxanthin from camelina seed from the 20 runs that were generated by the RSM (Table 3.2). Table 3.3 shows the statistical analysis of the quartic polynomial model based on ANOVA. After examining the lack of fit, a quartic polynomial model was found to be adequate to explain the relationship between the astaxanthin yield and the extraction parameters. Backward-elimination was applied to refine the model by eliminating the insignificant terms. Model P-value < 0.0001, an insignificant lack of fit (P-value= 0.2351) and a higher coefficient of determination ($R^2$= 0.989) confirmed the suitability of the model to explain the relationship within the range of variables studied.

**Table 3.2.** Experimental variables ($X_1$, pressure; $X_2$, temperature; $X_3$, ethanol concentration) and responses.

| Run | $X_1$ | $X_2$ | $X_3$ | Astaxanthin concentration (µg/g oil) | |
|---|---|---|---|---|---|
| | | | | Actual | Predicted |
| **1** | -1 | +1 | -1 | 274 | 274 |
| **2** | 0 | 0 | 0 | 305 | 298 |
| **3** | -1 | -1 | -1 | 261 | 262 |
| **4** | -1 | +1 | +1 | 283 | 283 |
| **5** | 0 | 0 | 0 | 299 | 298 |
| **6** | +1 | -1 | +1 | 364 | 364 |
| **7** | -1.68 | 0 | 0 | 287 | 287 |
| **8** | +1 | +1 | +1 | 276 | 276 |
| **9** | 0 | -1.68 | 0 | 342 | 342 |
| **10** | 0 | 0 | -1.68 | 208 | 215 |
| **11** | 0 | 0 | 0 | 298 | 298 |
| **12** | +1 | +1 | -1 | 328 | 328 |
| **13** | +1.68 | 0 | 0 | 396 | 396 |
| **14** | 0 | 0 | 0 | 310 | 298 |
| **15** | -1 | -1 | +1 | 269 | 270 |
| **16** | 0 | 0 | +1.68 | 375 | 381 |
| **17** | 0 | +1.68 | 0 | 360 | 360 |
| **18** | +1 | -1 | -1 | 270 | 270 |
| **19** | 0 | 0 | 0 | 287 | 298 |
| **20** | 0 | 0 | 0 | 300 | 298 |

**Table 3.3.** ANOVA for the fitted quartic polynomial model for optimization of extraction conditions.

| Source | Sum of squares | Degrees of freedom | Mean of square | F-value | Prob>F | Significance |
|---|---|---|---|---|---|---|
| **Model** | 38016.86 | 13 | 2924.37 | 42.99 | < 0.0001 | *** |
| **$X_1$** | 5955.77 | 1 | 5955.77 | 87.55 | < 0.0001 | *** |
| **$X_2$** | 177.47 | 1 | 177.47 | 2.61 | 0.1574 | n.s. |
| **$X_3$** | 13804.57 | 1 | 13804.57 | 202.92 | < 0.0001 | *** |
| **$X_1X_2$** | 388.37 | 1 | 388.37 | 5.71 | 0.0541 | n.s. |
| **$X_1X_3$** | 76.76 | 1 | 76.76 | 1.13 | 0.3290 | n.s. |
| **$X_2X_3$** | 2606.42 | 1 | 2606.42 | 38.31 | 0.0008 | *** |
| **$X_1^2$** | 3067.75 | 1 | 3067.75 | 45.10 | 0.0005 | *** |
| **$X_2^2$** | 4509.25 | 1 | 4509.25 | 66.29 | 0.0002 | *** |
| **$X_1X_2X_3$** | 2766.19 | 1 | 2766.19 | 40.66 | 0.0007 | *** |
| **$X_1^2X_2$** | 118.24 | 1 | 118.24 | 1.74 | 0.2355 | n.s. |
| **$X_1^2X_3$** | 5848.17 | 1 | 5848.17 | 85.97 | < 0.0001 | *** |
| **$X_1X_2^2$** | 613.73 | 1 | 613.73 | 9.02 | 0.0239 | n.s. |
| **$X_1^2X_2^2$** | 6594.35 | 1 | 6594.35 | 96.94 | < 0.0001 | *** |
| **Lack of Fit** | 108.97 | 1 | 108.97 | 1.82 | 0.2351 | n.s. |
| **Pure Error** | 299.20 | 5 | 59.84 | | | |
| **Cor Total** | 38425.03 | 19 | | | | |

CV= 2.71%, $R^2$= 0.9894. ***P<0.001; n.s., not significant.

Regression coefficients were determined to predict the polynomial model for astaxanthin concentration and Eq. (2), expressed in coded variables, was obtained:

$$Y= -20175.12 + 1161.47X_1 + 925.47X_2 - 111.28X_3 – 52.32X_1X_2 + 5.61X_1X_3 + 0.60X_2X_3 – 16.28X_1^2 -9.50 X_2^2 -0.02 X_1X_2X_3 + 0.73X_1^2X_2 -0.06 X_1^2X_3 + 0.53X_1X_2^2 -0.007 X_1^2X_2^2 \ldots\ldots \quad (2)$$

where $X_1$ is pressure, $X_2$ is temperature, $X_3$ is ethanol concentration. The coefficients in front of every term ($X_1$, $X_2$, and $X_3$) illustrate the effect of a factor and the interaction among the factors, respectively. The positive sign in front of the terms indicates the synergistic effect, while the negative sign indicates the antagonistic effect. In the fitting model for this response variable, the pressure ($p< 0.001$) and ethanol concentration ($p< 0.001$) affected the astaxanthin concentration linearly. Besides, an interaction of temperature and co-solvent was found.

### 3.4.2 Effect of extraction parameters on the astaxanthin yield

Figure 3.3 presents the effects of extraction parameters on the astaxanthin concentration at -1, 0, and +1 levels of the three variables. Unlike the quadratic or cubic polynomial model, the RSM plots of a quartic model were complex. At lower pressure (-1 level), ethanol concentration and temperature did not have a significant effect on the astaxanthin concentration (Fig. 3.3a). At moderate pressures (0 level), increasing ethanol concentration increased the astaxanthin yield at lower temperatures. At higher pressure (+1 level), increasing ethanol concentration at lower temperatures increased the astaxanthin yield; however, increasing ethanol concentration resulted in lower astaxanthin yields at higher temperatures. Effect of ethanol concentration and pressure on the astaxanthin yield at all temperature levels were similar. Regardless of the temperature level, at high ethanol concentration level, moderate pressure resulted in the highest astaxanthin concentration,

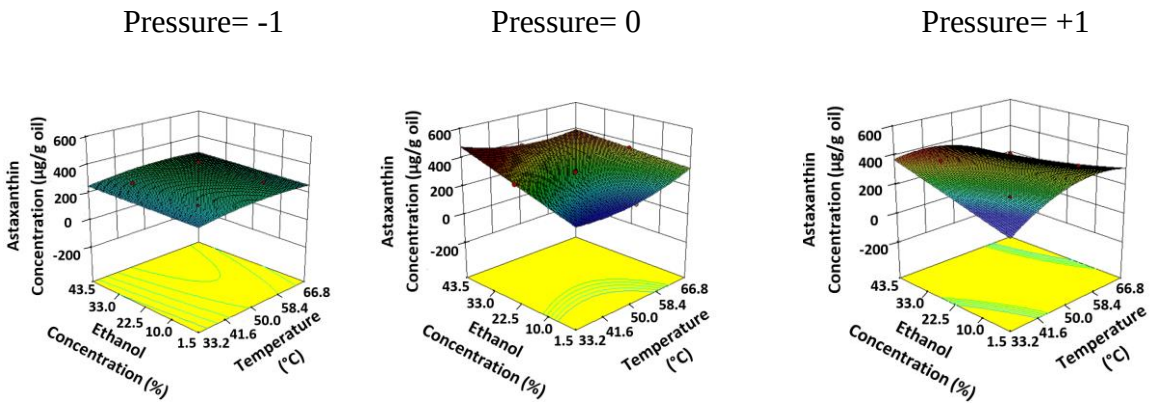
whereas moderate pressure led to the lowest astaxanthin concentration when ethanol concentration was low. At all ethanol levels, moderate pressures yielded higher astaxanthin at all temperatures. However, at lower and higher pressures, astaxanthin yield was lower at lower and higher temperatures at all ethanol levels. The temperature had two opposing effects; the density of $CO_2$ decreased with increasing temperature, which resulted in a reduced solvent power, on the other hand, increasing temperature led to an increase in the solute vapor pressure which affected the extraction positively. Furthermore, the temperature could affect the interaction between the solute and co-solvent molecules like hydrogen bond.

Extraction conditions were optimized to obtain the highest astaxanthin concentration using the RSM-developed model. The optimal extraction conditions were 41.6 MPa, 36.6 °C with 42.0% ethanol (wt.%) at 1 L/min $CO_2$ flow rate (measured at ambient conditions). The predicted optimum conditions were verified by three additional independent extractions at those optimum conditions. Optimum predicted astaxanthin concentration was 437 µg/g oil, while the oil yield was 28.3%. The actual astaxanthin concentration was 421 ± 14 µg/g oil, while the oil yield was 29 ± 2%. These results indicated that the experimental values were in good agreement with the predicted one.

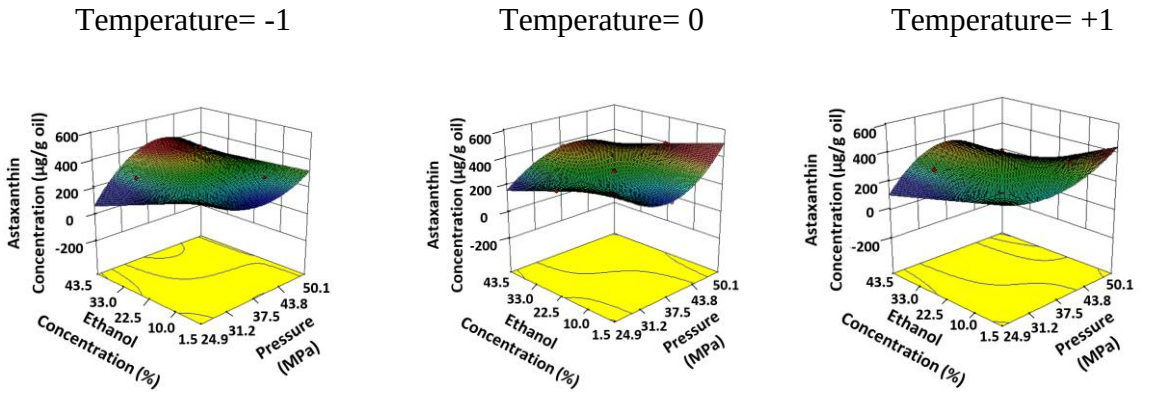
Once the optimum extraction conditions were obtained, an extraction curve of the camelina seed oil was generated (Fig. 3.4) to investigate the effect of extraction time. In the experimental design, extraction time was limited to 180 min to avoid long extraction times during optimization. However, it was found that an extraction time of 120 min at the optimized conditions is enough for the maximum recovery of the astaxanthin. It was found that the oil extracted in the first one hour of the extraction has the highest astaxanthin

concentration, meaning astaxanthin was extracted at a higher rate compared to camelina seed oil. This information is useful to obtain high purity products and to lower the processing costs with shorter extraction times.

**(a)**




**(b)**




**(c)**

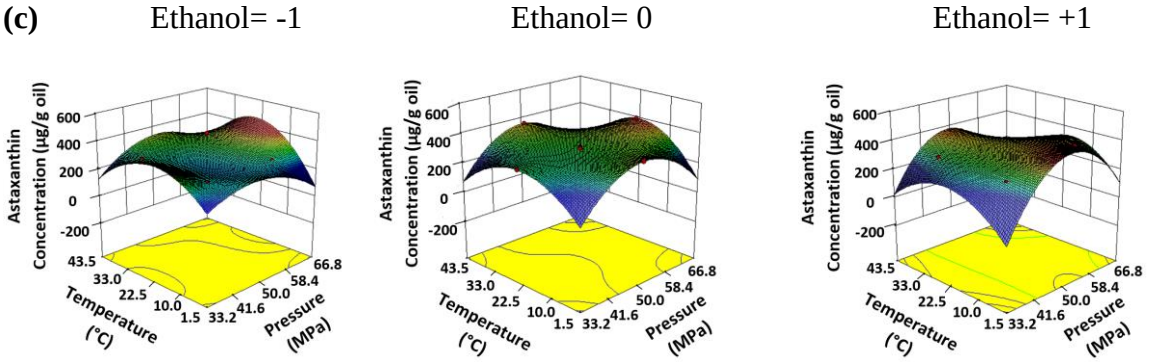



**Figure 3.3.** Response surface plots of astaxanthin concentration at -1, 0, and +1 levels of the independent variables.

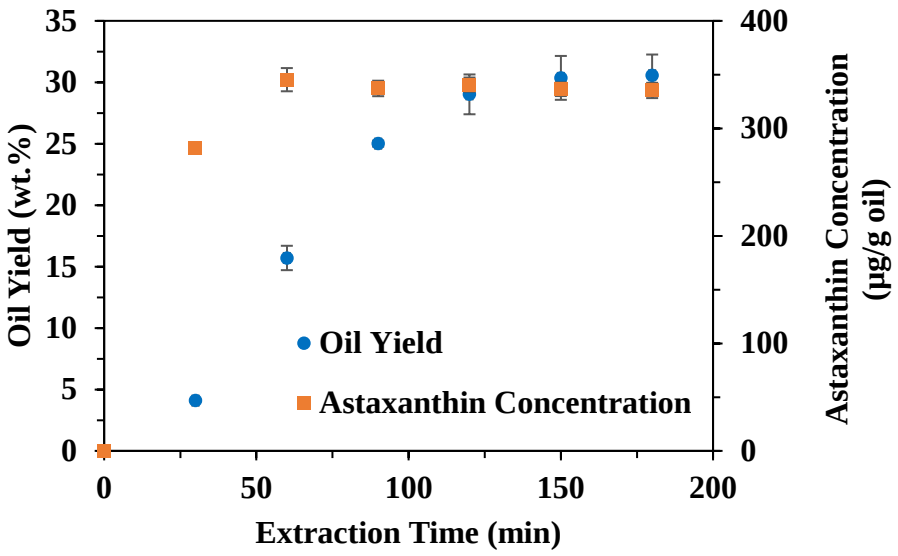



**Figure 3.4.** Experimental extraction curves for oil yield and astaxanthin concentration at RSM-optimized conditions (41.6 MPa pressure, 36.6 °C temperature, and 42.0% ethanol concentration, wt.%).

### 3.4.3 Comparison with other extraction methods

Figure 3.5 presents the comparison of the optimized ethanol-modified SC-$CO_2$ extraction (SC-$CO_{2,\ opt}$) with conventional hexane extraction and accelerated solvent extraction using hexane and ethanol as solvents. There was no significant difference between the oil yield and the astaxanthin concentration in oils extracted with SC-$CO_{2,\ opt}$ and hexane. Astaxanthin concentration of the oil extracted with SC-$CO_{2,\ opt}$ was 421 µg/g oil, whereas it was 418 µg/g oil in the hexane-extracted one. The lowest oil yield (23%) was obtained with accelerated hexane extraction (AHE), whereas the lowest astaxanthin concentration (75 µg/g oil) was obtained with accelerated ethanol extraction (AEE). For SC-$CO_{2,\ opt}$, the ethanol consumption was 166 mL, whereas it was 250 mL for the hexane (Soxhlet) extraction. ASE required approximately 50 mL solvent (ethanol or hexane),

which was much less than hexane and SC-$CO_{2,\ opt}$ extractions. Lower solvent consumption is desired due to processing costs related to solvent storage, pumping, and separation. Although ASE required a shorter extraction time and consumed less solvent, the astaxanthin concentration was very low compared to SC-$CO_{2,\ opt}$ and hexane extraction methods. The lowest astaxanthin yield with AEE was due to the high polarity of ethanol. Ethanol extracts polar compounds in the seed such as phospholipids and sugars but cannot extract slightly polar astaxanthin efficiently. A higher oil yield by AEE compared to AHE, but a lower β-carotene concentration in the oil was previously reported by Eller, Moser, Kenar, & Taylor (2010). Compared to the hexane extraction, a shorter extraction time of SC-$CO_{2,\ opt}$ allowed us to extract more astaxanthin, although two values were not significantly different. Reyes, Mendiola, Ibañez, & del Valle (2014) reported astaxanthin extraction recoveries from *Haematococcus pluvialis* up to 82.3% using ethanol-modified SC-$CO_2$.

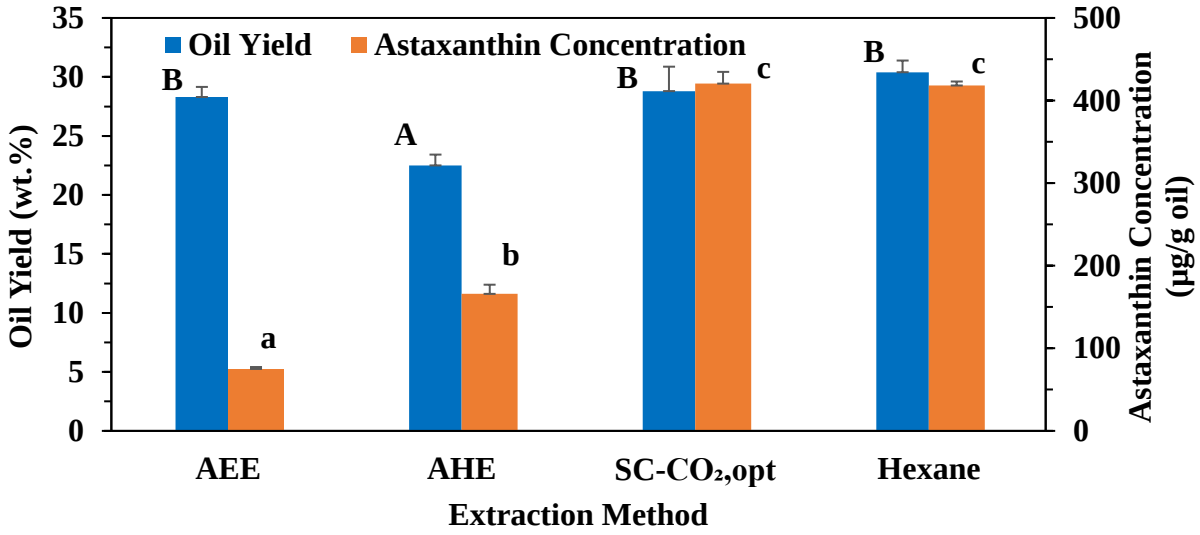



**Figure 3.5.** Oil yield and astaxanthin concentration obtained by SC-$CO_{2,\ opt}$, ASE and Hexane (soxhlet) extraction. SC-$CO_{2,\ opt}$: Optimized SC-$CO_2$ extraction; AEE:

Accelerated ethanol extraction; AHE: Accelerated hexane extraction. Different capital letters mean significant differences ($p< 0.05$) of the oil yield obtained from different extraction methods. Different lowercase letters mean significant differences ($p< 0.05$) of the astaxanthin concentration obtained from different extraction methods.

High ethanol concentrations in the optimized conditions suggested that better yields are obtained when $CO_2$-expanded ethanol was formed. Actually, the mechanism of extraction in a $CO_2$-expanded ethanol may be different than ethanol-modified SC-$CO_2$ extraction where pressure and temperature play the critical role by changing the solubility of astaxanthin in the supercritical phase, whereas when the ethanol content in the SC-$CO_2$ approaches 50%, the mixture behaves like a pressurized liquid (Golmakani, Mendiola, Rezaei, & Ibáñez, 2012). However, the low astaxanthin yields from AEE showed that the extraction mechanism of the AEE and the $CO_2$-expanded ethanol extraction is different. Both higher pressures and high ethanol contents at the optimized ethanol-modified SC-$CO_2$ extraction played a role by improving the mass transfer by higher pressures and improving polarity by the presence of ethanol. In must be noted that, in this study, the matrix contained a high amount of oil which acted as co-solvent; therefore, the extraction mechanism was different than that of extracted from high water-content marine animals. Previously, Krichnavaruk et al. (2008) used soybean oil, olive oil, and ethanol as co-solvent to extract astaxanthin from microalgae *Haematococcus pluvialis*. At the same extraction conditions, olive oil gave a comparable result to that with ethanol; however, soybean oil gave the lowest extraction efficiency. Those results suggested that the properties of the oil (e.g., fatty acid composition and viscosity) and the solubility of the oil in the SC-$CO_2$ could

affect the astaxanthin extraction efficiency. In another study, transgenic maize was extracted with ethanol in combination with added commercial maize oil, and it was found that this combination was as effective as tetrahydrofuran and chloroform (Breitenbach et al., 2016). In the study of transgenic maize (Breitenbach et al., 2016), the astaxanthin concentration of the final oil was 1 μg/g oil, which was considerably lower than the astaxanthin concentration obtained in this study.

In general, the polarity of the solvent used in the extraction should be the same as or similar with that of the target compounds, however, in some cases, using the mixture of polar and non-polar solvents may get higher recoveries (Sun, Ge, Lv, & Wang, 2012). For example, Yu et al. (2010) used a mixture of hexane and methanol as solvents to extract amitraz and 2,4- dimethylaniline (2,4- DMA) from animal tissues. Because amitraz is less polar than 2,4- DMA, a higher ratio of methanol in the mixture should result in a higher recovery for 2,4- DMA but not for amitraz. However, the highest recovery for both amitraz and 2,4- DMA was obtained when hexane/ methanol ratio was at a ratio of 1:9 (v/v) (Yu et al., 2010). Solvents used in the extraction not only act as the carrier of target compounds, but also have other functionalities. For example, the solvent can also swell the pores of the seeds or other materials. Thus, using the mixture of hexane and ethanol of ASE might result in higher recovery for oil, astaxanthin or both of them. However, mixing ethanol with hexane can be a concern for the manufacturers as the use of hexane cannot generate a product that complies with clean labeling.

### 3.4.4 Tocopherol content of the extracts obtained from different extraction methods

Tocopherols are important minor lipid components in camelina seed and are best known for their antioxidant activities. Table 3.4 presents the tocopherol composition of the oils obtained by four different extraction methods. The oils extracted with hexane contained the highest amount of tocopherols (763 mg/kg oil). Tocopherol content of the oils extracted with AEE, AHE, and SC-$CO_{2,\ opt}$ ranged between 633 and 650 mg/kg oil, and there was no significant difference among them ($p>0.05$). γ- and β- tocopherols were the dominant tocopherols in all oils, and their level ranged from 610 mg/kg oil (AEE) to 732 mg/kg oil (hexane). Low concentration of δ-tocopherol (17-20 mg/kg oil) followed by α-tocopherol (5-13 mg/kg oil) was detected in all samples. No significant difference was observed between the tocopherol contents of the oils extracted by AEE, AHE, and SC-$CO_{2,\ opt}$. Tocopherols are nonpolar compounds, and they show good solubility both in hexane and pure SC-$CO_2$; therefore, increasing the polarity of SC-$CO_2$ by ethanol decreased the extraction of tocopherols. Reports on the tocopherol content of camelina seed oil are scarce. Previously, tocopherol content of hexane- and SC-$CO_2$-extracted non-engineered camelina seed oil was reported as 653 and 766 mg/kg, respectively (Belayneh et al., 2017). It was shown that modification of SC-$CO_2$ with ethanol up to 10% ethanol at 35/45 MPa and 50/70 °C did not cause a significant difference in the extraction of tocopherols, but in this study it was shown that a drastic increase in the ethanol content of the SC-$CO_2$ decreases the extraction of tocopherols significantly compared to pure SC-$CO_2$.

**Table 3.4.** Effect of extraction method on the tocopherol composition of the oils.

| Extraction method | Tocopherol content (mg/kg oil) | | | |
|---|---|---|---|---|
| | **δ** | **γ and β** | **α** | **Total** |
| **AEE** | $20 \pm 0^{c}$ | $610 \pm 8^{a}$ | $5 \pm 0^{a}$ | $633 \pm 8^{a}$ |
| **AHE** | $17 \pm 1^{a}$ | $621 \pm 5^{a}$ | $12 \pm 1^{c}$ | $650 \pm 6^{a}$ |
| **SC-$CO_{2,\ opt}$** | $17 \pm 1^{ab}$ | $613 \pm 13^{a}$ | $9 \pm 0^{b}$ | $640 \pm 13^{a}$ |
| **Hexane** | $18 \pm 1^{bc}$ | $732 \pm 12^{b}$ | $13 \pm 0^{c}$ | $763 \pm 11^{b}$ |

Values followed by the different letters indicate significant differences ($p < 0.05$).

AEE: Accelerated ethanol extraction; AHE: Accelerated hexane extraction; SC-$CO_{2,\ opt}$: Optimized ethanol-modified SC-$CO_2$ extraction.

### 3.4.5 Antioxidant activity of the extracts obtained from different extraction methods

The ABTS radical scavenging activity of engineered camelina seed oils obtained from four extraction methods is shown in Table 3.5. The oil extracted from SC-$CO_{2,\ opt}$ showed the highest scavenging activity (65%), followed by hexane (48%), and AHE (39%). The lowest scavenging activity was obtained from AEE (32%). Results have shown that the ABTS scavenging activity increased with increasing astaxanthin content of the oils (Table 3.5 and Fig. 3.5). Tocopherols are natural antioxidants; however, it was found that astaxanthin content was the major factor determining the antioxidant activity of the oils. Even though the hexane-extracted oil had the highest tocopherol content, it did not have the highest antioxidant activity.

**Table 3.5.** Effect of extraction method on the ABTS radical scavenging activity of the oils.

| Extraction method | ABTS scavenging activity (%) |
|---|---|
| **AEE** | $32 \pm 1^{a}$ |
| **AHE** | $39 \pm 1^{b}$ |
| **SC-$CO_{2,\ opt}$** | $65 \pm 1^{d}$ |
| **Hexane** | $48 \pm 2^{c}$ |

Values followed by the different letters indicate significant differences ($p < 0.05$).

AEE: Accelerated ethanol extraction; AHE: Accelerated hexane extraction; SC-$CO_{2,\ opt}$: Optimized ethanol-modified SC-$CO_2$ extraction.

Previously, Reyes et al. (2014) stated that the antioxidant activity increased as the astaxanthin concentration in the extract from *Haematococcus pluvialis* increased regardless of the extraction method. However, Jaime et al. (2010) reported that the carotenoids in *Haematococcus pluvialis* extracted by AEE had higher antioxidant activity than those by AHE. The extraction condition and target compound could affect the selection of the appropriate solvent, resulting in different results. The oil extracted by SC-$CO_{2,\ opt}$ had higher antioxidant activity compared to that obtained by hexane extraction, even though their astaxanthin concentration in the oils were similar, showing the possible effect of other polar minor lipid compounds with antioxidant properties. Shao et al. (2014) compared the antioxidant activity of essential oils extracted from *Anoectochilus roxburghii* by hexane extraction and SC-$CO_2$ extraction and also reported that the oils extracted by SC-$CO_2$ showed higher antioxidant activity than those by hexane.

It should be noted that the material being investigated plays an important role in the antioxidant properties. The ABTS method measured the total antioxidant capacity of the extract; the specific chemical compositions of the extract could affect the antioxidant activity. Tocopherols are natural compounds that can have a synergistic effect on the antioxidant activity of the oils. Kang, Kim, & Moon (2016) reported that the highest antioxidant activity of paprika leave was achieved by the highest amount of lutein and γ-tocopherol in the extract. Our results suggested that tocopherols did not have a significant contribution to the antioxidant activity of the samples because the antioxidant activity of astaxanthin is much higher than that of tocopherols.

## 3.5 Conclusions

Engineered camelina seed was found to be a promising alternative source for astaxanthin. Ethanol-modified SC-$CO_2$ can be successfully used as a green extraction technique to extract astaxanthin. RSM was useful in optimizing the ethanol-modified SC-$CO_2$ extraction of astaxanthin from camelina oil seed. Ethanol-modified SC-$CO_2$ was more effective than AEE and AHE, and as effective as hexane to extract astaxanthin from camelina seed. Compared to hexane extraction, ethanol-modified SC-$CO_2$ extraction can provide protection to astaxanthin against oxidation and also eliminates hazardous solvents from processing.

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# Chapter 4. Encapsulation of astaxanthin-enriched camelina seed oil in ovalbumin/gum arabic- stabilized emulsion with/without crosslinking by tannic acid

## 4.1 Abstract

Engineered camelina seed is enriched in astaxanthin, a high-value carotenoid. The astaxanthin-enriched camelina seed oil extract can be a promising natural antioxidant and colorant. However, much effort is demanded to overcome the low oxidative stability and low water-solubility of the oil extract to broaden application fields. In this study, egg albumin (EA) and gum arabic (GA) were used as a combination to emulsify camelina oil. The resultant emulsions were further crosslinked by tannic acid, a potential natural phenolic cross-linker. The astaxanthin entrapment efficiency was around 70%. The size of EA/GA- stabilized emulsions at different pH conditions were investigated, and the smallest droplet diameter was observed at pH 7. But the best astaxanthin retention was achieved at pH 5 during storage at room temperature. After crosslinking by tannic acid, emulsions showed better protection of astaxanthin against UV light and heat. Our finding provided a promising emulsion system to encapsulate astaxanthin-enriched camelina oil.

**Keywords:** astaxanthin; tannic acid; emulsion; crosslinking; encapsulation.

## 4.2 Introduction

*Camelina sativa* is an underutilized oilseed in the USA, even though it has high oil content about 40% and high omega-3 fatty acids content (Belayneh, Wehling, Cahoon, & Ciftci, 2015). The engineered camelina seed used in this study was enriched with astaxanthin. Astaxanthin, a lipophilic bioactive compound, belongs to the carotenoid family which normally found in marine animals such as salmon, shrimp, and lobster (Shen & Quek, 2014). Due to the presence of hydroxyl and ketonic functional groups in its structure, astaxanthin has been reported to exhibit relatively higher antioxidant activity than vitamin E and beta-carotene (Khalid, Shu, Kobayashi, Nakajima, & Barrow, 2017; Shen & Quek, 2014). Several health benefits of astaxanthin have been reported, including protection against inflammation, cancer, and inhibition of oxidative stress and cardiovascular disease (Bustos-Garza, Yáñez-Fernández, & Barragán-Huerta, 2013; Liu, McClements, Cao, & Xiao, 2016). In aquaculture, astaxanthin is used as a natural colorant, particularly for salmonids (Khalid, Shu, Kobayashi, et al., 2017).

However, due to the high omega-3 fatty acid content, camelina oil has low oxidative stability. Besides, astaxanthin is highly unsaturated and likely to degrade due to the presence of light, oxygen, and heat stress. The low solubility of astaxanthin in water also limits its food applications. There is an increasing interest of using encapsulation strategies to protect astaxanthin against degradation, such as emulsification, incorporation into liposomes and solid lipid nanoparticles (Kamezaki et al., 2016; Khalid, Shu, Holland, et al., 2017; Li, Zahi, Yuan, Tian, & Liang, 2016). Emulsification is one of the most common methods to encapsulate oily material.

However, due to several physicochemical mechanisms, such as gravitational separation, flocculation, and Ostwald ripening, emulsion systems have a propensity to break down (Liu, Ma, Zhang, Gao, & Julian McClements, 2017). In order to stabilize the emulsion, emulsifiers are normally utilized, including both synthetic (e.g. Tween 80) and natural emulsifiers (e.g. proteins, polysaccharides, and phospholipids). Due to the safety concerns about those synthetic emulsifiers, there is a growing interest in utilizing natural emulsifiers in the food industry. Chicken egg albumin (EA), a common commercial albumin product, is the main constituent of egg white protein (~ 65%). It is an important food ingredient and frequently used as foam and emulsion stabilizing agent (Niu et al., 2016). Gum arabic (GA), an amphiphilic polysaccharide, is widely used in the food industry as stabilizer, thickening agent, and emulsifier (Ali, Ziada, & Blunden, 2009). Some properties, such as high water solubility, low viscosity, and persistent stability in a wide pH range, make GA an ideal biopolymer emulsifier in flavor beverage emulsion. The EA/GA- stabilized emulsions can be obtained from different preparation methods resulting in different emulsion structures (Niu et al., 2017). In this study, the oil droplet was coated by a single layer composed of EA/GA complexes.

However, the dissociation between EA and GA could occur due to environmental stresses resulting in an unstable emulsion (Anvari & Chung, 2016). Crosslinking techniques (e.g. chemical, physical, and enzymatic) of biopolymers in food emulsions have been shown effective at enhancing emulsion stability and functionality (Zeeb, Gibis, Fischer, & Weiss, 2012). Tannic acid, belonging to polyphenol family, is abundant with hydroxyl groups. It has been shown that tannic acid crosslinking improved gelling ability and mechanical properties of fish gelatin/GA gels (Anvari & Chung, 2016). In our

previous study, tannic acid showed strong interactions with EA, indicating a potential to be used as cross-linker for EA/GA- stabilized emulsions (Xie, Wehling, Ciftci, & Zhang, 2017).

Because of the lipophilic property of astaxanthin, emulsions were prepared from dissolving astaxanthin in additional organic solvents or commercial oils (Khalid, Shu, Holland, et al., 2017; Liu et al., 2016). However, in our study, astaxanthin is naturally dissolved in camelina oil extract, so there is no need for additional oil and toxic organic solvents. To our knowledge, there is no report using EA/GA complex to encapsulate camelina oil extract containing astaxanthin and utilizing tannic acid as the cross-linker. Therefore, the objectives of this study were to fabricate EA/GA- stabilized emulsion to encapsulate astaxanthin enriched camelina oil extract, characterize the emulsion systems at three pH conditions (pH 3, 5, and 7), and evaluate their physical properties. Furthermore, the effects of crosslinking by tannic acid on EA/GA- stabilized emulsion structures and functionalities were investigated.

## 4.3 Material and methods

### 4.3.1 Materials

Camelina oil extract was previously extracted from astaxanthin-enriched camelina seeds provided by the Plant Innovation Center at the University of Nebraska-Lincoln (Xie, Cahoon, Zhang, & Ciftci, 2019). EA, GA, tannic acid powders, and astaxanthin from *Haematococcus pluvialis* (≥ 97% purity) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). All other reagents and solvents were of the analytical grade.

### 4.3.2 Emulsion preparation

EA and GA stock solutions at 1% (w/v) and tannic acid stock solution at 10% (w/v) were obtained by dissolving powders in deionized water respectively and mechanically stirred at room temperature for 8 hrs. Mixtures of EA and GA were prepared by mixing appropriate volumes of stock solutions, and adjusted to pH 7 using 0.5N NaOH. A primary oil-in-water emulsion was prepared by homogenizing 1% (w/v) camelina oil extract with EA/GA mixture using an Ultra-Turrax T25 high-speed homogenizer (IKA Works, Inc., Wilmington, NC, USA) at 10, 000 rpm for 2 min. The fine emulsions were then obtained after ultrasonic treatment using the ultrasonic homogenizer (Thermofisher, Waltham, MA, USA) at 20 kHz for 2 min in an ice bath. The whole emulsion preparation process was conducted in the dark to minimize degradation by light. The pH of the emulsion was adjusted with 1N HCl or 0.5N NaOH to the desired pH. All final emulsions had a total biopolymer concentration of 1% (w/v) and oil content of 1% (w/v). For cross-linking, an appropriate volume of tannic acid stock solution was added to the emulsion at pH 5 to achieve a final concentration of 1, 2, 3% (w/v), respectively. Then the pH of the emulsion was adjusted back to 5 using 0.5N NaOH. All emulsions were stored at room temperature in the dark until further analysis.

### 4.3.3 Droplet size and zeta-potential measurements

The droplet size and zeta-potential of the emulsions were measured by dynamic light scattering (Nano Zetasizer, Malvern Instruments, UK) at 25 °C. To avoid multiple scattering, samples were diluted 1:10 using the citrate buffer solution (0.1M) at

corresponding pH. Samples were vortexed for 30s and settled for 30s before measurement. Reported mean droplet diameter and zeta-potential were the average of three independent replicates.

### 4.3.4 Quantification of astaxanthin

Quantification of astaxanthin content in EA/GA- stabilized emulsion was performed according to Khalid, Shu, Holland, et al. (2017) with minor modifications. Astaxanthin in emulsion samples was extracted using solvent: 1.0 mL of emulsion samples were mixed with 4.0 mL organic solvent (dichloromethane: methanol = 2:1 (v/v)), and then centrifuged at 4000 g for 20 min at 25 °C. Astaxanthin was quantified using an Evolution 201 UV-Visible Spectrophotometer (ThermoFisher, Waltham, MA, USA) at 474 nm. The pure dichloromethane and methanol solution (2:1, v/v) was used as a blank. The measurements were performed in duplicate. The standard curve of astaxanthin was obtained by dissolving astaxanthin standard in pure dichloromethane and methanol solution (2:1, v/v) ($R^2$=0.992). Then the astaxanthin entrapment efficiency in the emulsions could be calculated from the standard curve.

The astaxanthin retention was calculated according to the Eq. (1):

$$Astaxanthin\ retention\ (\%) = \frac{C_t}{C_0} \times 100 \qquad (1)$$

where $C_t$ is the concentration of astaxanthin in the emulsion at a specific time, while $C_0$ is the initial concentration of astaxanthin in oil extract added into emulsions. The measurements were conducted in triplicate.

### 4.3.5 Confocal fluorescence microscopy

The microstructure of the emulsions was examined using a confocal fluorescence microscope (Olympus FV500, Olympus Corporation, PA, USA). Twenty µL of the emulsion was stained with 4 µL Nile red and Fast green solution at 0.05 mg/mL, respectively to dye oil or protein. Then, the mixture was vortexed for 10s, and stained for 15 min before image collection. An aliquot (10 µL) of the stained emulsion was placed on a microscope slide, covered by a coverslip. Nile red and Fast green were excited at 543 nm and 488 nm, respectively.

### 4.3.6 UV light exposure

To evaluate the stability of the emulsified astaxanthin against UV light, 3 mL of oil control and emulsion samples in 5 mL glass vials were placed in a chamber illuminated by a white/UV transilluminator (UVP, LCC, Upland, CA). Astaxanthin retention was measured at an interval of 30 min as described in 4.3.4. Astaxanthin-enriched oil dissolved in ethanol was used as the control.

### 4.3.7 Measurement of antioxidant activity

$ABTS^{+}$ radical cation scavenging activity change of the emulsion due to the heating was measured according to (Wang, Gulati, Santra, Rose, & Zhang, 2018). Briefly, potassium persulfate was dissolved at an overall concentration of 2.45 mM in the aqueous solution of 7 mM ABTS. The mixture ($ABTS^{+}$) was allowed to stand in the dark

(25 °C) for at least 12 h. Then, the $ABTS^+$ solution was diluted with PBS buffer to achieve an absorbance of 0.700 (± 0.02) $cm^{-1}$ at 734 nm and equilibrated at ambient temperature for 30 min. Two mL of emulsions before and after heat treatment (80 °C for 5min) were added to 1 mL of $ABTS^+$ solution and incubated in the dark at room temperature for 6 min; the absorbance was then recorded at 734 nm using an Evolution 201 UV-Visible Spectrophotometer (ThermoFisher, Waltham, MA, USA). Each measurement was conducted in triplicate. The scavenging of free radical was calculated according to the Eq. (2):

$$Scavenging\ activity\ (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \quad (2)$$

### 4.3.8 Flow behavior of emulsions

The rheological behavior of emulsion was measured using an MCR 301 rheometer (Anton Parr, Graz, Austria) equipped with a 27 mm inner diameter concentric cylinder (CC27) at 25 °C. For the oscillatory shear measurements, the linear viscoelastic range of the emulsions was determined by a strain sweep (0.01-20%) at a constant frequency of 6.28 rad/s. Then, the dynamical oscillatory frequency sweep tests were performed. The frequency (f) was set from 0 to 125 rad/s, and the constant strain amplitude was fixed at 1% of the linear viscoelastic range. The storage modulus (G'), loss modulus (G"), and loss factor (G"/G') were measured in duplicate.

### 4.3.9 Statistical analysis

All the measurements were performed in duplicate or triplicate. SAS software (Version 9.4, SAS Institute, Cary, NC, USA) was used for the statistical analysis. The results were subjected to one-way analysis of variance (ANOVA), and the significance level was set at $p< 0.05$ for all analyses.

## 4.4 Results and discussions

### 4.4.1 Characterization of EA/GA- stabilized emulsions

The freshly prepared emulsions showed milky appearances and an orange color due to the presence of astaxanthin. Table 4.1 summarized the mean hydrodynamic diameter, zeta-potential, and entrapment efficiency of all emulsions. EA/GA- stabilized emulsions showed the biggest droplet size at pH 3. Niu et al. (2017) also reported the EA/GA- stabilized emulsion showed larger mean hydrodynamic diameter at pH 3.3. EA and GA can form the insoluble complexes, and then the EA/GA insoluble complexes assembled into larger particles and adsorbed onto the surface of the oil droplets. Besides, emulsions showed the lowest magnitude of zeta-potential (12.7 mV) at this pH, indicating that the flocculation might occur. The emulsion at pH 7 showed smaller diameter and larger magnitude of zeta-potential than other two pH conditions. At pH 7, both EA and GA carried significant negative charges. The magnitude of zeta-potential over 30 mV could stabilize the emulsion by strong electrostatic repulsion (Wang & Zhang, 2017). In this case, the aggregation of oil droplets was prevented by the repulsive electrostatic interaction resulting in the smallest droplet diameter. Niu et al. (2017) reported that EA/GA- stabilized sunflower oil emulsions showed the same mean droplet diameter at

pH 5 and 7. The different results obtained in our study might be due to the different oil property and homogenization methods. The entrapment efficiency of astaxanthin in the initial emulsions at three pH conditions was ranged from 71 to 75% (Table 4.1). The loss of about 30% astaxanthin during emulsion preparation may have been a result of oxidation happened during two-steps of homogenization. Some free radicals might be generated during the homogenization resulting in a 20% loss of astaxanthin (Liu et al., 2016). The different homogenization methods used in different studies could influence astaxanthin entrapment efficiency results. The pH environment did not have a significant effect on astaxanthin in the freshly prepared emulsion.

**Table 4.1.** Mean hydrodynamic diameter, zeta-potential, and astaxanthin entrapment efficiency of EA/GA- stabilized emulsion.

| pH | Diameter (μm) | Zeta-potential (mV) | Entrapment Efficiency (%) |
|---|---|---|---|
| **3** | $5.4 \pm 0.2^{c}$ | $-12.7 \pm 1.9^{a}$ | $71 \pm 1^{a}$ |
| **5** | $1.0 \pm 0.0^{b}$ | $-17.0 \pm 1.7^{b}$ | $72 \pm 2^{a}$ |
| **7** | $0.2 \pm 0.0^{a}$ | $-37.2 \pm 1.5^{c}$ | $75 \pm 2^{a}$ |

Values followed by the different letters indicate significant difference ($p < 0.05$).

Confocal fluorescence microscopy is a practical tool to observe the microstructure of the emulsions, therefore to investigate the interaction between oil and protein. The images obtained by confocal fluorescence microscopy were shown in Figure 4.1. In all images, the oil droplets were shown in red and proteins were indicated in green. The inter-fat droplet distribution mainly depended on the pH condition. At pH 3 and 5, protein aggregation seemed to be the dominating phenomenon. However, at pH 7, the oil droplets

were coated by protein individually. The results supported the droplet size observation that the strong electrostatic repulsion prevented the protein aggregation at pH 7.

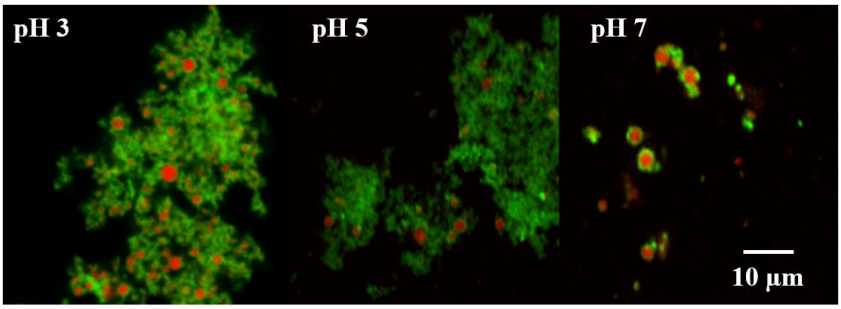



**Figure 4.1.** Confocal fluorescence microscopy images of EA/GA- stabilized emulsions at three pH conditions. Lipid in red and protein in green.

The flow behavior of EA/GA- stabilized emulsion at pH 5 and 7 was also studied (Figure 4.2). The flow behavior test of the emulsion at pH 3 was not recorded because of precipitation occurring during the test. The flow behavior is one of the most important properties of emulsions (Feng, Cai, Wang, Li, & Liu, 2018). Appearance, texture and shelf life of emulsion products are often related to the rheological properties. Small amplitude oscillatory shear tests were performed to provide information about the fluid-like and solid-like characteristics of emulsions (Feng et al., 2018; Liu, Wang, Sun, & Gao, 2016). The storage modulus G' is a measure of the energy stored reversibly within in the system, characterizing the elastic behavior, while the loss modulus G" represents the viscous behavior. The loss factor (G"/G') describes the relationship between the viscous and elastic portion of the sample. As shown in Figure 4.2, in both cases, the G' were higher than G", and loss factor values were less than 1, representing a more elastic

or gel-like system. The emulsion at pH 5 showed higher G' value compared to pH 7. Niu et al. (2016) also reported that EA/GA stabilized emulsion possessed a higher G' value at pH 4.8 than pH 7 because the emulsion droplets might have better rigidity at pH 4.8. The loss factor of the emulsion at pH 5 remained relatively stable within the range of angular frequency, which might reflect better emulsion stability (Niu et al., 2016). From the frequency study, the stability of emulsions may follow the order: pH 5> pH 7> pH 3.

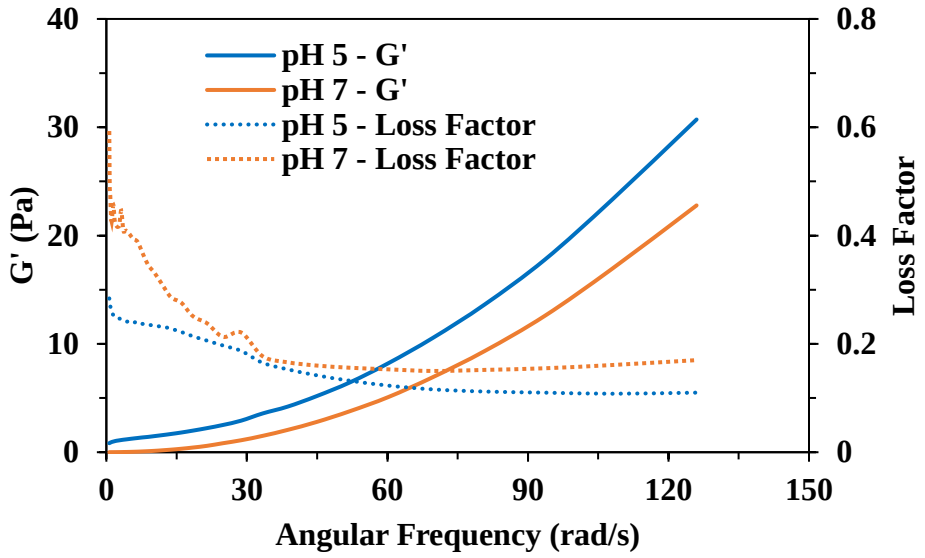



**Figure 4.2.** Storage modulus (G') and loss factor curve of EA/GA stabilized emulsions at pH 5 and 7.

### 4.4.2 Droplet size, zeta-potential, and astaxanthin retention change during storage

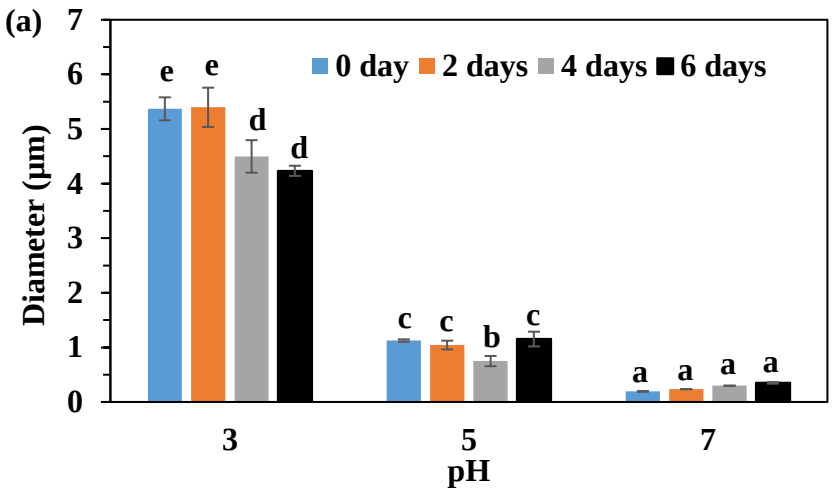
Figure 4.3 shows the change of droplet size (a), zeta-potential (b), and astaxanthin retention (c) of EA/GA- stabilized emulsions during storage for 6 days at room temperature at three pH conditions. As shown in Figure 4.3 (a), at pH 3, the mean droplet diameter slightly decreased as the storage time increased, but the size was still above 4

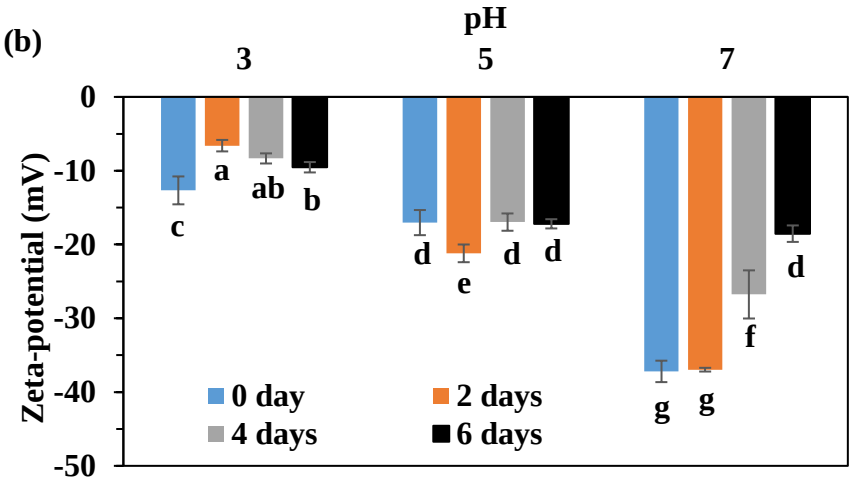
μm. At pH 5, the mean droplet diameter and zeta-potential remained stable during storage. But when pH changed to 7, the droplet size increased due to the decreased zeta-potential during storage. Niu et al. (2017) reported that EA/GA- stabilized sunflower oil emulsion remained stable after storage for 7 days at pH 3.5-7.0. The different homogenization method was one of the main factors leading to different observations.

At pH 3, due to the large droplet size, gravity-induced particle precipitation was the main factor resulting in the decrease of astaxanthin retention. Although the insoluble EA/GA complexes absorbed onto the surface of the oil droplets, they were not stable enough to form the Pickering emulsion in this case. At pH 5 and 7, EA and GA carried similar net charges and no electrostatic attractive interactions occurred, resulting in a thin and loose interfacial layer (Niu et al., 2017). However, after 6 days' storage, emulsion at pH 5 showed the highest astaxanthin retention. The phenomenon might be explained by relative stable droplet size, zeta-potential, and flow behavior compared to the emulsion at pH 7.

Another point was that the astaxanthin retention was not only affected by the emulsion stability, but also the degradation of astaxanthin. The degradation of astaxanthin could still occur during the storage even though the emulsions were stored in the dark. In addition, Takeungwongtrakul & Benjakul (2016) reported that the degradation of astaxanthin might be associated with lower oxidative stability of fatty acids in the oil. If lipid oxidation could take place due to the high omega-3 oil content of camelina oil, the formation of highly reactive compounds, like peroxyl radicals, could increase the degradation of astaxanthin. Lavecchia & Zuorro (2008) observed that the oxidation of vegetable oil increased the degradation of lutein. Limiting the oxidation of

camelina oil and decreasing astaxanthin degradation could be achieved by flushing the astaxanthin emulsion with nitrogen gas resulting in an oxygen-free atmosphere. However, exclusion of oxygen from food beverage during processing and storage is often not practical, and once the bottle is open, it would become exposed to oxygen (Anarjan & Tan, 2013).


(a)
Diameter (µm)
0 day
2 days
4 days
6 days
e e d d
c c b c
a a a a
3
5
7
pH


(b)
pH
3
5
7
Zeta-potential (mV)
c a ab b
d e d d
g g f d
0 day
2 days
4 days
6 days

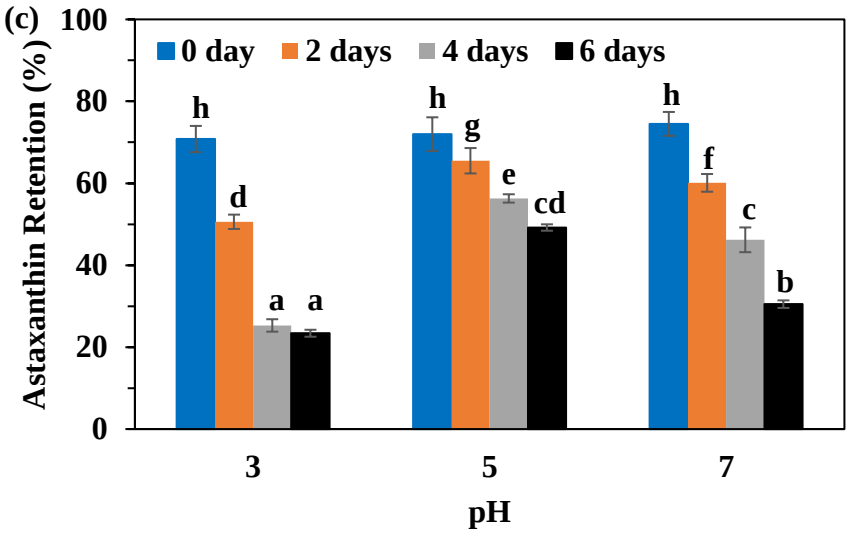

(c)
Astaxanthin Retention (%)
0 day
2 days
4 days
6 days
h d a a
h g e cd
h f c b
3
5
7
pH

**Figure 4.3.** Mean droplet diameter (a), zeta-potential (b), and astaxanthin retention (c) change of EA/GA- stabilized emulsions during room temperature storage. Different lowercase letters mean significant differences ($p < 0.05$).

### 4.4.3 Effect of crosslinking by tannic acid on EA/GA- stabilized emulsion at pH 5

#### 4.4.3.1 Droplet size and entrapment efficiency

EA/GA- stabilized emulsion at pH 5 was further crosslinked by tannic acid. The effect of crosslinking on droplet size and entrapment efficiency change was studied, and the results are shown in Figure 4.4. After the crosslinking, tannic acid formed linkages with EA/GA complexes. Only 2% and 3% tannic acid increased the mean droplet diameter of the emulsions. An appropriate tannic acid: biopolymer ratio should also be considered for the utilization of tannic acid as cross-linker. The additional tannic acid did not affect the entrapment efficiency, further confirmed our hypothesis that loss of astaxanthin mainly happened during emulsification. As a cross-linker, tannic acid did not interact with astaxanthin directly because the oil droplets are surrounded by the interfacial layer consisting of EA/GA complexes. In general, tannic acid can interact with proteins through non-covalent interactions (such as hydrogen bonding, electrostatic interaction, and hydrophobic interaction) (Xie et al., 2017). Based on the previous study, hydrogen bonds might be the main bonding force between EA and tannic acid (Xie et al., 2017); however, in this study, tannic acid interacted with EA/GA complexes as shown in Figure 4.5. Thus, further experiments will be needed to determine the binding mechanism between tannic acid and EA/GA complex. Anvari & Chung (2016) reported that the

hydroxyl groups or aromatic rings of tannic acid might be more reactive toward protein molecules into the fish gelatin-GA complex coacervates.

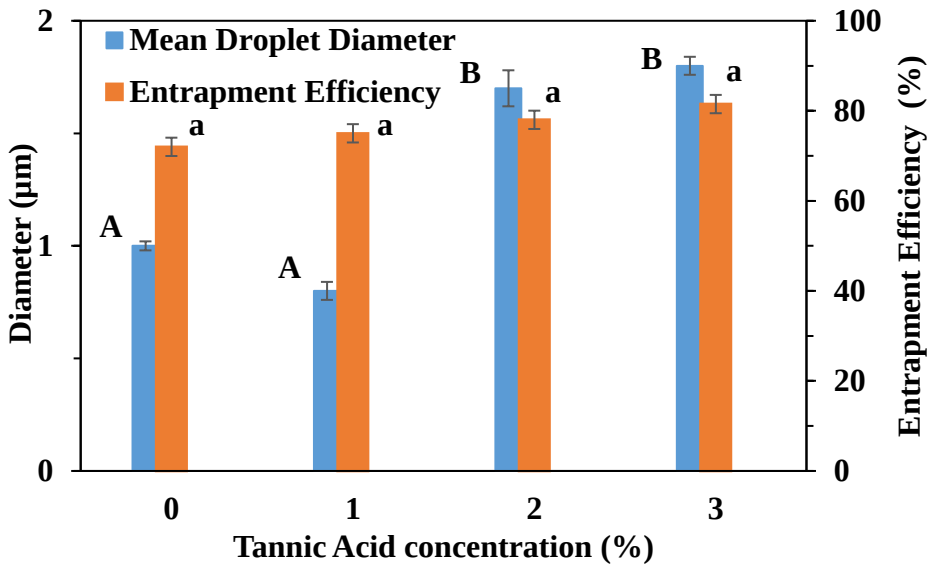



**Figure 4.4.** Effect of tannic acid on droplet size and entrapment efficiency of EA/GA-stabilized emulsion at pH 5. Different uppercase letters mean significant differences ($p < 0.05$) of the mean droplet diameter. Different lowercase letters mean significant differences ($p < 0.05$) of the entrapment efficiency.

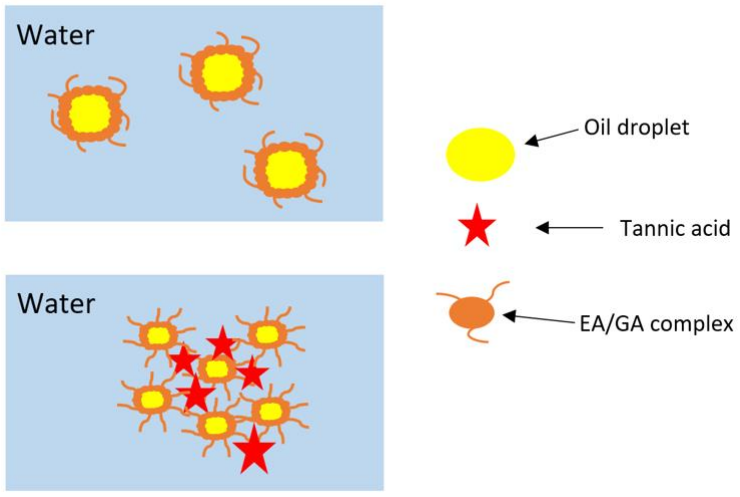



**Figure 4.5.** A proposed mechanism diagram of EA/GA- stabilized emulsion with and without tannic acid crosslinking.

#### 4.4.3.2 Protection of astaxanthin against UV light

Several mechanisms can result in carotenoids degradation in different systems due to UV light exposure. One possible mechanism is that the light may produce carotenoid radicals because of hydrogen abstraction, which caused the bleaching of carotenoids (Anarjan & Tan, 2013; Liu et al., 2016). The retention of astaxanthin in emulsions at pH 5 after UV light exposure was shown in Figure 4.6. Boon, McClements, Weiss, & Decker (2009) reported that the oil droplets in emulsion could scatter light, resulting in limited penetration of light waves into the sample. The solution showed milky appearance in the emulsion system, whereas the control solution was clear. The milky appearance of emulsion and scattering effect of oil could reduce the penetration of damage wavelength leading to higher astaxanthin retention, whereas, in optically transparent systems, the light waves can penetrate more easily resulting in the photodegradation of astaxanthin.

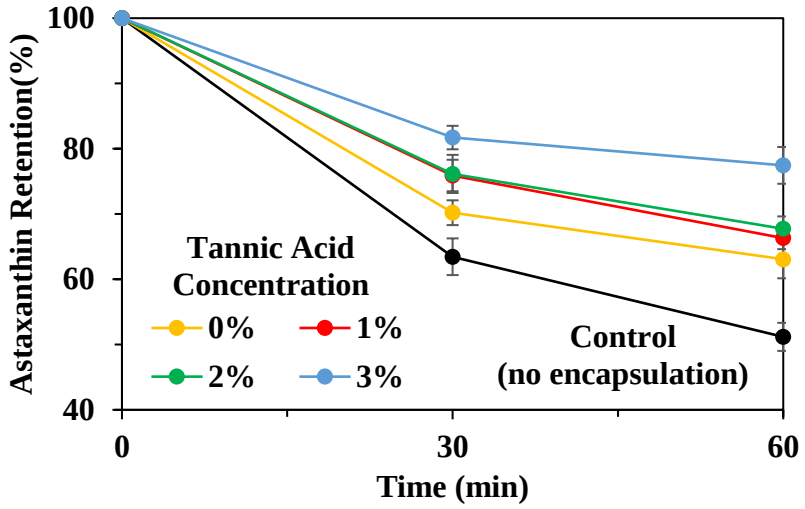



**Figure 4.6.** Effect of tannic acid on astaxanthin retention against UV light of EA/GA-stabilized emulsion at pH 5.

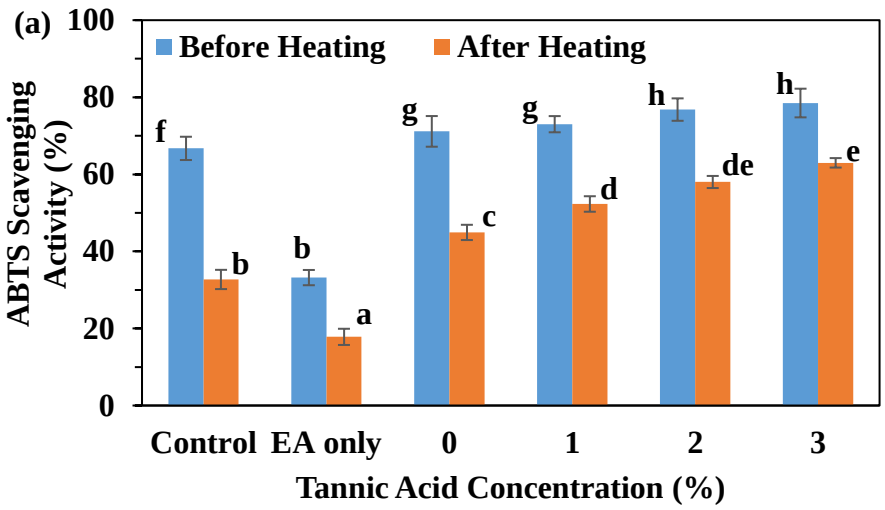
As shown in Figure 4.6, astaxanthin retention after UV light exposure was improved by tannic acid crosslinking, and the 3% tannic acid gave the best protection of astaxanthin against UV light. As the tannic acid concentration increased, the emulsion formed a more compact network, which could reduce light penetration. In addition, in the presence of singlet oxygen generators, light can degrade carotenoids very effectively (Choe & Min, 2009). Phenolic compounds are also oxidized by singlet oxygen. In this case, the oxidation of tannic acid could also occur. Once the tannic acid oxidized, the color of emulsion became intensive which could limit the light penetration. However, if there were not any singlet oxygen generators in the system, the decomposition of carotenoid through this pathway could be neglected (Anarjan & Tan, 2013).

#### 4.4.3.3 Protection of astaxanthin against heat stress

Thermal processing is very common for commercial food and beverage emulsions. The denaturation temperature of EA was around 80 °C (Lara, Gourdin-Bertin, Adamcik, Bolisetty, & Mezzenga, 2012). The denaturation of protein can reveal reactive groups such as non-polar and sulfhydryl, which could further affect the encapsulation system (Niu et al., 2017). In addition, the thermal treatment can increase the degradation of astaxanthin and camelina oil resulting in low antioxidant activity. Thus, it is very important to study the influence of heat on the antioxidant activity of EA- stabilized and EA/GA- stabilized emulsions. The effect of additional tannic acid was also examined in this study. As shown in Figure 4.7 (a), before heating, the EA- stabilized emulsion had the lowest antioxidant activity, because the emulsion was not stable at pH 5. Other treatments did not show any significant difference in antioxidant activity before heating. After the thermal processing, the EA- stabilized emulsion still had the lowest antioxidant activity (~17.8%), and it was even worse than the control (no encapsulation). The pI of EA was around 5, so the protein precipitation was more likely to occur resulting in unstable emulsion and less antioxidant activity. However, the incorporation of GA improved the emulsion stability at pH 5. In the EA/GA- stabilized emulsions, EA/GA complexes formed the interfacial layer, which overcame the limitation of using EA at pH 5. The EA/GA- stabilized emulsion system was more effective than control and EA to protect astaxanthin against heat stress. In addition, as the concentration of tannic acid in the emulsion increased, the antioxidant activity also increased. Tannic acid belongs to the polyphenol group and acts as an antioxidant through single electron transfer (Goiris et al., 2012). ABTS method detects antioxidant activity from both carotenoids and polyphenols.

In this case, astaxanthin and tannic acid both contributed to the antioxidant activity of the system.

Tocopherols are important minor lipid components in camelina seed, and best known for their antioxidant activities (Xie et al., 2019). Non-engineered camelina seed oil was also used in this study in order to examine the contribution to the antioxidant activity from tocopherols. Compare Figure 4.7 (a) with (b), either with or without encapsulation, regular camelina seed oil contained much lower antioxidant activity ($P<0.05$), which agreed with the results from (Xie et al., 2019). After the crosslinking by tannic acid, the antioxidant activity of emulsions was improved.



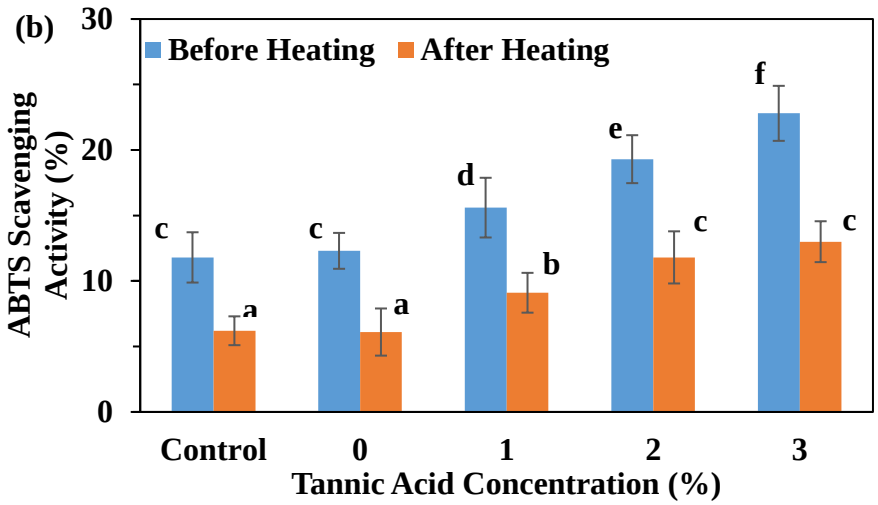



**Figure 4.7.** Effect of tannic acid on ABTS scavenging antioxidant activity against the heat of EA/GA- stabilized emulsion at pH 5. (a) astaxanthin-enriched camelina seed oil; (b) non-engineered camelina seed oil. Control: no encapsulation. EA only: EA stabilized emulsion. Different lowercase letters mean significant differences ($p < 0.05$) of the ABTS scavenging activity.

#### 4.4.3.4 Flow behavior

The effect of tannic acid on emulsion flow behavior was studied by frequency sweeping test. As shown in Figure 4.8, all the emulsions exhibited a predominantly elastic gel-like behavior for all the frequencies tested because the loss factor (G"/G') was less than 1. The loss factor is greater than 0.1, typical behavior of dressings and emulsions (Mandala, Savvas, & Kostaropoulos, 2004). Therefore, they were characterized as weak gels. However, after the crosslinking by 3% tannic acid, emulsion at pH 5 showed different flow behavior compared to other emulsions. As the frequency increased, the loss factor of emulsion with 3% tannic acid at pH 5 was also increased,

whereas the loss factor of other emulsions decreased. Although the elastic gel-like behavior was still dominant in all emulsions, the viscous portion increased in the emulsion with 3% tannic acid at pH 5. Generally, the crosslinking by tannic acid increased the loss factor at each pH condition, which indicated that after the crosslinking, the network structure became easily to rearrange to accommodate the strain (Bortnowska, Balejko, Tokarczyk, Romanowska-Osuch, & Krzemińska, 2014). Because of the weak gel structure, macromolecules interconnections and entanglements could be disrupted by applying high shear rates (Mandala et al., 2004). Our rheological analysis results partially agreed with the results from (Niu et al., 2016). The properties of oil, homogenization method, and rheological behavior determination system all could affect the final analysis results.

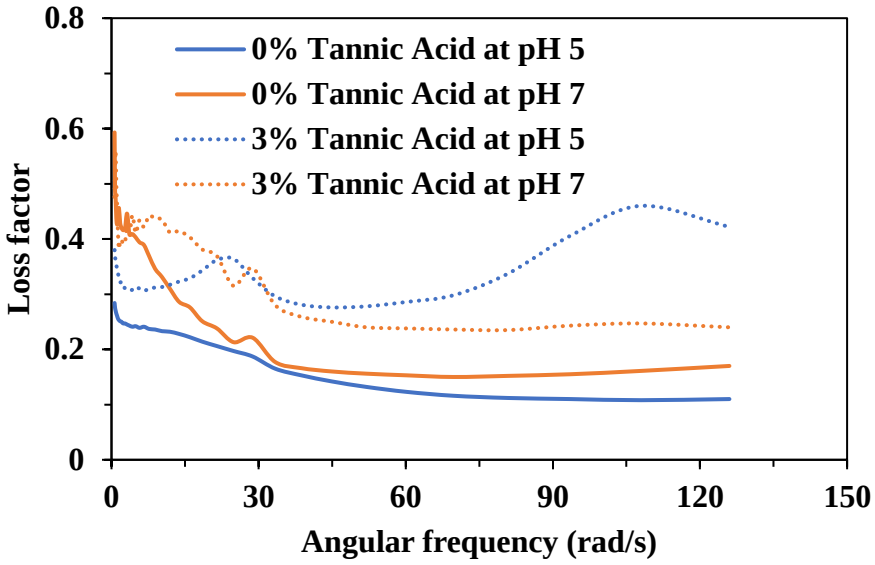



**Figure 4.8.** Flow behavior of EA/GA- stabilized emulsions at pH 5 and 7 with and without crosslinking by 3% tannic acid.

## 4.5 Conclusions

Astaxanthin-enriched camelina seed oil, as a potential astaxanthin source, was emulsified using a mixture of EA/GA with and without tannic acid cross-linking. The emulsion showed the smallest droplet size at pH 7, but astaxanthin showed the highest retention at pH 5 during storage. EA/GA- stabilized emulsion was able to protect astaxanthin against UV light effectively and reduce the deduction of antioxidant activity after heating. The crosslinking by tannic acid further improved the protection of astaxanthin and increased the antioxidant activity. But it also changed the droplet size and flow behavior of the emulsion. The resultant emulsion product may have the potential to be used as a food ingredient in food applications.

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# Chapter 5. Summary, Conclusions and Recommendation

## 5.1 Summary and conclusions

This thesis has reported that ethanol-modified SC-$CO_2$ is a green extraction technique suitable for extraction of natural astaxanthin from a high oil, low water content material. EA/GA stabilized emulsion crosslinking by tannic acid was effective in protecting astaxanthin against UV light and heat stresses.

In Chapter 3, it has been found that ethanol-modified SC-$CO_2$ was as effective as hexane at extracting astaxanthin. At optimized extraction conditions, RSM predicted model showed good agreement with actual experimental results. For the astaxanthin-enriched camelina seed oil, astaxanthin content was the major factor determining the antioxidant activity of the oils. In addition, the oil extracted from SC-$CO_{2,opt}$ showed higher ABTS scavenging activity compared to that obtained from hexane. These results suggest that astaxanthin obtained by ethanol-modified SC-$CO_2$ has a higher antioxidant activity compared with that obtained by hexane, and can be labeled as a clean product.

In Chapter 4, astaxanthin-enriched camelina oil was emulsified by using a mixture of EA/GA. The properties of the emulsion such as droplet size, zeta-potential, and entrapment efficiency of astaxanthin at different pH conditions were studied. After the encapsulation, astaxanthin showed higher stability against UV light and heat than control. The addition of tannic acid crosslinking provided better protection and increased the antioxidant activity of the emulsion system. These results indicate that a EA/GA-stabilized emulsion with tannic acid crosslinking could effectively protect astaxanthin and further broaden food applications of the astaxanthin-enriched camelina oil.

## 5.2 Recommendations

In this thesis, we effectively used ethanol-modified SC-$CO_2$ to extract astaxanthin from a new engineered material. And we further incorporated the astaxanthin-enriched extract into a natural biopolymer based emulsion system. However, the emulsion system was susceptible to pH changes, and stability during long term storage should be further improved. In the future, some other techniques such as spray-drying could be applied to this emulsion system, to extend the shelf-life of this system. The modification on emulsifiers used in this system could also be considered. For example, using protein-polyphenol conjugates instead of non-covalent complexation could be a possible modification method.

Because the health benefits and high value of astaxanthin, natural astaxanthin may have a huge market potential in food, nutraceutical, and pharmaceutical industries. But the bioavailability of astaxanthin from this engineered camelina seed has not been studied before. Thus, another future research that should be conducted is to investigate the bioavailability of astaxanthin from camelina seed *in vitro* and compare the result with astaxanthin from other sources. Since camelina oil contains high omega-3 oil content, the lipid oxidation may occur in the emulsion system. A work which could provide information on whether lipid oxidation or astaxanthin degradation is the most important factor in determining the shelf-life of an astaxanthin-enriched emulsion system can also be considered in the future.