IMPROVING THE CHEMICAL STABILITY OF BETA-CAROTENE AND CITRAL BY NANOEMULSIONS

by

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ABSTRACT OF THE DISSERTATION

Improving the stability of beta-carotene and citral by nanoemulsions

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In this study, various nanoemulsion formulations were prepared to encapsulate non-polar beta-carotene and partly polar flavor molecule citral.

For the encapsulation of beta-carotene, the nanoemulsion core material was a mixture of both solid lipid (palm kernel fat) and liquid oil (medium chain triglyceride, MCT). Aqueous nanoemulsion dispersions were obtained by the combination of high speed and high pressure homogenization processes and the solid nanoemulsion powders were also obtained by lyophilization using sucrose as the cryoprotectant. For the aqueous dispersions, the formulation using pure palm kernel fat as the lipid phase was the best one to protect beta-carotene from degradation. For the freeze-dried samples, the incorporation of liquid MCT oil is necessary to disturb solid lipid crystallization to protect beta-carotene. The in *vitro* digestion experiments were performed to evaluate the

bioaccessibility of beta-carotene in palm kernel fat nanoemulsion and MCT nanoemulsion; the results showed that the bioaccessibility of beta-carotene was greatly improved after encapsulation. The palm kernel fat had better performance than the MCT to release beta-carotene from the nanoemulsion formulations.

For the encapsulation of citral, two strategies were developed to improve citral stability at acidic condition (pH = 3.0). The first strategy was to incorporate six different natural antioxidants (black tea extract, ascorbic acid, naringenin, tangeretin, beta-carotene and tanshinone) with citral together in the palm kernel fat nanoemulsions. The second strategy was to construct multilayer nanoemulsions to encapsulate citral. The multilayer emulsions were prepared by the layer-by-layer deposition technique between oppositely charged emulsion droplets and two polymer coatings: chitosan (CS) and ε-polylysine (EPL). The stability of citral as well as the production of the off-flavor compounds was analyzed by solid phase microextraction gas chromatography (SPME-GC). The results suggested that encapsulation of citral in combine with the appropriate antioxidants (beta-carotene, tanshinone and black tea extract) could greatly enhance citral's chemical stability during storage; and the additional cationic chitosan interfacial layer was also effective to improve the stability of citral.

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1 Introduction

1.1 Encapsulation of food ingredients

Food ingredients perform various roles in food industry, for example, they may contribute to the flavor, texture, mouth-feel and nutritive value of the food products. However, many of these food ingredients (*e.g.* vitamins, botanical extracts, flavors) are sensitive and easy to decompose under environmental stresses, such as heat, light and oxygen. Another problem is that some food ingredients (*e.g.* polyphenols, carotenoids, lipophilic vitamins) have low water solubility and can hardly be applied to food products. Therefore, encapsulation in food industry is of great importance to protect, solubilize and stabilize active food ingredients. One challenge of formulating food encapsulation systems is the requirement of completely using food grade ingredients. The usually used encapsulating or wall materials are starch, gums, lipids and so on. The wall materials help to entrap the food ingredients in a matrix, which could isolate the encapsulated food ingredients from the surrounding environment. There are a variety of encapsulation methods to be used, such as emulsions, coacervates, micelles, liposomes and so on. Among these methods, encapsulation systems at the nanometer scale begin to attract more and more attention due to their novel functionalities. This chapter reviews the encapsulation systems for active food ingredients formulated by nanotechnology, such as nanoemulsions, solid lipid nanoparticles and multilayer emulsions.

1.1.1 Nanoemulsions

Nanoemulsions are colloidal systems usually with droplet size smaller than 200 nm (Tadros and others 2004). The very small droplet size provides nanoemulsions various advantages to be used in the food, drug and cosmetic industry:

(1) The very small particle size could reduce gravity/buoyancy of the emulsion droplets, so Brownian motion might be sufficient to overcome gravity/buoyancy therefore creaming or sedimentation could be avoided;

(2) Nanoemulsion droplets are rigid to resist surface fluctuations, therefore coalescence could be avoided;

(3) Due to the large interfacial area of the nanoemulsion system, the encapsulated active compounds could gain enhanced gastrointestinal absorption with improved release profile.

Compare to conventional macroemulsions, which are considered to be thermodynamically unstable because of the inherent creaming, flocculation, coalescence and sedimentation problems, nanoemulsions show kinetic stability which imparts long shelf-life to the product. The enhanced absorption profile of nanoemulsions helps improve the oral bioavailability of many functional food ingredients. For example, lutein, a lipophilic carotenoid, was formulated into a stable nanoemulsion and showed significantly greater bioavailability than the supplement-pill forms (Vishwanathan and others 2009). Kuo and Subramanian et al.

investigated the anti-inflammation and bioavailability properties of an antioxidant synergy nanoemulsion system containing delta, alpha and gamma tocopherol in CD-1 mice, and proved that the formulation had enhanced anti-inflammator properties and increased bioavailability compare to the antioxidants suspensions (Kuo and others 2008).

1.1.1.1 Preparation methods

High energy input and low energy input methods are usually used to produce nanoemulsions:

1. High energy input method

Energy input is required due to the equation:

$$\Delta G = \Delta A\gamma - T\Delta S$$

where ΔA is the increase of the interfacial area and γ is the interfacial tension and both of them have positive values. The entropy term $T\Delta S$ is also positive but its value is too small to compensate the term of $\Delta A\gamma$. Thus energy input is required to produce emulsion with very small droplet size.

The most commonly used devices to provide energy for producing nanoemulsions are high speed and high pressure homogenizers. Both of them could produce high mechanical shear stress to break down the dispersed phases and lead to the formation of small emulsion droplets. Usually, high speed homogenizer is used to prepare "coarse" emulsion with relatively large particle size, while the following use of high pressure homogenizer could effectively reduce the particle size to the nanometer

range. For example, Wang and Jiang et al. (Wang and others 2007) prepared medium chain triacylglycerols (MCT) oil-in-water (O/W) emulsion using both high speed and high pressure homogenizers; when only high speed homogenizer was used, the particle size of the emulsion was 683.2 nm and this number was reduced to 82.1 nm when high pressure homogenizer was applied (1500 bar for 6 cycles).

Microfluidizer is another mechanical device to provide high energy for making nanoemulsions due to the interaction and auxiliary chambers with microchannels inside the device. These chambers provide cavitations, shear and impact forces to form emulsions with narrower particle size distribution than the conventional high pressure homogenizer (Sanguansri and Augustin 2006). However, "overprocessing" may happen simultaneously also due to the generation of the cavitations, shear and impact forces, which occur in a very short time (~ 10^{-4} s), especially when biopolymers (*e.g.* proteins and polysaccharides) are involved in the emulsion production process (Paquin 1999).

Ultrasonic generator has also been widely used to prepare nanoemulsions. During emulsification, imploding cavitation bubbles are formed due to the mechanical vibrations generated from the sonicator probe. The cavaitation bubbles collapse to produce intensive shock waves, which lead to the formation of high velocity liquid jets to break down the dispersed phases and form emulsion droplets with small size (Maa and Hsu 1999). The disadvantage of ultrasonic generator is that it is only appropriate for small batches (Sanguansri and Augustin 2006).

2. Low energy input method

Studies have shown that nanoemulsions sometimes could form spontaneously with little or no energy input (Bouchemal and others 2004). Commonly used methods are phase inversion method, Ouzo emulsification method and membrane emulsification method. The most commonly studied low energy method is the phase inversion temperature (PIT) method, which is based on the fact that some surfactants, usually polyoxyethylene-type surfactants, dehydrate with the increase of temperature to change from hydrophilic to lipophilic. During this process, an oil-in-water (O/W) emulsion invert to water-in-oil (W/O) emulsion.

1.1.1.2 Applications

Nanoemulsions are promising to encapsulate and deliver active food ingredients due to their excellent properties and functionalities (*e.g.* kinetic stability and improved absorption profile). A major issue that limits many nutrients and nutraceuticals applications is their low water solubility, and nanoemulsions help solubilize them in food applications. Chen and Wagner (Chen and Wagner 2004) produced a vitamin E (an extreme lipophilic vitamin) nanoemulsion with an average particle size of around 100 nm stabilized by starch using ultra high-pressure homogenization method; and the vitamin E product showed good stability in beverage. When the solubility of the active compound is enhanced by encapsulation, its bioavailability or biological efficacy can also been improved due to the increase of its contact area with the intestinal membrane, especially for nanoemulsion with very large interfacial area. For

example, Date and Nagarsenker (Date and Nagarsenker 2007) developed self-emulsifying nanoemulsions to encapsulate and deliver cefpodoxime proxetil (CFP, an antibiotic with low bioavailability); the results showed that the nanoemulsions could increase the CFP solubility as well as protect CFP from degradation, while at the same time, the optimized formulation completely released CFP within 20 min irrespective of the pH of the dissolution medium. Wang and Wang et al. (Wang and others 2009) used nanoemulsions to encapsulate polyphenols including epigallocatechin gallate (EGCG) and curcumin, which are good food grade antioxidants with limited solubility and low oral bioavailability; the anti-inflammation and anti-tumor functions of nanoemulsion-encapsulated polyphenols were tested in mice and the results showed that nanoemulsions improved the oral bioavailability of polyphenols (EGCG and curcumin).

In summary, it has been proved by many studies that nanoemulsions are effective encapsulation and delivery systems to improve the solubility, stability and the bioavailability of active compounds. However, there are also some disadvantages associated with nanoemulsions applications. For instance, many studies have emphasized the various advantages of nanoemulsions, and interest from both academic and industrial areas have greatly increased for several years, but real nanoemulsion products for parenteral use are still limited on today's market. The requirement for high energy input to produce nanoemulsions increases the cost of production; the use of non-food-grade ingredients of the low energy methods poses

safety issues of nanoemulsions; and the lack of direct evidence to prove the health benefits of nanoemulsions also limits its applications, although intensive research have been done on nanoemulsions in various bioassays. Therefore the work in the nanoemulsions area is still on going and researchers are continuously making efforts to explore the benefits of nanoemulsions to be used in real applications.

1.1.2 Solid lipid nanoparticles

The concept of solid lipid nanoparticles/nanoemulsions (SLN) has been introduced as a new generation of edible emulsion delivery system by the research groups of Gasco (Gasco 1993b) in Italy and Müller in Germany (Müller and others 1996). SLN represent a colloidal system produced by similar method as that of conventional emulsions (*e.g.* macroemulsion and nanoemulsion). The main difference between SLN and conventional nanoemulsion is that solid lipid, which is solid at room temperature, is used instead of liquid oil as the lipid phase. SLN system shares the advantages with liquid nanoemulsion, such as high dissolution rate and excellent storage stability; at the same time, SLN system has other advantages (Mehnert and Mäder 2001), such as possible targeting and controlled release profile as well as improved chemical stability of the encapsulated compound. Major components of SLN are solid lipids, emulsifiers and water. Yang and Huang et al. summarized the commonly used SLN ingredients as listed in Table 1-1 and Table 1-2 (Yang and others 2011a).

Table 1-1 Commonly used emulsifiers for SLN.

Emulsifiers	Literatures
Soy bean lecithin (Lipoid S100, S75)	(Westesen and others 1997; Bunjes and others 1996; Westesen and Bunjes 1995; Schwarz and Mehnert 1997)
Soy phosphatidylcholine 95% (Epikuron 200)	(Venkateswarlu and Manjunath 2004; Cavalli and others 1997)
Egg lecithin	(Morel and others 1996; Sznitowska and others 2001)
Poloxamer 182	(Almeida and others 1997; Liedtke and others 2000)
Poloxamer 188	(Venkateswarlu and Manjunath 2004; Jores and others 2004; Cavalli et al. 1997; Liedtke et al. 2000)
Poloxamer407	(Müller et al. 1996)
Poloxamer908	(Müller et al. 1996; Goppert and Müller 2003)
Tween 20	(Charcosset and others 2005)
Tween 60	(Charcosset et al. 2005)
Tween 80	(Mei and others 2003; Goppert and Müller 2003; Müller and others 2000; Almeida et al. 1997; Liedtke et al. 2000)
Span 20	(Mei and others 2005)
Span 60	(Patravale and Ambarkhane 2003)
Span 80	(Müller et al. 2000)
Span 85	(Asasutjarit and others 2007)
Sodium cholate	(Müller et al. 1996; Liu and others 2007; Almeida et al. 1997)
Sodium glycocholate	(Westesen and Bunjes 1995)

Table 1-2 Commonly used lipids for SLN.

Lipids	Literatures
Trimyristin	(Westesen et al. 1997; Bunjes et al. 1996; Venkateswarlu and Manjunath 2004)
Tristearin	(Bunjes et al. 1996; Venkateswarlu and Manjunath 2004)
Tripalmitin	(Bunjes et al. 1996; Venkateswarlu and Manjunath 2004)
Trilaurin	(Bunjes et al. 1996; Westesen and Bunjes 1995)
Cetyl palmitate	(Sznitowska et al. 2001; Wissing and Müller 2003)
Stearic acid	(Yang and others 1999b; Cavalli et al. 1997)
Palmitic acids	(Jenning and Gohla 2001)
Behenic acid	(Cavalli et al. 1997)
Dynasan 112 (glycerol trilaurate)	(Schwarz and Mehnert 1997)
Hard fat (Witepsol E85, W35, S58 etc.)	(Almeida et al. 1997; Manjunath and others 2005; Liedtke et al. 2000)

1.1.2.1 Preparation methods

Since the lipid phase used in SLN is solid, the preparation method for SLN is slightly different from that of conventional nanoemulsion.

1. Hot and cold homogenization methods

The general procedures of both hot and cold homogenization methods are shown in Figure 1-1. The temperature of hot homogenization method is usually kept 5 to 10 ^{o}C higher than the melting point of the solid lipid used in the formulation. This is to avoid solid lipid crystallization during the product preparation process.

For the cold homogenization method, the solid lipid phase is grinded first and then dispersed in the emulsifier(s)/stabilizer(s) aqueous solution for further

homogenization. Cold homogenization method is suitable for encapsulating heat active compounds. However, particles produced by this method usually have larger size with wider size distribution than those obtained from hot homogenization method (Mehnert and Mäder 2001).

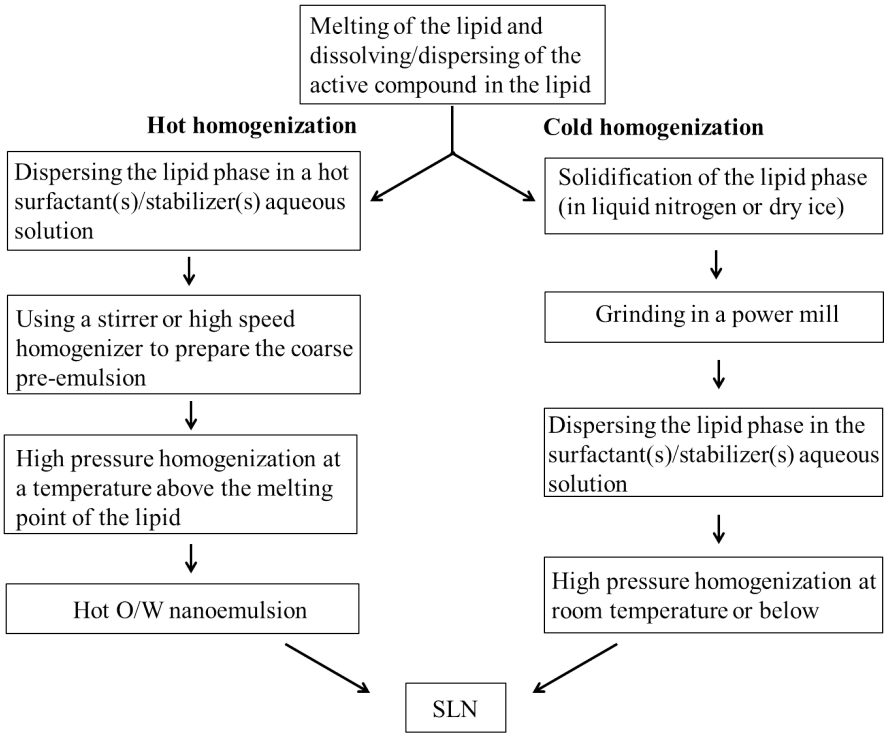


Figure 1-1 Schematic procedure of hot and cold homogenization methods (Mehnert and Mäder 2001).

2. Solvent emulsification – evaporation and solvent diffusion method

Solvent emulsification – evaporation and solvent diffusion method is a low energy method that can be used for the production of SLN (Sjöström and Bergenståhl 1992). Basically the lipid phase is dissolved in water immiscible organic solvent(s) such as hexane and chloroform and then emulsified in the aqueous phase containing surfactant(s) by high energy input methods (*e.g.* high speed homogenization, high

pressure homogenization and sonication) as described above. The organic solvent(s) will then be removed under reduced pressure. Then the lipid phase will precipitate to form SLN automatically. The avoidance of heat treatment during the SLN production process makes this method suitable for the encapsulation of heat sensitive molecules. However, the possible organic solvent residues in the final product also pose a safety issue which limits the application of this method.

In the solvent diffusion method, partially water-miscible solvents are used instead of the water-immiscible solvents in the solvent emulsification-evaporation method. At first, water and the solvents are mixed together to saturate each other. Then the lipid phase is dissolved in the water-saturated solvent while surfactant(s) are dissolved in the solvent-saturated water; and both phases are emulsified together also by high energy input methods (*e.g.* high speed homogenization, high pressure homogenization and sonication) as described above to form pre-emulsion. At last, a large amount of water is added into the pre-emulsion and the lipid phase will also precipitate to form SLN automatically. Although the solvent diffusion method could produce SLN with small particle size and narrow size distribution, the use of solvents and the production of diluted dispersion also limit its application.

3. Dilution of microemulsion

The Gasco group in Italy developed the microemulsion dilution method to produce SLN (Gasco 1993a). The term “microemulsion” is defined as “a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable

liquid solution" (Danielsson and Lindman 1981). The preparation of microemulsion does not require high energy input; only mixing and stirring the surfactant(s), water and lipid together could produce thermodynamically stable microemulsion. A thorough review paper described the structure, dynamics and transport behaviors of microemulsion (Moulik and Paul 1998).

The microemulsion dilution method is a simple way to produce SLN. At first, the melted lipid, water, surfactant(s) and co-surfactant(s) are mixed together and stirred at a temperature slightly higher than the melting point of the solid lipid. Then the microemulsion is dispersed in a large amount of water, and during this dispersion process, SLN will precipitate automatically. Although very low energy is required in the microemulsion dilution method, the addition of co-surfactants and the removal of excess water limit its application.

1.1.2.2 Applications

SLN is considered to be a promising new generation edible carrier and delivery system for active compounds because the solid matrix is meant to provide better protection for the compounds. The lipid phase of SLN is usually composed of physiologically related lipids, which undergo enzyme degradation reactions once the SLN reach the gastrointestinal tract (GIT) of human beings and the degradation rates can be controlled by adjusting the lipids compositions as well as surfactants compositions (Mehnert and Mäder 2001). Olbrich and Müller developed an enzymatic degradation assay to study the degradation behaviors of SLN (Olbrich and Müller

1999); and the results showed that the SLN degradations can be affected by many factors, such as the length of the fatty acid chains and the surfactant types. In general, as regard to the fatty acids, the longer the chain length, the slower the degradation rate; as regard to the surfactants, some of them can promote degradation (such as cholic acid sodium salt) while some them hinder the degradation (such as polyoxyethylene – polyoxypropylene block polymers) (Olbrich and Müller 1999). This may allow optimizing the controlled release profiles of the SLN formulation.

The studies of SLN mainly focus on the use of SLN as drug encapsulation and delivery systems, such as camptothecin (Yang and others 1999a), insulin (Zhang and others 2006) and diclofenac sodium (Attama and others 2008). Although the patent of SLN has already entered the pharmaceutical industry in 1999 by SkyePharma AG (Muttenz, Switzerland), the application of SLN in food industry is still lacking on the market. Due to the many advantages that SLN share with nanoemulsions, and the possible controllable release profile that SLN possess, both academia and industry are still interested in this promising delivery system to be used in pharmaceutical and nutraceutical industry.

1.1.3 Multilayer emulsions

Layer-by-layer (LBL) technique was introduced by Decher's group in 1997 which provides an easy bottom-up method to fabricate a multilayer structure based on the electrostatic interaction between oppositely charged molecules (Decher 1997). Recently, this technique has been utilized in colloidal carrier systems, for example, to

coat the emulsion droplets with one or more than one extra layer(s) and under certain conditions these layers have been found to offer the emulsion droplets better stability to environmental stresses (Ogawa and others 2003; Klinkesorn and others 2005b; Surh and others 2005; Aoki and others 2005; Klinkesorn and others 2005a; Güzey and McClements 2006; Gu and others 2005). The general procedure to produce multilayer coated colloidal carrier system is shown in Figure 1-2.

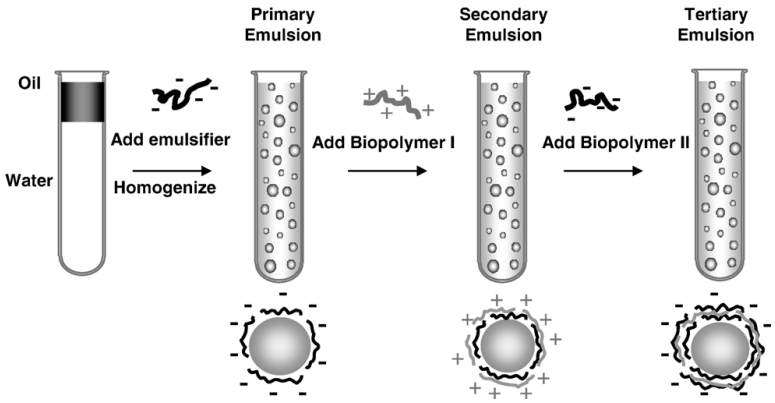


Figure 1-2. Schematic representation of LBL procedure for producing multilayer emulsion droplets (Guzey and McClements 2006).

First, the suspension of charged colloidal particles is produced by using charged emulsifiers and the colloidal particles can be solid (*e.g.* SLN) or liquid (*e.g.* traditional emulsion droplets). Next, an oppositely charged polyelectrolyte solution is introduced to mix with the charged particles and the polyelectrolyte will adsorb onto the particle surfaces due to the electrostatic attraction between the opposite charges; if there is enough polyelectrolyte present in the solution, the surface charge signal would reverse thus facilitate the adsorption of the next layer. Before the next adsorption step, it is

necessary to remove excess polyelectrolyte in case they will interact with the other oppositely charged polyelectrolyte. It is also necessary to add enough polyelectrolyte to prevent bridging effect between coated and un-coated particles. Basically, there are several strategies to remove the excess polyelectrolyte (Voigt and others 1999; Guzey and McClements 2006):

(1) Saturation method. Saturation here means to adjust the polyelectrolyte concentration to the point that just the right amount of molecules are present in the solution to adsorb onto the particle surface with no or litter free molecules left in the aqueous phase after the adsorption process. Usually, numbers of experiments are needed to find out this optimal concentration. For example, ξ-potential data is a good indicator to observe the surface charge reversal phenomenon and to determine the right polyelectrolyte concentration. The problem of this method is that too much work is needed to get the data.

(2) Centrifugation method. When more than enough polyelectrolyte is used in the adsorption process, centrifugation is an easy way to remove excess non-adsorbed molecules. The particles will settle at the bottom of the centrifuge tube while the polyelectrolyte will be in the upper supernatant. The bottom part can be collected and re-suspended in proper buffers for further experiments. The problem of this method is that particle aggregation may happen during centrifugation.

(3) Filtration method. The same as in centrifugation method, when more than enough polyelectrolyte is used in the adsorption process the non-adsorbed polyelectrolyte is

removed by filtration. The colloidal suspension is put under pressure to make it easy for the polyelectrolyte to pass through the membrane filter and a buffer which is used to prepare the suspension can be added to maintain a constant concentration of the colloidal particles so to prevent possible aggregation.

1.2 Background information of beta-carotene

1.2.1 Basic information

The term carotene refers to a class of molecules with a general formula of $C_{40}H_x$. The molecules are composed of eight isoprene units to provide a C40 skeleton. The two primary isomers of carotene are α-carotene and β-carotene, differ in the position of the double bonds in the cyclic group of the molecular structure. Other forms (γ, δ, ε, and ζ-carotene) also exist. Among all these isomers, β-carotene is the most common form. Figure 1-3 shows the structure of all-trans-β-carotene. Major physical properties of β-carotene are listed in Table 1-3 (Eitenmiller and others 2008).

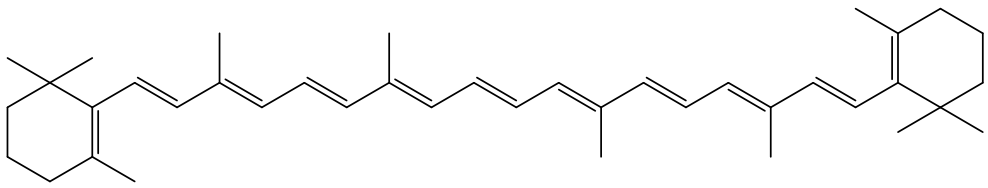


Figure 1-3. Structure of all-trans-β-carotene

Table 1-3. Physical properties of β-carotene (Eitenmiller et al. 2008)

Substance	Molar Mass	Formula	Melting point (^{o}C)	Crystal form	Solubility
β-carotene	536.88	$C_{40}H_{56}$	183	Red rhombic/ square leaflets	Soluble in limited organic solvents, such as chloroform and THF

1.2.2 Biological efficacy

Epidemiological studies have consistently associated diets rich in fruits and vegetables especially carotenoids with a reduced prevalence of several diseases with their clearly demonstrated antioxidant properties (Tyssandier and others 2001; Peto and others 1981; Block and others 1992). Although β-carotene is only one of the many carotenoids, it is one of the most intensively studied carotenoid since it was approved for human consumption by the U.S. Food and Drug Administration (FDA). Liebler and McClure et al. provided the first mechanistic explanation for the radical scavenging reactions of β-carotene that were unambiguously associated with its antioxidant property (Liebler and McClure 1996). Many other studies were also done to try to relate the antioxidant property of β-carotene with its possible health benefits. Besides the many cell culture and animal experiments, several large clinical trials were conducted to evaluate the possible effect of β-carotene on reducing chronic diseases (Ziegler 1993). However, different trials have led to different results with controversy and ambiguous conclusions. For example, the 12 years Physician's Health Study (PHS) found that the supplemental β-carotene intake produced neither health benefit nor harm with regard to the incidence of malignant neoplasms and

cardiovascular disease (Hennekens and others 1996). However, Hennekens and Eberlein et al. did a randomized, placebo-controlled, double-blind clinical trial to prove the preventive effects of beta-carotene on cancer incidence (Hennekens and Eberlein 1985). And a similar trial conducted by Omenn and Goodman et al. proved that oral administration of beta-carotene can decrease the incidence of lung cancer in high risk populations, namely, heavy smokers and asbestos-exposed workers (Omenn and others 1994). Another 18 years study including over 4000 physicians demonstrated that 50mg of beta-carotene every other day prevented cognitive decline due to the oxidative stress caused brain aging (Grodstein and others 2007).

Although whether taking β-carotene supplement can reduce the risk for chronic diseases remains unclear, consuming small amount of β-carotene by no means is unwise for any population (Pryor and others 2000). The confusion and controversy between different studies require further comprehensive study to get clear understanding of the biological efficacy and health benefits of β-carotene and other botanical antioxidants.

1.2.3 Issues associated with the application of β-carotene

The pure hydrogen-carbon skeleton of the molecule endows β-carotene lipophilic property which greatly limits its bioavailability. The term bioavailability is used to describe the fraction of an ingested active molecule, such as drugs and nutrients, which reaches the systemic circulation and is available for utilization in normal physiological functions (MJ 1997) . In order to improve the bioavailability of

β-carotene, various colloidal systems, such as oil in water emulsions or dispersions, are promising formulations for the encapsulation of carotenes, and decreasing particle size of the carriers usually results in a better uptake of encapsulated actives (Schubert and others 2003) . It has been reported that β-carotene dissolved in oil is absorbed far more readily than β-carotene from foods (Castenmiller and West 1998). Borel reviewed the factors affecting intestinal absorption of highly lipophilic food nutrients including β-carotene, and all the evidences show that lipid phase is a necessary condition for the digestion and utilization of β-carotene for human bodies (Borel 2003).

Besides bioavailability, the high unsaturation degree of β-carotene structure makes it easy to undergo degradation reactions such as isomerization, photosensitization, thermal and chemical oxidations (Jia and others 2007). Studies have shown that similar volatile degradation products (*e.g.* dihydroactinidiolide and β-ionone) were found for different types of β-carotene degradation which originated from the cleavage at bonds 7-8, 8-9 and 9-10 of the β-carotene molecule (Glória and others 1993).

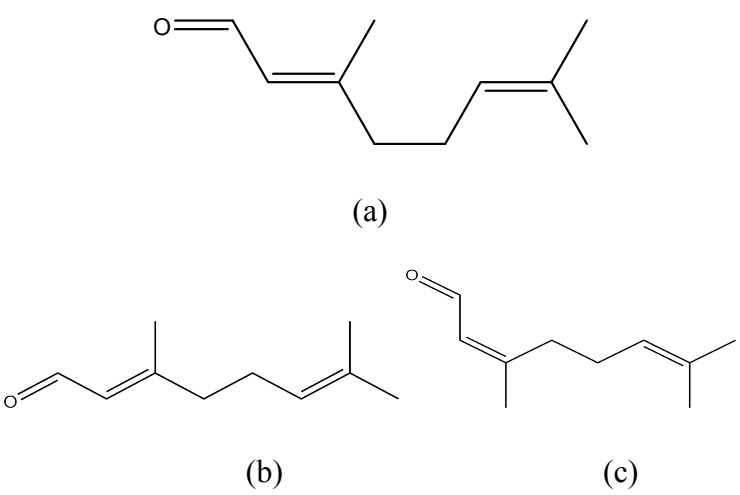
Many studies have been done concerning issues mentioned above to improve the bioavailability and stability of β-carotene. For example, administration of β-carotene encapsulated in liposomes to hamsters showed significant higher anti-tumor activity (approximately 5,000-fold difference) than the control tumor group (Schwartz and others 1991). Yin and Chu et al. investigated β-carotene nanodispersions stabilized by

different emulsifiers and discovered that sodium caseinate is the best physical barrier to protect the encapsulated β-carotene against free radicals and the stability of β-carotene was also improved due to the antioxidative activity of sodium caseinate (Yin and others 2009). Stearyl ferulate-based solid lipid nanoparticles were formulated to encapsulate β-carotene and the results proved that encapsulation could improve the stability of β-carotene as well as maintain its antioxidant activity at the same time (Trombino and others 2009). Li and Nguyen et al. used various surfactants (*e.g.* polyoxyethylene sorbitan fatty acid esters) to solubilize lipophilic β-carotene in the form of emulsion or micelle and obtained promising results for the encapsulated actives with improved stability and bioavailability (Li and others 2005).

1.3 Background information of citral

1.3.1 Basic information

Citrus oil is one of the most popular flavor ingredients used in foods and citral is among the most important flavor molecules in citrus oil. Citral (3,7-dimethyl-2,6-octadienal, Mw 152.24) contains two geometrical isomers, neral and geranial (structures shown in Figure 1-4) and the major physical properties of neral and geranial are listed in Table 1-4.



(a)

(b) (c)

Figure 1-4 Chemical structures of (a) citral (3,7-dimethyl-2,6-octadienal); (b) geranial and (c) neral.

Table 1-4 Physical properties of geranial and neral (Surburg and Panten 2006)

	b_p(2.7kPa)	d^{20}	n_D^{20}	Odor-description	Retronasal threshold (μg/L)	Amount in fresh orange juice (μg/L)	Amount in processed orange juice (μg/L)
Geranial	118-119˚C	0.8888	1.4898	Lemongrass, lemon-like, citrus, minty	N/A	45	N/A
Neral	120˚C	0.8869	1.4869	Citrus-like, green, minty	40	45	270

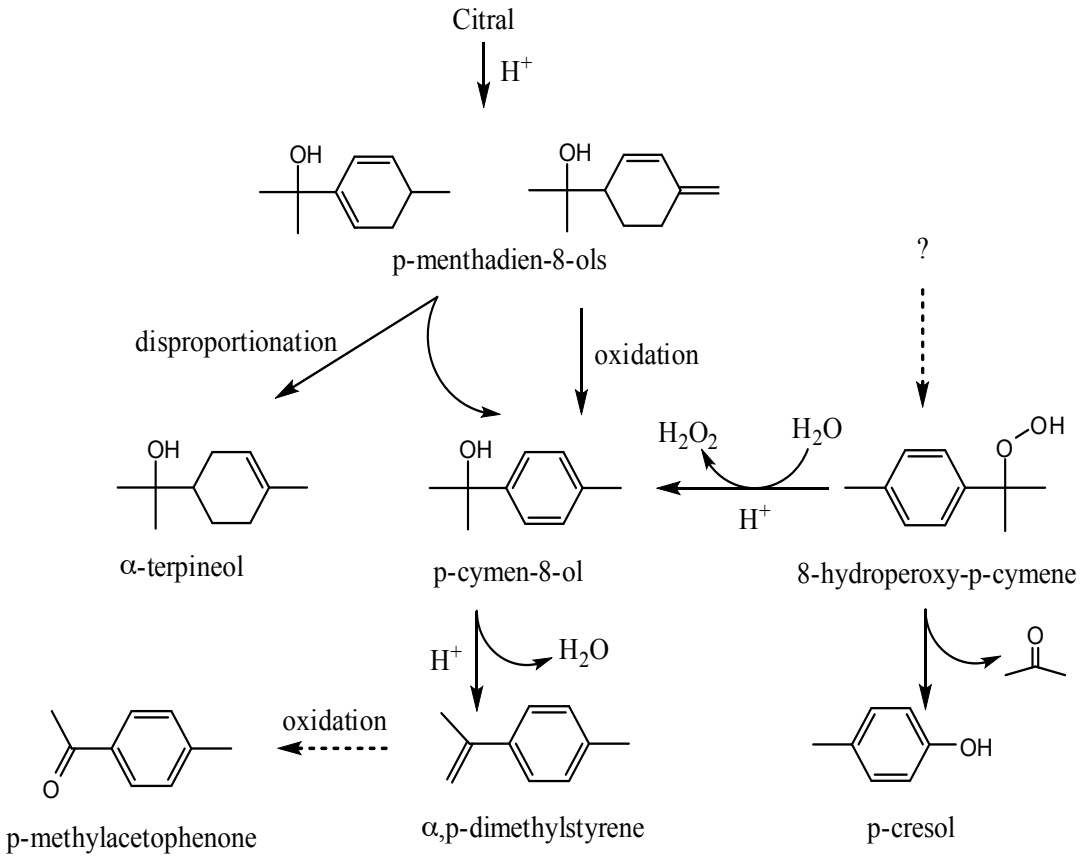
1.3.2 Problems associated with the application of citral

Citral is an α,β-unsaturated aldehyde with one additional double bond, so both of its two isomers can easily undergo a series of degradation reactions especially under low pH condition and with the presence of oxygen (Schieberle and Grosch 2002). Citral is of high consumer acceptance with lemon-like flavor in food and beverage industry, however, it is very unstable and can easily undergo various chemical reactions to

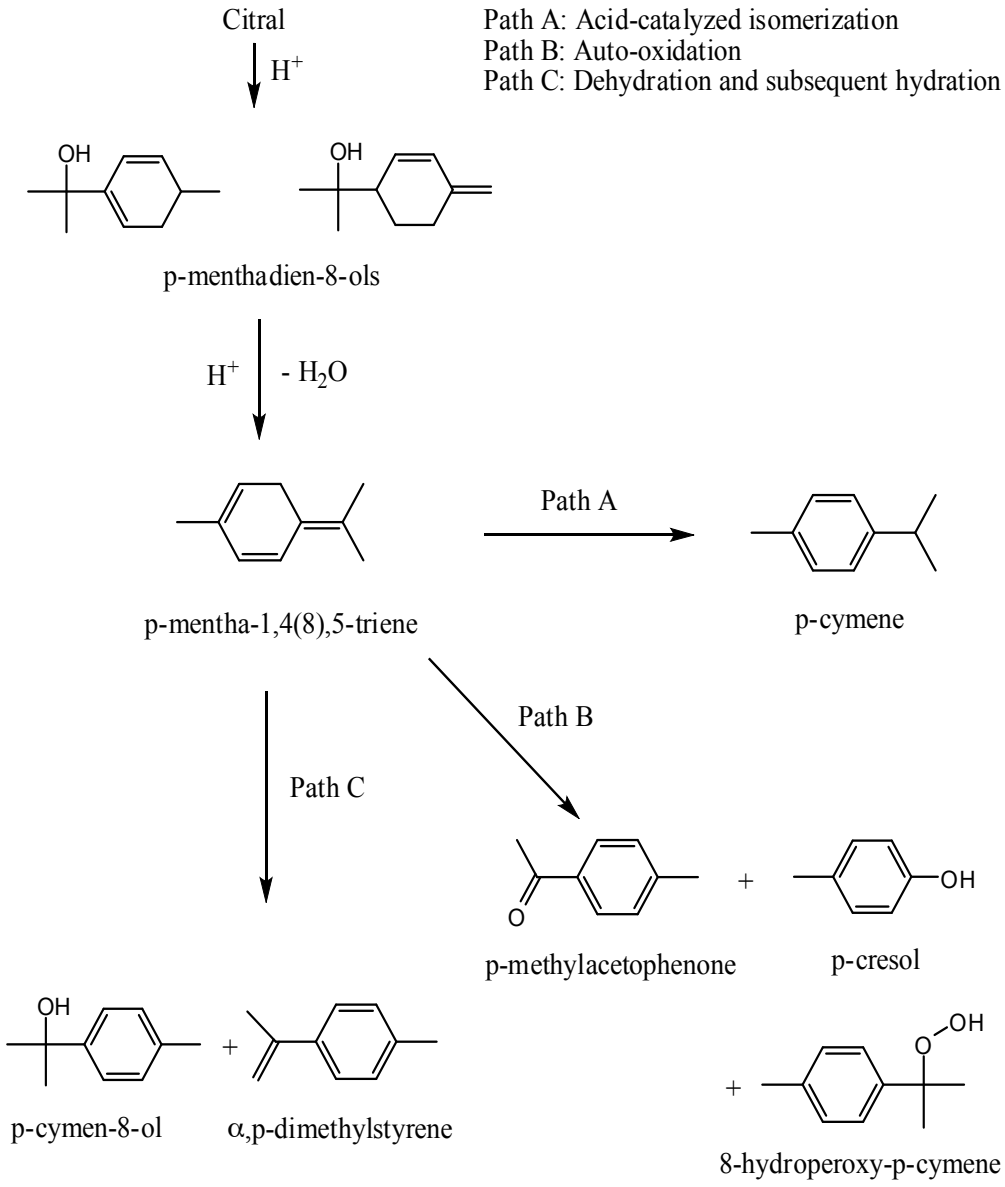
produce undesirable off-flavors which cause the loss of product quality. Therefore, numerous works have been done to stabilize citral.

Degradation of citral will lead to the loss of lemon like flavor and the production of various off-flavor compounds (Ueno and others 2004). Among all these compounds, *p*-cresol, *p*-methylacetophenone and *p*-cymene are the most potent off-odorants with phenolic, gasoline-like and bitter almond-like odors, respectively, thus these three molecules are always used as indicators for the assessment of the citral degradation products (Schieberle and Grosch 2002).

The degradation mechanism is still unclarified now. Previous studies proposed that the reaction under acidic aqueous conditions started from isomerization of geranial to neral which then undergoes cyclization to form *p*-menthadien-8-ols and/or *p*-menthadien-4-ols; oxidation of the monoterpene alcohols then take place to produce *p*-cymene-8-ol and its dehydration products α,*p*-dimethylstyrene, *p*-cymene and *p*-cresol; and *p*-methylacetophenone was suggested to be formed by further oxidation of α,*p*-dimethylstyrene (Schieberle and others 1988; Kimura and others 2002; Peacock and Kuneman 2002; Kimura and others 1983). Scheme 1-1 shows the previously proposed pathways of citral degradation products under acidic aqueous conditions and Scheme 1-2 shows the summary of different pathways for the formation of citral off-odorants by isomerization, autoxidation and dehydration.



Scheme 1-1 Previously proposed citral degradation pathways under acidic aqueous conditions (Ueno et al. 2004; Schieberle et al. 1988; Kimura et al. 2002; Peacock and Kuneman 2002; Kimura et al. 1983).



Scheme 1-2 Summary of different pathways for the formation of citral off-odorants (Barton and Parekh 1989b; Ueno et al. 2004; Ueno and others 2006).

1.3.3 Inhibition of citral degradation

There are mainly two strategies to inhibit citral degradation which are (1) to reduce the production and storage temperature and (2) to change the environmental parameters such as to increase the pH and reduce the oxygen in the product. Unfortunately, these strategies are not always practical in food industry so new methods need to be developed to increase citral stability under severe conditions.

Numerous works have been done to stabilize citral by encapsulating it in the form of emulsion due to the fact that the encapsulation could isolate the encapsulated active compound (*i.e.* citral) from the reactive molecules in the aqueous medium, such as protons and free radicals. For example, Choi and Decker et al. (Choi and others 2009) encapsulated citral in both medium train triacylglycerols emulsion droplets and triacetin microemulsion droplets, and the results showed great improvement of citral's stability stored at 20^{o}C under acidic condition (pH 3.0).

Antioxidants have also been used to inhibit citral's degradation and reduce the generation of the off-flavor compounds, such as *p*-cymene and *p*-cresol. Kimura et al. attempted to prevent citral from deterioration by dissolving several antioxidants and citral together: 2,6-di-tert-butyl-p-cresol (BHT), 2- and 3-tert-butyl-4-hydroxyanisoles(BHA), n-propyl-gallate, *dl*-α-tocopherol, nordihydroguaiaretic acid and n-tritriacontan-16,18-dione isolated from the leaf wax of Eucalyptus leaves were used as the antioxidants and the deterioration products were quantitatively determined by gas-liquid chromatography; unfortunately, none of

these compounds was effective in decreasing the amount of oxidative products (Kimura et al. 1983). Peacock and Kuneman discovered that iso-ascorbic acid could react with oxidizing agents (*e.g.* dissolved oxygen and oxygen in the headspace) in carbonated citral-containing beverage system so to protect citral from oxidation (Peacock and Kuneman 2002). Liang and Wang et al. used four different commercially available plant extracts from black tea, pomegranate seed, grape seed and green tea to successfully inhibit the off-ordor formation from citral degradation at pH3.0 (Liang and others 2004). Ueno and Kiyohara et al. studied the effect of pure theaflavin extracted from black tea on the formation of off-odorants *p*-cresol and *p*-methylacetophenone and discovered that the odor activity values of thoses off-odorants were greatly reduced (Ueno et al. 2006).

1.4 Background information of lecithin

1.4.1 Basic information

Phospholipids (general structure shown in Figure 1-5) are a class of fat derivatives which refers to lipids containing phosphoric acid residues (Hanahan 1997). And lecithin is mainly composed of various phospholipids, such as phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and other glycerol phospholipids of complex fatty acid compositions.

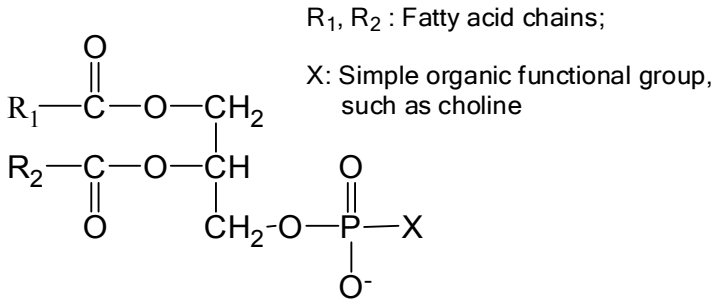


Figure 1-5 General structure of phospholipids.

1.4.2 Applications

Lecithin is a very vital constituent of all living cells in animal and plant tissues by acting as the phospholipids portion of the cell membrane. Besides its biological meanings, such as maintaining the integrity of cell membranes, lecithin is widely used as an emulsifying, wetting and dispersing agent due to its special structure: the co-existence of the hydrophobic fatty acid chains and the hydrophilic phosphorous containing functional group makes lecithin amphiphilic molecules.

Commercially used lecithins are mixtures of phospholipids extracted from animal and vegetable origins. Animal lecithins are mainly derived from milk and eggs, while vegetable lecithins are mainly derived from oil-bearing seeds such as soybeans and rapeseeds. Lecithin is always chosen when natural emulsifier and/or lubricant is needed. For example, lecithin was used to improve the wettability and dispersibility of instant cocoa beverage powders and to prevent them from agglomeration (P. Hla 1999). Since it is an integral part of living organisms and can be totally metabolized without being excreted via kidneys like other emulsifiers, lecithin is non-toxic to human beings. In the United States, lecithins are classified in the Food Chemical

Codex (FCC III). The US Food and Drugs Administrations (FDA) has regulated lecithin under Title 21, part 184, direct food substances affirmed as generally recognized as safe (GRAS). Lecithin is listed in § 184.1400, enzyme-modified lecithin in § 184.1063.

Besides being used as a surfactant and/or lubricant, lecithin has been shown to possess important physiological significance, and they also exhibit well-documented nutritional and/or therapeutic benefits, thus can be used in pharmaceutical and functional food products. What's more, choline, the vital component of lecithin, is also an essential nutrition factor for animals and human beings. It plays a significant role in prevention of fatty livers, the metabolism of fat, and the transmission of nerve impulses (Ensminger 1994). There has been research shown that lecithin can induce a reduction in plasma cholesteroal, possibly through an increase formation of high density lipoprotein (HDL, the "good cholesterol") (Jimenez and others 1990). It was proved that by providing hyper-cholesterolemic diet as well as dietary phospholipids, such as safflower phospholipids and soybean phospholipids to rats, the phospholipid diets induced a significant increase of HDL and decrease in concentrations of liver cholesterol, which suggested that the vegetable lecithin can markedly inhibit the adsorption of dietary cholesterol in small intestine (Iwata T 1993). A 10-year study showed that polyunsaturated lecithin can reduce alcohol-induced hepatic fibrosis and repair damaged liver tissue (Lieber and others 1990). And the remarkable physiological and pharmacological actions of phosphatidyl serine (a component of

lecithin) have been clearly and consistently demonstrated on the brain and immune system, and also for the repair of the age-dependent behavioral impairment associated with few side effects (Pepeu and others 1996). In recent years, a large number of diet integrators and/or pharmaceuticals containing lecithin have come on the market, which are administered to consumers or patients suffering from different disorder diseases, such as hypercholesteremia, hypertriglyceridemia and hyperlipoproteinemia, as well as acute and chronic hepatitis and various types of hepatoxicosis (Campanella and others 1998). Because of its GRAS status and physiological/nutritional benefits, lecithin is a promising emulsifier used in food and pharmaceutical products. Moreno and Frutos et al. developed lecithin stabilized oil-in-water microemulsions as potential amphotericin B (AmB) delivery systems and evaluated their in-*vivo* acute toxicity (Moreno and others 2001). Due to the low solubility of AmB in most solvents, its bioavailability is extremely low through oral route, thus it is parenterally administered when solubilized in sodium deoxycholate (Fungizonet, Bristol- Myers Squibb). However, a series of adverse effects are associated with this formulation. Moreno's research proved that the novel microemulsion using lecithin as the emulsifier had higher stability and lower level of toxicity. Fukui and Kurohara et al. discovered an enhancing effect of lecithin dispersed medium chain triglycerides on intestinal absorption of tocopherol acetate in rats compared to the same delivery system using polysorbate-80 as the emulsifier (Fukui and others 1989). Wajda did research on 24 human volunteers, and the results showed that the NanoSolve (a phospholipids-based

emulsifier, Lipoid GmbH, Ludwigshafen, Germany) formulations greatly enhanced the bioavailability of Coenzyme Q10 and vitamin E for 5-fold and 10-fold compare to the control formulations respectively (Rudi Wajda 2007). Peters and Brain summarized that soy-lecithin-based technology can (1) protect the encapsulated active from degradation in the GI tract; (2) prolong the active's transit time in the small intestine thus to enhance its bioavailability; and (3) facilitate the permeation of the active molecules through the morphological barriers in small intestine thus to further enhance the molecules bioavailability (Peters and Brain 2009). Since lecithin can greatly enhance the bioavailability of lipophilic substances, such as carotenoids and vitamins, elaborately designed formulations in combine with lecithin will be attractive for the future market of pharmaceutical and functional food products.

2 Scientific Rationales and hypotheses

2.1 Using solid lipid to formulate the nanoemulsion system

Nanoemulsions made from solid lipids instead of liquid oils have attracted more and more attention recently. The solid lipid nanoemulsions/nanoparticles (SLN) share the advantages of traditional nanoemulsions, such as long shelf life with good physical as well as chemical stability. While at the same time, SLN is a better carrier system than traditional nanoemulsions, by providing more protection for the encapsulated labile molecules (*i.e.* drugs and active food ingredients) from degradation and better controlled release profile. The main reason is that the molecules being encapsulated have lower mobility in a solid matrix than in a liquid matrix. Intensive studies have been done to show the various advantages of SLN (Charcosset et al. 2005; Heiati and others 1997; Jee and others 2006). Therefore the first hypothesis of this research is that using SLN to encapsulate beta-carotene could slow down beta-carotene degradation.

2.2 Incorporation of liquid oil into the solid lipid matrix

Despite the many advantages that SLN has, the major issues related with SLN is the low drug loading capacity as well as drug expulsion during storage. The loading capacity of a SLN system is limited mainly by three factors (Wissing and others 2004):

(1) The limited active compounds solubility in the lipid melt;

(2) The molecular structure and properties of the solid lipid;

(3) The polymorphic transition of the solid lipid during storage.

For a SLN system, if the solid lipid core material is composed of single or multiple similar lipid molecules, the lipid matrix tends to form crystal with ordered structures and few imperfections. During the SLN production process, the active compounds being encapsulated are dissolved in the lipid melt first and then stabilized by the surfactant(s)/stabilizer(s) and solidified; therefore, after the SLN production, the active compounds (especially the lipophilic compounds) are mainly located between the fatty acid chains and the lipid layers (Wissing et al. 2004). When the fatty acid chains and the lipid layers tend to arrange into an ordered crystal structure, the formed SLN core structure will not be able to accommodate many encapsulated molecules.

The lipid molecules polymorphic transition also causes the expulsion of the encapsulated molecules. The lipid molecules in the freshly made SLN usually exist as a mixture of stable β' form with unstable polymorphs (α and sub α) (Hernqvist 1988; Freitas and Müller 1999). These polymorphs possess more free space and imperfections in the crystal structure to entrap the molecules being encapsulated. If however these crystal forms transform to a more stable β form with lower energy and more ordered crystal structure, the molecules being encapsulated will be expelled out thus lose protection from encapsulation.

Solution to solve these problems is to interrupt the formation of lipid crystals. The

easiest way to achieve this goal is to use lipid mixture (e.g. lipids with different chain lengths) as the core material in the SLN formulation. Different lipids with different fatty acid chains and chain structures could result in the formation of crystal imperfections thus create more space available to accommodate the encapsulated molecules (as shown in Figure 2-1).

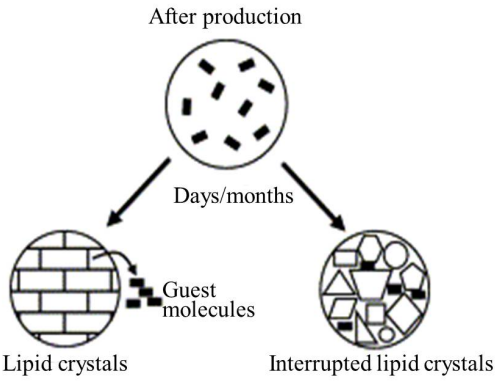


Figure 2-1 Schematic representation of different lipid structures of SLN (Müller and others 2002).

Therefore in this study, both solid lipid (palm kernel fat) and liquid oil (medium chain triglyceride, MCT) were used as the core material of SLN. Based on the scientific rationale described above, the hypothesis is that the combination of both solid lipid and liquid oil could increase the entrapment efficiency for beta-carotene in this study. However, the incorporation of liquid oil may also increase the mobility of the guest molecules, therefore the SLN formulation with the incorporation of liquid oil may lose its advantage to provide better protection for the guest molecules as described in section 2.1. In this study, SLN with different liquid oil contents were prepared and the beta-carotene stability was measured to test the hypotheses in this section.

2.3 Encapsulation system with improved release profile

It is generally accepted that emulsions are effective to improve the bioavailability for the lipophilic active compounds, such as nutrients and nutraceuticals. The review paper by Acosta summarized several recent studies on the uptake of active ingredients delivered by nanoparticles as a function of the particle size (Acosta 2009). It was found out that the smaller the particle size of the emulsion, the more the active compounds can be absorbed by human body. Based on current knowledge and research, the hypothesis of this study is that the bioaccessibility of the encapsulated beta-carotene can be greatly improved after being encapsulated in nanoemulsions.

2.4 Incorporation of antioxidants to improve citral stability

Numerous works have been done to stabilize citral by encapsulating it in the form of emulsion because encapsulation could isolate the active compound (*i.e.* citral) from the reactive species in the aqueous medium, such as protons and free radicals. For example, Choi and Decker et al. (Choi et al. 2009) encapsulated citral in both medium chain triacylglycerols emulsion droplets and triacetin microemulsion droplets, and the results showed great improvement of citral's stability stored at 20^{o}C under acidic condition (pH 3.0). Mei and Choi et al. (Mei and others 2009) evaluated the stability of citral in oil-in-water emulsions at pH 3.0 with solid and liquid octadecane, and it was found out that citral's stability could be improved in oil-in-water emulsions.

Antioxidants have also been used to inhibit citral's degradation and to reduce the generation of the off-flavor compounds, such as *p*-cymene and *p*-cresol. Kimura and Nishimura et al. (Kimura et al. 1983) attempted to inhibit the formation of undesirable off-flavors produced by citral in acidic aqueous solution by the use of butylated hydroxytoluene (BHT), butylated hydroxyanisoles (BHA), n-propyl gallate, α-tocopherol, nordihydroguaiaretic acid and n-tritriacontane-16,18-dione; however, these compounds were found to be not effective on reducing the production of oxidative products from citral. In contrast, Liang and Wang et al. (Liang et al. 2004) showed that the antioxidative phenolic compounds (from grape seed, pomegranate seed, green tea and black tea extracts, respectively) were able to inhibit the formation of *p*-cymene, *p*-cresol, *p*-methylacetophenone and 8-hydroperoxy-*p*-cymene from citral degradation at pH 3.0. Ueno and Kiyohara et al. (Ueno et al. 2006) also discovered the inhibitory effects of black tea theaflavins on the formation of *p*-cresol and *p*-methylacetophenone for citral in acidic buffer solutions at pH 3.0. Peacock and Kuneman (Peacock and Kuneman 2002) used isoascorbic acid to inhibit the formation of *α,p*-dimethylstyrene and *p*-cymen-8-ol in a carbonated beverage system containing citral. All these studies were conducted in acidic aqueous buffers and no work has been done to investigate the inhibition of citral degradation in an emulsion system by antioxidants. Due to the scientific rationales described above, the hypothesis is that encapsulating citral in nanoemulsions with antioxidants together could improve the stability of citral under acidic condition (pH = 3.0).

2.5 LBL modification of SLN to improve citral stability

For the emulsion system, amphiphilic emulsifiers create the interfacial region separating the polar bulk aqueous phase from the non-polar hydrocarbon tails for the lipid phase. The interfacial region consists of water, hydrophilic head-groups of the emulsifiers and counter-ions (0.6 – 0.9/ionic head group) (Bunton and others 2002). This region is very anisotropic with intermediate properties between water and lipid phases; and high amount of ions will exert an influence on many of the physical and chemical reactions at this region. Studies of ion reactivity at charged interface showed that for a positively charged surface, the concentration of protons in the interfacial region is significantly lower (10 – 100 times or more) than in the surrounding bulk solutions, for example, when the pH of the aqueous solution is 3, even in the presence of buffers, the pH in the interfacial region may range from 4-6 depending upon experimental conditions; and anionic and cationic emulsifiers have opposite effects on the rates of chemical reactions of organic molecules in micelles with hydronium or hydroxide ions (Bunton et al. 2002; Romsted and others 1997; Bunton and Romsted 1999; Bunton 2006). The concentrations of anions, to the contrary, may be 10-100 or even 1000 times higher than in the surrounding aqueous phase. For example, letting the black dots in Figure 2-2 represent anionic counter-ions to the cationic headgroups (white dots attached to hydrophobic tails) of the surfactant molecules, the local interfacial concentration of anions typically varies from 1-3 M while the

stoichiometric concentration is on the order of 1-100 mM; anionic surfactants have the opposite effect. They increase the local hydrogen ion concentration by ca. 10-1000 times (Gruen 1985; Bunton et al. 2002) .

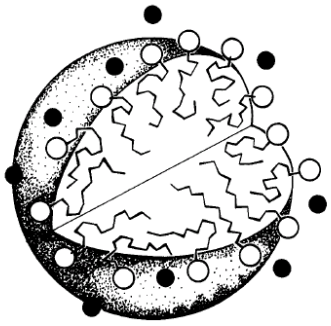


Figure 2-2 Cartoon of a spherical cationic micelle representing SDS micelle with white dots attached to tails as the surfactant molecules and black dots as the counterions (Gruen 1985; Bunton et al. 2002).

Therefore, the hypothesis is that multilayer nanoemulsion with cationic surface could be fabricated through the LBL technique and the multilayer nanoemulsion could provide improved protection for the encapsulated citral. Because for the protection of citral, a cationic emulsion system is favorable due to the positively charged interfacial layer which can repel reactive species (*e.g.* protons and metal ions) away from the emulsion droplets, therefore the degradation of citral, which is induced and accelerated by protons, metal ions and free radicals, could be inhibited.

3 Objectives

1. To improve beta-carotene stability using solid lipid nanoemulsions. The lipid phase is composed of both solid lipid (palm kernel fat) and liquid oil (medium chain triglyceride, MCT). Lecithin is used as the emulsifier. High speed and high pressure homogenizers will be used to produce various nanoemulsions. At the same time, lyophilization will be used as the secondary production method to prepare solid nanoemulsion powders. The influence of the freeze-drying process on both the physical and chemical stabilities of all the formulations will be evaluated.

2. To develop an in *vitro* digestion model to examine the release profile and the bioaccessibility of beta-carotene before and after encapsulation in two nanoemulsion formulations: palm kernel fat and MCT nanoemulsions, respectively. The extent of digestion and the amounts of beta-carotene stabilized in digestion fluids will be evaluated.

3. To improve citral stability under acidic condition (pH = 3.0) using solid lipid nanoemulsions in combination of various antioxidants. The nanoemulsions will be stabilized by the emulsifier lecithin and produced by high speed and high pressure homogenizers. The effects of six different antioxidants will be tested: black tea extract, ascorbic acid, naringenin, tangeretin, beta-carotene and tanshinone. GC and GC-MS will be used to examine citral stability as well as the production of the off-flavor compounds.

4. To improve citral stability by multilayer nanoemulsions fabricated by LBL technique. Cationic chitosan and ε-polylysine will be used as the cationic polymer coatings. Zeta-potential, particle size and QCM-D measurements will be conducted to monitor the LBL process and to determine the optimal multilayer nanoemulsion formulations. GC and GC-MS will also be used to examine citral stability as well as the production of the off-flavor compounds.

4 Materials and methods

4.1 Encapsulation of beta-carotene

4.1.1 Materials

Palm kernel fat was a gift from Firmenich (Princeton, NJ). Lecithin was a gift from American Lecithin Co. (Oxford, CT). Medium chain triglyceride (MCT 1053) was a gift from Stepan (Northfield, IL). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification.

4.1.2 Beta-carotene encapsulation

Palm kernel fat was heated to 50^{o}C when completely melted into liquid and then mixed with MCT oil at the same temperature with different ratios. Then beta-carotene was added into the lipid mixture under magnetic stirring. The beta-carotene loaded lipid dispersion was then mixed with the hot lecithin aqueous solution by using an Ultra-Turrax T-25 homogenizer (IKA Works Inc., Wilmington, DE) for 20 min to produce pre-emulsions. The pre-emulsions were further homogenized by using a high-pressure homogenizer (EmulsiFlex-C3, Avestin Inc., Ottawa, Canada) for 6 cycles at the pressure of 150 MPa. The final concentrations of lecithin and lipid were 5 and 10 wt%, respectively. Each of the emulsion dispersions (25 mL) was stored in a 50 mL polypropylene centrifuge tubes (with 25 mL air as the headspace) for further characterizations.

4.1.3 Lyophilization of beta-carotene nanoemulsions

Emulsion dispersions with various MCT oil contents were first prepared as described above and then mixed with sucrose aqueous solution (1:1, v/v) by using the Ultra-Turrax T-25 homogenizer (IKA Works Inc., Wilmington, DE) for 1 min. The final concentrations of lipid and sucrose were 5 and 15 wt%, respectively. The emulsion dispersions with sucrose as the cryoprotectant were then lyophilized by a Freezone 4.5 Liter benchtop freeze-dry system (Missouri, USA) for 24 h at the temperature of -40˚C under vacuum. Reconstitution of the lyophilized powders was performed by adding the powder into water and vortexing for 1 min to achieve the original concentration before lyophilization.

4.1.4 Characterization of beta-carotene nanoemulsions

4.1.4.1 Physical stability

Physical stability of all the samples were evaluated by measuring their particle size changes at different storage temperatures (25^{o}C and 50^{o}C) with time. The particle sizes were measured by photon correlation spectroscopy (PCS) – based BIC 90 plus particle size analyzer equipped with a Brookhaven BI-9000AT digital correlator (Brookhaven Instrument Corp., New York, NY, USA). The light source of the particle size analyzer is a solid state laser operating at 658 nm with 30 mW power, and the signals were detected by a high sensitivity avalanche photodiode detector. All measurements were made at a fixed scattering angle of 90° and temperature of 25.0 ±

0.1^{o}C. Two methods were used to analyze the autocorrelation function G (q, t) data obtained from the PCS measurements. The first one was the Cumulant analysis method, where G (q, t) was decomposed into a distribution of the decay rate $\Gamma = 1/\tau$ given by

$$G(q, t) = \int G(\Gamma) \exp(-\Gamma t)\, d\Gamma \quad (1)$$

And the second method was William-Watts (WW) single stretched exponential function given by

$$G(q, t) = \exp[-(t/\tau)^{\beta}] \quad (2)$$

where τ is the relaxation time and β is the distribution parameter.

Then the particle diffusion coefficient D could be calculated by:

$$D = 1/\tau q^2 \quad (3)$$

where q is the amplitude of scattering vector defined as:

$$q = (4\pi n/\lambda) \sin(\theta/2) \quad (4)$$

where n is the solution refractive index, λ is the laser wavelength and θ is the scattering angle. The diffusion coefficient D can be converted into mean particle diameter d using the Stokes-Einstein equation:

$$d = kT/3\pi\eta D \quad (5)$$

where k is the Boltzmann constant, T is the absolute temperature and η is the solvent viscosity.

4.1.4.2 Entrapment efficiency

After sample preparation, the beta-carotene loaded emulsions were 1:10 (v/v) diluted by DI water and filtered through a 0.45μm filter to remove the non-encapsulated beta-carotene. Then 100 μL of aliquots filtered solution was dissolved in 900 μL ethanol and vortexed for 1 min. After dissolving completely, 100 μL of the ethanol solution was mixed with 900 μL hexane and then subjected to HPLC to quantify the amount of the encapsulated beta-carotene. Entrapment efficiencies were calculated by the equation below:

$$Entrapment\ \ Efficiency = \frac{Encapsulated\ \ \beta - carotene}{\beta - carotene\ \ added} \times 100\%$$

4.1.4.3 Chemical stability

The beta-carotene loaded emulsion dispersions were divided into two groups. One group was put in transparent glass tubes and the other group was put in lightproof polypropylene tubes. The first group was placed under a light bulb with 800 lumenes and illuminated up to 17 days and the second group was stored in dark. Then 100 μL of aliquots emulsion dispersion was taken from each sample at designated time intervals and dissolved in 900 μL ethanol and vortex-mixed for 1 min.

The beta-carotene content was quantified by HPLC, using an UltiMate 3000 HPLC system with a 25D UV/Vis absorption detector (Dionex, Sunnyvale, CA). A 250 × 4.6 mm YMC C30 column (particle size = 5 μm, Waters Inc., Wilmington, NC) was used.

The mobile phase gradient procedure was as follows: (1) initial conditions of 90% solvent A (97% methanol and 3% water) and 10% solvent B (100% methyl tert-butyl ether); (2) increasing solvent B to 40% from 0 to 4 min; (3) increasing solvent B to 85% from 7 to 15 min; (4) maintaining 85% solvent B and 15% solvent A for 2 min and then decreasing solvent B to 10%.

4.1.4.4 Thermal properties

Differential scanning calorimetry (DSC) analysis was performed to evaluate the melting points of various samples by using a METTLER DSC 821^e instrument (Mettler Toledo, Germany). 5 to 10 mg of the sample was weighted and placed in 40µl aluminum pan and an empty aluminum pan was used as a reference. The scan rate of 5˚C/min was used for all the measurements.

4.1.4.5 In-vitro digestion

The *in vitro* digestion procedure was a modification of that previously described by Garett et al. (Garrett and others 1999) and Liu et al. (Liu and others 2004). The temperature for the whole digestion procedure was kept at 37^oC by using oil bath. Briefly, 2 mL of the sample was mixed with 8 mL basal saline (140 mM NaCl, 5 mM KCl and 150 µm $CaCl_2$) and the pH was adjusted to 2.0 by adding 0.1 M/ 1.0 M HCl. Then 0.5 mL pepsin solution (0.2 g of pepsin in 5 mL 0.1 M HCl) was added to initiate the gastric digestion. After 1 h, the pH of the sample was adjusted to 6.5 with 0.2 M/1.0 M NaOH. Then the intestinal digestion was initiated by adding 2.5 mL pancreatin – bile solution (0.075 g of pancreatin and 0.45 g bile extract in 37.5 mL

0.1M NaOH). During 2 h of the intestinal digestion process, 0.2 M NaOH was added to maintain the pH of the sample at 7.5.

The extent of digestion is defined as the percentage of lipid digested in the experiment calculated by the equation:

$$Extent_{digestion} = \frac{V_{NaOH} \times C_{NaOH}}{2Mw_{lipid}}$$

where V_{NaOH} is the volume of NaOH consumed during the digestion, C_{NaOH} is the concentration of NaOH (0.20 M) and Mw_{lipid} is the average molecular weight of the lipids (503 g/mol for MCT and 765 g/mol for palm kernel fat).

The kinetic paramters Vm (the maximum product conversion) and Km (the product concentration at which the reaction rate is equal to half Vm) were obtained by fitting the titration curves during digestion.

Isolation of the micellar fraction containing beta-carotene. Aliquots (15.0 g) of the digestate samples were transferred to ultracentrifuge tubes (Beckman Coutler, Inc., Brea, CA, USA) and placed in a type 60 Ti rotor to centrifuge at 4^{o}C and 40,000 rpm (113,613 *g*) for 40 min (Beckman Coutler, Inc., Brea, CA, USA). For beta-carotene dissolved in lipids (parlm kernel fat and MCT oil, respectively), small amounts of undigested lipid phase was adhered to the edge of the unltracentrifuge tube; and there was no such lipid phase for beta-carotene encapsulated in nanoemulsions. The middle aqueous fraction was collected by using an 18 gauge needle attached to a 10 mL syringe. The aqueous sample was then filtered through a 0.45 μm filter and transferred to amber glass vials under nitrogen at -45^{o}C until analysis (*i.e.,* within 2

weeks).

4.2 Encapsulation of citral

4.2.1 Materials

Palm kernel fat was a gift from Firmenich (Princeton, NJ, USA). Lecithin was a gift from Lipoid GmbH (Ludwigshafen, Germany). Narigenin, tangeretin, tanshinone were purchased from Quality Phytochemicals, LLC (Edison, NJ, USA). Black tea extract standardized to 30% theaflavins was a gift from Wellgen (New Brunswick, NJ, USA). ε-Polylysine (EPL) was purchased from Zhejiang Silver-Elephant Bioengineering Co. (Zhejiang, China). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification.

4.2.2 Citral encapsulation

Hot homogenization method (Schwarz and others 1994) was used to encapsulate citral. Palm kernel fat (10 wt%) was heated to 45^{o}C when completely melted into liquid. 0.1 wt% Citral and 0.01 wt% undecane (as the internal standard) were dissolved in the lipid phase and mixed with 5 wt% lecithin aqueous buffer solution (10 mM citric acid/sodium hydroxide/sodium chloride pH 3.0 buffer) at the same temperature by using a ULTRA–TURRAX T-25 homogenizer (IKA Works Inc., Willmington, USA) for 5 min to obtain a coarse emulsion. The coarse emulsion was then homogenized by using a high-pressure homogenizer (EmulsiFlex-C3, AVESTIN Inc., Ottawa, Canada)

for 6 cycles at the pressure of 150 MPa. The temperature was kept at 45^{o}C during the whole sample preparation process to avoid lipid crystallization.

4.2.3 Incorporation of antioxidants

Due to the antioxidants' different solubility, they were encapsulated in the SLN with citral together as follows: 0.1 wt% ascorbic acid was dissolved in the surfactant aqueous buffer solution before mixing with the lipid phase; 0.1 wt% beta-carotene and tanshinone were dissolved in the melted palm kernel fat with citral, respectively; 0.1 wt% naringenin, tangeretin and black tea extract were dispersed in 2 wt% polyether glycol by vortex for 5 min and then mixed with the melted lipid with citral together, respectively. Then high speed and high pressure homogenizers were used to produce homogeneous SLN as described above. Citric acid was added when necessary to maintain the pH value (pH 3.0) of all the samples (citral loaded SLN with and without antioxidants). 10 mL of each SLN dispersion was stored in a 20 mL amber glass vial with 10 mL headspace. All the samples were divided into two groups, with one group stored at 25^{o}C and the other group stored at 50^{o}C.

4.2.4 LBL modification of citral nanoemulsions

Chitosan (CS) was dissolved in 10 mM pH 3.0 sodium acetate/acetic acid buffer solution by magnetic stirring for 30 min and filtered by 0.45 μm filter to remove any possible impurities. ε-Polylysine (EPL) was dissolved in 10 mM citric acid/sodium hydroxide/sodium chloride pH 3.0 buffer solution by magnetic stirring for 30 min and

filtered by 0.45 μm filter to remove any possible impurities. Same amount of the 10 mM citric acid/sodium hydroxide/sodium chloride pH 3.0 buffer solution was also added into the primary emulsion to make sure all the samples (lecithin, lecithin-CS and lecithin-EPL stabilized emulsions) have the same contents of lipid (10 wt%), lecithin (5 wt%), citral (0.1 wt%) and undecane (0.01 wt%, as the internal standard). After sample preparation, citric acid was added when necessary to maintain the pH value (3.0) of all the samples. 10 mL of each emulsion dispersion was kept in a 20 mL amber glass vial with 10 mL headspace. All the samples were divided into two groups, with one group stored at 25^{o}C and the other group stored at 50^{o}C.

4.2.5 Characterization of citral nanoemulsions

4.2.5.1 Surface charge measurements

The surface charges of the lecithin, lecithin-CS and lecithin-EPL stabilized emulsions were measured using a Zetasizer Nano ZS-90 instrument (Malvern Instruments Ltd., Southboro, MA). All the samples were diluted by pH 3.0 citric acid buffer before each measurement and all the measurements were performed at 25^{o}C.

4.2.5.2 Particle size measurements

Particle sizes of all the samples were evaluated by using the photon correlation spectroscopy (PCS) – based BIC 90 plus particle size analyzer equipped with a Brookhave BI-9000AT digital correlator (Brookhaven Instrument Corp., New York, NY, USA). The light source of the particle size analyzer is a solid state laser operating

at 658 nm with 30 mW power, and the signals were detected by a high sensitivity avalanche photodiode detector. All measurements were made at a fixed scattering angle of 90° and temperature of 25.0 ± 0.1^{o}C and same models were used to process the data as described above.

4.2.5.3 Adsorption measurements

The adsorption processes between CS, EPL and lecithin stabilized primary emulsions were monitored using a commercial QCM-D apparatus (Q-Sense AB, Sweden) with a Q-Sense D300 electronic unit, which was controlled by Q-Sense software (Q-Soft, Q-Sense). QCM-D is sensitive to small changes caused by deposited molecules on a gold quartz crystal. A voltage is applied to the crystal to make it oscillate at a constant frequency, thus any structure changes and/or mass changes on the crystal surface will induce a change in its resonant frequency. The experiments were performed at 25.00 ± 0.02^{o}C. Before each measurement, the gold-coated quartz crystal was soaked in 2 wt% sodium dodecyl sulfate solution for 30 min, then rinsed with Milli-Q water and dried with nitrogen gas; the crystal was then cleaned in an UV/ozone chamber for 10 min, followed by soaking in a 1:1:5 (v:v:v) mixture of ammonia hydroxide (NH_4OH, 25%), hydrogen peroxide (H_2O_2, 30%) and Milli-Q water for 15 min at 75^{o}C; it was then rinsed with Milli-Q water, dried with nitrogen gas, and finally cleaned in an UV/ozone chamber for another 10 min. This cleaning procedure could remove the possible contaminants on the crystal surface. The crystal was then mounted in the QCM-D chamber and excited at their fundamental frequency (about 5 MHz) as well

as the third, fifth, and seventh overtones (denoted by n=3, 5, and 7, corresponding to frequencies of 15, 25 and 35 MHz, respectively). All the samples were 10 times diluted before injecting into the QCM-D chamber to avoid clogging the instrument tubes and the samples were injected only when a stable Δf signal was obtained (Δf < 1 Hz in 5 min of the 3rd overtone).

4.2.5.4 Physical stability

Physical stability of all the samples were evaluated by measuring their particle size changes at different storage temperatures (25°C and 50°C) with time (day 0, day 30 and day 60, respectively) using the same instrument and models as described in section 4.1.4.1.

4.2.5.5 Chemical stability

Analysis of citral and its degradation products were conducted on an Agilent 6850 gas chromatography (GC) equipped with a J & W DB-5MS capillary column (30 m×0.25 mm i.d.; 0.25 μm film thickness) and a flame ionizing detector (FID). The oven temperature was increased from 60 °C to 150 °C at 4 °C/min, then increased to 230 °C at 20 °C/min, and held at 230 °C for 5 min. The gas flow was controlled as follows: hydrogen flow at 40.0 mL/min, air flow at 45.0 mL/min, and helium as carrier gas flow at 45.0 mL/min. FID detector temperature was 250 °C.

A 0.75 mm i.d. solid phase microextraction (SPME) injection sleeve was employed to minimize the broadening effect compare to a 2.0 mm injection glass liner. For SPME analysis, 10 mL of each SLN dispersion was stored in a 20 mL amber glass vial

containing a magnetic stir bar under stirring. The glass vial was sealed with a PTFE (polytetrafluoro ethylene)/silicone speta and a screw cap. The 65 μm PDMS-DVB (polydimethylsiloxane-carboxen) SPME fiber was exposed to the sample headspace manually for 30 min at 25^{o}C (for the 25^{o}C storage samples) and 50^{o}C (for the 50^{o}C storage samples), respectively. After the absorption process, the fiber was inserted immediately into the injection port of GC and held for 5 min to ensure a complete thermal desorption. The quantification of citral and the degradations products were analyzed by computing their peak areas versus the internal standard (undecane) peak area.

An Agilent 6890 gas chromatograph equipped with an Agilent 5973 mass detector and a J & W DB-5MS capillary column (30 m×0.25 mm i.d.; 0.25 μm film thickness) was used. The gas flow was controlled as follows: hydrogen flow at 40.0 mL/min, air flow at 45.0 mL/min, and nitrogen flow 45.0 mL/min. The injection port was kept at 230^{o}C. The oven temperature was increased from 60 ^{o}C to 150 ^{o}C at 4 ^{o}C/min, then increased to 230 ^{o}C at 20 ^{o}C/min, and held at 230 ^{o}C for 5 min. The ionization voltage was held at 70 eV and the ion temperature was at 280^{o}C.

5 Results and Discussion

5.1 Encapsulation of beta-carotene

Storage stability of beta-carotene loaded nanoemulsions. When only high speed homogenization was performed to stabilize the emulsion, even at the maximum speed 65,000 rpm for 20 min, emulsion creaming and phase separation occurred within 12 h at room temperature. And the particle size measurement for the freshly made emulsion sample indicated that the emulsion droplets were bigger than 300 nm. To further reduce the particle size of the emulsion, high pressure homogenizer was used and detailed particle size results will be shown and discussed below.

Stability of beta-carotene loaded emulsions was evaluated by measuring the particle size changes at day 0, 30 and 60, respectively. All the samples with various MCT contents from 0 – 40% were stored in 50 mL polypropylene centrifuge tubes at 4 ^{o}C and 25^{o}C, respectively. The particle size results were shown in Figure 5-1. Different lipid compositions had no significant influence on the particle size. Therefore lipid is not the determining factor to affect the particle size of emulsions in this study, which also agrees well with other people's work (Teeranachaideekul and others 2008). However, Hu et al. (Hu and others 2005) discovered that when combining liquid oil (oleic acid) with solid lipid (stearic acid) together to produce O/W emulsion, increasing the liquid oil content to some extent could greatly reduce the particle size; and this result was probably related with the different viscosity of solid and liquid oil.

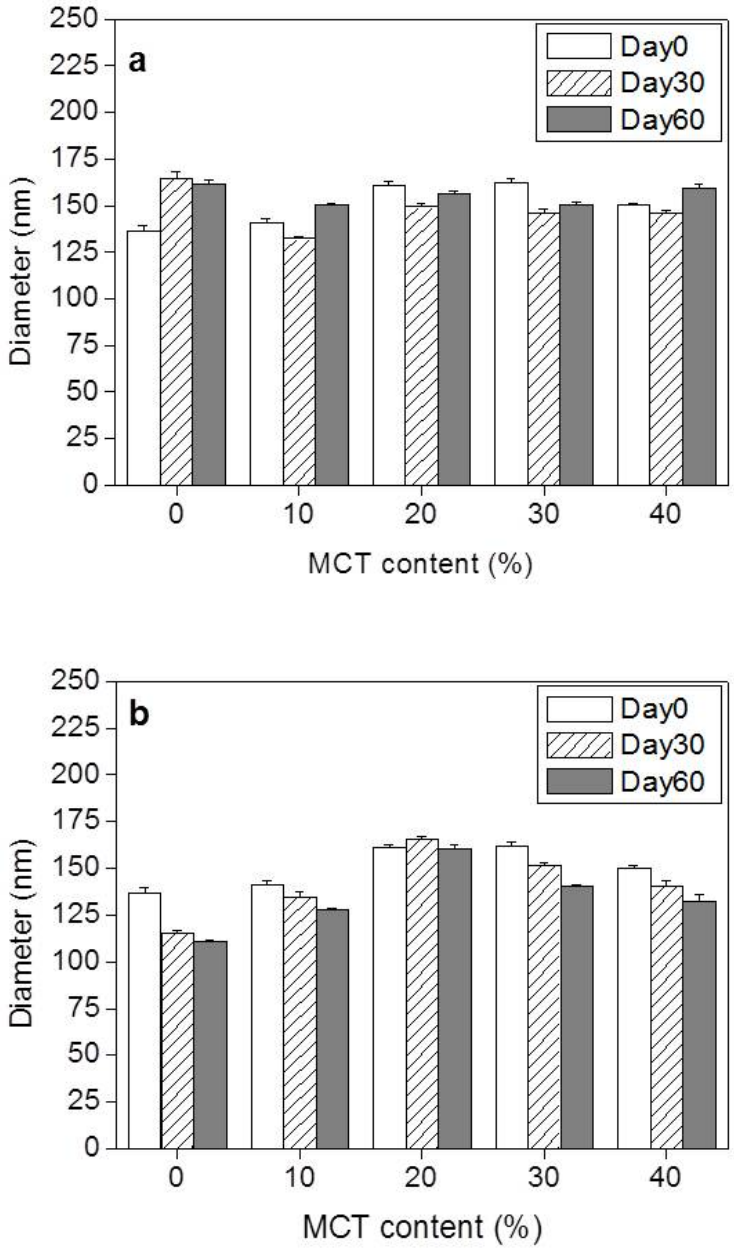


Figure 5-1 Particle size of beta-carotene loaded palm kernel fat nanoemulsions with various MCT oil contents ranging from 0 to 40% in the lipid phase (lipid phase in the emulsion: 10 wt%) stored at (a) 4 ^{o}C and (b) 25 ^{o}C .

In this study, no obvious particle size difference between different formulations (MCT contents ranging from 0 to 40%) was observed and the most possible reason might be that the solid lipid palm kernel fat used in this study was a low melting lipid, therefore when making into nanoemulsions, the viscosity difference between the solid lipid and

the liquid MCT oil is not significant enough to cause the particle size changing trend. During 60 days of storage, the fluctuations of the particle size were only several nanometers, which proved that the formulations in this study had good storage stability to avoid creaming.

Freeze-thaw stability. For emulsions using sucrose as the cryoprotectant to produce solid state samples by lyophilization, the freeze-thaw cycling stability was also examined by measuring the particle size changes for 10 continuous freeze-thaw cycles. Freshly made emulsions were mixed with aqueous cryoprotectant solutions and frozen at -40^{o}C, dried under vacuum simultaneously in a freeze-dryer for 24 hrs. Solid emulsion powders were obtained after the freeze-drying process.

Lyophilization was a secondary production step to convert liquid emulsion dispersions to solid state samples. The mostly used secondary production equipments to produce solid state products are freeze-dryer and spray-dryer. Although spray-dryer is more cost-effective than the freeze-dryer, due to the incorporation of heat sensitive beta-carotene and the avoidance of heat treatment of the freeze-drying process, the freeze-dryer was chosen here in this research. Powders obtained after the freeze-dry process were reconstituted in water and then stored in refrigerator (-18^{o}C) for 18 hrs until totally freezed into ice and thawed under room temperature for 6 hrs until totally thawed. Then the particle size of the thawed dispersions were examined by PCS and the results were shown in Figure 5-2.

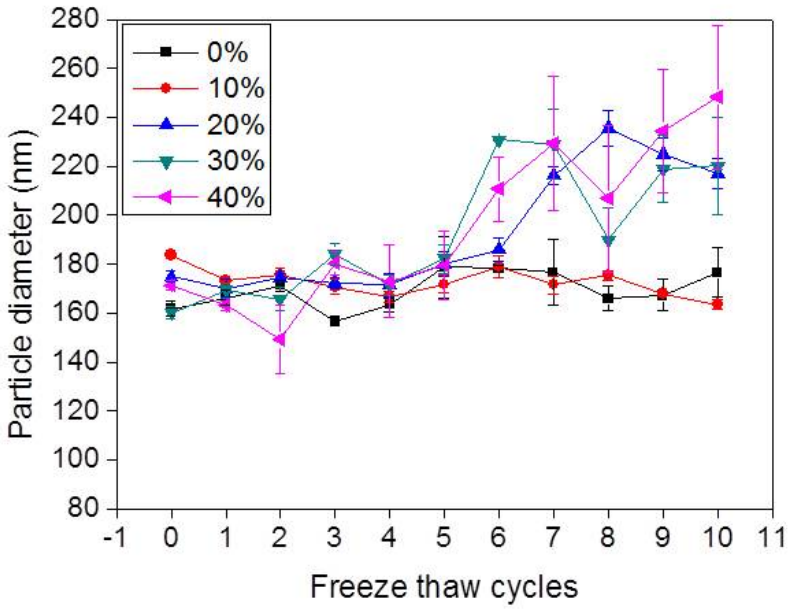
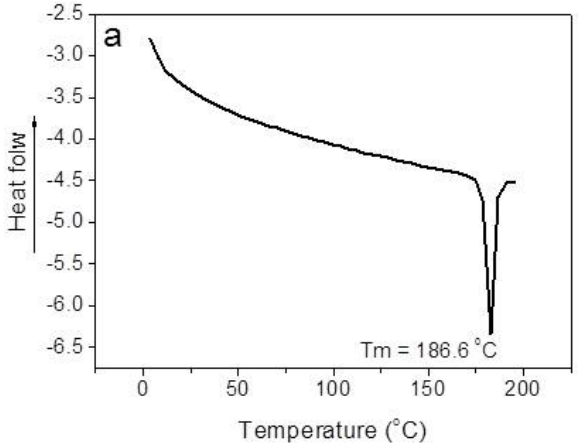


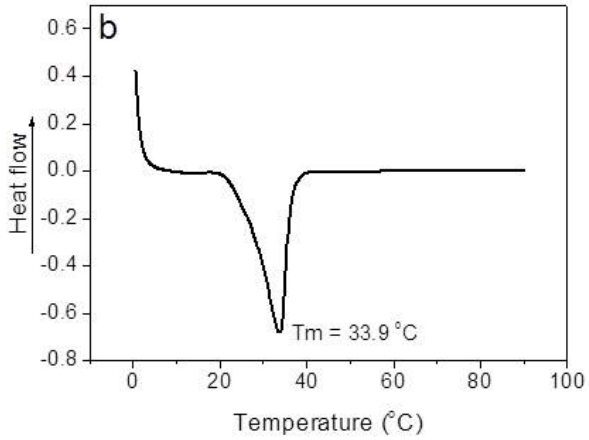
Figure 5-2 Influence of freeze-thaw cycles on the particle size of lyophilized palm kernel fat emulsions with various MCT oil contents in the lipid phase ranging from 0 to 40% (the emulsion contains 5.0 wt% lipid, 15 wt% sucrose and 2.5 wt% lecithin).

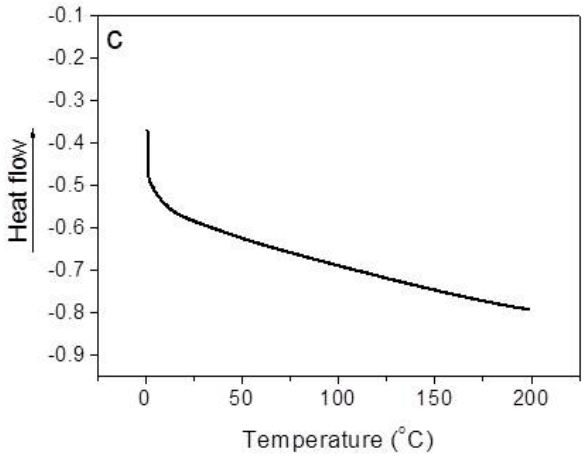
In general, all the formulations showed good freeze-thaw stability during the first five cycles, while particle aggregation became more obvious starting from the sixth cycle, especially with the increase of the MCT oil content. For example, for the formulations with 0 and 10% MCT oil, particle size hardly changed during the 10 cycles while for the formulations with 20 – 40% MCT oil, the particle size greatly increased as shown in Figure 5-2. The results provided information on the extent to which the incorporation of MCT oil interferes with the lyophilization process and crystallization played an important role in this process. Schwarz and Mehnert et al. studied the freeze-dried drug-free and drug-loaded SLN and discovered that the loading drug could greatly promote particle aggregation due to the free drug in the dispersion medium (Schwarz and Mehnert 1997). In this study, palm kernel fat crystallized

during the freezing process to expel the MCT oil as well as the loading beta-carotene, which might lead to the aggregation of the emulsion droplets/particles. The crystallization of palm kernel fat will be proved in the DSC measurement section.

Melting points measurements. DSC was used to measure the melting points of various emulsions with and without lyophilization. The DSC curves for all the ingredients are shown in Figure 5-3. The DSC curve for lecithin is smooth with no peaks thus lecithin can be recognized as a non-crystalline surfactant at room temperature; and MCT oil is a non-crystalline oil for the same reason. The melting points of beta-carotene, palm kernel fat and sucrose are 186.6, 33.9 and 192.0 ^{o}C, respectively.


a
Heat folw
-2.5
-3.0
-3.5
-4.0
-4.5
-5.0
-5.5
-6.0
-6.5
Tm = 186.6 °C
0
50
100
150
200
Temperature (°C)


b
Heat flow
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
Tm = 33.9 °C
0
20
40
60
80
100
Temperature (°C)


c
Heat flow
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
-0.8
-0.9
0
50
100
150
200
Temperature (°C)

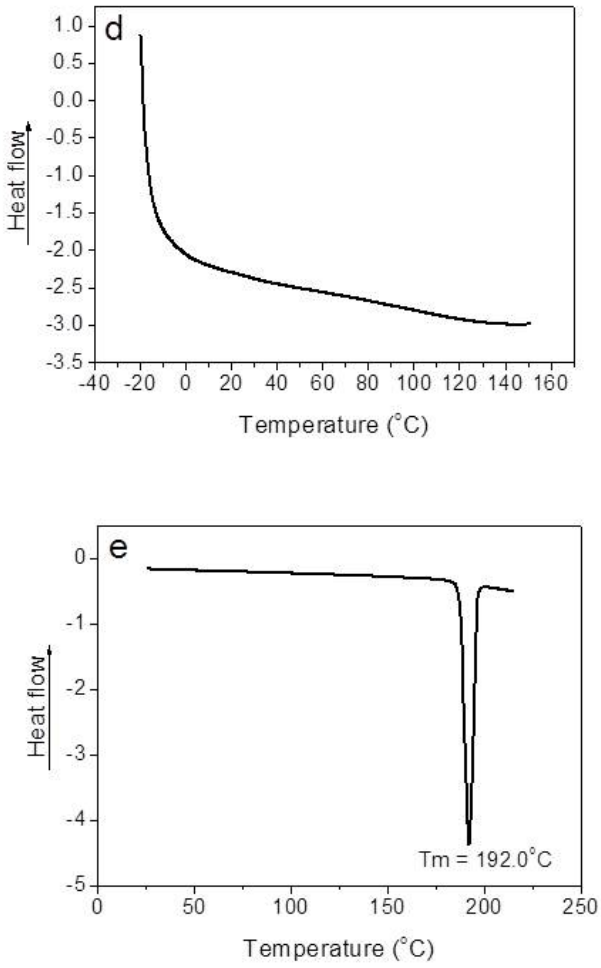


Figure 5-3 DSC curves of the ingredients used in the SLN formulations: (a) beta-carotene; (b) palm kernel fat; (c) MCT 1053 oil; (d) lecithin and (e) sucrose.

Figure 5-4 shows the DSC curves for the liquid SLN dispersions loaded with beta-carotene and Figure 5-5 shows the DSC curves for the freeze-dried SLN powders loaded with beta-carotene. All the measurements started from 0°C with a scan rate of 5°C/min. This starting temperature was set to avoid gel formation for the emulsion dispersions, because it was already proved that cooling could induce lipid crystallization and emulsion gelation (Awad and others 2009). In the temperature range studied, only the palm kernel fat has a melting point at 33.9°C and no melting points were observed for the MCT oil and the surfactant lecithin. The beta-carotene

loaded emulsion dispersions with no cryoprotectant had no melting points, while the lyophilized powders showed clear endothermic peaks (Figure 5-5) indicating the melting points and the formation of ordered crystal structures.

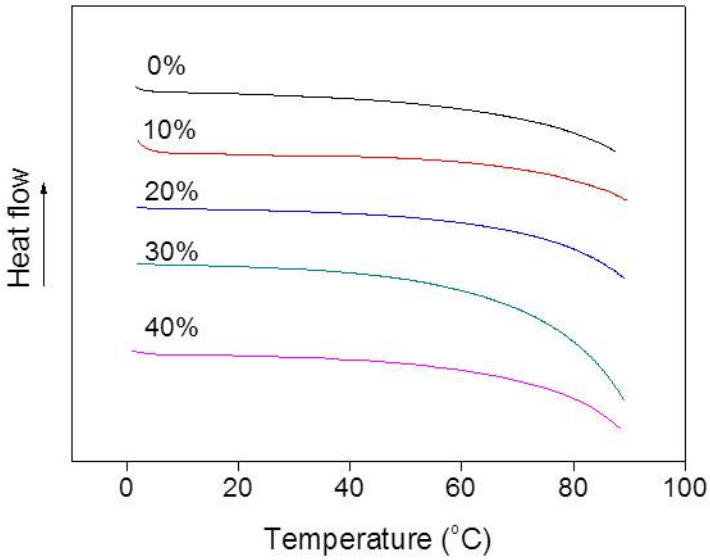


Figure 5-4 DSC thermograms of SLN with various MCT oil contents in the lipid phase ranging from 0 to 40% (each emulsion sample contains 10 wt% lipid phase, 5 wt% lecithin and 85 wt% water).

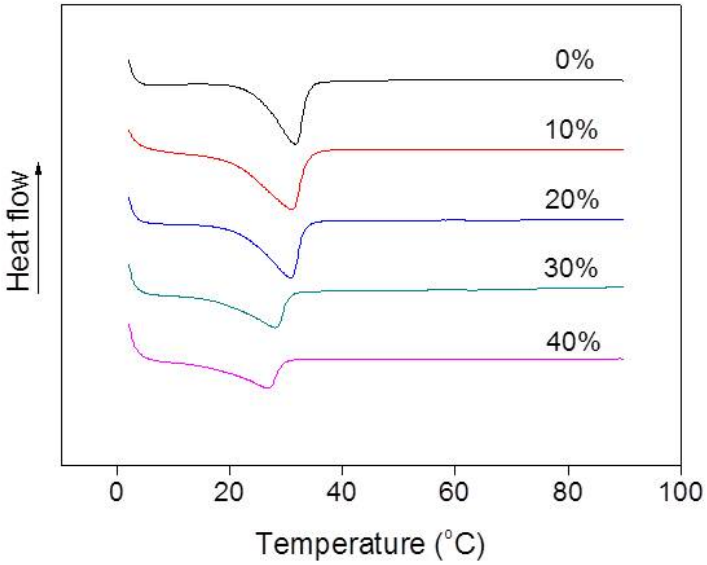


Figure 5-5 DSC thermograms of lyophilized SLN powders with various MCT oil contents ranging from 0 to 40% in the lipid phase (each powder sample contains 22.2 wt% lipid, 66.7 wt% sucrose as the cryoprotectant and 11.1 wt% lecithin).

An interesting phenomenon is that the melting points for the SLN powders decreased compare to the melting point of pure palm kernel fat (33.9 ^{o}C); furthermore, with the increase of the MCT oil content from 0 to 40%, the melting points gradually decreased. For example, for the SLN with no MCT oil included, the melting point is 31.6 ^{o}C and it decreased to 26.7 ^{o}C when the lipid phase contained 60% palm kernel fat and 40% MCT oil. This melting points depression phenomenon was also observed in other groups works (Hu et al. 2005; Jenning and others 2000) and it was suggested that the inclusion of liquid oil into the solid lipid matrix could disturb the ordered solid lipid crystal structure to create lattice defects. Awad and Helgason et al. (Awad et al. 2009) examined the crystallization and polymorphic transition behaviors of a series SLN suspensions with different lipid matrixes, which were composed of different amounts of both high melting lipid (tripalmitin) and low melting lipid (fish oil). The results showed that the crystallization, melting, polymorphic transition and stability of SLN were greatly influenced by the incorporation of fish oil into the lipid phase. For the emulsion dispersions without lyophilization, no melting points were observed in the temperature range studied. The thermotropic phase behavior of the lipid matrix of an emulsion system can be highly affected by the guest molecules/additives, which is the surfactant lecithin in this case. The surfactant lecithin used in this study is an oil-free soy lecithin and is solid at room temperature (25^{o}C). It is a mixture of various phospholipids and fatty acids and from the DSC measurement in this study, there was no peak for the lecithin during the temperature

range studied therefore it has less ordered structure compared to other emulsifiers such as Tween 60 (Tm ≈ 57 °C) and Phospholipon 80H (lecithin, Tm ≈ 52 °C) (Helgason and others 2009). Studies have found that the crystallized emulsifiers could work as templates (as shown in Figure 5-6) to influence the solid fat crystallization (Helgason et al. 2009; Helgason and others 2008). For this study, no melting peaks were found for emulsion dispersions which imply that the existence of non-crystallized lecithin disturbed the ordered structure of the fat molecules. For the freeze-dried emulsion powders, all the samples went through a severe freezing condition with the temperature as low as -40^{o}C under vacuum for 24 hrs which greatly promoted the crystallization process. Therefore the different thermal properties between lyophilized and non-lyophilized SLN samples might prove that the emulsion crystallization is a kinetic process, because the severe experimental conditions could accelerate this process. The mechanism of how different surfactants influence the crystallization behavior of solid lipid emulsions is still not fully understood and requires further in depth study by using other techniques, such as X-ray and neutron scattering techniques (Bunjes et al. 1996).

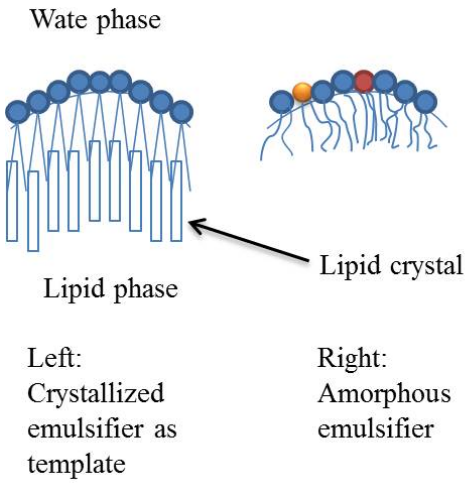


Figure 5-6 Schematic representation of the effects caused by different emulsifiers on the crystallization behaviors of the SLN lipid phase.

Entrapment efficiency. In order to evaluate the influence of different MCT oil contents on the entrapment efficiency (EE) of beta-carotene in different formulations, HPLC was used to quantify the beta-carotene being encapsulated and the results were shown in Figure 5-7. The EE of beta-carotene gradually increased from 43.7% to 54.2% with the increasing amount of MCT oil from 0 to 40%. Similar results have also been obtained in other group's work (Hu et al. 2005; Souto and others 2004). For example, Hu and Jiang et al. (Hu et al. 2005) discovered that with the increase of oleic acid (the liquid lipid composition) in the stearic acid SLN, the EE of the model drug clobetasol propionate was increased at the same time. Souto and Wissing et al. (Souto et al. 2004) also found that the binary mixture of liquid and solid lipids formulated colloidal system did work to improve the EE of a lipophilic drug clotrimazole. Therefore for the production of SLN, a heterogeneous lipid phase might be favored to encapsulate lipophilic compound, due to more space being available to accommodate the encapsulated molecules.

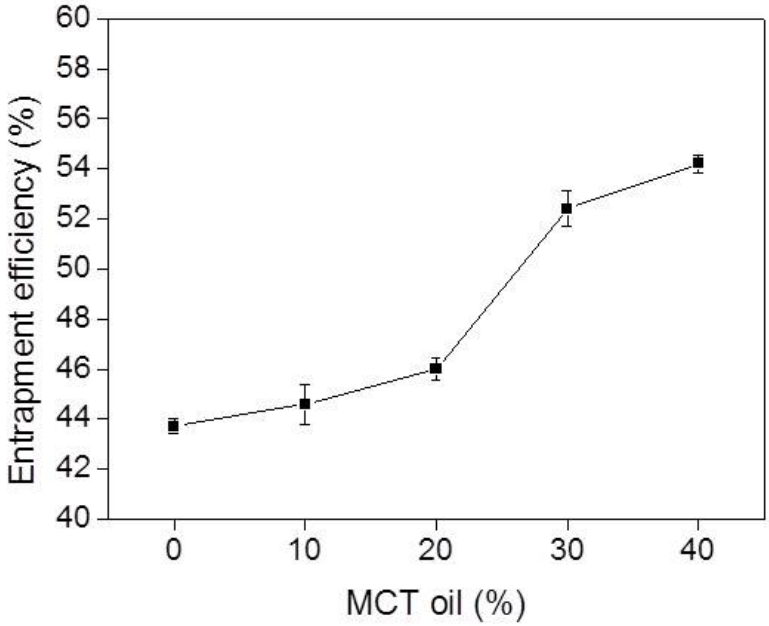


Figure 5-7 Entrapment efficiency of beta-carotene in SLN with various MCT oil contents ranging from 0 to 40% in the lipid phase (each emulsion sample contains 10 wt% lipid phase, 5 wt% lecithin and 85 wt% water).

Chemical stability of beta-carotene. The chemical stability of beta-carotene encapsulated in aqueous SLN dispersions and freeze-dried powders were examined by HPLC for 17 continuous days (Figure 5-8) and the results were shown in Figure 5-9 and Figure 5-10. The samples were divided into two groups: one group was put under light exposure and the other one was stored in dark. For pure beta-carotene dissolved in ethanol as the control, 24.9% and 23.5% were lost after 1 day when stored under light and dark conditions, respectively; and at the end of the measurement (after 17 days), 81.5% and 77.1% of beta-carotene were gone for storage under light and dark conditions, respectively. The degradation rate of beta-carotene was reduced after encapsulation. For example, when stored in dark, only ~ 2% beta-carotene degraded

for all the emulsion formulations with different MCT oil content ranging from 0 to 40% after 1 day of storage, ~4.5% after 2 days of storage and ~7% after 3 days of storage. After 5 days of storage, the beta-carotene degradation difference between different formulations gradually appeared. For example, 13% and 21% beta-carotene degraded for the emulsion formulations containing 0 and 40% MCT oil, respectively. At the end of the measurements (17 days), 33.8%, 39.4%, 48.3%, 52.7% and 53.4% beta-carotene were lost for the emulsions containing 0 to 40% MCT oil. Beta-carotene loaded samples stored under light exposure degraded faster than beta-carotene in the samples stored in dark. For example for the formulation with no MCT oil included, 41.8% beta-carotene was lost in light condition compare to 33.8% in dark condition at the end of the measurement. The promotion effect of light on beta-carotene degradation was within expectation because it was well known that illumination could induce the isomerization and degradation of beta-carotene (Chen and Huang 1998) and the data agreed well with previously reported research (Scita 1992).

At the same time, the lyophilized powders were reconstituted in water and the beta-carotene stability was also measured under different storage conditions (dark and light, respectively) and the results are shown in Figure 5-10. The beta-carotene degradation behavior in this group of samples showed significant differences compared to beta-carotene in emulsion dispersions. First, the degradation rates of beta-carotene in all the lyophilized samples were faster than that of beta-carotene in the aqueous SLN dispersions. For example, when stored in dark, 75.2% and 34.8%

beta-carotene were lost for the emulsion with no MCT oil with and without lyophilization at the end of the measurement; when stored under light exposure, 96% and 41.8% beta-carotene were lost for the emulsion with no MCT oil with and without lyophilization at the end of the measurement. The lyophilization process obviously promoted the degradation of beta-carotene under both storage conditions (with and without light exposure). Second, for the lyophilization samples stored in dark, all the formulations (emulsions with various MCT oil contents from 0 to 40%) protected beta-carotene from degradation compare to the control (beta-carotene dissolved in ethanol); however, for the lyophilization samples exposed to light, only the formulation with the highest MCT oil content (40%) showed some protection effect for beta-carotene. Third, for the samples without lyophilization, the formulation with the lowest MCT oil content had better protection for beta-carotene in liquid dispersions while for the lyophilized samples, the formulation with the highest MCT oil content had better protection for beta-carotene.

There are mainly two factors that affect the stability of beta-carotene in different formulations: mobility and crystallization. The prominent and unique advantage of using solid fat as the emulsion lipid matrix is that the solid state of lipid can confine the movement of the encapsulated compounds (such as beta-carotene in this study), thus keep them from contacting with other active molecules (such as oxygen and metal ions) in the aqueous medium. The incorporation of liquid MCT oil could increase the mobility of these compounds; therefore the degradation reactions of the

encapsulated compounds can be accelerated. Another factor that accelerates beta-carotene degradation is crystallization, which could be affected by many parameters, such as crystallized surfactant with ordered tails and severe processing procedure. Crystallization is a process that solid fat molecules form homogeneous ordered structure which expels the foreign guest molecules (such as beta-carotene and liquid MCT oil) out so beta-carotene gradually loses protection from encapsulation.

Besides these considerations, it is also likely that the freeze-drying process itself could promote the degradation of the active compounds. Desobry and Netto et al. encapsulated pure beta-carotene in 25 dextrose equivalent maltodextrin by spray-drying, drum drying and freeze-drying and they found out that both spray-drying and freeze-drying didn't provide protection for beta-carotene compare to drum drying; for example, the actual time to 50% beta-carotene loss was 6 weeks for both spray drying and freeze-drying and 24 weeks for drum drying (Desobry and others 1997). Cannac and Ferrat et al. evaluated the effects of different prehandling methods on the flavonoid concentrations in the plant *Posidonia oceanica*, and the results showed that freeze-drying caused more loss of the flavonoids even than oven drying (a loss of 71% of total (pro)anthocyanidins, 87% of total flavonols, and 95% of all simple flavonols) (Cannac and others 2007). Papageorgiou and Mallouchos et al. also compared the influence of air-drying and freeze-drying on the phenolic contents on several plants, and found out that freeze-drying significantly decreased the total phenolic content than air-drying (Papageorgiou and others 2008). Although similar

phenomenon has been discovered by several different studies, the reasons why different drying methods led to different active compounds (*i.e.* antioxidants in plant tissues) stability behaviors were not discussed and remained unknown till now. Taking these factors into consideration, lyophilization is not a favorable method to transform aqueous emulsion dispersion into dry product in this research since it can promote the lipid crystallization and beta-carotene degradation.

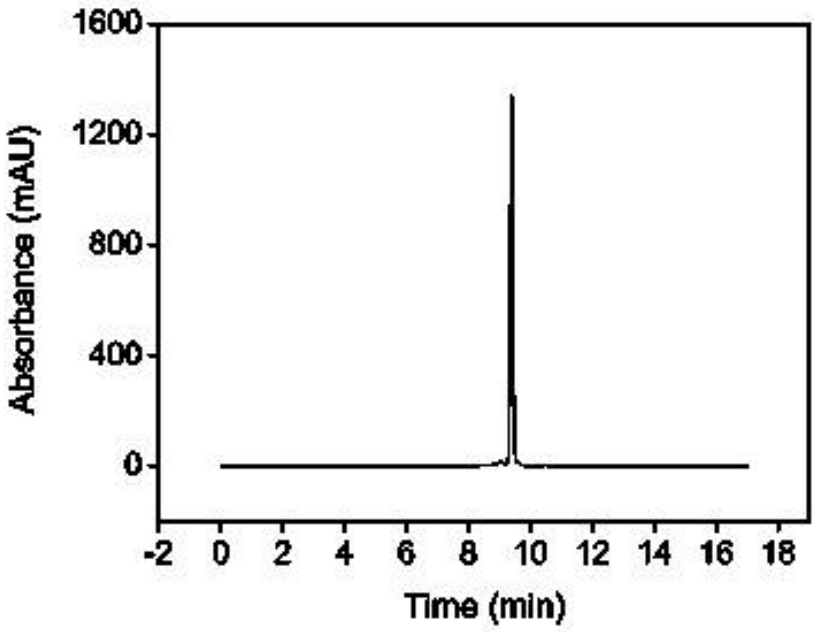


Figure 5-8 Representative HPLC chromatography of beta-carotene loaded emulsion.

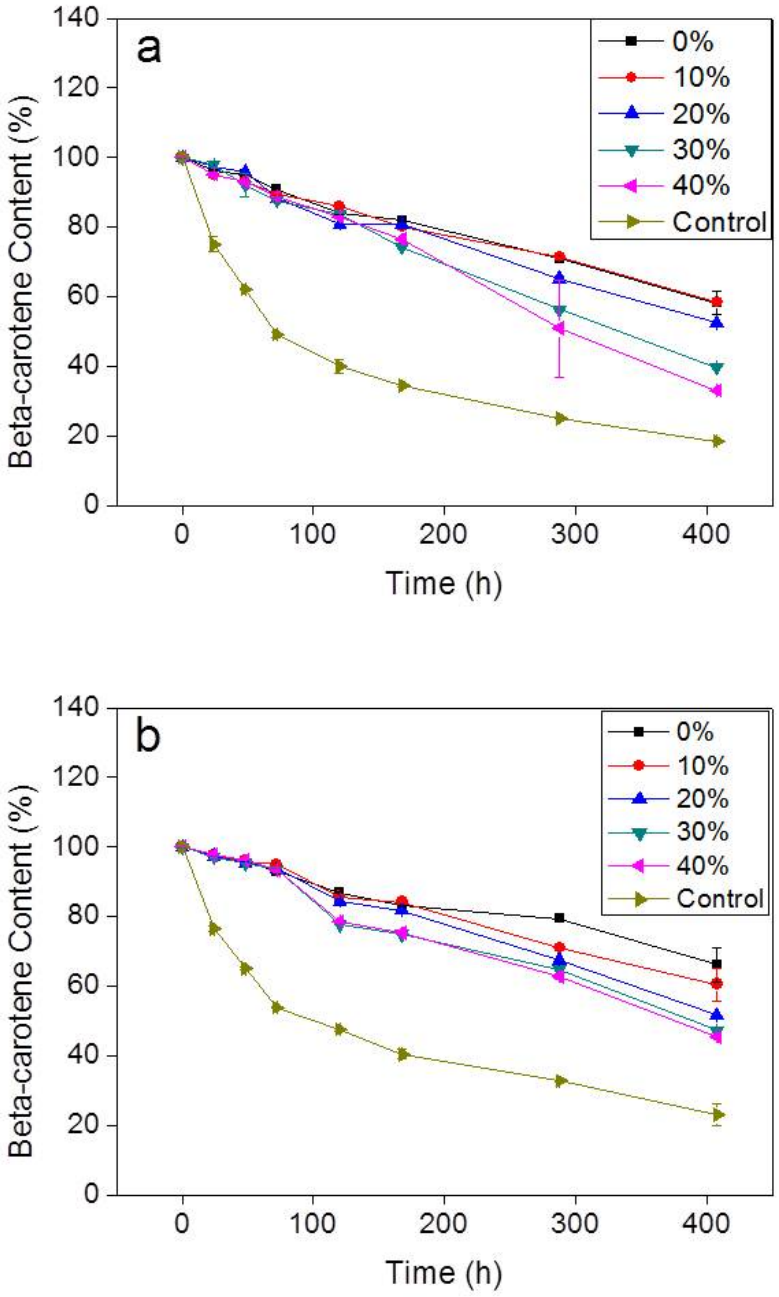


Figure 5-9 Stability of beta-carotene (A) under exposure to light and (B) in dark in SLN with various MCT oil contents during storage (each emulsion sample contains 10 wt% lipid phase, 5 wt% lecithin and 85 wt% water).

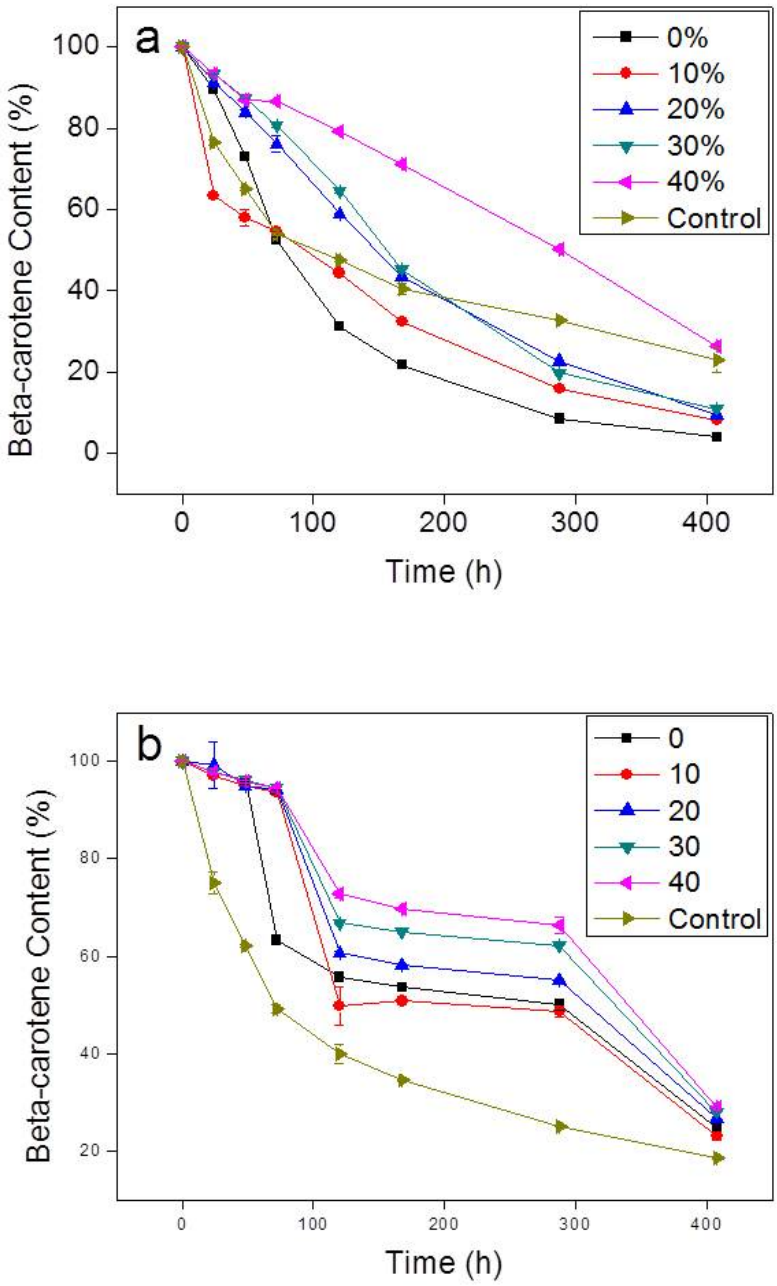


Figure 5-10 Stability of beta-carotene (A) under exposure to light and (B) in dark in lyophilized SLN powders with various MCT oil contents reconstituted in water during storage (each sample contains 5.0 wt% lipid, 15 wt% sucrose and 2.5 wt% lecithin).

In summary, nanoemulsions using a combination of both solid lipid (palm kernel fat) and liquid lipid (MCT oil) were prepared to encapsulate beta-carotene. Without lyophilization, the emulsions showed great effect to protect beta-carotene from degradation. Lyophilization promoted the crystallization of palm kernel fat and the inclusion of liquid MCT oil could disturb the crystallization to some extent by showing a melting point depression phenomenon. The incorporation of liquid MCT oil could increase the entrapment efficiency of beta-carotene; however, this increment effect is limited and could accelerate beta-carotene degradation in the aqueous SLN dispersions. The aqueous SLN dispersions showed excellent stability during storage and the reconstituted freeze-dried powders also showed good freeze-thaw stability within 5 freeze-thaw cycles.

5.2 In vitro digestion of beta-carotene

The in *vitro* digestion experiments were conducted for 4 groups of beta-carotene loaded samples, which were: (1) SLN formulated by palm kernel fat; (2) nanoemulsion formulated by MCT; (3) beta-carotene dissolved in palm kernel fat; and (4) beta-carotene dissolved in MCT. The purposes of this study were first to examine the effects of in *vitro* digestion (the combination of simulated gastric juice and then small intestine juice digestion) process on oil-in-water nanoemulsions in comparison to bulk lipids with dissolved beta-carotene; and second to compare the

bioaccessibility of beta-carotene in different formulations as described above. This investigation may provide a background for understanding the parameters that influence the digestibility and the influence of long chain (palm kernel fat) and medium chain lipid (MCT) based nanoemulsion formulations on the bioaccessibility of the encapsulated lipophilic compound (*i.e.* beta-carotene in this study).

It has been reported that carotenoids (including beta-carotene) intake with dietary fat is absorbed far more readily than carotenoids from foods without fat (Castenmiller and West 1998; Williams and others 1998). Borel reviewed the factors affecting intestinal absorption of highly lipophilic food nutrients including beta-carotene, and all the evidences show that lipid phase is a necessary condition for the digestion and utilization of beta-carotene for human bodies (Borel 2003). Schubert and Ax et al. reviewed the use of various colloidal systems, such as oil in water emulsions and dispersions, to improve the carotenoids bioavailability; they concluded that oil-in-water emulsions "combine the advantages of the good bioavailability of an oily carotenoid solution and applicability as water-dispersible system" (Schubert et al. 2003).

Although the most accurate way to evaluate the efficacy of various drug and food ingredients encapsulation and delivery systems in human gastrointestinal tract (GIT) is animal study and human feeding study, there are many limitations which make it unrealistic, such as ethical issues and economic problems. Therefore various in *vitro* digestion models have been developed which create a more cost-effective,

time-effective and convenient way to examine the digestibility of various lipids, drugs and food ingredients (Kaukonen and others 2004; White and others 2009; Bonnaire and others 2008). It is difficult to accurately simulate the real digestion process of human beings, since large variations exist of the digestion fluids properties (such as composition and pH) between different individuals and a schematic diagram of the physicochemical conditions in the different regions of the human GIT is shown in Figure 5-11.

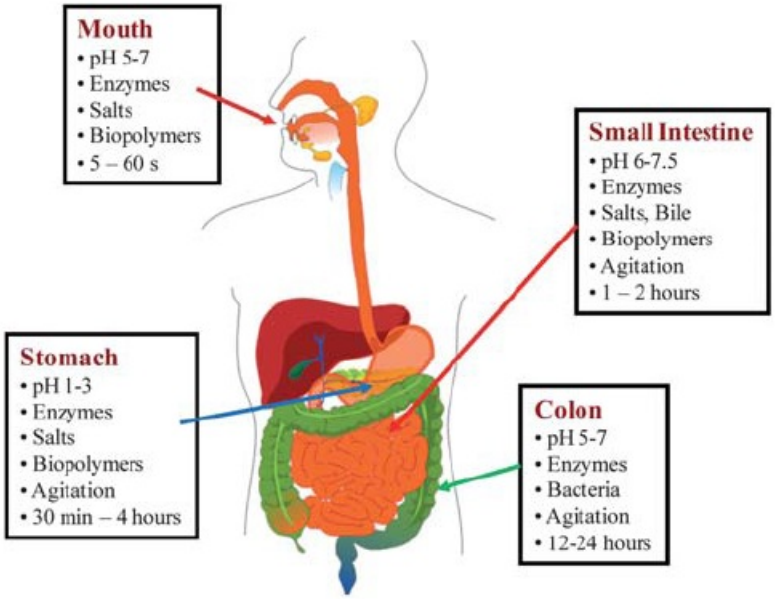


Figure 5-11 Schematic diagram of the physicochemical conditions in the different regions of the human GIT. (McClements and Li 2010a)

In this study, two steps were undertaken to simulate the digestion process: simulated stomach digestion at pH 2.0 for 1h and simulated small intestine digestion at pH 7.5 for 2h. The final products were analyzed after the simulated small intestine digestion process, because it was already proved that the absorption of beta-carotene by human

bodies is through the lymphatic route (Goodman and others 1966) in small intestine. Most of the lipid digestion and absorption processes happen in small intestine: during digestion, the lipid molecules are degraded by the enzymes (*i.e.* lipase) to form other small molecules, such as free fatty acids, monoglycerides and diglycerides (McClements and Li 2010a). With the release of free fatty acids, the pH of the simulated small intestine juice could decrease therefore alkali (NaOH) must be added to maintain the pH value (*i.e.* 7.5 in this study). The titration curves of NaOH addition versus digestion time results for different formulations are shown in Figure 5-12 and the extents of digestion for different samples are shown in Figure 5-13. From the results it can be seen that the MCT emulsion consumed more NaOH than the SLN, which means more free acids were released during the MCT emulsion digestion. Different lipid compositions led to this phenomenon. For the lipolysis of triglyceride, a two-step reaction takes place: at first, the ester bond in position 1 is cleaved to release one diglyceride and one free fatty acid molecule; and then the further degradation of diglyceride produces one more free fatty acid molecule. The weights of the lipids in all the formulations were calculated to be the same in all the experiments so the MCT formulation could release more free fatty acids due to its lower average molecular weight (503 g/mol) than the SLN formulation (the average molecular weight of palm kernel fat is 765 g/mol); consequently, more NaOH was needed to neutralize the fatty acids for the MCT formulation than for the SLN formulation. The MCT formulation also showed higher digestion extent than SLN

(Figure 5-13), which should also be attributed to different triglyceride compositions of different lipids. For example, the MCT oil used in this research was composed of caprylic/capric triglyceride while palm kernel fat was composed of mainly lauric and myristic triglyceride. Therefore different digestion products (free fatty acids and monoglycerols) could be produced due to the different lipids compositions (long chain fatty acids and long chain monoglycerols for palm kernel fat; while medium chain fatty acids and medium chain monoglycerols for MCT). Since long chain fatty acids are more prone to accumulate at the emulsion droplets surface to inhibit the subsequent enzyme reactions, lower digestion extent should be expected for the palm kernel fat formulation (SLN) (Sek and others 2002).

In order to establish a better understanding of the effects of different formulations on different digestion profiles, the kinetic parameters Vm (the maximum production converstion) and Km (the product concentration at which the reaction rate is equal to half Vm) were obtained by fitting the titration curves during digestion and the results are listed in Table 5-1. The Km values reflect the digestion rates related to different formulations, and the order in this research is: MCT emulsion (6.78) > SLN (18.3) > MCT oil (66.2) > palm kernel fat (156.8). The Vm values are also related to the enzyme reaction rates as well as the digestions extents and different samples showed same order as MCT emulsion > SLN > MCT oil > palm kernel fat. The reasons for faster digestion and the production of more fatty acids of the MCT emulsion than SLN have already been discussed above; and if compared between the same lipids

formulations (SLN *versus* palm kernel fat and MCT emulsion *versus* MCT oil), the digestion took place much faster and more fatty acids could be released for the emulsions than the lipids only (SLN > palm kernel fat and MCT emulsion > MCT oil). It has been suggested that the lipid composition of a colloidal system has a major impact on lipid digestion and release of the encapsulated active compound. For example, MCT was proved to have faster digestion rate and higher digestion extent than long chain triglyceride (LCT) (McClements and Li 2010b; Porter and others 2007). The free fatty acids and other molecules released during lipid digestion tend to accumulate at the lipid-water interface, therefore the presence of these molecules inhibit further enzyme reactions until they are dispersed and solubilized in the aqueous phase of the digestion fluid by bile-salt micelles (Porter et al. 2007). Porter et al. reviewed several in *vivo* as well as in *vitro* studies on how lipids and lipid-based formulations can be used to improve oral delivery of lipophilic drugs and they suggested that further studies are needed to examine how the factors, such as the lipid composition and phase changes during the digestion of lipid-based formulations, affect the release and absorption of the lipophilic drug (Porter et al. 2007). The results suggested that the colloidal emulsion systems with much larger interfacial areas could result in more thorough digestion processes than the lipids only.

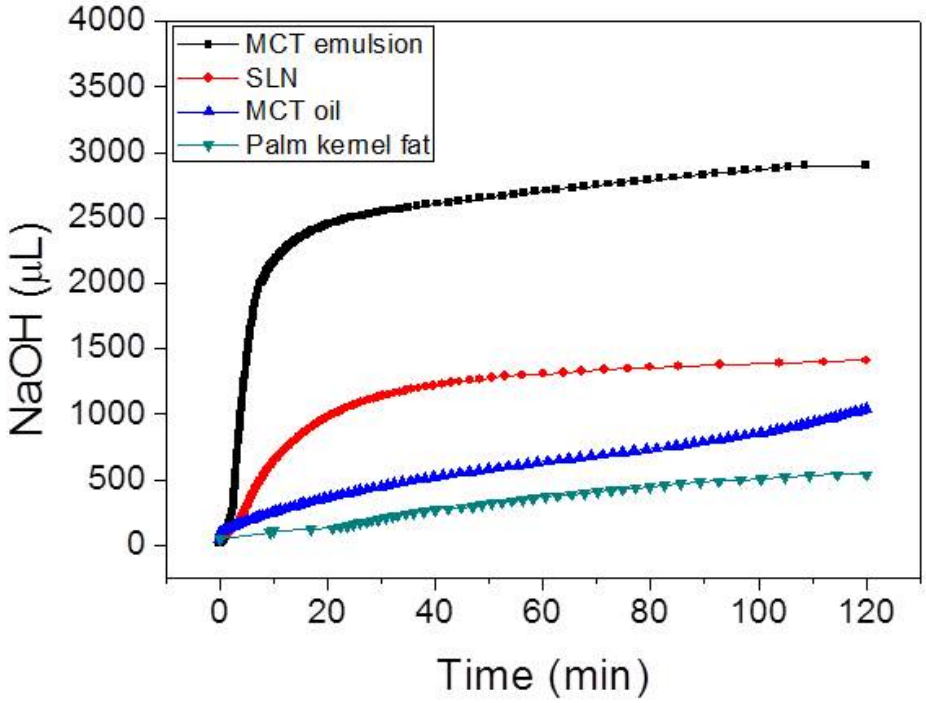


Figure 5-12 Representative titration curves (volume of NaOH added to maintain the pH of the simulated small intestine juice at 7.5) for different formulations during a 2h small intestine digestion process.

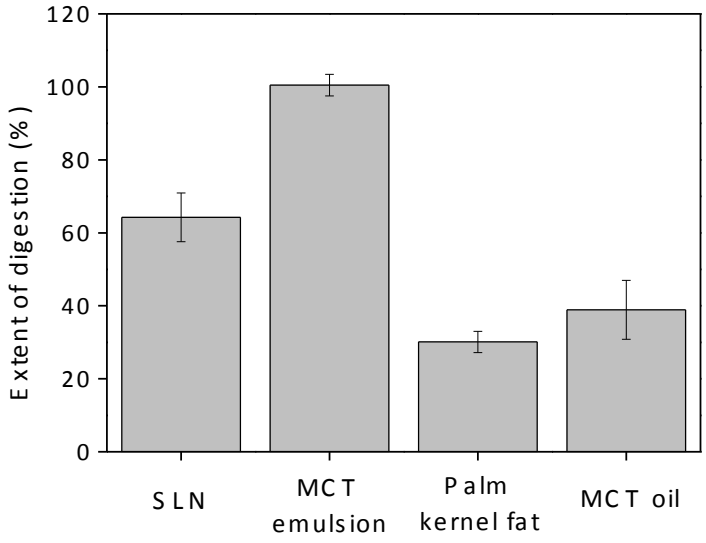


Figure 5-13 The extent of digestion for nanoemulsions formulated by using palm kernel fat and MCT; and also for palm kernel fat and MCT oil only.

Table 5-1 The kinetic parameters (Vm and Km) of various formulations during the in *vitro* digestion experiments.

	Vm (mmol mL^{-1})	Km (mmol mL^{-1} min^{-1})
MCT emulsion	0.635 ± 0.0116	6.78 ± 0.417
MCT oil	0.289 ± 0.0107	66.2 ± 5.20
SLN	0.351 ± 0.00519	18.3 ± 0.619
Palm kernel fat	0.261 ± 0.0125	156.8 ± 10.9

After the digestion process, the released beta-carotene and the compounds produced from lipids degradation such as free fatty acids were solubilized in the bile-salt micelles followed by absorption (Staggers and others 1990; Hernell and others 1990). Therefore the digested samples were ultracentrifuged at 4^{o}C and 40,000 rpm (113,613 g) to get the micellar fraction and then HPLC was used to quantify the beta-carotene concentrations. The bioaccessibility (% transfer of beta-carotene from the digested samples to the micellar fractions) was calculated and the results were shown in Figure 5-14. The term "bioaccessibility" represents the amount of the food ingredient released from the food matrix, such as emulsions, that is available for absorption (White et al. 2009). For the in *vitro* digestion studies, the active compounds that are released from the samples to the aqueous micellar fractions are considered bioaccessible for the subsequent small intestine absorption (White et al. 2009; Acosta 2009). In SLN, 95.3 ± 4.5% beta-carotene was transferred to the digestion fluids and this number for the MCT emulsion was 80.3 ± 2.5%; both lipids with dissolved beta-carotene showed poor efficacies for beta-carotene micellization: 1.8 ± 0.17% for palm kernel fat and 4.1 ± 0.14% for MCT oil, respectively. The results suggested that the bioaccessibility of beta-carotene in colloidal emulsions (both SLN and MCT

emulsions in this study) is significantly higher than in bulk lipids (both palm kernel fat and MCT oil in this study).

The reason that more encapsulated beta-carotene can be released from the colloidal formulations than from the bulk lipids is that the colloidal systems have much larger surface area than the bulk lipids. Lipid digestion is an interfacial phenomenon when surface active bile salts and various enzymes adsorb to the lipid-water interface. Lipases then start hydrolyze the lipid molecules and bile salts help stabilize the produced free fatty acids and other molecules by forming micelles. For the bulk lipids digestion experiments, the interfacial area is determined by the experimental condition, such as the beaker used. For the nanoemulsions, the water dispersible emulsion droplets have sufficient opportunity to react with the enzymes and bile salts in the water phase, therefore the digestion efficacy is much higher for emulsions than for the bulk lipids. McClements and Li reviewed the digestion and release of lipophilic food components in emulsion-based delivery systems and summarized the influence of surface area on the digestion rate (McClements and Li 2010b). White and Fisk et al. discovered similar phenomenon when to assess the bioaccessibility of tocopherol and fatty acids in sunflower seed oil bodies and oil emulsions stabilized by Tween 20; in their experiments, the mean bioaccessibility of tocopherol and fatty acids was significantly increased from 0.6 and 8.4% to 35 and 52%, respectively, by using the emulsion systems (White et al. 2009).

Another phenomenon that can be observed from the current results is that the

beta-carotene bioaccessibility in the SLN (95.3 ± 4.5%) is better than in the MCT emulsion (80.3 ± 2.5%). Although the MCT emulsion has been proved to have higher digestion extent than SLN, the released beta-carotene that can be stabilized in the micellar fraction was lower than SLN. The most possible reason is that more molecules were produced from the MCT emulsion (such as fatty acids and monoglycerols as discussed above) than from the SLN, so the amount of bile salts in the aqueous digestion buffer might be insufficient to stabilize all the enzyme reaction products, including beta-carotene. The bioaccessibility results in this study showed that palm kernel fat may be better to improve the release of beta-carotene probably due to the formation of less fatty acids as already proved in the NaOH titration results discussed above. However, the data is not sufficient enough to prove the advantage of using palm kernel fat over MCT; and further in-depth study is needed to evaluate the influence of lipid composition on the in *vitro* digestion profiles, such as the aqueous phase (micellar) composition and different physical phases formed during digestion.

In summary, the in *vitro* digestion behavior of two lipids and their corresponding nanoemulsions (MCT oil, MCT nanoemulsion, palm kernel fat and palm kernel fat nanoemulsion) were assessed and the micellization efficacies of beta-carotene in the 4 formulations were also determined by HPLC. The results suggested that for food ingredients and/or drugs with poor solubility in both water and lipid, nanoemulsions may be beneficial to improve their solubilization capacity and bioaccessibility. Furthermore, palm kernel fat nanoemulsion showed better capacity to improve the

beta-carotene bioaccessibility than the MCT nanoemulsion. This study demonstrates the potential utility of using the cost-effective and time-effective in *vitro* digestion model to evaluate the performance of lipids and lipid-based nanoemulsion formulations of lipophilic drugs and food ingredients, such as oil-soluble vitamins and carotenoids. The results may provide useful information for developing encapsulation and delivery systems with desired controlled release profile. And further in-depth study is needed to obtain more detailed information about various parameters that influence the digestion and release behavior of those systems.

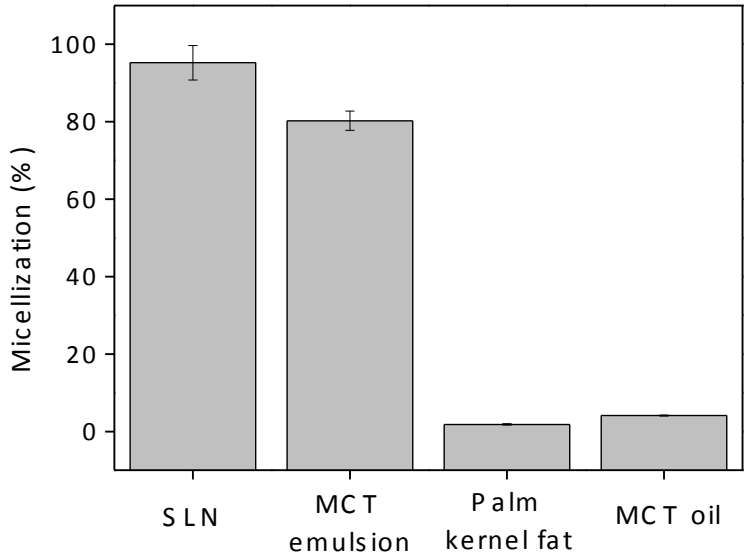


Figure 5-14 Bioaccessibility (shown as % transfer of beta-carotene from the digested samples to the micellar fractions) in different samples.

5.3 Encapsulation of citral

5.3.1 Incorporation of antioxidants

In this section, the effects of six different antioxidants on citral stability under acidic condition (pH = 3.0) were evaluated by GC and GC-MS. The antioxidants used were:

(1) Tanshinone II-A (structure shown in Figure 5-15). Tanshinone II-A is a derivative of phenanthrenequinone with a molecular formula of $C_{19}H_{18}O_3$. It is extracted from a plant Tanshen (*Salvia miltiorrhiza* Bunge), which has been used in traditional Chinese medicine due to its antioxidant properties to scavenge free radicals (Weng and Gordon 2002).

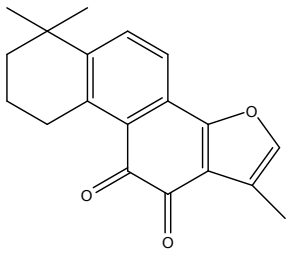
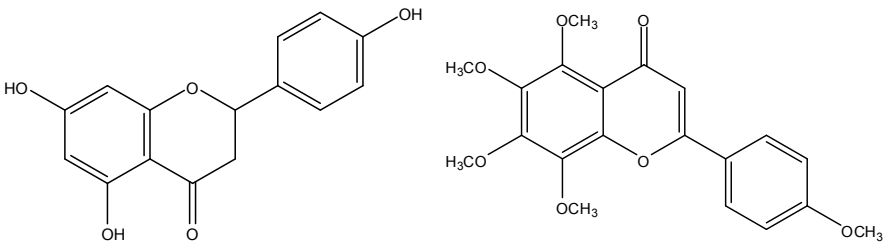


Figure 5-15 Structure of tanshinone II-A.

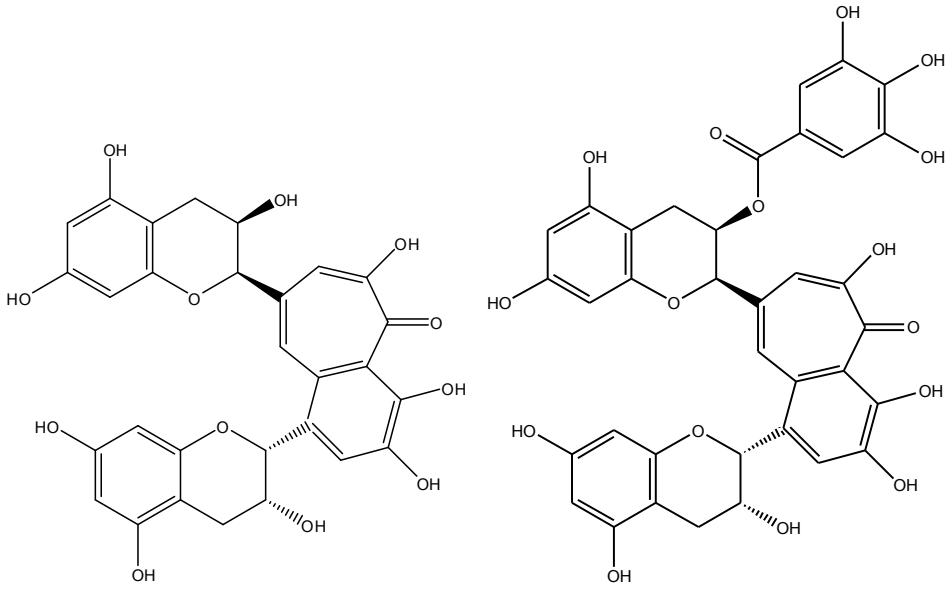
(2) & (3) Naringenin and tangeretin. Both naringenin and tangeretin are polyphenolic flavonoids with the diphenylpropane ($C_6C_3C_6$) skeleton (structures are shown in Figure 5-16). They are found in citrus and grapefruits and known to have a bioactive effect on human health as antioxidants (Saija and others 1995; Zbarsky and others 2005; Yi and others 2008).



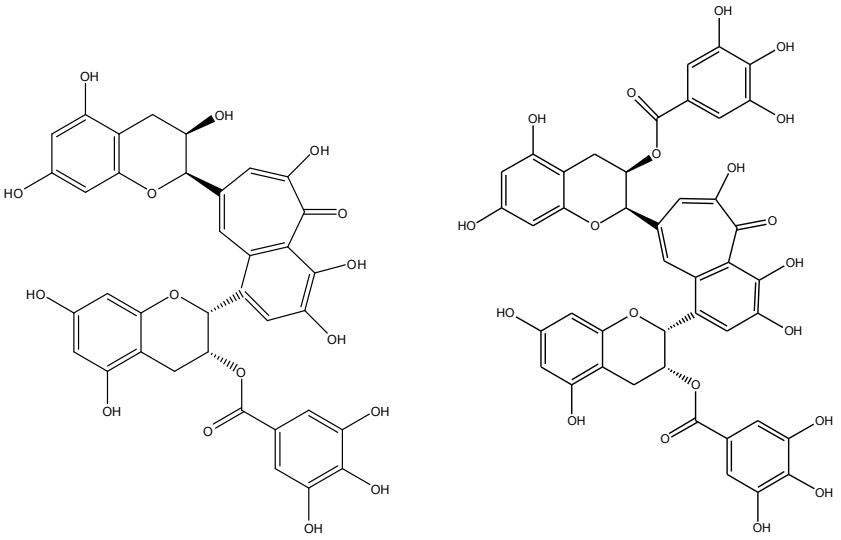
(A) (B)

Figure 5-16 Structures of (A)naringenin and (B) tangeretin.

(4) Black tea extract. Black tea consists of the fermented leaves of *Camellia sinensis*, family Theaceae. The fermentation process leads to the formation of oligomers such as theaflavin and its derivatives (Lee and others 2004) (Figure 5-17). Black tea has many health benefits for human body due to its antioxidant and anti-inflammatory properties (Zaineb and Suham 2002).



(A) theaflavin (B) theaflavin 3-gallate



(C) theaflavin 3'-gallate (D) theaflavin-digallate

Figure 5-17 Structures of theaflavin and its derivatives in black tea extract.

(5) Ascorbic acid. Ascorbic acid (also known as vitamin C, structure shown in Figure 5-18) is a water soluble antioxidant. A review paper (Arrigoni and De Tullio 2002) summarizes its functionalities, especially its capability to scavenge free radicals and other reactive oxygen species.

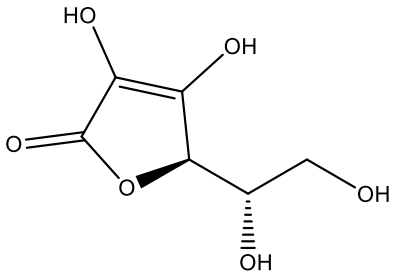


Figure 5-18 Structure of ascorbic acid.

(6) Beta-carotene. Refer to section 1.2 for detailed information of beta-carotene.

Physical stability of citral loaded emulsions with and without antioxidants. Particle sizes of the citral loaded emulsions (with and without antioxidants) were

measured at day-0, day-30 and day-60 during storage under 25^{o}C and 50^{o}C, respectively. The representative PCS Cumulant analysis and single stretched exponential fit results are shown in Figure 5-19a and Figure 5-19b, respectively. Comparable particle sizes could be obtained from both data analysis methods (difference < 10%), therefore particle sizes calculated from the Cumulant method were used for different emulsion formulations during storage as shown in Figure 5-19c and Figure 5-19d, respectively. Freshly made emulsions had the particle size between 109 to 129 nm at day-0, while the samples without antioxidant had the smallest size and the incorporation of different antioxidants increased the particle size. Due to the density difference between the lipid phase and the aqueous medium, lipid particles showed the tendency to grow due to creaming or sedimentation (Sjöblom 2006). In this study, the particle sizes increased slightly during storage. The particle size for the emulsions stored at 25^{o}C showed an increment between 10 to 20 nm; and since the particles moved faster at higher temperature, the size increment for samples stored at 50^{o}C was ~70 – 80 nm. No obvious phase separation or creaming was observed for all the emulsion samples even at high storage temperature (50^{o}C). Therefore, the results proved that very small particles possessed kinetic stability (Tadros et al. 2004) especially at relatively lower temperature (25^{o}C in this study) and the emulsion formulations in this study showed excellent physical stability to avoid creaming.

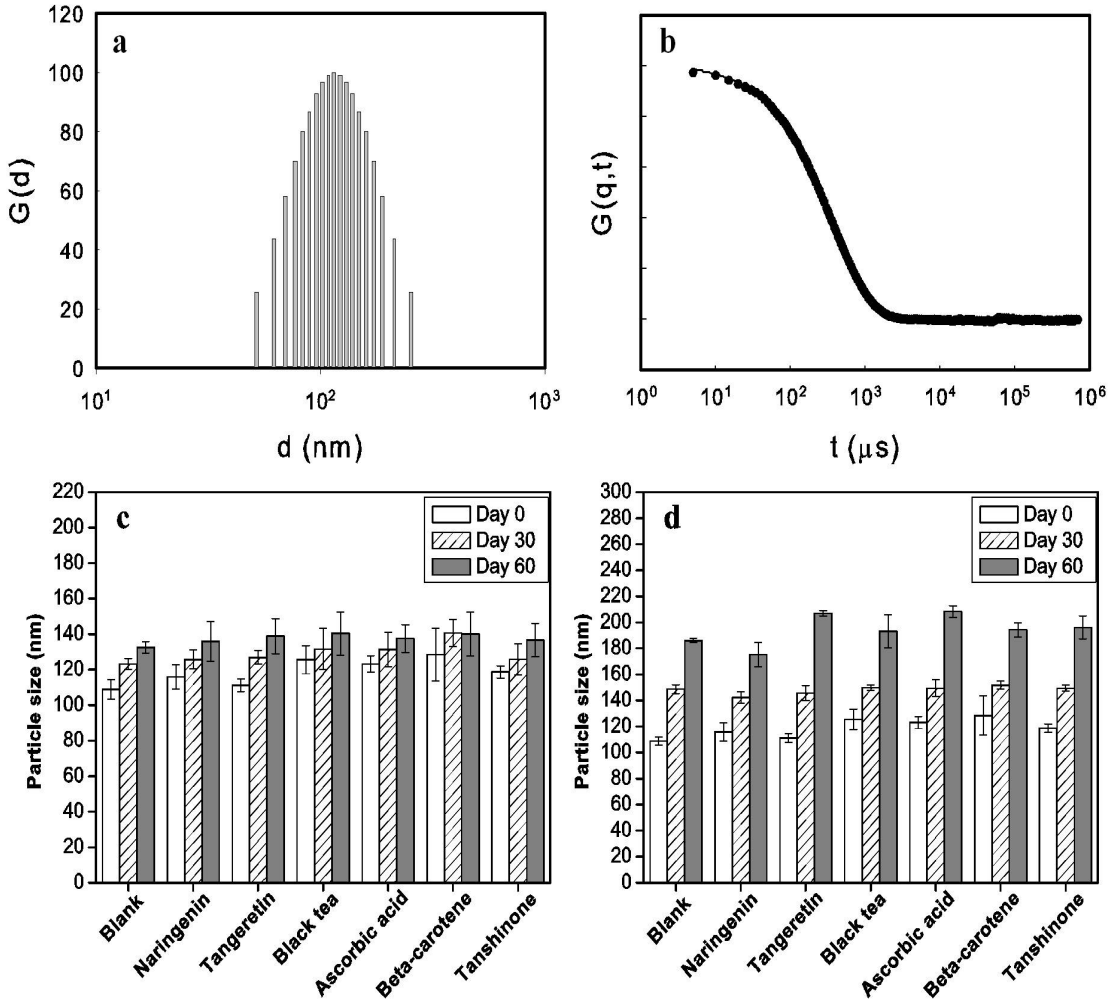


Figure 5-19 Representative photon correlation spectroscopy (PCS) resutls of emulsions analyzed by (a) Cumulant analysis and (b) single stretched exponential fit method; as well as the mean particle diameter changes for citral loaded emulsions with and without antioxidants stored under (a) 25^{o}C and (b) 50^{o}C. Data represent means ± standard deviations (n = 3). (each emulsion sample contains 10 wt% lipid phase, 5 wt% lecithin and 85 wt% pH 3.0 citric acid buffer).

Stability of Citral in emulsions with and without antioxidants. The stability of citral was evaluated by calculating the loss of both its two isomers (neral and geranial) during storage at different temperatures. Both neral and geranial showed similar degradation trends at 25^{o}C and 50^{o}C, respectively (Figure 5-20 and Figure 5-21). At 25^{o}C, 33.5% neral and 32.1% geranial were left in the emulsion without antioxidant after 28 days; while 0.225% neral and 0.235% geranial were left in the same formulation at 50^{o}C after 28 days. It can be clearly seen that at low temperature the incorporation of certain antioxidants could slow down citral (both neral and geranial) degradation. Compare to the blank sample (emulsion without antioxidant), almost two times of citral was left in the formulations with beta-carotene and tanshinone, respectively. For instance, citral degradation was not observed for the formulation with beta-carotene for the first week; there were still 95.5% neral and 94.6% geranial left after two weeks; and 75.4% neral and 74.0% geranial remained in the sample at the end of the measurement (four weeks). As for the emulsion with tanshinone stored at 25^{o}C, 65.2% neral and 58.7% geranial remained in the sample at the end of the measurement (four weeks). Several previous studies also evaluated the capabilities of various emulsions to inhibit citral degradation, for example, Choi and Decker et al. (Choi and others 2010) discovered the dependence of citral's degradation rates on different surfactant types and Djordjevic and Cercaci et al. (Djordjevic and others 2008) proved the ability of whey protein isolate (WPI) as the emulsifier to inhibit the oxidative deterioration of citral. Due to different test conditions, such as GC

measurement methods and storage temperatures and pH values of the samples, it is difficult to compare different works directly. The formulations used in this research may have better performance to inhibit citral's degradation compare to other's work. For example, at similar storage conditions (pH 3.0, 20^{o}C and 29 days) in the work of Mei et al. (Mei et al. 2009), more than 90% of citral degraded in the sodium dodecyl sulfate (SDS) stabilized liquid octadecane emulsion while more than 50% of citral degraded in the Brij 35 (polyoxyethylene lauryl ether) stabilized liquid octadecane. The performances of other four antioxidants narigenin, tangeretin, black tea extract and ascorbic acid were in between the blank sample (emulsion without antioxidant) and the emulsions with beta-carotene and tanshinone, although their effects to inhibit citral degradation were not as significant as beta-carotene and tanshinone. Furthermore, no obvious off-flavor products produced from citral, such as *p*-cresol and *α,p*-dimethylstyrene, were detected for all the formulations (both with and without antioxidants) stored under 25^{o}C for four weeks (data not shown).

The high temperature (50^{o}C) storage samples were also measured by GC to evaluate citral's stability as well as the generation of possible off-flavor compounds (Figure 5-21). As expected, citral degraded much faster for all the formulations stored at 50^{o}C than at 25^{o}C, therefore it is difficult to differentiate between various formulations. For example, about 50% of both neral and geranial were lost for all the samples only after 1 day and almost no citral was left in them after four weeks of storage at 50^{o}C. Since the purpose of the high temperature storage measurements was to evaluate the

production of off-flavor compounds from the citral loaded emulsions, the results will be shown in the next section.

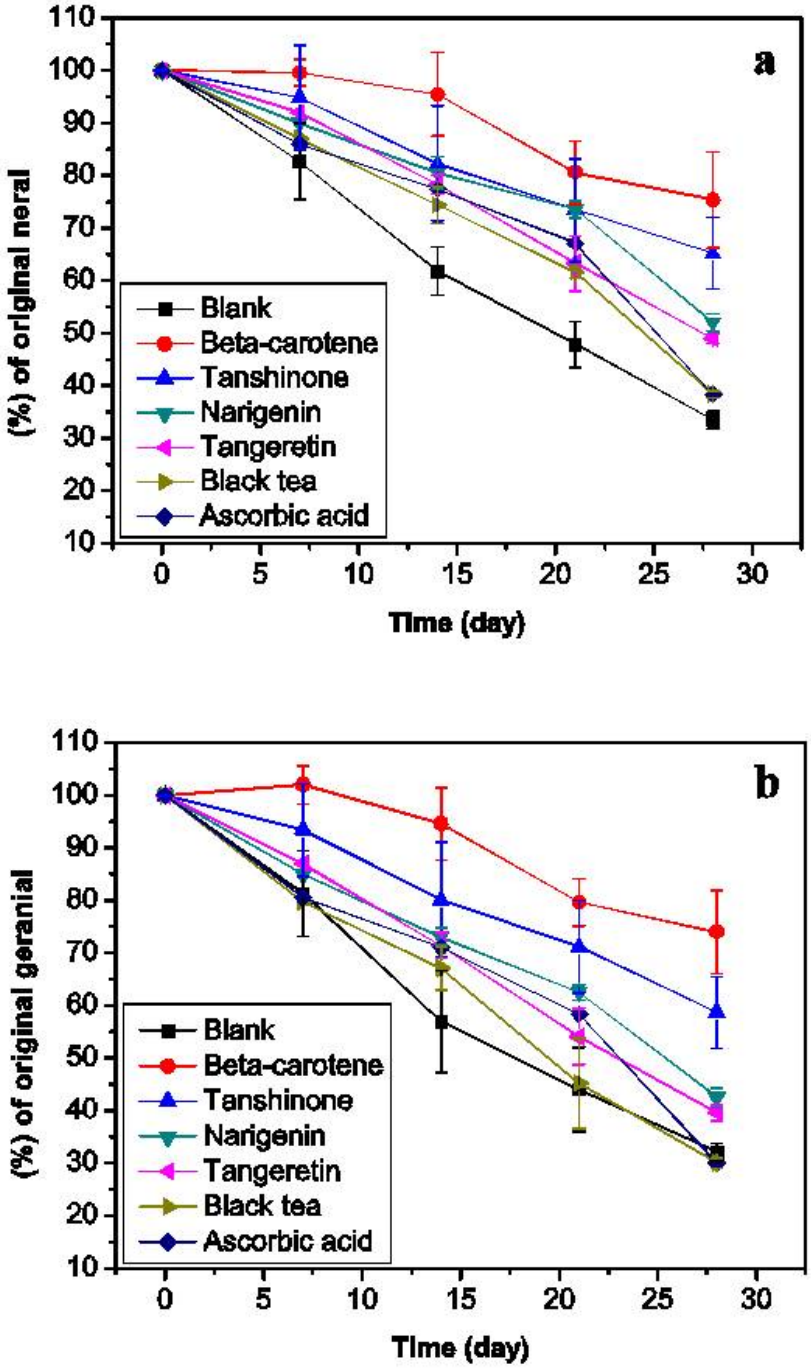


Figure 5-20 Degradation of (a) neral and (b) geranial in emulsions with and without different antioxidants stored at 25^{o}C (each emulsion sample contains 10 wt% lipid phase, 5 wt% lecithin and 85 wt% pH 3.0 citric acid buffer).

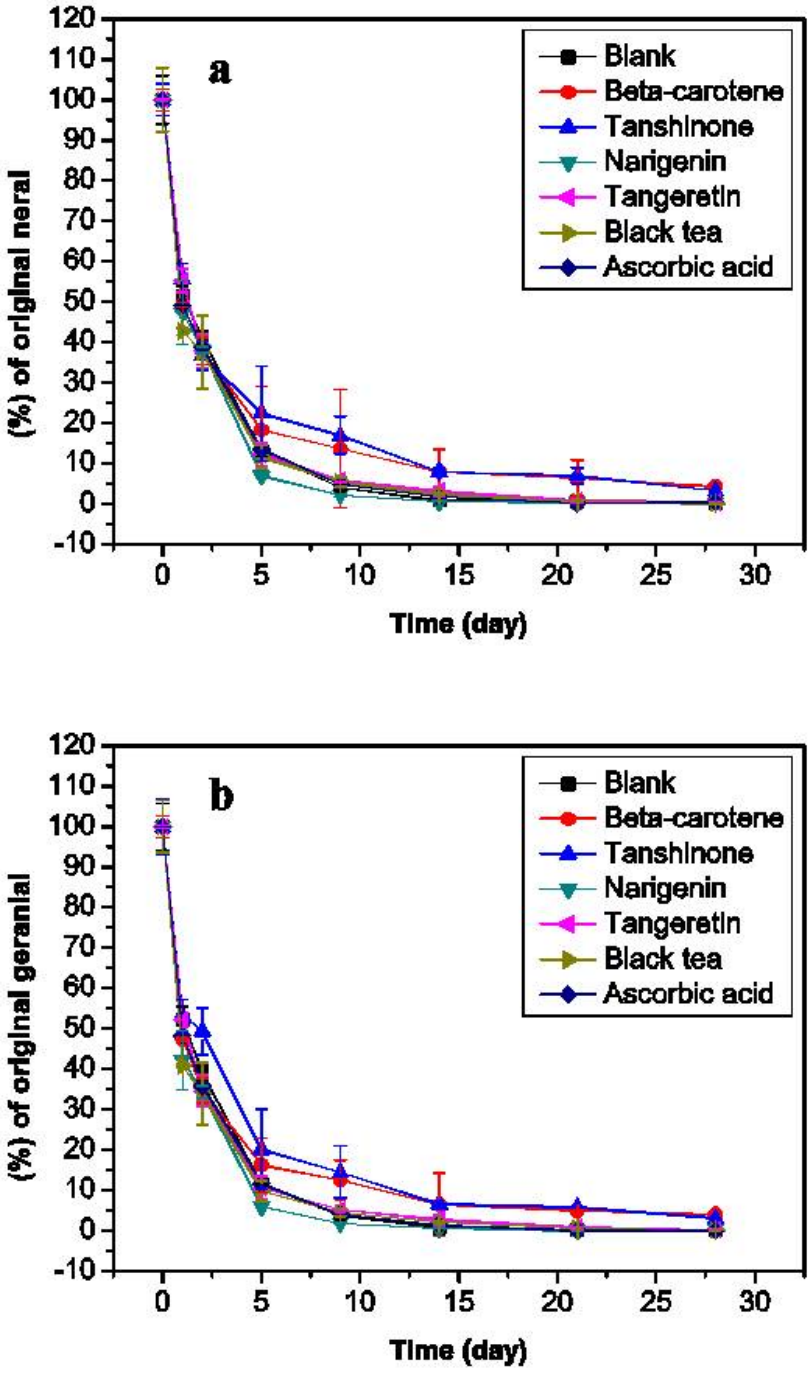


Figure 5-21 Degradation of (a) neral and (b) geranial in emulsions with and without different antioxidants stored at 50^{o}C (each emulsion sample contains 10 wt% lipid phase, 5 wt% lecithin and 85 wt% pH 3.0 citric acid buffer).

Evaluation of off-flavor compounds for citral loaded emulsions. During storage at 50^{o}C for four weeks, citral was completely degraded as shown in Figure 5-22 and Table 5-2. The major products generated from citral loaded emulsions could be divided into two groups. One was the commonly detected citral degradation products, such as *p*-cresol (peak 4), *α,p*-dimethylstyrene (peak 5), *p*-metha-1,5-dien-8-ol and *p*-methylacetophenone (peaks 7 and 8). The most significant point is that most of the citral degradation products (Liang et al. 2004; Kimura et al. 1983; Peacock and Kuneman 2002) cannot be detected in all the emulsion formulations, such as *p*-cymene, *p*-cymen-8-ol, 8-hydroperoxy-*p*-cymene and many monoterpene alcohols. Besides, among four of the detected compounds mentioned above, only *p*-metha-1,5-dien-8-ol is the acid-catalyzed reaction product and all the others are oxidation products (Ueno et al. 2006). Therefore the encapsulation of citral in emulsions effectively inhibited its degradation, especially the acid-catalyzed reactions. The reason is that citral could be isolated from the protons in the aqueous medium after encapsulation, which proves to be a useful way to reduce the acid-catalyzed reactions. The other group was the lipid degradation products, such as 2-heptanone, 1-octen-3-ol, and butanoic acid (peaks 1, 2 and 6) (Figure 5-22 and Table 5-2). The generation of these compounds is inevitable due to the presence of lipid and phospholipids (palm kernel fat and lecithin) in the emulsions. Although the study of lipid oxidation is beyond the scope of this research, the effects of antioxidants on the production of all the major emulsions degradation products will be discussed in this

section.

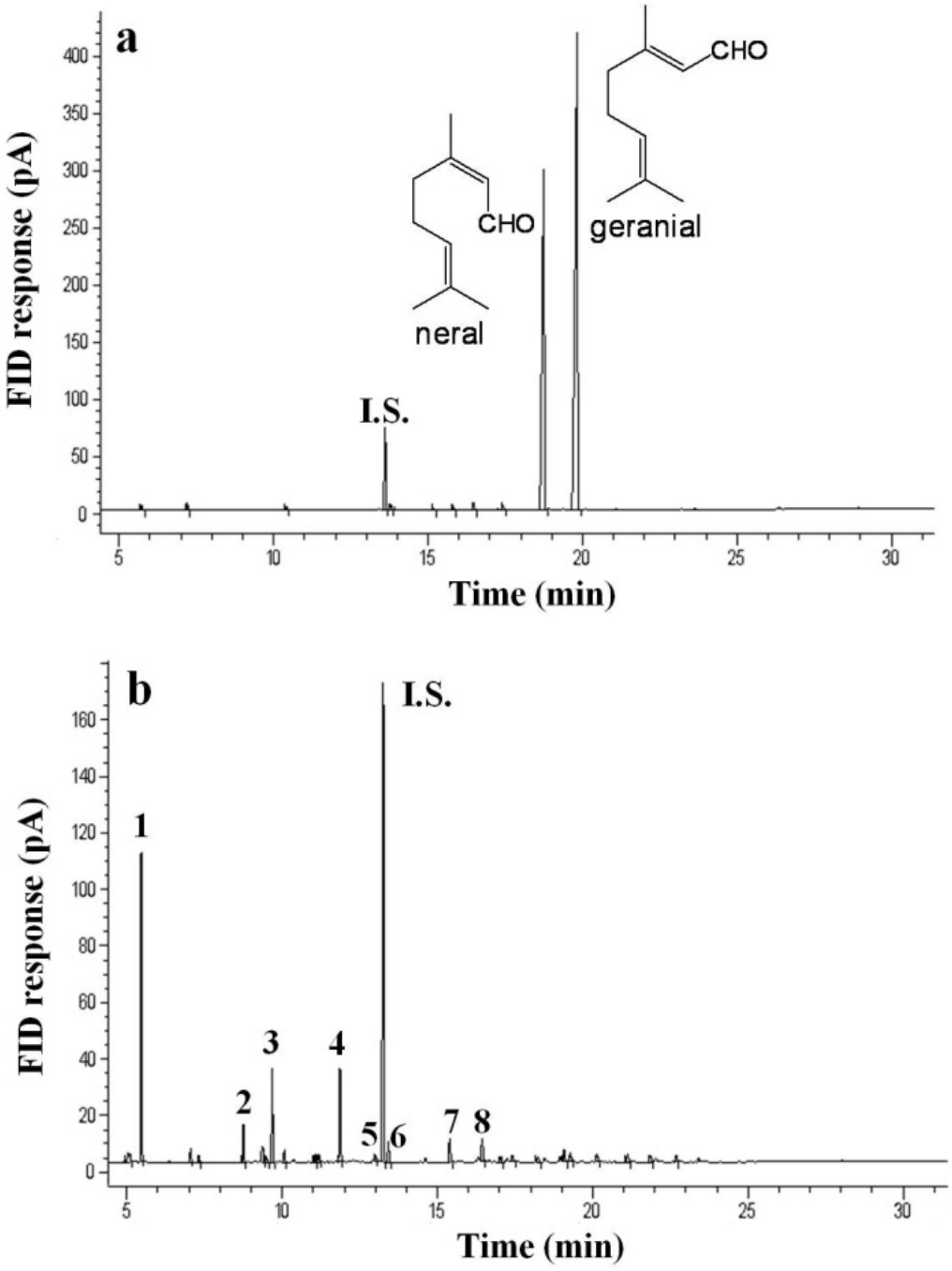


Figure 5-22 Representative gas chromatogram of encapsulated citral under acidic condition (pH 3.0) stored under 50^{o}C (a) at day-0 and (b) at day-28. Numbers correspond to those in Table 5-2. Undecanse was used as the internal standard (I.S.).

Table 5-2 Degradation products formed from citral loaded emulsions stored at 50°C for 28 days

Compound No.[a]	Compound	ID Method
1	2-Heptanone	A
2	1-Octen-3-ol	A
3	Delta-2-carene	A
4	*p*-Cresol	B
5	*α,p*-Dimethylstyrene	B
6	Butanoic acid	A
7	*p*-Metha-1,5-dien-8-ol	B
8	*p*-Methylacetophenone	B

[a] Numbers correspond to those in **Figure 4**.

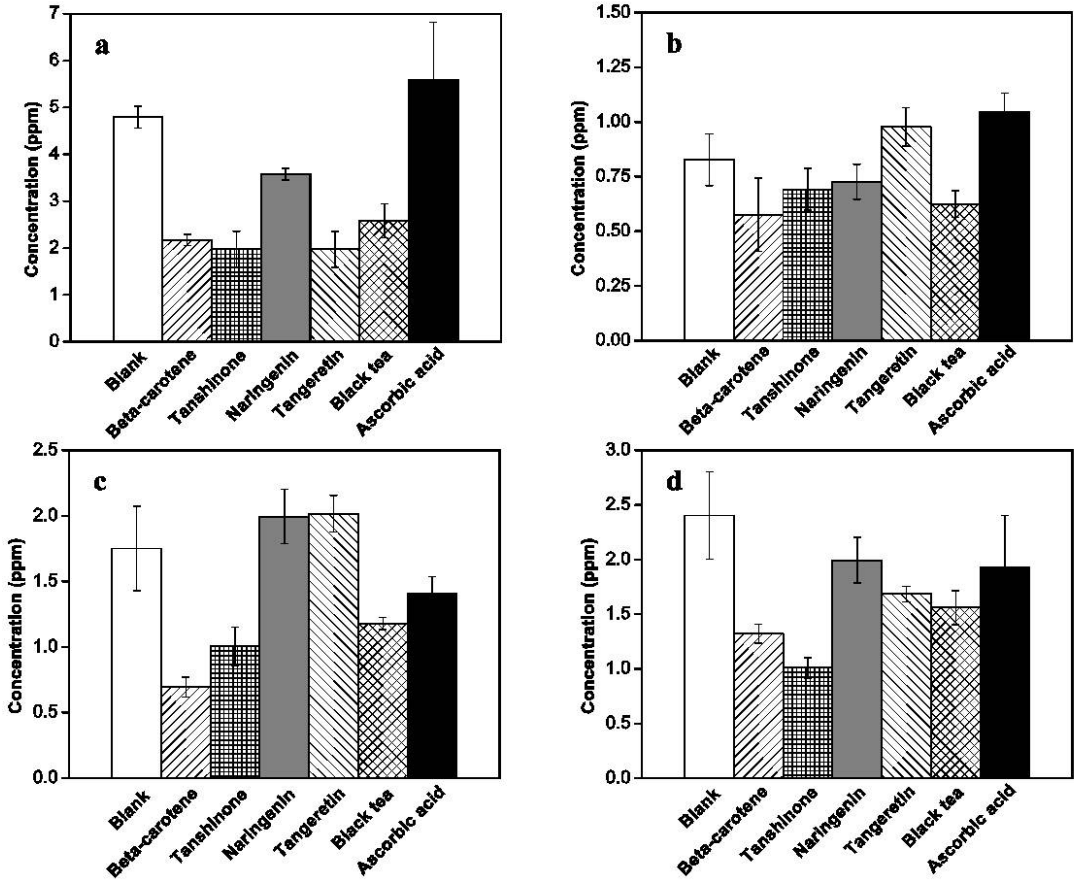
[b]Compounds were identified on the basis of the following criteria: A, mass spectrum agrees with that of Wiley mass spectral database and the compounds can only be considered as "tentatively identified"; B, mass spectrum and retention index agree with those of authentic compounds purchased from Sigma-Aldrich (St. Louis, MO, USA).

The effects of the six antioxidants on the formation of all the major degradation products showed different and complicated results (Figure 5-23). For example, beta-carotene inhibited the production of compounds **1**, **2**, **3**, **4** and **7** while had similar concentration levels of compounds **5**, **6** and **8**; tanshinone inhibited the production of compounds **1**, **3**, **4** and **7**, promoted the production of compounds **5** and **8**, while had similar concentration levels of compounds **2** and **6**; black tea extract inhibited the production of compounds **1**, **2**, **3**, **4**, **6** and **7** while promoted the production of compounds **5** and **8**; ascorbic acid was the worst among all the antioxidants due to its promotion effect on compounds **1**, **2**, **5**, **7**, and **8** while only slightly inhibited the production of compounds **3**, **4** and **6**. In general, beta-carotene, tanshinone and black tea extract did well to inhibit both citral and lipid degradation;

the performances of the two citrus flavonoids naringenin and tangeretin fluctuated with the production of different compounds; and although ascorbic acid is an excellent antioxidant with good metal chelating capability, it was the worst one to be used in the emulsion formulation since it promoted the production of most of the degradation products.

Previous studies (Liang et al. 2004; Ueno et al. 2006) also found that the use of antioxidants (phenolic plant extracts and pure catechins) could inhibit the generation of several citral off-odor compounds, such as *p*-methylacetophenone and *p*-cresol; however, the production of monoterpene alcohols, *p*-cymen-8-ol and *α,p*-dimethylstyrene were greatly induced. Although details of citral degradation and how the antioxidants work are still not fully understood, it is generally accepted that there are free radicals produced during citral degradation as well as lipid oxidation especially at high temperature. For example, it was suggested (Ueno et al. 2004) that *p*-mentha-1,5-dien-8-ol produced from citral could undergo dehydration and subsequent isomerization to form an intermediate compound *p*-mentha-1,4(8),5-triene, which soon resulted in the generation of peroxy radical to facilitate the production of other oxidation products, such as *p*-methylacetophenone and *p*-cresol (Figure 5-24). Lipid oxidation has been widely studied for many years and it is known that the generation of highly reactive peroxyl, alkoxyl radicals and other pro-oxidants are responsible for lipid oxidation in emulsion systems (McClements and Decker 2000). It has been widely reported that the addition of antioxidants into emulsions could

retard lipid oxidation through inactivating the free radicals, scavenging oxygen and other oxidative molecules (Decker 1998; Reische and others 1998).



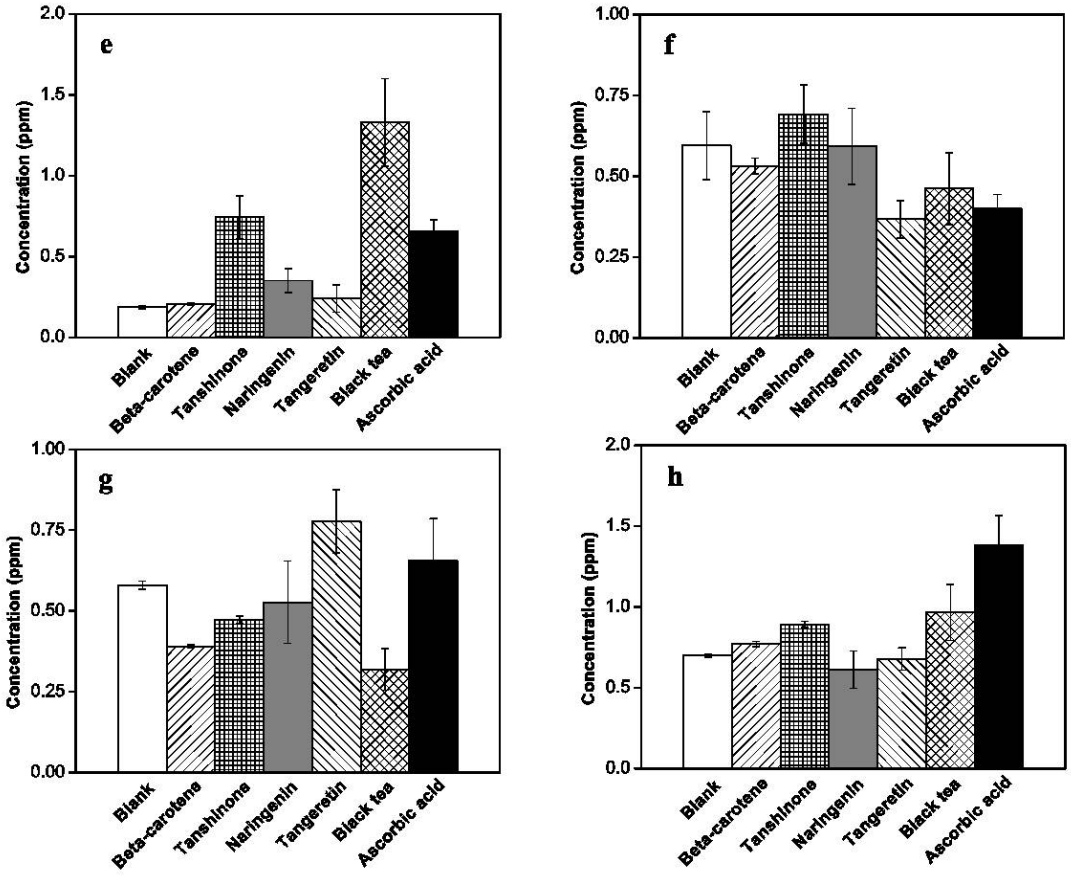


Figure 5-23 Concentrations for all the major degradation compounds from citral loaded emulsions (each emulsion sample contains 10 wt% lipid phase, 5 wt% lecithin and 85 wt% pH 3.0 citric acid buffer) stored at 50^{o}C for 4 weeks: (a) 2-heptanone; (b) 1-octen-3-ol; (c) delta-2-carene; (d) *p*-cresol; (e) *α,p*-dimethylstyrene; (f) butanoic acid; (g) *p*-metha-1,5-dien-8-ol and (h) *p*-methylacetophenone.

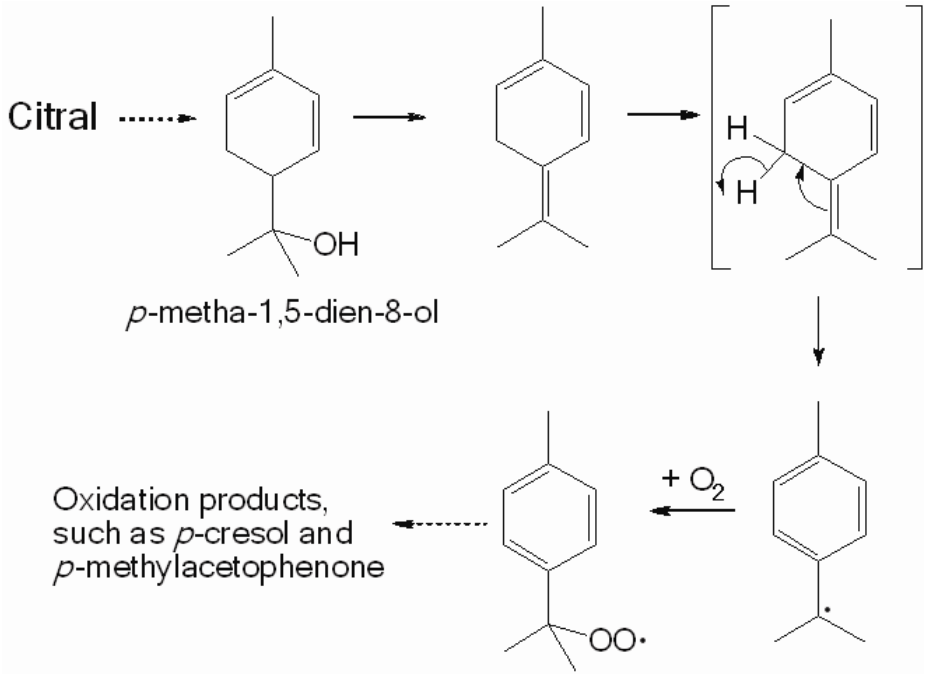


Figure 5-24 Previously proposed free radical and oxidation products formed from citral (Ueno et al. 2004).

In this study, beta-carotene and tanshinone had better performance than the others probably due to their extremely non-polar characteristics, because it has been stated that non-polar lipophilic antioxidants are more effective in emulsions than the polar hydrophilic antioxidants (McClements and Decker 2000). Ascorbic acid was the worst antioxidant in this study due to many possibilities: first, it is the most polar compound with very high water solubility among the six antioxidants; when both citral and lipid oxidation happened inside or in the interfacial region of the emulsion droplet, it is within expectation that water soluble antioxidant is much less effective to inhibit the degradation reactions. Second, the hydroxyl group in ascorbic acid behaves like an acid which may further promote the production of *p*-menthadien-8-ols and *p*-mentha-1,4(8),5-triene and the followed dehydration and/or oxidation reactions. Third, ascorbate anion radical may have lower reduction potential than the other

antioxidants (Williams and Yandell 1982). Black tea extract is rich in various theaflavins and besides its inhibition effect on most of the citral degradation products, it greatly promoted the production of *α,p*-dimethylstyrene, which agreed well with previous study (Ueno et al. 2006); it was reported that theaflavins could produce a quinine compound (theanaphthoquinone), which might be involved in the dehydrogenation of *p*-mentha-1,4(8),5-triene. The two citrus flavonoids naringenin and tangeretin were not good enough to protect either citral or lipid compare to beta-carotene, tanshinone and black tea extract (Figure 5-23). Although they are also phenolic compounds like theaflavins, they have much simpler structures with less substitute moieties. Very few research has been done to reveal the antioxidant activities of both naringenin and tangeretin related with citral or lipid oxidation, therefore further study is needed to investigate the mechanisms of how the flavonoids work to influence the flavor and/or lipid degradation pathways.

In summary, emulsions were used to encapsulate citral and the effects of six different antioxidants (beta-carotene, tanshinone, naringenin, tangeretin, black tea and ascorbic acid) on citral's chemical stability under acidic condition (pH 3.0) were evaluated. Based upon the current research results, encapsulation of citral in emulsions could improve its chemical stability and reduce the production of many off-flavor compounds. For example, *p*-cymene and most of the monoterpene alcohols were completely suppressed. In addition, the incorporation of the appropriate antioxidants (i.e. beta-carotene, tanshinone and black tea extract) with citral together

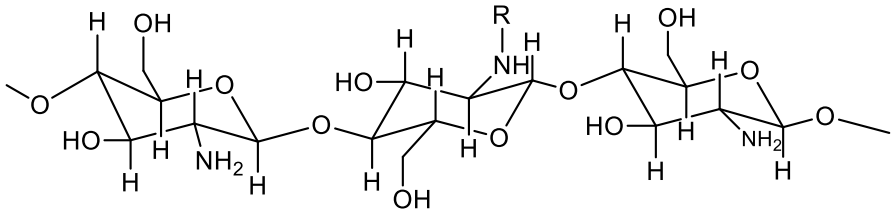
could further inhibit citral degradation as well as lipid oxidation. Future work is needed to study the mechanisms of how encapsulation and antioxidants work to increase citral's stability. The knowledge might be promising to design new strategies to improve the stability for many sensitive flavor molecules as well as to reduce lipid deterioration in food emulsions.

5.3.2 LBL modification

In this section, chitosan (CS) and ε-polylysine (EPL) (structures shown in Figure 5-25 and Figure 5-26) were used as cationic biopolymers to produce multilayer nanoemulsions for the encapsulation of citral. CS is a linear polysaccharide comprising copolymers of D-glucosamine and N-acetyl-D-glucosamine. It has attracted increased attention due to its potential wide range of industrial applications (Shahidi and others 1999). Due to the basic amine groups ($pK_a \approx 6.5$) (Allan and Peyron 1995) along the molecular chain, CS is soluble in water at low pH with positive charges. Another cationic biopolymer, EPL, is a homopolymer of L-lysine characterized by the isopeptide bond between ε-amino and α-carboxyl groups. Compared to the chemically synthesized poly(L-lysine), EPL is naturally produced by the bacterium *Streptomyces albulus* and its characteristics depend on the specific bacteria strains and culture conditions (Shih and others 2006). The isoelectric point of EPL is around 9.0 (Yoshida and Nagasawa 2003) therefore it is also positively charged at low pH. The emulsifier lecithin used in this study contains approximately 75% phosphatidylcholine (PC), 25% phosphatidylethanolamine (PE) and

lysophosphatidylcholine (LPC), among which PE is the one that contains both acidic and basic functional groups (phosphate and amine groups, respectively), therefore it is the major component that contributes to the negative charge of lecithin used in this study.

Three citral encapsulation systems were prepared in this study: anionic lecithin stabilized emulsion, cationic lecithin-CS and lecithin-EPL stabilized emulsions. The objectives of this research were to: (1) investigate the influence of different polymer concentrations on the emulsions physical properties (surface charge and particle size); (2) compare the stability of citral in three different formulations and (3) evaluate the effects of different interfacial layers on the production of off-flavor compounds from citral under acidic condition (pH 3.0).



R = H or $COCH_3$

Figure 5-25 Structure of chitosan

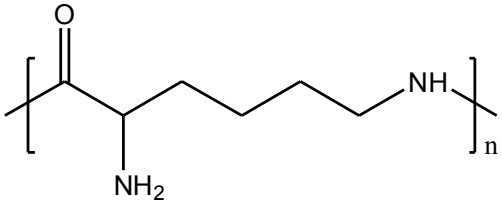


Figure 5-26 Structure of ε-polylysine.

Influence of CS and EPL concentrations on emulsion droplets surface charges. The surface charges of lecithin-CS and lecithin-EPL stabilized emulsions with different CS and EPL concentrations (at pH 3.0) were measured by ζ-potential. There are two purposes for the ζ-potential measurements: first, changes of the surface charge could prove the adsorption between the oppositely charged emulsion droplets and the polymers (i.e. CS and EPL, respectively); second, the optimum concentrations for both CS and EPL could be identified to prepare stable lecithin-CS and lecithin-EPL coated secondary emulsion dispersions in the following experiments to encapsulate citral. A series of secondary emulsion dispersions were prepared with various CS (0 – 1.75 mg/mL) and EPL (0 – 8 mg/mL) concentrations and the dependence of the surface charges on different CS and EPL concentrations at pH 3.0 was shown in Figure 5-27.

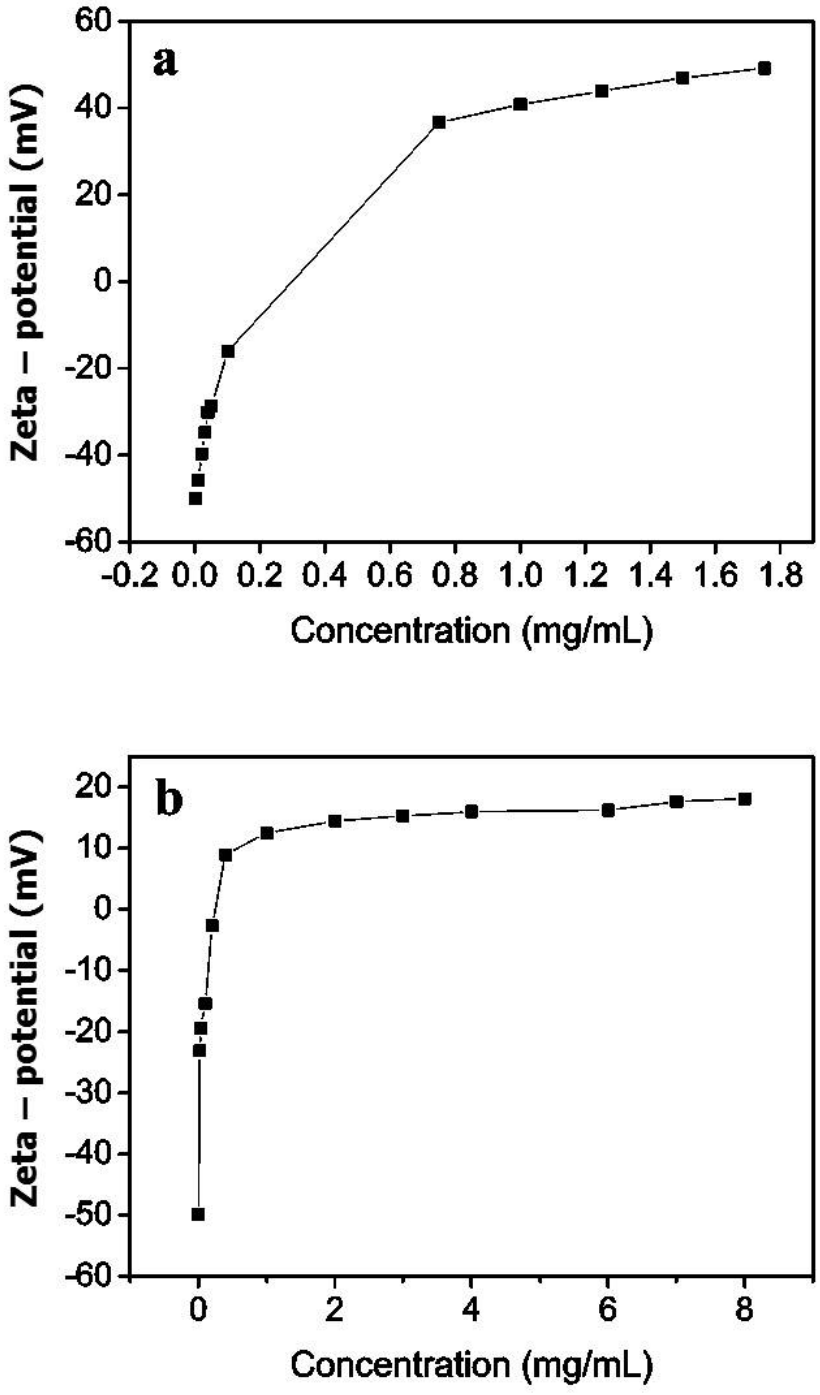


Figure 5-27 Dependence of the particle surface chage (ζ -potential) on (a) chitosan and (b) ε-polylysine concentrations (each emulsion sample contains 10 wt% lipid phase, 5 wt% lecithin and 85 wt% pH 3.0 citric acid buffer with dissolved polymer; some error bars lie within data points).

In the absence of either CS or EPL, the surface charge of lecithin stabilized emulsion was -49.9 mV. For the emulsion droplets coated with CS, the surface charge became less negative with the increase of CS concentration, which suggested that the positively charged CS adsorbed onto the negatively charged emulsion droplets surfaces forming an extra CS layer around the droplets. There is a CS concentration gap between 0.1 to 0.75 mg/mL as shown in Figure 5-27a because ζ-potential cannot be measured for samples coated with CS in that concentration range. During the sample preparation process, when the CS aqueous solution (with concentration between 0.1 to 0.75 mg/mL) was mixed with the lecithin stabilized emulsion, thick gel formed within hours therefore no ζ-potential can be measured. The most possible explanation for this phenomenon is that particle aggregation substantially dominated within that CS concentration range, and consequently gel formed due to the bridging flocculation between the negatively charged emulsion droplets and positively charged CS molecular chains. When the CS concentration was high enough, the surface charge values reached a plateau at ~ 45 mV, indicating that the negatively charged emulsion was saturated with CS.

For the EPL coated samples, the surface charge also switched from negative to positive with the increase of EPL concentration, and then reached a plateau around 15 to 18 mV when the EPL concentration was high enough (Figure 5-27b). The surface charges of the EPL coated emulsions were much smaller than the CS coated

emulsions. For example, the EPL coated emulsions showed lower surface charges (~18 mV) than that of the CS coated emulsions (~45mV). This was due to the different polymer characteristics, such as molecular chain structures and charge densities along the molecular chains between CS and EPL. Besides the surface charges, another marked difference between CS and EPL coated emulsions is that no concentration gap was observed for the EPL coated emulsions. For the CS coated emulsions, there existed a CS concentration range where bridging flocculation dominated to cause the emulsion droplets to aggregate and form gel as discussed above; however, there was no such concentration range for EPL as shown in Figure 5-27b. During the preparation of EPL coated samples, phase separation happened very fast when the EPL concentration was between 0.2 to 4 mg/mL. Therefore within this concentration range, the EPL coated samples were vortexed for 30s prior to the ζ-potential measurement. Different polymer coatings resulted in different emulsion phenomenon such as gelation for CS coated samples and phase separation for EPL coated samples, which could be related to different polymer characteristics.

Influence of CS and EPL concentrations on particle size. The mean particle diameters of lecithin-CS and lecithin-EPL stabilized emulsions with different CS and EPL concentrations were measured at pH 3.0 and the results are shown in Figure 5-28. In the absence of either CS or EPL, the mean particle diameter of the lecithin stabilized emulsion droplets was ~100 nm. For the CS coated emulsions, three phases could be observed for the particle size changing trend as shown in Figure 5-28a: (1)

particle size slightly increased when the CS concentration increased from 0 to 0.1 mg/mL; (2) particle size greatly increased when the CS concentration increased from 0.1 to 1 mg/mL; (3) particle size reached a constant value of ~450 nm when CS concentration exceeded 1 mg/mL. Similar trend could also be observed for the EPL coated emulsions as shown in Figure 5-28b, where the mean particle diameter first slightly and then dramatically increased with the increase of EPL concentration, and finally decreased and reached a plateau of ~600 nm when the EPL concentration was high enough (≥ 6 mg/mL). Similar particle size changing trends were also reported in several previous studies (Surh et al. 2005; Aoki et al. 2005). Before the coating processes of the cationic biopolymers CS and EPL, lecithin stabilized emulsions had relatively smaller particle size and good physical stability during storage (will be discussed in the next section). During the initial stage of adding polymers (both CS and EPL) into the lecithin stabilized emulsion, polymer chains began to adsorb onto the emulsion droplets surfaces due to the electrostatic interaction between the negative charges on the emulsion droplets and the positive charges on the polymer molecular chains, which caused the increase of the particle size. For example, at the concentration of 0.1 mg/mL for both CS and EPL, the mean diameter for CS coated emulsion was 289.4 nm while for EPL coated emulsion was 474.5 nm. In the second stage with further increase of the polymer concentrations, most of the emulsion droplets were partially coated with polymers, therefore bridging flocculation happened. At this stage, the polymer chains could act as “bridges” between the

opposite charges on different emulsion droplets, which means one CS or EPL molecular chain could adsorb onto more than one droplet surface simultaneously. The bridging effect resulted in the sharply increase of the particle size. For example, the CS coated emulsion droplets became larger than 600 nm when CS concentration exceeded 0.1 mg/mL; and the mean diameter for the EPL coated emulsion showed a ~5 times increase when EPL concentration increased from 0.1 to 0.2 mg/mL. At the final stage when the polymer concentrations were high enough, the polymer coated emulsion droplets gradually became saturated, consequently bridging disappeared and the electrostatic repulsion was strong enough to avoid emulsion aggregation.

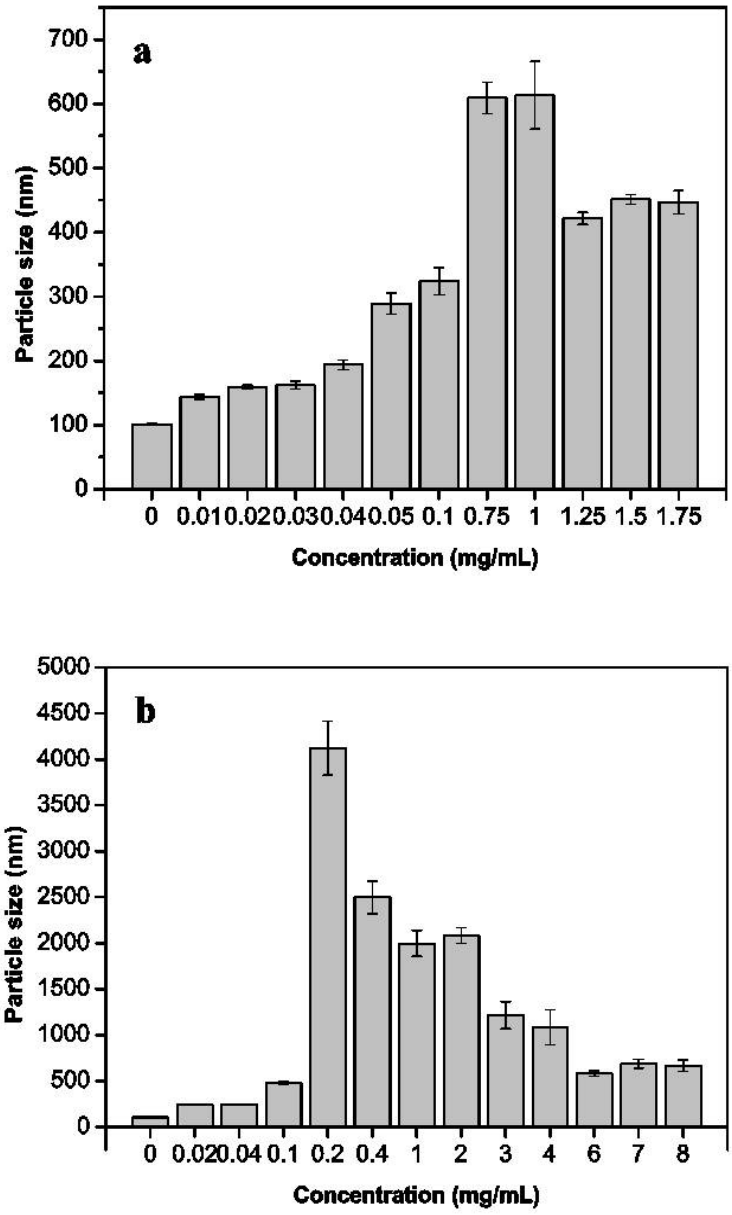


Figure 5-28 Dependence of the mean particle diameter on (a) chitosan and (b) ε-polylysine concentrations (each emulsion sample contains 10 wt% lipid phase, 5 wt% lecithin and 85 wt% pH 3.0 citric acid buffer with dissolved polymer; some error bars lie within data points).

Adsorption between CS and lecithin stabilized emulsion studied by QCM-D. Both the ζ-potential and the particle size results proved the adsorption between the emulsion droplets and the polymers to a certain extent, however, direct evidence is lacking in most of the similar studies. In this research, QCM-D was used to monitor the adsorption process between the emulsion and polymers directly. Figure 5-29a showed the frequency shift for the adsorption between CS and lecithin stabilized emulsion starting with the deposition of CS on the quartz crystal sensor surface. With the injection of emulsion into the QCM-D chamber, the frequency decreased immediately which was associated with the strong interaction between CS and the emulsion. Furthermore, an additional buffer wash did not cause significant frequency change which indicated that the interaction between CS and emulsion was robust enough to resist washing. In order to prove that the frequency shift was not caused by the physical deposition of emulsion due to gravity, a control measurement was taken as shown in Figure 5-29b. During the control measurement, the frequency remained at 0 which suggested that the frequency shift was resulted from the interaction between CS and emulsion, not from the physical deposition of the emulsion. The same adsorption measurements were also taken for EPL coated emulsions; however, the frequency shift soon exceeded the instrument limitation even at very low EPL concentration, therefore only data from CS coated emulsions are shown here. In this study, QCM-D was used as supplementary means to determine the optimal concentration of CS. In combination of the ζ-potential and particle size measurement

results, five different CS concentrations were chosen for the QCM-D measurements and the fitting results of the frequencies are shown in Figure 5-30. When the CS concentration increased from 0.75 to 1.75 mg/mL, the frequency shifts also increased at first and then reached a plateau. Consequently, the ultimate CS concentration 1.5 mg/mL was used to prepare citral loaded emulsion; and the final EPL concentration 6 mg/mL was chosen based on the ζ-potential and particle size results.

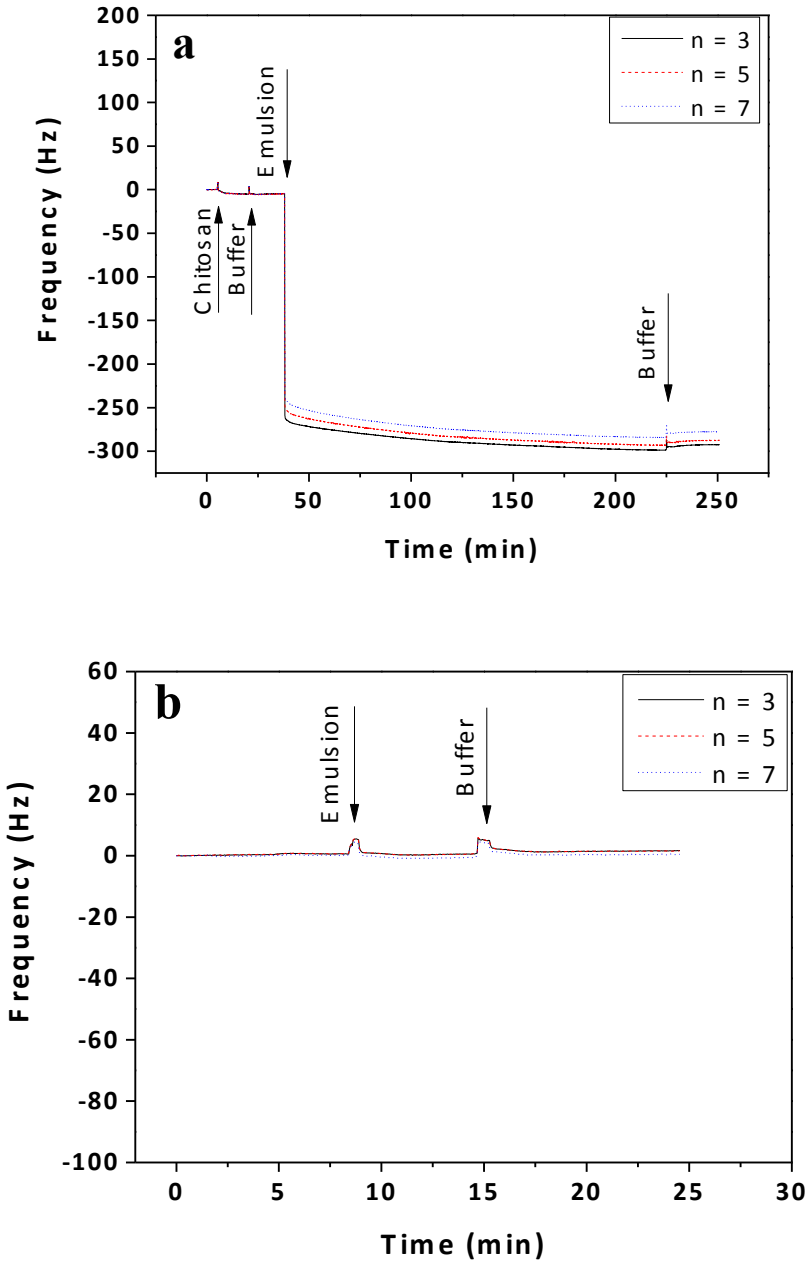


Figure 5-29 Representative QCM-D frequency shifts for (a) the adsorption between chitosan and lecithin stabilized emulsion and (b) the adsorption of lecithin stabilized emulsion onto the crystal sensor surface without chitosan at three frequency overtones at pH 3.0 (n = 3, 5 and 7).

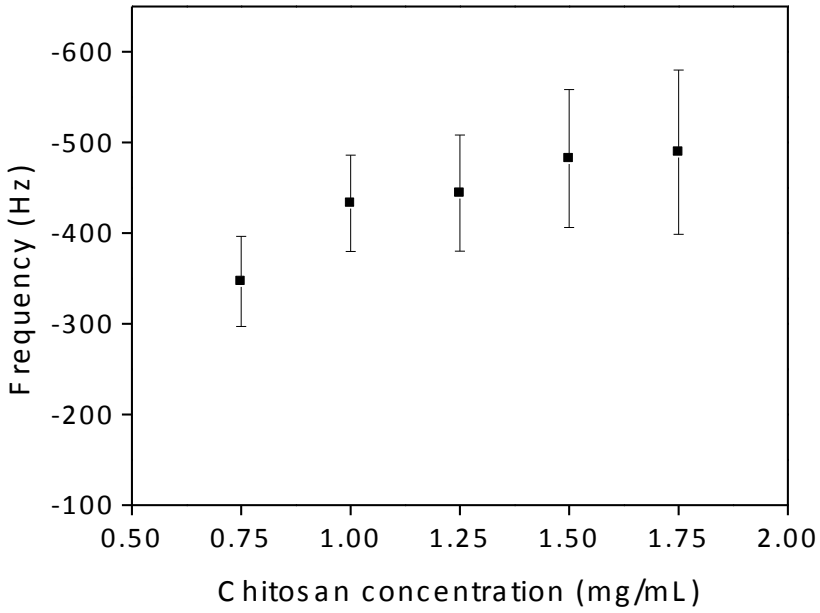


Figure 5-30 Dependence of the frequency shifts on chitosan concentrations at 25 ^{o}C and pH 3.0.

Physical stability of citral loaded emulsions with different polymer coatings. Particle sizes of citral loaded emulsions stabilized by lecithin, lecithin-CS and lecithin-EPL were measured at day-0, day-30 and day-60 during storage under 25^{o}C and 50^{o}C, respectively. The mean particle diameter data are listed in Figure 5-2.

Table 5-3 Mean particle diameter changes during storage for citral loaded emulsions stabilized by lecithin, lecithin-chitosan (CS) and lecithin-EPL stored under 25^{o}C and 50^{o}C, respectively.

T (^{o}C)	Samples	Day 0	Day 30	Day 60
25	Lecithin	109.2 ± 3.0	123.3 ± 4.7	132.7 ± 9.8
	Lecithin-CS	451.6 ± 7.4	494.7 ± 12.5	512.3 ± 15.7
	Lecithin-EPL	580.5 ± 28.1	-	-
50	Lecithin	109.2 ± 3.0	148.9 ± 3.3	186.1 ± 7.9
	Lecithin-CS	451.6 ± 7.4	601.8 ± 38.3	646.7 ± 41.2
	Lecithin-EPL	580.5 ± 28.1	-	-

Lecithin stabilized emulsion had the smallest particle size of 109.2 nm while both CS and EPL coatings increased the particle size to 451.6 and 580.5 nm, respectively. EPL coated emulsion was very unstable and phase separation happened during storage even at 25^{o}C therefore no particle size was measured at day-30 and day-60. The most possible reasons are that first, EPL coated emulsion droplets had the largest size among all the formulations; and second, this group of sample also had the smallest surface charge (~ 18 mV). It has long been proved that the addition of polymers in an emulsion system could exert a great influence on the emulsion stability (Yu and Somasundaran 1996; Chuah and others 2009; Dickinson 1992). The charge density, molecular concentration and structure conformation of the polymer are the major factors that could influence the emulsion stability. Chitosan has been proved to stabilize an emulsion system (Güzey and McClements 2006; Ogawa et al. 2003) while the use of EPL as a second interfacial layer has never been studied before. The possible reason that caused the unstability problem of the EPL coated emulsion is probably the EPL induced depletion flocculation, which is a common but poorly understood phenomenon, with little quantitative data available. Since the driven forces that work to prevent emulsion droplets flocculation are mainly electrostatic repulsion and the steric hindrance (Dickinson 1992; Jenkins and Snowden 1996), it has been proved in the zeta-potential results that the chitosan coated emulsion droplets had much higher surface charge than the EPL coated droplets, therefore not sufficient electrostatic repulsion for the EPL system might be one reason that caused the

emulsion flocculation. And flocculation could result in the subsequent coalescence as well as emulsion creaming to cause phase separation of the emulsion (Dickinson 1992). For the other two formulations (lecithin and lecithin-CS stabilized emulsions), particle size increased during storage with time especially at higher temperature (50^{o}C); however, both of them still showed good physical stability because the particle size increments were not significant enough to caused obvious creaming or phase separation, which might be associated with the high surface charges and the consequent large repulsive force between the emulsion droplets.

Stability of citral during storage. The stability of citral was determined in various formulations (lecithin, lecithin-CS and lecithin-EPL stabilized emulsions) by calculating the loss of citral's isomers, neral and geranial during storage. At 25^{o}C, both neral and geranial showed similar degradation trends as shown in Figure 5-31. Lecithin-EPL stabilized emulsion was the worst formulation since both neral and geranial degraded very fast compare to the other two groups of samples. Only ~3% citral was left after 3 weeks and no citral could be detected after 4 weeks. The fast degradation of citral in the lecithin-EPL stabilized formulation was caused by phase separation of the emulsion. Therefore, the emulsion's protection for citral no longer existed. Lecithin stabilized emulsion showed certain protection for citral when 82.7% neral and 81.3% geranial were left after the first week and 33.5% neral and 32.1% geranial were still remained in the sample after 4 weeks. Compare to the lecithin stabilized emulsion, more than two times of citral was left in the lecithin-CS stabilized

formulation. For example, almost no neral and geranial degraded after the first week, and 77.6% neral and 75.6% geranial remained in the sample at the end of the measurement (4 weeks). The extra CS coating had positive effect to inhibit citral from degradation. Similar to our previous study (Yang and others 2011b), all the samples were also stored at a relatively higher temperature (50^{o}C) to accelerate citral degradation (Figure 5-32). As expected, both neral and geranial degraded much faster than the samples stored at 25^{o}C so that the difference between different formulations could not be differentiated. For instance, ~ 48% citral was lost for lecithin stabilized emulsion, ~ 43% citral was lost for CS coated emulsion and ~ 60% citral was lost for the EPL coated emulsion only after 1 day of storage at 50^{o}C. Several off-flavor compounds were also detected and the analysis results will be discussed in the following section.

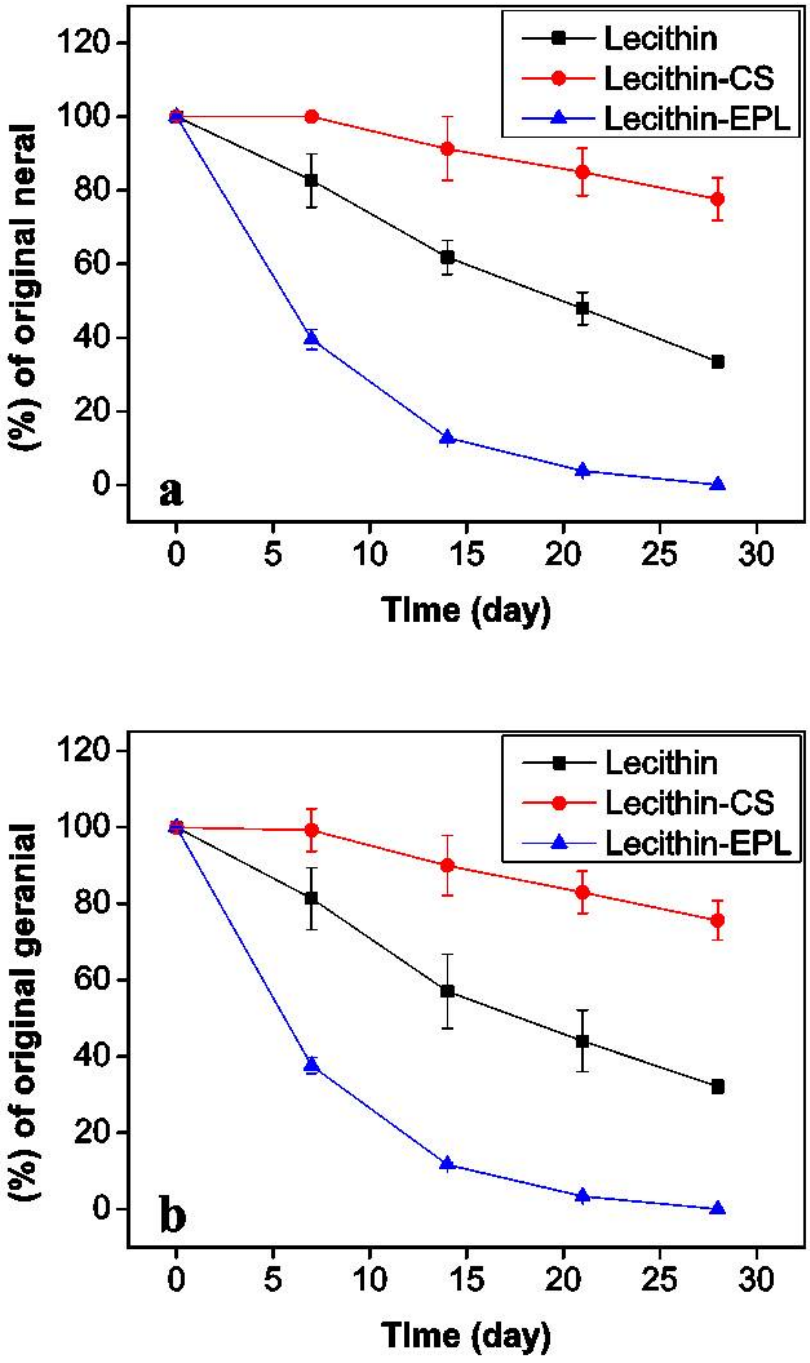


Figure 5-31 Degradation of (a) neral and (b) geranial in lecithin, lecithin-CS and lecithin-EPL stabilized emulsions stored at 25°C (each emulsion sample contains 10 wt% lipid phase, 5 wt% lecithin and 85 wt% pH 3.0 citric acid buffer with dissolved polymer).

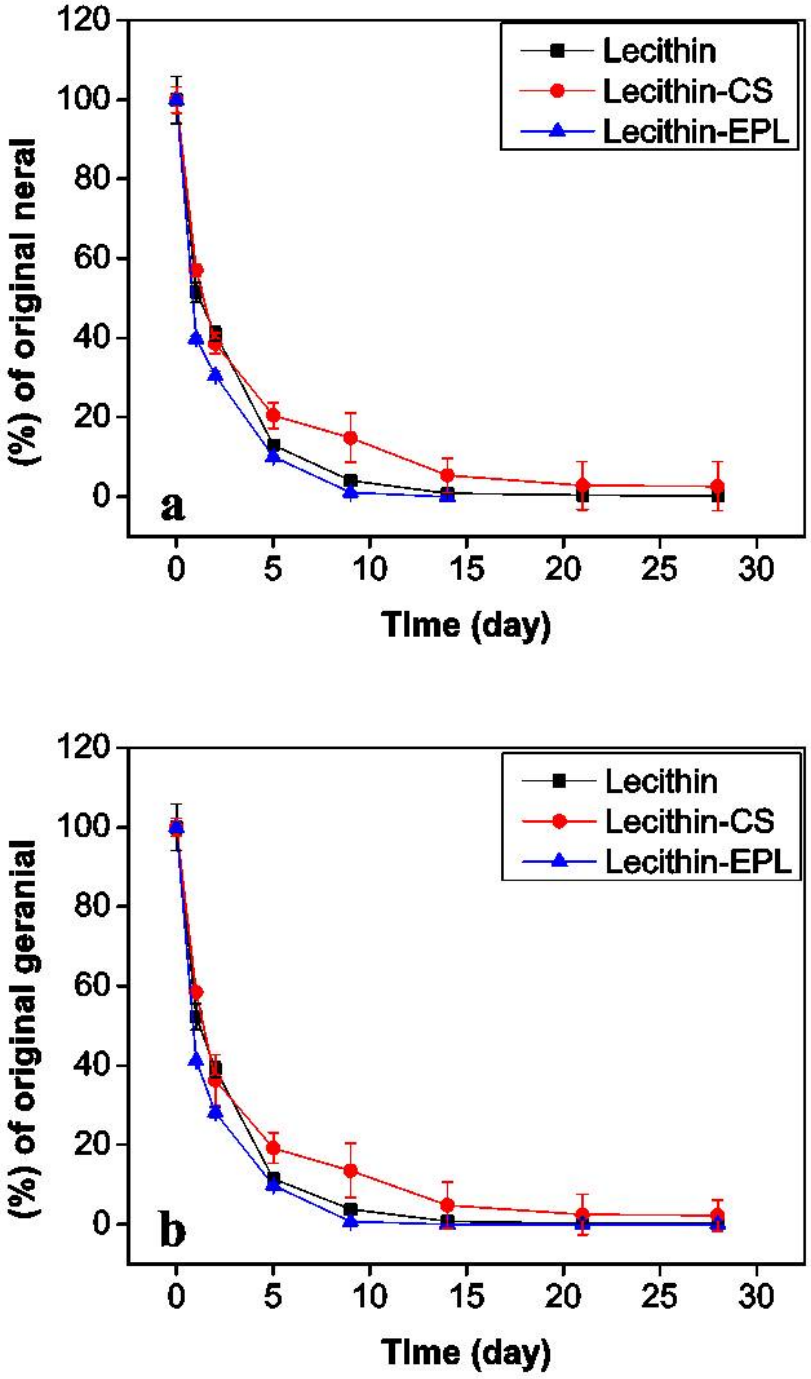


Figure 5-32 Degradation of (a) neral and (b) geranial in lecithin, lecithin-CS and lecithin-EPL stabilized emulsions stored at 50^{o}C (each emulsion sample contains 10 wt% lipid phase, 5 wt% lecithin and 85 wt% pH 3.0 citric acid buffer with dissolved polymer)..

Evaluation of off-flavor compounds for citral loaded emulsions. Citral was completely degraded and almost totally converted to its degradation products during storage at 50^{o}C for 4 weeks. As shown in our previous study (Yang et al. 2011b), similar GC spectrum were obtained in this study and the major off-flavor compounds were listed in Table 5-2. Both citral and lipid degradation products were detected from all the formulations. It is well known that lipid degradation will generate many off-flavor compounds especially at high temperature, such as 2-heptanone, 1-octen-3-ol, and butanoic acid in this study. A cationic interfacial membrane is supposed to increase the lipid oxidative stability in an oil in water emulsion system based on several previous studies (Klinkesorn and others 2005c; Shaw and others 2007; Silvestre and others 2000), however, both polymer coatings did not show significant effects to inhibit lipid oxidation in this study. For the citral degradation, it has been accepted that citral degradation started from acid catalyzed reactions first to form several compounds such as *p*-cymene, *p*-menthadien-8-ols, *p*-menth-2-ene-1,8-diols (Kimura and others 1982; Clark and others 1977; Ueno et al. 2004). In this study, only *p*-metha-1,5-dien-8-ol was produced as the acid catalyzed reaction product from citral after encapsulation. While the EPL coating had no effect to reduce the production of *p*-metha-1,5-dien-8-ol, the CS coating worked to inhibit its production as shown in Figure 5-33. The other citral degradation products, such as delta-2-carene, *p*-cresol, *α,p*-dimethylstyrene and *p*-methylacetophenone, were

produced from citral's oxidation reactions. Compare to the lecithin stabilized emulsion, the CS coating reduced the production of delta-2-carene and *p*-cresol, and had the similar concentration level of *p*-methylacetophenone, but unfortunately it also promoted the production of *α,p*-dimethylstyrene; while the EPL coating increased the production of delta-2-carene, *α,p*-dimethylstyrene and *p*-methylacetophenone and interestingly it also decreased the production of *p*-cresol as the CS coating. Most of the citral oxidation products were not detected in all the formulations, such as *p*-cymen-8-ol and 8-hydroperoxy-*p*-cymene (Kimura et al. 1983; Ueno et al. 2004). There might be two reasons to explain this phenomenon: first, previous studies proposed that most of citral's oxidation products were produced from a series of dehydration and/or oxidation reactions of many intermediate compounds, such as *p*-menthadien-8-ols and *p*-mentha-1,4(8),5-triene (Ueno et al. 2004; Barton and Parekh 1989a). These intermediate compounds were resulted from the acid catalyzed reactions of citral induced by protons in the acidic aqueous medium. It had been shown that most of the acid catalyzed products were inhibited after citral was encapsulated in this study, because encapsulation was an effective way to isolate citral from the reactive species, such as protons and metal ions, in the acidic aqueous medium. Consequently the oxidation reactions pathways were interrupted to produce the commonly detected oxidation products of citral; and the second reason might be the presence of lipid and phospholipids (palm kernel fat and lecithin) in all the formulations. However, the mechanisms are still unknown and more research is

needed to study how the other ingredients, such as lipid and phospholipids, influence the reaction pathways of citral's degradation.

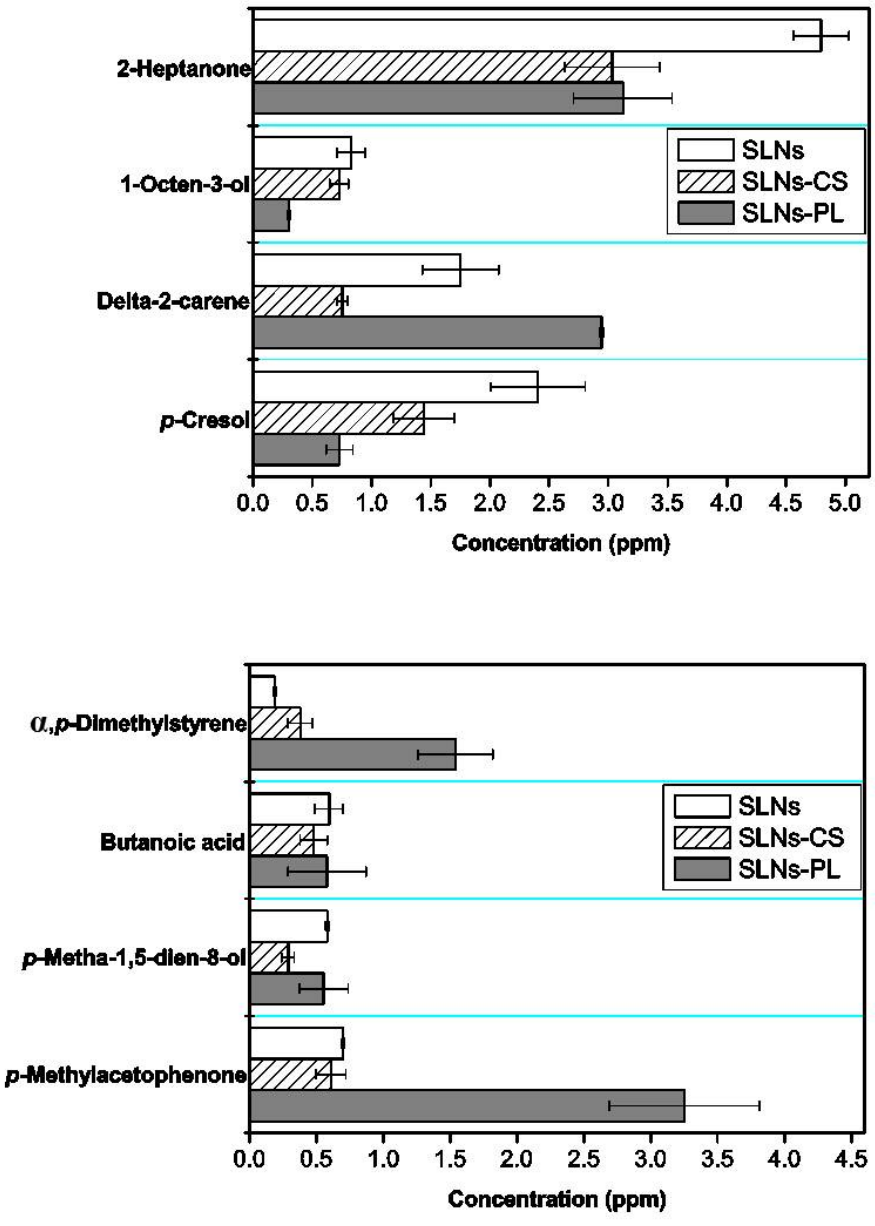


Figure 5-33 Concentrations for the major degradation compounds produced from citral loaded emulsions stored at 50^{o}C for 4 weeks (each emulsion sample contains 10 wt% lipid phase, 5 wt% lecithin and 85 wt% pH 3.0 citric acid buffer with dissolved polymer).

In summary, citral was encapsulated in anionic lecithin stabilized emulsion and two cationic lecithin-CS and lecithin-EPL stabilized emulsions, respectively. ζ-Potential, particle size and QCM-D measurements have confirmed the feasibility to prepare different emulsion systems with different polymer coatings based on the electrostatic interaction between oppositely charged emulsion droplets and polymers. EPL coated particles were not stable enough to protect citral due to the small surface charge and the formation of very large particles. The lecithin-CS stabilized emulsion dispersion not only possessed good physical stability during storage, it also worked better than the solely lecithin stabilized particles to inhibit citral from degradation.

6 Conclusions and future works

Nanoemulsions formulated by solid lipid palm kernel fat, MCT and stabilized by lecithin were prepared to encapsulate beta-carotene (as the non-polar molecule) and citral (as the partially polar molecule). The research mainly focused on improving the stability and enhancing the in *vitro* release profile of the encapsulated compounds.

6.1 Encapsulation of beta-carotene

Beta-carotene was encapsulated in aqueous SLN dispersions as well as lyophilized SLN powders. The lipid phase was composed of two different lipids: palm kernel fat (solid at room temperature with melting pint of 33.9^{o}C) and MCT (liquid at room temperature). The influence of different palm kernel fat and MCT mixtures with different lipids ratios on beta-carotene stability was investigated.

For physical stability, all the formulations showed good stability (as measured by particle size analyzer) in 60 days and no obvious phase separation or creaming was observed. For the lyophilized powders using sucrose as the cryoprotectant, the reconstituted freeze-dried samples in water also showed good freeze-thaw stability within 5 freeze-thaw cycles.

For chemical stability, beta-carotene contents in all the formulations were measured by HPLC under two conditions: exposed to light and in dark, while light was proved to promote beta-carotene degradation. In the aqueous SLN dispersions, the higher the palm kernel fat content, the better the SLN formulation can protect beta-carotene from

degradation. The most probable reason is that the guest molecules (lecithin and beta-carotene) disrupted the ordered structure of the solid lipid; therefore the presence of liquid MCT increased the mobility of beta-carotene which increased its degradation.

In lyophilized SLN powders, the degradation rates of beta-carotene in all the lyophilized samples were faster than that of beta-carotene in the aqueous SLN dispersions under both storage conditions (with and without light exposure). Lyophilization promoted the crystallization of palm kernel fat and the inclusion of liquid MCT oil could disturb the crystallization to some extent by showing a melting point depression phenomenon. The crystallization disruption effect caused by MCT exerted some protection for beta-carotene in this group of samples. However, lyophilization was proved not to be a favored secondary production method to convert liquid emulsion samples into solid.

The cost-effective and time-effective in *vitro* digestion model was also developed in this study to evaluate the digestion behavior of two lipids (MCT oil and palm kernel fat) and their corresponding nanoemulsions (MCT nanoemulsion and palm kernel fat nanoemulsion); and at the same time, the micelization efficacies of beta-carotene in the 4 formulations were also determined by HPLC. The results showed that both nanoemulsion systems had better solubilization capacity for the encapsulated beta-carotene than bulk lipids, therefore the bioaccessibility of beta-carotene was greatly improved after encapsulation. Furthermore, more beta-carotene was released

from the palm kernel fat nanoemulsion than from the MCT nanoemulsion, which was probably influenced by different lipid composition of palm kernel fat and MCT oil. The results may provide useful information for developing encapsulation and delivery systems with desired controlled release profile.

6.2 Encapsulation of citral

The stability of citral was improved by two strategies: (1) citral was encapsulated in SLN in combine with six different antioxidants (beta-carotene, tanshinone, naringenin, tangeretin, black tea and ascorbic acid) and (2) citral was encapsulated in multilayer SLN with two different polymer coatings chitosan (CS) and ε-polylysine (EPL), respectively. The chemical stability of citral under acidic condition (pH 3.0) was examined by GC and its major degradation products were examined by GC – MS at 25 and 50 ^{o}C, respectively. The results proved that encapsulation of citral could improve its stability and reduce the production of many off-flavor compounds, such as *p*-cymene and most of the monoterpene alcohols.

For the incorporation of various antioxidants with citral together, certain antioxidants (*i.e.* beta-carotene, tanshinone and black tea extract) could slow down citral degradation as well as lipid oxidation (since there was lipid in all the SLN formulations).

CS and EPL were used to fabricate cationic multilayer SLN systems through the LBL technique. ζ-Potential, particle size and QCM-D results were used to confirm the

optimal formulation; however, due to the low charge density of EPL, the EPL coated SLN was not stable enough during storage to protect citral. The CS coated SLN system showed excellent stability during storage at both temperatures (25 and 50 ^{o}C), and this system also worked to further improve citral stability.

In summary, this dissertation provides a study of the effectiveness of the nanoemulsion in improving the stability of both the non-polar beta-carotene and the partially polar citral molecules. Meanwhile, the in *vitro* release profile was greatly enhanced for the nanoemulsions compare to the bulk lipids. The information obtained from this research showed that through engineering the emulsions formulations, such as the composition of the lipid phase and the interfacial membrane properties, was an effective way to prepare encapsulation and delivery systems for food ingredients.

6.3 Future works

1. In *vivo* animal study to examine the effectiveness of nanoemulsions on delivering active food ingredients. Although the current study has proved that nanoemulsions have excellent release profile in *vitro*, the correlation between the in *vitro* and in *vivo* study is needed in the future.
2. Future work is needed to study the mechanism of how encapsulation, antioxidants, and extra cationic biopolymer layers work to increase citral stability. The information obtained from the mechanism study may be useful to establish protocol to further improve the nanoemulsions capabilities to protect the encapsulated molecules.

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Curriculum Vitae

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Publications

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